CD44 and vimentin, markers involved with epithelial-mesenchymal transition: A proteomic analysis of sequential proteins extraction of triple-negative breast cancer cells after treatment with all-trans retinoic acid

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Abstract. This work aimed to provide, in one isolation and separation step, an overview of the content of proteins with different solubility after treatment with all-trans retinoic acid, which is considered to be an important therapeutic agent, predominantly in acute promyelocytic leukemia. Breast, ovarian, bladder, and skin cancers have been demonstrated to be suppressed by retinoic acid, as well. The bottom-up proteomic strategies were applied for the analysis of proteins extracted from triple-negative breast cancer MDA-MB-231 cells utilizing a commercially manufactured kit. The gel electrophoresis followed by MALDI-TOF MS analysis was used for protein determination. By employing PDQuest™ software, we identified several proteins affected by all-trans retinoic acid. Two proteins, vimentin and CD44, which are associated with the epithelial-mesenchymal transition, were selected for a detailed study. We have found that all-trans retinoic acid results in significantly reduced levels of vimentin and CD44 in both the cytoplasmic and membrane fractions. A significant effect was particularly evident in CD44, where protein level in the cytoplasmic fraction was almost completely suppressed.

Key words: Breast cancer — All-trans retinoic acid — Proteins — Biomarker — Sequential protein extraction
research groups analyzing the same neoplastic disease came to conflicting conclusions about the correlation between CD44 expression and disease prognosis, probably due to differences in methodology (Eibl et al. 1995; Naor et al. 2002). These problems need to be solved and further studies are needed to determine the prognostic value of CD44 and its variant isoforms.

MDA-MB-231, a triple-negative breast cancer model, was used in this study to evaluate and compare membrane and cytoplasmic proteins after retinoic acid isomer treatment. Proteins were extracted using a commercially available kit, separated on SDS-PAGE, and characterized by MALDI-TOF/TOF MS/MS.

The cancer cell culture was purchased from the HPACC (Salisbury, Great Britain). Cells were grown and passaged routinely as monolayer culture. For experiments, the cells were seeded into Petri dishes (6 cm diameter, TPP, Switzerland) at 1.2 × 10^6 cells/dish density in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin, streptomycin, gentamicin) and treated for 48 h with 1 μmol/l all-trans retinoic acid (ATRA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. A stock solution of ATRA was originally dissolved in ethanol, and an equal volume of ethanol (final concentration < 0.02%) was added to the control cells. Then cells were washed twice with ice-cooled PBS. The sequential protein extraction was made according to an instruction manual of ReadyPrep™ Protein Extraction Kit Bio Rad.

Proteomic analysis of membrane proteins is challenged by the protein solubility and detergent incompatibility with MS analysis. Here, we used the ReadyPrep protein extraction kit which is a simple, rapid, and reproducible method to prepare protein fractions highly enriched in the membrane and cytoplasmic proteins. This approach makes it possible to reduce the complexity of the sample in order to improve the chances of identifying low-occurring proteins and to simplify proteomic studies. The advantage of this procedure is that the kit does not require the use of ultracentrifugation, as the kit does not require the use of ultracentrifugation. Its variant isoforms.

In this work, we analyzed and compared the protein profiles of the membrane and cytoplasmic fractions of MDA-MB-231 cells after treatment with ATRA.

Our previous studies have shown that the natural retinoid ATRA, a cognate ligand of nuclear retinoic acid receptors (RARs), is a promising agent that affects the proteomic profile of cancer cells (Fiodrova et al. 2015, 2017). ATRA belongs to a class of retinoids that are known to have a wide range of functions (Alizadeh et al. 2014). Breast, lung, prostate, ovarian, bladder, and skin cancers have been demonstrated to be suppressed by ATRA (Chen et al. 2014). ATRA is known for several decades for its therapeutic effects due to antiproliferative and apoptosis-inducing action, and thus to act in the treatment or prevention of cancer (Carlberg et al. 1993). Furthermore, they inhibit carcinogenesis and suppress tumour growth and invasion in various tissues (Shi et al. 2019), which was the main reason, why ATRA has been used in our studies. The presented study consists of several aims: i) to perform a basic comparison of the membrane and cytoplasmic fractions of MDA-MB-231 cells based on SDS-gels protein profiles; ii) to identify the major proteins related to EMT; iii) to quantify VIME and CD44 after treatment of ATRA.
First, we compared the membrane and cytoplasmic fractions of both control cells and cells after treatment. Figure 1A shows the protein profiles of the non-treated (control) fractions. Visual inspection of the protein pattern indicated that the membrane and the cytoplasmic fraction share some similarities. On the other hand, significant differences in the electrophoretic profiles of both fractions were observed at molecular weights of approximately 30–50 kDa (Fig. 1A, B). In addition, considerable changes between fractions after treatment in the higher molecular masses (80–110 kDa) were observed (Fig. 1B). The bands showing differences between fractions were cut off and used for further proteomic evaluation including gel tryptic digestion, MALDI-TOF MS. Additional MS/MS analysis of individual peptides and following database searching resulted in the identification of the proteins summarized in Table 1 and Table 2. Selected important proteins are indicated on the SDS gels and are also listed in bold in the tables.

Among proteins with regard to EMT, of importance are predominantly VIME and CD44. As expected, these well-known human breast cancer markers were identified in mass area about 55 kDa (VIME) and about 85 kDa (CD44) in our experiment. In addition, a high molecular weight form of VIME (VIME HMW) has also been identified, the occur-

Figure 1. SDS-PAGE separation of the membrane and cytoplasmic proteins of MDA-MB-231 cells. A. Non-treated sample (control sample). B. Sample after 48-h treatment with ATRA. The bands showing differences between fractions were cut off and used for further proteomic evaluation including gel tryptic digestion, MALDI-TOF MS. Selected important proteins are listed on the SDS gel and are shown in bold in the tables. MW, molecular weight.
### Table 1: Summary of identified proteins found in non-treated sample in individual cell fraction

| Region I | MEMBRANE FRACTION | CYTOPLASMIC FRACTION |
|----------|-------------------|----------------------|
| Accession | Mass (Da) | Description | Accession | Mass (Da) | Description |
| TASO2_HUMAN | 271697 | Protein TASOR 2 | KI21B_HUMAN | 184316 | Kinesin-like protein KIF21B |
| ITA2_HUMAN | 130468 | Integrin alpha-2 | ARHG1_HUMAN | 103056 | Rho guanine nucleotide exchange factor 1 |
| SMCA1_HUMAN | 123211 | Probable global transcription activator SNF2L1 | ENPL_HUMAN | 92696 | Endoplasmin |
| GANAB_HUMAN | 107263 | Neutral alpha-glucosidase AB | CD44_HUMAN | 82001 | CD44 antigen |
| ITB1_HUMAN | 91664 | Integrin beta-1 | SFPQ_HUMAN | 76216 | Splicing factor, proline- and glutamine-rich |
| CD44_HUMAN | 82001 | CD44 antigen | GRP75_HUMAN | 73920 | Stress-70 protein, mitochondrial |
| BIP_HUMAN | 72402 | Endoplasmic reticulum chaperone BiP | BIP_HUMAN | 72402 | Endoplasmic reticulum chaperone BiP |
| GRP75_HUMAN | 73920 | Stress-70 protein, mitochondrial | HSP7C_HUMAN | 71082 | Heat shock cognate 71 kDa protein |
| CALX_HUMAN | 67982 | Calnexin | VIME_HUMAN | 53676 | Vimentin |
| H90B3_HUMAN | 68624 | Putative heat shock protein HSP 90-beta-3 | TBA1A_HUMAN | 50788 | Tubulin alpha-1A chain |
| ACTB_HUMAN | 42052 | Actin, cytoplasmic 1 | ROA1_HUMAN | 38837 | Heterogeneous nuclear ribonucleoprotein A1 |
| 1A02_HUMAN | 41181 | HLA class I histocompatibility antigen, A-2 alpha chain | TCP10_HUMAN | 38358 | T-complex protein 10A homolog |
| HLAC_HUMAN | 41136 | HLA class I histocompatibility antigen, C alpha chain | ROA2_HUMAN | 37464 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| HLAA_HUMAN | 41100 | HLA class I histocompatibility antigen, A alpha chain | G3P_HUMAN | 36201 | Glyceraldehyde-3-phosphate dehydrogenase |
| MPCP_HUMAN | 40525 | Phosphate carrier protein, mitochondrial | MDHM_HUMAN | 35937 | Malate dehydrogenase, mitochondrial |
| STML2_HUMAN | 38624 | Stomatin-like protein 2, mitochondrial | HNRPC_HUMAN | 33707 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| CY1_HUMAN | 35741 | Cytochrome c1, heme protein, mitochondrial | NPM_HUMAN | 32726 | Neolipin |
| C1QBP_HUMAN | 31742 | Complement component 1Q subcomponent-binding protein |
| NB5R3_HUMAN | 34441 | NADH-cytochrome b5 reductase 3 OS=Homo sapiens |
| PHB2_HUMAN | 33276 | Prohibitin |
| VDAC1_HUMAN | 30868 | Voltage-dependent anion-selective channel protein |
| NDKB_HUMAN | 31724 | Voltage-dependent anion-selective channel protein |

Selected important proteins are listed on the SDS gel and are shown in bold in the table.
Table 2. Summary of identified proteins found after ATRA treatment in individual cell fraction

| Accession     | Mass (Da) | Description                          | Accession     | Mass (Da) | Description                          |
|---------------|-----------|--------------------------------------|---------------|-----------|--------------------------------------|
| **MEMBRANE FRACTION** |           |                                       | **CYTOPLASMIC FRACTION** |           |                                       |
| PRR36_HUMAN   | 132748    | Proline-rich protein 36              | ENPL_HUMAN    | 92469     | Endoplasmnin                         |
| ITA2_HUMAN    | 129925    | Integrin alpha-2                     | MTSS2_HUMAN   | 80460     | Protein MTSS2                        |
| GANAB_HUMAN   | 107263    | Neutral alpha-glucosidase A           | CALX_HUMAN    | 67990     | Calnexin                             |
| CD44_HUMAN    | 82009     | CD44 antigen                         |               |           |                                      |
| CALX_HUMAN    | 67990     | Calnexin                             |               |           |                                      |
| **Region I.** |           |                                       |               |           |                                       |
| 5NTD_HUMAN    | 63908     | 5’-nucleotidase                       | K2C1_HUMAN    | 66173     | Keratin, type II cytoskeletal 1       |
| VIME_HUMAN    | 53677     | Vimentin                             | MED26_HUMAN   | 65446     | Mediator of RNA polymerase II transcrip |
| TBA1A_HUMAN   | 50800     | Tubulin alpha-1A chain               | EIF2D_HUMAN   | 65304     | Eukaryotic translation initiation factor 2D |
| ACTB_HUMAN    | 42058     | Actin, cytoplasmic 1                  | K2C1B_HUMAN   | 62154     | Keratin, type II cytoskeletal 1 b     |
| PHB2_HUMAN    | 33276     | Prohibitin-2                          | K1C10_HUMAN   | 59024     | Keratin, type I cytoskeletal 10      |
| VDAC2_HUMAN   | 32069     | Voltage-dependent anion-selective protein 2 | VIME_HUMAN | 53677     | Vimentin                             |
| PHB_HUMAN     | 29804     | Prohibitin                           | TBA1A_HUMAN   | 50800     | Tubulin alpha-1A chain               |
| **Region II.** |           |                                       | KPSH2_HUMAN   | 43027     | Serine/threonine-protein kinase H2   |
|               |           |                                       | ROA1_HUMAN    | 38837     | Heterogeneous nuclear ribonucleoprotein A1 |
|               |           |                                       | ROA2_HUMAN    | 38542     | Heterogeneous nuclear ribonucleoproteins A2/B1 |
|               |           |                                       | G3P_HUMAN     | 36201     | Glyceraldehyde-3-phosphate dehydrogenase |
|               |           |                                       | HNRPC_HUMAN   | 33708     | Heterogeneous nuclear ribonucleoproteins C1/C2 |
|               |           |                                       |               |           |                                      |
| **Region III.** |         |                                       | SODM_HUMAN    | 24906     | Superoxide dismutase [Mn], mitochondrial |
| PRDX3_HUMAN   | 27693     | Thioredoxin-dependent peroxide reductase | CALL3_HUMAN | 16891     | Calmodulin-like protein 3            |
| COX2_HUMAN    | 25722     | Cytochrome c oxidase subunit 2        | H2A1A_HUMAN   | 14225     | Histone H2A type 1-A                |
| RAB5C_HUMAN   | 23696     | Ras-related protein Rab-5C            | H2B1B_HUMAN   | 13950     | Histone H2B type 1-B                |
| RAB7A_HUMAN   | 23490     | Ras-related protein Rab-7a            | H4_HUMAN      | 11360     | Histone H4                          |
| H2A1A_HUMAN   | 14225     | Histone H2A type 1-A                  |               |           |                                      |
| H4_HUMAN      | 11360     | Histone H4                            |               |           |                                      |

Selected important proteins are listed on the SDS gel and are shown in bold in the table.
rence of which may explain its ability to form a dimer and possible modifications (Qin and Buehler 2010).

For deeper analysis, the obtained 1D maps were processed by PDQuest software™ and compared with a focus on quantitative and qualitative changes. The changes were monitored in the control samples and the samples after treatment. The comparative data were obtained from three independent replicates from each sample (control and ATRA treated) where the chosen spots were used for the pairwise comparisons of relative protein amount. The quantification of chosen proteins in spots was then expressed as the sum of pixel intensities in a given spot. To eliminate some of the image differences caused by the gel staining and de-staining process, the normalization between individual gel images was performed. Obtained data based on PDQuest software analyses were presented as mean ± SD (standard deviation) from three independent experiments. Statistical analyses were performed with Student’s t-test. Differences between more than two groups were assessed by one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls method. Differences with \( p < 0.05 \) were considered as statistically significant.

Based on the results obtained, the identified proteins were compared according to their expression influenced by ATRA. Significant effects were seen mainly in VIME as well as in its HMW form, where the protein levels in the membrane and cytoplasmic fraction were almost completely suppressed after treatment with ATRA (see Fig. 2).

The reduction effects of ATRA were also visible in cases of CD44. The obtained PDQuest data report the most significant decrease of cytoplasmic CD44 level for treatment by the ATRA, where the amount of protein was almost impossible to identify. However, this finding does not correspond to the results obtained with membrane CD44 of which concentration was decreased only partially (Fig. 2).

Recently, several types of treatment with ATRA were carried out on the cell lysis of human triple-negative MDA-MB-231 cells. ATRA, 9-cis retinoic acid, and a mixture of these two retinoic receptor ligands were tested by Flodrova et al. (2017). The treatment of MDA-MB-231 cells with triorganotin compounds together with ATRA resulted in an additional reduction of annexin 5, nucleoside diphosphate kinase B and VIME (Strouhalova et al. 2019, 2020). In this work, the hypothesis of these studies which stated that ATRA led to a significant reduction in VIME as well as CD44 protein level was confirmed. Moreover, our findings verified that although alternative splicing can produce a large number of different isoforms of CD44, they all retain a common transmembrane and cytoplasmic domain (Thorne et al. 2004) and therefore it is important to study its presence in both the membrane and cytoplasmic fractions obtained by sequential protein extraction.

In conclusion, this work provides first insights into the presentation of VIME and CD44 in the cytoplasmic and membrane protein fraction in the MDA-MB-231 cells after ATRA treatment. Some types of cancer can become more invasive and malignant after undergoing the EMT process. VIME is one of the types of protein markers of EMT that is present in mesenchymal cells and is involved in cancer progression (Kalluri and Weinberg 2009; Zeisberg and Neilson 2009). Also, some findings suggest that CD44 may provide some growth benefits to some neoplastic cells and therefore could be used as a cancer treatment target (Naor et al. 1997). We are convinced that the present data can help reveal additional aspects of the mechanism of action of all-trans retinoic acid in breast cancer, which we consider a highly desirable.

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