Comparative proteome profiling of breast tumor cell lines by gel electrophoresis and mass spectrometry reveals an epithelial mesenchymal transition associated protein signature†

Daniele Vergara,‡aab Pasquale Simeone,‡c Piero del Boccio,ª Claudia Toto,b Damiana Pieragostino,ª Andrea Tinelli,ª Raffaele Acierno,‡b Saverio Alberti,c Michel Salzet,ª Gianluigi Giannelli,b Paolo Sacchettad and Michele Maffia*ab

The epithelial to mesenchymal transition (EMT) is a cellular program associated with the organ morphogenesis but also with the disease progression. EMT in the cancer field fuels neoplastic progression promoting the resistance to cell death, the resistance to chemotherapy and the acquisition of stem cell properties. Considering the crucial role of EMT in breast cancer metastasis, a better understanding of this process may provide new therapeutic options. Here, by using a proteomic approach we identified a set of proteins differentially expressed between an epithelial and a mesenchymal breast cancer cell line. The protein–protein network of these identified proteins was determined by an in silico analysis highlighting, in the EMT program, the role of proteins involved in cell adhesion, migration, and invasion, together with protein kinases involved in proliferation and survival, with many of these emerging as possible targets of novel biological agents. Finally, the pharmacological inhibition of some of these kinases was able to reverse the mesenchymal phenotype to an epithelial phenotype.

Introduction

Cancer metastasis is the major cause for breast cancer mortality. Patients diagnosed with late stage cancer have reduced survival chances compared to early stage patients, making the identification of molecular pathways underlying tumor progression an important goal. However, the mechanisms by which tumor cells invade and escape from the primary tumor are not entirely understood. The establishment and growth of metastases is considered the result of the activation of several cellular pathways and underlying genes and proteins. In the case of breast cancer, several lines of evidence suggest that an epithelial to mesenchymal transition (EMT) process, and the reverse process mesenchymal to epithelial transition (MET), is one of the main mechanisms involved in metastasis.¹⁵

Both EMT and MET have been thoroughly studied in mammalian development. EMT is a critical event during embryogenesis, required for morphogenetic movements during gastrulation and formation of migratory neural crest cells.⁶ During EMT, apico-basolateral polarity is lost, cell-cell junctions dissolve and the actin cytoskeleton is remodelled thus enabling the mobility and invasiveness of cells. The reactivation of this development program has also been observed during tumor progression, which may represent a way for epithelial-derived tumors to become invasive and metastasize.⁷ EMT has been documented in several cancer models and associated with the induction of stem cell properties and with the development of chemoresistance by altering the...
expression of genes involved in drug transport and apoptosis. This process that mainly occurs at the invasive front of the tumor is coordinated primarily through growth factor- and cytokine-dependent activation of transcription factors of the Snail, ZEB, and Twist families and a number of signaling pathways that converge on these transcription factors to induce EMT such as transforming growth factor (TGF)-β, epidermal growth factor (EGF), and Wnt/β-catenin. These transcription factors drive EMT by repressing the expression of genes coding for epithelial markers such as E-cadherin and activating the expression of mesenchymal genes such as N-cadherin, Vimentin, and Smooth-Muscle-Actin (SMA). Upon completion of EMT, cells have acquired a complete fibroblast-like (spindle) morphology and invasive and metastatic behaviour.

A loss of epithelial markers and an over-expression of mesenchymal markers have been observed in patients with breast cancer and significantly associated with poor patient outcomes and tumor aggressiveness. As these breast samples express some markers associated with “basal like” or “claudin low” subtypes of breast carcinomas, EMT changes are proposed to correlate with these particular tumor subtypes both in vitro and in vivo. Moreover, the mesenchymal phenotype has also been identified in residual breast tumor cells surviving after endocrine therapy and chemotherapy suggesting that EMT-program strongly correlates EMT with tumor aggressiveness, treatment resistance and metastatic spread.

The inherent drug resistance of many advanced cancers that have undergone EMT indicates the need for more effective therapy. Identifying pathways that regulate the EMT process could provide a basis for new therapeutic strategies. In these years, gene-expression methods have been extensively used to characterize this process but only few studies adopted a proteomic strategy to study EMT.

To better understand the mechanisms that control EMT in breast cancer cells we characterized proteins isolated from two breast cancer cellular models, MCF-7 (luminal type) and MDA-MB-231 (mesenchymal type), by two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) to define at a molecular level the proteome changes that occur as a consequence of EMT. We identified an EMT protein expression signature that was validated by western blot and RT-PCR. Moreover, we extended the set of identified proteins to other possible EMT markers by an in silico network analysis. Molecular pathways that regulate EMT include proteins involved in a variety of biological functions such as cell adhesion, migration, invasion, metabolism, survival and proliferation. The pharmacological inhibition of some of these signalling pathways, more notably Src, protein kinase B (Akt), mitogen-activated protein kinase (MAPK), and epidermal growth factor receptor (EGFR) can reverse the mesenchymal phenotype to an epithelial phenotype.

### Experimental methods

#### Cell culture and materials

HBL-100, MCF-7, MDA-MB-231, MDA-MB-361, and MDA-MB-435 human breast cancer cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Euroclone), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Sigma) in humidified 37 °C chambers with 5% CO₂.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was from Sigma-Aldrich. The following reagents were obtained from GE Healthcare: IPGs pH 3–10 NL, IPG buffer 3–10, dry strip cover fluid and the Hybond ECL membrane and ECL western blotting detection reagents. Primary antibodies used in this work are listed in Table S1 (ESI†). All other reagents were from standard commercial sources and were of the highest grade available.

#### Cell viability assay

Cells were seeded at a density of 5 × 10³ per well in a 96-well plate containing 100 μl of full medium and allowed to adhere to the plate overnight. For determining cell viability, the MTT assay was used. After treatment, the culture medium was aspirated and 100 μl of RPMI-phenol free medium containing 10 μl of MTT stock solution, 5 mg ml⁻¹ in phosphate-buffered saline (PBS) solution, was added to each well. After two hours of incubation, the MTT solution was removed and 100 μl of DMSO were added to the wells maintaining in agitation for 15 min to dissolve MTT-formazan crystals. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. The relative cell viability was expressed as a percentage of the untreated control wells. The concentration of drug required to inhibit cell proliferation by 50% (IC50) was calculated using the Microsoft Excel software. Data were exported and analyzed using an Excel sheet and IC50 values were calculated by linear interpolation. IC values are expressed as mean ± standard deviation from at least ten independent experiments.

#### 2-DE and image analysis

Cell pellets were resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, protease inhibitor cocktail, 1 mM sodium orthovanadate, 10 mM sodium fluoride and sonicated on ice for three rounds of 10 s. The lysates were centrifuged for 15 min at 12,000 rpm to separate the unsolved cell debris. Protein concentration was determined using the Bradford protein assay. Protein 2-DE was performed as described before. In detail, protein samples (80 μg) were diluted up to 250 μl with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and 0.5% v/v IPG buffer) and applied to IPG strips (13 cm, pH 3–10 NL). IEF and second dimension was performed with IPGPhor IEF and Hoefer SE 600 Ruby electrophoresis (GE Healthcare). The IPG-strips were loaded and run on a 12% SDS-PAGE gel at a constant current of 20 mA per gel until the bromophenol blue dye front had run off the base of the gel. Gels were fixed overnight in fixing solution (30% v/v ethanol, 10% v/v acetic acid and 0.05% naphthalene disulfonic acid), and stained according to the protocol of Chevallet and collaborators. Gels were scanned by the Image Master scanner at 300 dpi and analysed by Image Master software 5.0. Spot detection and matching were carried out by the software tools and corrected manually when necessary. After spot detection,
editing, matching and normalization, 1189 ± 59 and 1215 ± 41 spots were detected in the MDA-231 and MCF-7 gels, respectively. The analysis was performed by comparing the volume% of each spot, expressed as percentage of the spot volume over the total volume of all spots in the gel. Significant differences in protein levels were determined by Student’s t-test with a set value of \( p < 0.05 \). Experiments were performed three times independently.

Mass spectrometry analysis

**Protein identification by nHPLC ESI-trap analysis.** The protein spots were manually picked from 2D-gels, destained with \( \text{H}_2\text{O}_2 \) and subjected to trypsin digestion followed by identification using nLC-MS/MS. The nano-HPLC used was a Proxeon Easy-nLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a NS-AC-10 analytical column, 5 \( \mu \text{M} \), C18, 375 \( \mu \text{M} \) OD \( \times 75 \mu \text{M} \) ID \( \times 10 \text{ cm} \) length, protected by a NS-MP-10 guard column, 5 \( \mu \text{M} \), C18, 375 \( \mu \text{M} \) OD \( \times 100 \mu \text{M} \) ID \( \times 2 \text{ cm} \) length (Nano Separations, Nieuwkoop, The Netherlands). The gradient was (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) 5% to 35% B from 0 to 10 min, 35% to 100% B from 10 to 12 min, 100% B from 12 to 20 min, with a flow of 0.3 \( \mu \text{L} \) min\(^{-1} \). An HCT ESI trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with an ESI nanosprayer, was used to record peptides over the mass range of 100 to 1800 \( \text{m/z} \), at a speed of 8000 \( \text{m/z s}^{-1} \); MS/MS spectra were automatically obtained from the 3 precursor ions with highest intensity over the mass range of 100 to 2800 \( \text{m/z} \), at a speed of 23000 \( \text{m/z s}^{-1} \). Repeatedly, each MS spectrum was recorded followed by three data-dependent CID MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.5 min after two spectra was used to detect low abundant peptides. The voltage between a ion spray tip and a spray shield was set to 3500 V. Nitrogen was used as dry gas, abundant peptides. The voltage between a ion spray tip and a trap column, by a carrier solvent (water with 0.2% of formic acid) at 20 \( \text{L min}^{-1} \) for 3 min and subsequently, it was eluted at 0.3 \( \text{L min}^{-1} \) on a Thermo C18 EASY-Column\(^{34} \), L 10 cm, ID 75 \( \mu \text{M} \), 3 \( \mu \text{M} \), 120 \( \text{Å} \) with a water/acetonitrile gradient in the presence of 0.2% of formic acid. Argon was used as collision gas. The collision energy was as a function of the precursor ion mass. MS/MS spectra were acquired by automatic switching between MS and MS/MS mode. Acquired MS/MS data were processed by FlexAnalysis and analyzed using MASCOT MS/MS ion search engine (http://www.matrixscience.com) with Swiss-Prot human database (release 2010_09 of UniProtKB/Swiss-Prot, 519348 sequence entries). Peptide tolerance was set as 0.6 \( \text{Da} \), searching peptide charges of 2+, 3+ and 4+. At most one miss-cleavage for tryptic peptides was allowed. Carbamidomethylation of cysteine was set as fixed modification while methionine oxidation was set as variable modification. Identifications were accepted based on significant MASCOT Mowse score >37 as described above.

**Protein identification by ESI-Q-TOF analysis.** Protein identification by nanoESI-Q-TOF was carried out through a Proxeon Easy-nLC (Thermo Fisher Scientific, Waltham, MA, USA) system coupled on-line with a nano-ESI-Q-TOF instrument Maxis 4G (Bruker Daltonics). The sample was first concentrated into a Thermo EASY-Column\(^{35} \), C18, L 2 cm, ID 100 \( \mu \text{M} \), 5 \( \mu \text{M} \), 120 \( \text{Å} \), trap column, by a carrier solvent (water with 0.2% of formic acid) at 20 \( \text{L min}^{-1} \) for 3 min and subsequently, it was eluted at 0.3 \( \text{L min}^{-1} \) on a Thermo C18 EASY-Column\(^{36} \), L 10 cm, ID 75 \( \mu \text{M} \), 3 \( \mu \text{M} \), 120 \( \text{Å} \) with a water/acetonitrile gradient in the presence of 0.2% of formic acid. Argon was used as collision gas. The collision energy was as a function of the precursor ion mass. MS/MS spectra were acquired by automatic switching between MS and MS/MS mode. Acquired MS/MS data were processed by FlexAnalysis and analyzed using MASCOT MS/MS ion search engine (http://www.matrixscience.com) with Swiss-Prot human database (release 2010_09 of UniProtKB/Swiss-Prot, 519348 sequence entries). The maximal tolerance for peptide masses was 100 ppm and for MS/MS data was 0.3 Da, searching peptide charges of 2+, 3+ and 4+. At most one miss-cleavage for tryptic peptides was allowed, and the variable modification accepted was oxidation of methionines. Identifications were accepted based on significant MASCOT Mowse score >37 as described above.

**Statistical analysis**

Data were analysed using the statistical software GraphPad (version 4.0). The two-tailed Mann–Whitney t-test was used to test statistical significance. Differences were considered statistically significant for \( p \)-values <0.05.

Spearman correlation analysis, principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed using the SIMCA 13 software package (Umetrics, Sweden)\(^{23} \) and the MetaboAnalyst 2.0 software (www.metaboanalyst.ca).\(^{24,26} \) The analysis was conducted on the autoscaled protein density values\(^{27} \) applied to make each variable more comparable in magnitude to the others.\(^{24} \)

To define the optimal number of principal components (PCs), “7-fold cross-validation” (CV) was applied.\(^{28,29} \) The number of components yielding the lowest percentage of miss-classification was chosen as the optimal model.\(^{30,31} \) Two parameters were used to evaluate the model: \( R^2 \) (goodness of fit) and \( Q^2 \) (goodness of prediction). Generally, a model with a \( Q^2 > 0.5 \) is considered good, \( Q^2 > 0.9 \) excellent.\(^{32} \)

Since cross-validation assesses only the predictive power without a statistical validation, the performance of the PLS-DA model was also validated by a permutation test.\(^{36} \) This method uses randomly reordered Y-data. For the new PLS permuted models \( Q^2 \) and \( R^2 \) are calculated.\(^{28} \) The process of permutation was reiterated 200 times. The original model is significant if the \( Q^2 \) and \( R^2 \) values of the permuted model are lower than the \( Q^2 \) value of the original dataset and if the regression line, in the validation plot, gives a negative intercept on the Y-axis.\(^{32} \)

For the PLS-DA model, the “VIP score” (Variable Importance in the Projection) was analyzed to identify which descriptors
explain better most of the differences between the two groups. The VIP score is a weighted sum of squares of the PLS loading weights taking into account the amount of explained Y-variance in each dimension.24,25

Pathway analysis

Functional protein association analysis was performed using the software STRING (http://string-db.org/). This program builds protein networks based on known direct and indirect interactions described in the literature. A confidence limit of 95% was considered the cut-off for the analysis.

RT-PCR

Total cellular RNA was isolated by the Illustra TriplePrep extraction kit following manufacturer’s instruction and immediately used. Purified DNA and protein pellets were stored at −80 °C for further analysis. Total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). PCR was conducted using a MyCycler thermal cycler (Bio-Rad). The final volume of 25 μl included 1 μl of cDNA template, 12.5 μl of PCR Master Mix (Promega), and 1 μl of a mix containing primers. PCR was carried out under the following conditions: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. PCR samples were loaded onto a 1.2% agarose gel containing ethidium bromide and analysed. The primers used for PCR amplification were designed using the Primer blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table S2 (ESI†).

Western blot analysis

Whole proteins were extracted in RIPA buffer (Cell Signaling) and quantified by the Bradford protein assay (BIORAD). Samples were separated by 12% SDS-PAGE and transferred to the Hybond ECL nitrocellulose membrane. The membranes were blocked overnight in Blotto A (Santa Cruz) at 4 °C, and subsequently probed by the appropriately diluted primary antibodies for 2 h at room temperature. After three washes with a solution of 0.1% (v/v) tween 20 in TBS, the blots were incubated with secondary antibody HRP-conjugated for 2 h at room temperature (1 : 2000 dilution). Immunoblots were developed using the ECL system. Images shown in the paper are representative of at least three replicates with similar results.

Results and discussion

To identify putative markers associated with the EMT program, we performed a proteomic analysis on the MCF-7 and MDA-231 cancer cell lines, representing two of the most common cell models for studying breast cancer pathology. These two cell lines were well profiled at the genomic level and clustered into the luminal, MCF-7, and the mesenchymal subtype, MDA-231.16,31

We first analyzed the expression of epithelial and mesenchymal markers in the used cell models to monitor their correspondence to the epithelial/mesenchymal phenotype. We evaluated the expression of E-cadherin, N-cadherin, Vimentin, Snail, Slug, and Twist. MCF-7 cells express E-cadherin, while MDA-231 cells lack E-cadherin and N-cadherin expression (Fig. S1, ESI†). Extensive data show that loss of E- and N-cadherin expression is an epigenetic event directly regulated by methylation and repression of promoter activity.34 In MDA-231, a marked up-regulation of Snail, Slug and Twist, different repressors of E-cadherin transcription, correlate with E-cadherin down-regulation. Also, high expression of Vimentin, a marker of mesenchymal cells, occurs in MDA-231 with respect to MCF-7 [Fig. S1, ESI†], supporting the usability of this in vitro model. Despite loss of expression of E-cadherin often coincides with the expression of N-cadherin, a process generally referred as cadherin switching, MDA-231 cells lack N-cadherin expression but express high levels of another mesenchymal protein, Cadherin 11, as described elsewhere.35

We prepared lysates from cell lines maintained under a normal growth condition, during their exponential growth phase. Whole proteins were isolated using 2-DE and visualized by silver staining (Fig. 1). Differentially expressed spots with at least a 1.5-fold change in normalised spot volume between the two cell lines were excised and in-gel digested for MS/MS analysis. Among the total number of differentially expressed proteins, 28 proteins were identified after database searching; the results are listed in Table 1 and Table S3 (ESI†). Fig. S2 (ESI†) shows the differences between the expression levels of the 28 spots in the two cell lines, as represented by the mean spot volume percentage on the 2D-gels. Proteins found to be differentially expressed between the two cell lines are involved in multiple cellular processes including ion transport, cell motility, glycolysis, cytoskeleton organization, cell proliferation and other biological functions (see Table S4, ESI† for a detailed description).

Some of the identified proteins were further validated by western blot and RT-PCR as shown in Fig. 2b. In the MDA-231 high basal levels of ANXA1, ANXA3, LDH-B, and CLIC1 mRNA expression was observed. Increased expression of Keratin 8, 18, and 19 occurred in MCF-7. Cofilin overexpression was confirmed by western blot but not by RT-PCR suggesting a possible post-translational regulation.

Spot volume dataset generated by the image analysis software was analysed through the Spearman correlation analysis. This approach allows analysing data values in a global overview highlighting negative or positive correlations between identified proteins (Fig. 2a and Table S5, ESI†). Highest positive correlation values can be evidenced between COF1, PRDX1, K1C19; between VINC, K2C7, COF1_2, ACTB/ANXA1/ACTC; and between ACTB/ANXA1/ACTC, 2AAA, GDIR2, ANXA3, PLST, ENOA, CLIC1. Other important correlations are: GRP75/ANXA6 with ANXA2 and HS90A; LDHB with THIO; MOES with LMNA; LMNA with ANXA5; TBA1B/VIME with GDIR2, 2AAA, PLST, ANXA3, CLIC1, ACTB/ANXA1/ACTC; ANXA2 with HS90A; HS90A with ANXA5.

Moreover keratins show important correlation with other identified proteins and each other. K2C6B positively correlates with ANXA3, LMNA, MOES, CLIC1, GDIR2, 2AAA, PLST, ENOA. K2C8_1 correlates with K2C8_2; K2C8_1 with K1C18; K2C8_3 with CH60 (for a complete overview see Table S5, ESI†).
In contrast COF1, PRDX1, and K1C19 present a negative correlation with VINC, K2C7, COF1_2, ACTB/ANXA1/ACTC. The K1C19 has a negative correlation with THIO; K1C18 with LMNA, HS90A and ANXA5; K2C8_1 with K2C6B, LMNA, MOES, ANXA5; K2C8_2 with K2C6B, LMNA, MOES; K2C8_3 with GRP75/ANXA6, ANXA2, HS90A, CH60 with GRP75/ANXA6, ANXA2, HS90A (Fig. 2a).

PCA is an unsupervised method adopted to find the directions of maximum variance in a dataset (X) without

**Table 1** Protein spots differentially expressed between MDA-231 and MCF-7 cell lines

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Swiss Prot accession no.</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Matched peptides</th>
<th>Coverage (%)</th>
<th>Protein score</th>
<th>Fold variation</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 000299</td>
<td>P08727</td>
<td>Chloride intracellular channel protein 1 (CLIC1)</td>
<td>CLIC1</td>
<td>4</td>
<td>28</td>
<td>104</td>
<td>2.3</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P05783</td>
<td>Keratin, type I cytoskeletal 19 (K1C19)</td>
<td>KRT19</td>
<td>21</td>
<td>44</td>
<td>237</td>
<td>-4.5</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P05783</td>
<td>Keratin, type I cytoskeletal 18 (K1C18)</td>
<td>KRT18</td>
<td>4</td>
<td>17</td>
<td>52</td>
<td>-3.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P52566</td>
<td>Rho GDP-dissociation inhibitor 2 (GD1R2)</td>
<td>ARHGDB</td>
<td>2</td>
<td>13</td>
<td>46</td>
<td>2.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P30153</td>
<td>Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (2AAA)</td>
<td>PPP2R1A</td>
<td>6</td>
<td>11</td>
<td>125</td>
<td>1.6</td>
<td>*</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P10809</td>
<td>60 kDa heat shock protein, mitochondrial (CH60)</td>
<td>HSPOD1</td>
<td>4</td>
<td>12</td>
<td>59</td>
<td>-1.8</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P38646</td>
<td>Stress-70 protein, mitochondrial (GRP75)/ANXA6</td>
<td>HSPA9/ANXA6</td>
<td>13/8</td>
<td>23/11</td>
<td>92/62</td>
<td>3.5</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P08133</td>
<td>Annexin A6 (ANXA6)</td>
<td>ANXA6</td>
<td>52</td>
<td>94</td>
<td>7</td>
<td>2.4</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P13797</td>
<td>Plastin 3 (PLST)</td>
<td>PL53</td>
<td>3</td>
<td>9</td>
<td>62</td>
<td>3.8</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P06733</td>
<td>Alpha-enolase (ENOA)</td>
<td>ENO1</td>
<td>15</td>
<td>37</td>
<td>99</td>
<td>2</td>
<td>*</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P23522</td>
<td>Cofilin-1 (COF1)</td>
<td>CFL1</td>
<td>6</td>
<td>42</td>
<td>57</td>
<td>-2.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P10599</td>
<td>Thioredoxin (THIO)</td>
<td>TXN</td>
<td>1</td>
<td>12</td>
<td>48</td>
<td>3.5</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P08729</td>
<td>Keratin, type II cytoskeletal 7 (K2C7)</td>
<td>KRT7</td>
<td>4</td>
<td>7</td>
<td>86</td>
<td>3</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P035787</td>
<td>Keratin, type II cytoskeletal 8 (K2C8) (G)</td>
<td>KRT8</td>
<td>6</td>
<td>14</td>
<td>77</td>
<td>-19.1</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P035787</td>
<td>Keratin, type II cytoskeletal 8 (K2C8) (E)</td>
<td>KRT8</td>
<td>17</td>
<td>24</td>
<td>133</td>
<td>-4.9</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P035787</td>
<td>Keratin, type II cytoskeletal 8 (K2C8) (F)</td>
<td>KRT8</td>
<td>8</td>
<td>18</td>
<td>92</td>
<td>-6.5</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P12429</td>
<td>Annexin A3 (ANXA3)</td>
<td>ANXA3</td>
<td>5</td>
<td>21</td>
<td>59</td>
<td>2.4</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P07195</td>
<td>L-Lactate dehydrogenase B chain (LDHB)</td>
<td>LDHB</td>
<td>11</td>
<td>33</td>
<td>98</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P60709/P68363/P68032</td>
<td>Actin, cytoplasmic 1 (ACTB)/Annexin A1/Actin, alpha cardiac muscle 1 (ACTC)</td>
<td>ACTB/ANXA1/ANXA3</td>
<td>6/5/3</td>
<td>25/23/11</td>
<td>81/64/55</td>
<td># # #</td>
<td># # #</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P07355</td>
<td>Annexin A2 (ANXA2)</td>
<td>ANXA2</td>
<td>9</td>
<td>27</td>
<td>165</td>
<td>1.5</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P08670/P68363</td>
<td>Vimentin (VIME)/Tubulin alpha-1B chain (TBA1B)</td>
<td>VIME/TUBA1B</td>
<td>8/7</td>
<td>21/21</td>
<td>115/92</td>
<td>2.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P08670/P68363</td>
<td>Vimentin (VIME)/Tubulin alpha-1B chain (TBA1B)</td>
<td>VIME/TUBA1B</td>
<td>8/7</td>
<td>21/21</td>
<td>115/92</td>
<td>2.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P20826</td>
<td>Vinculin (VINC)</td>
<td>VCL</td>
<td>9</td>
<td>10</td>
<td>105</td>
<td>2.9</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P06830</td>
<td>Peroxiredoxin 1 (PRDX1)</td>
<td>PRDX1</td>
<td>5</td>
<td>29</td>
<td>47</td>
<td>-2.4</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P04259</td>
<td>Keratin, type II cytoskeletal 6B (K2C6B)</td>
<td>KRT6B</td>
<td>1</td>
<td>1</td>
<td>46</td>
<td>2.75</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P02545</td>
<td>Lamin A/C (LMNA)</td>
<td>LMNA</td>
<td>12</td>
<td>19</td>
<td>343</td>
<td>2.2</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P23528</td>
<td>Cofilin-1 (COF1)</td>
<td>CFL1</td>
<td>2</td>
<td>25</td>
<td>48</td>
<td>3.4</td>
<td>**</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P26038</td>
<td>Moesin (MOES)</td>
<td>MSN</td>
<td>5</td>
<td>10</td>
<td>166</td>
<td>10</td>
<td>#</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P07900</td>
<td>Heat shock protein HSP 90-alpha (HS90A)</td>
<td>HSP90A1/ANXA5</td>
<td>8</td>
<td>12</td>
<td>70</td>
<td>32</td>
<td>***</td>
</tr>
<tr>
<td>Spot 000299</td>
<td>P07875</td>
<td>Annexin A5 (ANXA5)</td>
<td>ANXA5</td>
<td>11</td>
<td>35</td>
<td>59</td>
<td>4.6</td>
<td>***</td>
</tr>
</tbody>
</table>

p < 0.05; *p < 0.01; **p < 0.001; *** # expressed only in the cell line MDA-231. A positive fold change indicates greater expression in the cancer cells line MDA-231 while a negative fold change indicates a greater expression in MCF-7.
referring to the class labels (Y). PCA was used to cluster the experimental groups on the basis of protein spot expression, in the form of principal components. In the PCA score plot (Fig. S3a, ESI†), samples with similar protein expression cluster together because the principal component scores generated are very similar, so the relative nearness for samples indicates similarity in protein expression. In our model the first principal component (PC1) explained 94% of the variance and the second (PC2) explained a further 4%, the two-component model use 97.77% of X for predicting 94.70% of the response variation (Fig. S3c and Table S6a, ESI†).

The loading plot (Fig. S3b, ESI†) gives an immediate overview of proteins related to the different cell lines. The overexpression of K1C18, CH60, K2C8_1, K2C8_2, K2C8_3, K1C19, COF1 and PRDX1 is correlated with MCF-7 while the overexpression of ENOA, 2AAA, TBA1B/VIME, COF1_2, K2C7, PLST, ANXA3, GDIR2, ACTB/ANXA1/ACTC, VINC, CLIC1, LDHB, THIO, K2C6B, MOES, HS90A, ANXA5, GRP75/ANXA6, LMNA, ANXA2 correlates with MDA-231.

The analysis of the Variable Importance in the Projection score (VIP score) of PLS-DA allowed us to identify which variables explain most of the differences between the two cell lines. Partial least square discriminant analysis (PLS-DA) was used to create a supervised prediction model. This technique is adopted when the responses are groups of categorical variables, and when the goal is to define the relative importance of a set of descriptors. Considering the 28 variables used initially according to the criterion for VIP statistics (VIP > 1), 16 variables were identified as stronger discriminants. Compared with PCA the supervised PLS-DA model preserves the same clustering among groups (data not shown). Furthermore, the PLS-DA model is highly significant according to cross-validation ($R^2 = 1$, $Q^2 = 0.99$) (Table S6b, ESI†) and a permutation test (MDA-231: $R^2 = 0.506$, $Q^2 = 0.282$; MCF7: $R^2 = 0.489$, $Q^2 = 0.302$) (Fig. S3d and e, ESI†). Variables, which mostly contributed to discriminating the two groups are: MOES, K2C6B, K2C8_2, K2C8_3, THIO, and CLIC1 (Fig. S3f, ESI†). In detail, a significant upregulation of cytokeratins was observed in MCF-7, while moesin, thioredoxin and CLIC1 were upregulated in MDA-231. This observation, notably for keratins and moesins, agrees with the previous gene and protein expression data that associates the expression of these genes/proteins with the luminal and mesenchymal subtypes, respectively. Other markers previously correlated with the two subtypes, in particular proteins that codify for membrane receptors, were not identified by 2-DE. This represents a group of proteins notoriously difficult to analyse by this approach. A prefractionation procedure will be necessary in the future for the investigation of these proteins.
Cytokeratins are the major structural proteins expressed in cells of epithelial origin. These filaments form a cytoplasmic network of intermediate filaments thus controlling cellular shape and mechanical properties. In addition to their scaffolding function, keratins interact with various kinases thus modulating associated signalling pathways. Dynamic cells require a structural reorganization to acquire spindle-like morphology, increased motility and invasiveness, for this reason the functional significance of decreased keratin expression in the context of EMT is well clear if we consider that under basal conditions the established keratin cytoskeleton restricts tumor cell motility. Busch and colleagues confirmed this observation demonstrating that depletion of keratin 8 or keratin 18 resulted in a significantly enhanced random migration of tumor cells. The Snail transcriptional factor has a role in the regulation of keratin expression. Using chromatin immunoprecipitation, De Craene and colleagues demonstrated that the expression of keratins 18 and 19 is regulated by Snail, which binds the E-boxes binding sites in the promoter region of both genes. In breast cancer, cytokeratin expression has been shown to have prognostic relevance. In particular, suppression of keratin 18 expression was significantly associated with overall survival of breast cancer patients, whereas loss of keratin 8 expression was related to cancer-specific survival. At least three distinct processes drive the acquisition of a mesenchymal phenotype: loss of junctions, loss of adherens junctions and desmosomes and cytoskeletal changes. In line with this program, several proteins that drive changes in cell adhesion, morphology and cytoskeleton architecture are among the identified proteins including Moesin, Vimentin, Vinculin, Actin and Cofilin (Table 1). Moesin, a member of the ezrin/radixin/moesin (ERM) family of actin-binding proteins, is among the highly upregulated proteins in MDA-231 and the top-ranking VIP score protein. Haynes and colleagues demonstrated that increased moesin expression is necessary for morphological changes and efficient actin filament remodelling during EMT. Moreover, elevated moesin expression is observed to be associated with metaplastic carcinomas and correlated positively with Snail, suggestive of a role of moesin in EMT. Another group of proteins showing increased expression in MDA-231 are annexins, a family of calcium/phospholipid-binding and actin regulatory proteins. Five different members of this family were identified by 2-DE. The overexpression of two of these proteins, Annexin A1 and Annexin A3, was also confirmed by RT-PCR (Fig. 2b). There is growing evidence that annexins may be important mediators of EMT, the best characterized in this process are Annexin A1 and Annexin A2. Direct evidence for the involvement of Annexin A1 in EMT emerged from a study on basal breast tumors and breast cancer cell lines. Like Annexin A1, also Annexin A2 has been proposed to be associated with the EMT process. Although there is not a precise role for ANXA3, ANXA5 and ANXA6 in EMT, it is clear that these proteins have an important function in tumor development, metastasis and drug resistance making them targets for therapeutic intervention. Proteins involved in metabolic processes were also found differentially expressed. Alpha-enolase and LDH-B were found increased and detected only in MDA-231 respectively. These results appear consistent with the important role of glycolysis during the process of EMT and, in general, with the alterations of the glycolytic pathway including elevation of lactates that are thought to be hallmarks of cancer cells. High levels of LDH-B, which kinetically favour the conversion of lactate to pyruvate, seem to shift tumor energy metabolism towards the lactate–pyruvate direction more than the reverse reaction. However, the role of LDH-B can extend beyond this metabolic function. Zha and colleagues reported that LDH-B is a downstream target of a mammalian kinase target of rapamycin (mTOR) and found to be pivotal for the proliferation and tumor formation of the cells with hyperactive mTOR signalling. Since the activation of the mTOR pathway has been associated with EMT, mTOR pathway-based therapeutic approaches may have a role in blocking EMT. For instance, Metformin, an AMP-activated protein kinase (AMPK) stimulator, seems to be a possible therapeutic choice to limit EMT. In this context, LDH-B holds a potential role in a novel cancer therapy. We tried to find a correlation in vivo between the expression of LDH-B and breast tumor subtypes. For this reason we interrogated the oncomine database (www.oncomine.org) for the expression of LDH-B in breast tumors with different histological subtypes. In the Farmer et al. dataset LDH-B is highly expressed in the basal-like invasive breast samples compared with luminal-like cancers (fold change 2.584). This may suggest a possible association between LDH-B and EMT. However, we did not observe in vitro any correlation between the expression of LDH-B and the expression of other mesenchymal markers across the breast cancer cell lines (HBL-100, MCF-7, MDA-231, MDA-361, MDA-435) used in this study (data not shown). The protein–protein interaction network of identified proteins was determined using the online software STRING. Results obtained are expected to give us information about other possible actors of the EMT program. The network generated by STRING is shown in Fig. 3 and the complete list of interaction partners is reported in Table S7 (ESI†). All proteins were classified according to their biological functions using the software geneMANIA,50 gene ontology classifications of all identified proteins are listed in Table S7 (ESI†). Major categories include protein serine/threonine kinase activity, cell junction, response to growth factor stimulus, and actin filament-based process. To validate the results generated by STRING, we examined the expression of some of these proteins by western blot. As shown in Fig. 3, β-Catenin, Cyclin D1 and Ezrin, identified by in silico analysis, were all differentially expressed between the two cell lines. Cells undergoing EMT are characterized by alterations in gene expression patterns that regulate cell cycle progression and proliferation. In particular, the expression of cyclin D1 has been recently associated with the claudin-low subtype of breast cancer, which is enriched with EMT markers and with a low expression of cyclin D1. In the work of Vega and collaborators, the expression of Snail has been shown to inhibit phosphorylation of Rb, lower expression of cyclins D2
and D1 and increase expression of p21. Here, we showed an overexpression of another cell cycle regulator, p27, in MCF-7 cells (Fig. 3). To assess the possible relationship between cyclin D1 and p27 and EMT, we next determined the expression of these two proteins in a variety of breast cancer cell lines. We observed that protein levels were high in epithelial breast cancer MDA-361 and MCF-7 cells but lower in mesenchymal invasive breast cancer MDA-435 and MDA-231 cells (Fig. S4, ESI†). Similar results were observed for β-Catenin, as reported in a previous study.53

The dynamic remodeling of the actin cytoskeleton is necessary for EMT. As we previously demonstrated, actin cytoskeleton-disrupting agents (cytochalasin D) inhibit EGF-induced EMT in breast cancer cells.10 Consistent with the different migratory and invasive behaviour of the two cell lines, network analysis further strengthens the role of known cell motility regulators including members of the RhoA pathway (Fig. 3 and Table S7, ESI†). RT-PCR analysis revealed a significant increase in the expression of a member of this pathway, Lim domain kinase 1 (LIMK1), in MDA-231 compared to MCF-7 cells (Fig. 3). In addition to the proteins reported in Table S8 (ESI†), the expression of other actin binding proteins was investigated in the two cell lines. An increased expression of Fascin, Transgelin, Eplin was found in MDA-231 (Fig. 3), suggestive of a possible link between EMT and this class of proteins. It would be interesting to test if the individual knockdown of these proteins (with siRNA or specific inhibitors) resulted in inhibition of EMT and restoration of MET.

Multiple protein kinase networks were identified by String analysis including Akt, mitogen-activated protein kinase (MAPK), signal-transducer and activator of transcription (STAT), Src, and nuclear factor-kappaB (NF-κB). The EGF pathway has also emerged. In MDA-231 cells, an increase in the phosphorylation of Akt, Erk and Src was confirmed by western blot (Fig. 3).

The identification of these signalling networks led us to understand what functional consequence the inhibition of these kinases could have on the mesenchymal phenotype of MDA-231. In particular, we believe that the restoration of an epithelial phenotype could have a clinical impact considering that, in many tumor types, EMT has been shown to be associated with drug sensitivity.54–56 Firstly, we identified possible inducers of a MET program in MDA-231.
As previously described in the text, proteins that are involved in the remodelling of the cytoskeleton contribute largely to the proteomic differences observed in MCF-7 and MDA-231. This class of proteins represent possible candidates to regulate EMT. The c-Src protein, identified by in silico analysis, belongs to a family of nonreceptor tyrosine kinase proteins that regulate the integrity of both adherens junctions and focal adhesions thus exerting a profound effect on the reorganization of the cytoskeleton and the adhesion systems.57 We noticed that the treatment of MDA-231 with the Src inhibitor PP2 induced a modest reduction of cell viability (10% of reduction after 24 h of treatment) but an evident change in the morphology of MDA-231 cells, whereby the spindle morphology is switched to the cobblestone-like appearance of epithelial cells (Fig. 4). We guess if Src inhibition could be considered a therapeutic option for reverting the mesenchymal phenotype. However, the same effects were not observed in the mesenchymal cell line MDA-435 that does not exhibit high activation of Src compared

![Fig. 4](image-url)

Fig. 4 (a) MDA-231 cells were treated with different inhibitors and observed under an inverted microscope. (1) MDA-231 control; (2) MDA-231 treated with PP2 10 μM for 24 h; (3) MDA-231 treated with LY29004 10 μM and UO126 10 μM for 48 h; (4) MDA-231 treated with Vandetanib 10 μM for 24 h; (5) MDA-231 treated with Vandetanib 10 μM and Sorafenib 5 μM for 24 h. For every treatment, 5 random fields of vision were acquired. All studies were conducted in six triplicates and repeated at least three times independently, micrographs shown are representative of the average cell morphology upon treatment. (b) Cell viability after treatment of MDA-231 cells with Vandetanib and Sorafenib alone or in combination at the indicated concentrations for 24 h. (c) RT-PCR analysis of MDA-231 cells treated for 24 h with Sorafenib 5 μM and Vandetanib 10 μM. RT-PCR data are presented as an average fold change of selected genes compared to the control GAPDH (p < 0.001***). Error bars indicate the standard deviation of three independent experiments.
to MDA-231 (data not shown) suggesting that inhibition of Src could result in the activation of MET program in cell lines expressing elevated levels of Src activity.

The RhoA/Rho-associated kinase (ROCK)/myosin pathway emerged from in silico and 2-DE analysis. Potent and selective inhibitors exist for the kinases ROCK and myosin light chain kinase (MLCK), Y-27632 and ML-7 respectively. Since both ROCK and MLCK act on MLCK, a key regulator of cellular contraction, we found that treatment of MDA-231 with both inhibitors has profound consequences on cellular structures, modifications that were not associated with a MET program (data not shown). In contrast, the treatment of MCF-7 with Y-27632 is associated with a decrease in cell–cell adhesion and alterations in the biophysical properties that are suggestive of EMT (manuscript in preparation).

EGFR is a key chemotherapeutic target because it is overexpressed in the majority of basal breast tumors. Accumulating evidence suggests that EGFR and its downstream phosphoinositide 3-kinase (PI3K)/Akt and MEK/extracellular signal regulated kinase 1/2 (ERK) signalling pathways regulate EMT. As a result of these findings, EGFR signalling pathway-targeted therapies might have the potential to reverse the mesenchymal phenotype of MDA-231 cells. We used two inhibitors, LY29004 and UO126, to specifically block the PI3K and ERK pathway, respectively. UO126 treatment alone reduced spindle morphology, whereas LY29004 had no effect on cell morphology. The coadministration of LY29004 and UO126 markedly decreased cell proliferation and was more effective in inhibiting the mesenchymal phenotype than treatment with single agents (Fig. 4). Consistent with the involvement of different pathways, combination therapies or treatment with multikinase inhibitors may be the most effective treatment. For this reason, we evaluated the action of different small kinase inhibitors, Erlotinib, Fosfomatinib, Sorafenib, and Vandetanib in the restoration of MET. In MDA-231, treatment with Erlotinib, Fosfomatinib, and Sorafenib did not decrease mesenchymal features. In contrast, MDA-231 cells treated with Vandetanib lost their mesenchymal morphology suggesting that they had undergone a mesenchymal-to-epithelial transition (Fig. 4). Combination of Vandetanib and Sorafenib has demonstrated synergy in this context, cells, in fact, appeared tightly packed and formed clustered structures. Identical results were obtained with the cell line MDA-435. The epithelial conversion observed in MDA-231 after treatment with Vandetanib and Vandetanib plus Sorafenib was associated with decreased expression levels of Vimentin, Slug, Twist (mesenchymal markers), ANXA2 and ANXA3. A concomitant downregulation of the actin binding proteins Eplin, Fascin and Cofilin was also observed after treatment, the biological impact of this result could be suggestive of a reduced motile phenotype. Compared to single Vandetanib treatment, the expression of Eplin and Fascin was further decreased with drug combination (p < 0.001 and p < 0.01 respectively) raising some hypotheses about the class of genes/proteins that represent likely the most contributors of the synergistic effect of Vandetanib and Sorafenib on cell morphology.

To examine whether the synergism between Vandetanib and Sorafenib also occurs in reducing cell viability, MDA-231 cells were treated with drugs individually or in combination and examined by the MTT test. The IC50 values (inhibitory concentration 50%) of Vandetanib and Sorafenib after 24 h of treatment were calculated to be 17.26 ± 0.7 μM and 8.32 ± 0.4 μM, respectively. As shown in Fig. 4b, MDA-231 cells were treated with different concentrations of Sorafenib in combination with a fixed concentration of Vandetanib, at concentrations lower than their IC50 values. Based on MTT analysis we observed that these combinations were more effective than single agents in reducing viability with possible advantages compared with single-drug therapy in the treatment of breast cancer.

**Conclusion**

In recent years, EMT has been widely accepted as a hallmark of cancer progression, thus inhibition of this process can be considered a realistic therapeutic strategy. However, in vitro and in vivo data suggest that EMT is a complex cellular program and that the identification of a set of biomarkers for therapeutic intervention will require a more in depth comprehension of the biological mechanisms underlying the process. Comprehensive analysis of not only alterations in the level of mRNA but also proteome is likely to improve this process. Even though mRNA expression analysis was extensively used to describe and characterize EMT, there may not be a significant correlation between the level of mRNA and that of protein. Differences between mRNA and protein expression were also found in this work (e.g., cofilin, vinculin) suggesting that integrated genomics and proteomics approaches are complementary and more informative than single approaches alone.

As demonstrated here, in epithelial cells the acquisition of mesenchymal features is associated with the modulation in the expression of proteins involved in cell adhesion, migration, metabolism, cytoskeletal organization and survival/proliferation. Understanding if these proteins are directly involved in EMT will be a future strategic point. Furthermore, by multivariate analysis we explored the relationships between identified proteins in our dataset revealing distinct differences between them. Although expected, this analysis provided a statistical support for negative or positive correlation among proteins, which future studies may prove to be clinically important.

The protein–protein network of our proteins was investigated by in silico analysis. This approach disclosed signal transduction pathways that may contribute to EMT. This opens the possibility to block EMT and cancer progression by targeting these pathways and represents a rationale for combinatorial drug design. Candidate targets include receptor tyrosine kinases such as EGFR, non-receptor tyrosine kinases, especially Src, MAPK and AKT pathways. Here, we showed that the concurrent treatment with Vandetanib and Sorafenib, small molecules that inhibit the kinase activities of EGFR and RAF, respectively, could be a realistic therapeutic option to circumvent the chemotherapeutic resistance associated with EMT. The Rho/MLCK pathway should also be further investigated in this context because it regulates cell–cell adhesion and because potent inhibitors of this pathway are available. Other promising candidates for targeted
therapies including proteins involved in metabolism (e.g., LDH-B), cell cycle, molecular chaperones (e.g., HSP90), or ion channels (e.g., CLIC1) could also be useful.

MCF-7 and MDA-231 represent an appropriate cellular model for studying EMT but considering the molecular heterogeneity that occurs in breast cancer samples in vivo and considering that cellular variants may arise due to a prolonged culture of cell lines in vitro, the analysis of such limited number of cancer models represents an obvious limitation towards the definition of the key EMT-driving protein signature. This is well clear if we consider the results obtained with some of the inhibitors used in this work that showed efficacy in some cellular models but not in others, and highlights the complexity and the tumor specificity of EMT. It is true that many of the pathways involved in EMT are in common between tumors of different origins (e.g., EGFR, Src, Wnt) but it is less clear if the inhibition of these pathways is clinically useful for every type of tumor. To achieve this, we plan to enlarge the panel of breast cancer cell models and to extend the analysis to cultured tumour-derived cell lines. This is expected to improve our knowledge of the EMT program and to accelerate the development of specific therapies.

Acknowledgements

We thank Dr Antonio Danieli for his technical support. This work was supported by the PS105 ARTI strategic project “Development and realization of bio-chip for molecular diagnostic and typization of human pathogenic viruses (HPV, HCV)” of Apulia Region and supported by a motility grant “Bando contributo mobilita’” from the ITPA society. We also thank the “ANGELA SERRA” Foundation for Cancer Research, Parabita (Lecce), Italy. The authors acknowledge Dr Valdo Mellone, Dr Ottavio Narracci and Dr Antonio Vigna for providing support to the activity of the laboratory of clinical proteomics.

References