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Docosahexaenoic acid inhibits the invasion of MDA-MB-231 breast cancer cells through upregulation of cytokeratin-1

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Abstract. Docosahexaenoic acid (DHA), the main member of the omega-3 essential fatty acid family, has been shown to reduce the invasion of the triple-negative breast cancer cell line MDA-MB-231, but the mechanism involved remains unclear. In the present study, a proteomic approach was used to define changes in protein expression induced by DHA. Proteins from crude membrane preparations of MDA-MB-231 cells treated with 100 μ M DHA were separated by two-dimensional electrophoresis (2-DE) and differentially expressed proteins were identified using MALDI-TOF mass spectrometry. The main changes observed were the upregulation of Keratin, type II cytoskeletal 1 (KRT1), catalase and lamin-A/C. Immunocytochemistry analyses confirmed the increase in KRT1 induced by DHA. Furthermore, *in vitro* invasion assays showed that siRNA against KRT1 was able to reverse the DHA-induced inhibition of breast cancer cell invasion. In conclusion, KRT1 is involved in the anti-invasive activity of DHA in breast cancer cells.

Introduction

Docosahexaenoic acid (DHA) is a 22:6n-3, long-chain polyunsaturated fatty acid (PUFA) present in fat fish, fish oils (1) and in marine microalgae (2). Among numerous beneficial effects, such as in cardiovascular (3) or metabolic syndromes (4), DHA may play a preventive role in cancer (5,6). The mechanism by which DHA could prevent tumorigenesis or directly target cancer cells remains unclear (7). However, it has been

shown that DHA not only acts as an anti-proliferative agent by lengthening the cell cycle between the G2/M transition (8), but also it is capable of inducing apoptosis and reducing the invasive potential of the triple-negative breast cancer cell line MDA-MB-231 with an optimal amount of 100 μ M (9). DHA can modify the metastatic phenotype of cancer cells, emphasizing the anti-cancer potential of the omega-3 (n-3) PUFAs (10,11). This anti-cancer activity of DHA is promising and could partly result in a modification of the lipid contents of the plasma membrane and its fluidity (12).

Metastasis is a multifactorial process involving extracellular matrix remodeling, extra- and intravasation, and requiring the involvement of a variety of cell surface proteins. For example, the urokinase-type plasminogen activator (uPA) and metalloproteinases (MMPs) are involved in extracellular matrix disorganization leading to the release of angiogenic factors such as VEGF or FGF, allowing the sprouting of new blood vessels and ultimately extra- and intravasation (13-15). Several studies have shown that lipid rafts in the plasma membrane can play an important role in cancer cells when n-3 PUFAs were added, as it altered the cholesterol levels and consequently cell migration, invasion and angiogenesis (16,17). The DHA-induced decrease in breast cancer cell invasion may also be due to inhibition of voltage-gated Na⁺ channels (18,19). These voltage-gated channels also called neonatal Na_v1.5 are inhibited in a dose-dependent manner by DHA and the use of specific blockers, like tetrodotoxin, can reduce the migration of MDA-MB-231 at the same level to that observed with DHA (18). Thus, DHA-induced suppression of cellular migration may occur via downregulation of neonatal Na_v1.5 mRNA and functional protein expression (18). The localization of voltage-gated Na⁺ channel in lipid rafts (20) may be affected by n-3 PUFA (19).

In addition, DHA may change the biophysical properties of lipid rafts decreasing the content of cholesterol and the distribution of key proteins such as EGFR, Src, heterotrimeric G-protein subunits, or sphingomyelinase. Among these proteins, the Src kinase might play an important role by regulating the migration and invasion of the MDA-MB-231 cell line (21). Src was shown to play a role in cancer and invasive-

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ness (21,22) and was also related to other molecules such as Keratin, type II cytoskeletal 1 (KRT1) via integrin $\beta 1$ (23) or the voltage-gated Na^+ channels where one of its subunits can also be considered as an integrin (24). Together, plasma membrane and related cytosolic molecules appear to play an important role in the DHA-induced inhibition of breast cancer cell invasion.

In this context, we have decided to analyze changes in the protein content of crude membrane preparations from breast cancer cells treated with DHA. Two-dimensional electrophoresis (2-DE) and MALDI-TOF mass spectrometry were used and several proteins were identified as upregulated by DHA. Increase in the level of KRT1 was the major change and interestingly functional *in vitro* assays have shown its involvement in mediating the anti-invasive effect of DHA.

Material and methods

Cell culture. The triple-negative breast cancer cell line MDA-MB-231 was purchased from ATCC (Manassas, VA, USA) and routinely grown as monolayers at 37°C, in a humidified atmosphere with 5% CO_2 , in minimum essential medium (MEM) (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 10% fetal calf serum (FCS) (Gibco; Invitrogen, Cergy Pontoise, France), 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Sigma-Aldrich).

Crude membrane protein extraction. MDA-MB-231 cells were grown in T75 cm^2 flasks until reaching subconfluency. A set of 7×10^8 cells were treated, or not, with 100 μM DHA (Sigma-Aldrich Chimie S.a.r.l.) for 24 h. After treatment, cells were rinsed three times with 10 ml MEM and twice with 20 mM phosphate buffer pH 7.4 containing 150 mM NaCl. Cells were then detached with 2 ml of Versene (Sigma-Aldrich) and centrifuged at 200 x g for 10 min at 4°C. The supernatant was discarded and the cell pellet stored at -70°C. The membrane preparations were carried out on ice and at 4°C according to Venkateswaran *et al.* (25). Briefly, the cell pellets were defrosted and homogenized in 3 ml buffer A composed with 20 mM Hepes, 200 mM sucrose and 5 mM EDTA. The suspension was then transferred in a 7 ml Dounce and cells were disrupted with 40 strikes of pestle. The homogenate was centrifuged at 1,000 x g for 20 min at 4°C in order to pellet the nuclei. The supernatant containing the membranes was transferred in a tube for centrifugation. Prior to this step, four volumes of buffer B composed of 20 mM Hepes, 1 mM CaCl_2 , 1 mM MgCl_2 (all from Sigma-Aldrich), and 100 mM NaCl were added to the supernatant to decrease buffer density and to allow the best ionic environment for membrane proteins. The mixture was centrifuged at 60,000 x g for 90 min at 4°C in order to obtain crude membrane pellets. The supernatants were removed and the pellets suspended in 500 μl buffer B and washed twice in the same conditions. Aliquots of the pellets were taken for a protein assay using Bradford's method (Bio-Rad, Marnes-la-Coquette, France) with BSA (Sigma-Aldrich) as standard. Aliquots of 100 μg membrane proteins were stored in Eppendorf tubes and centrifuged at 20,000 x g for 60 min at 4°C. The pellets were then solubilized with 50 μl of a lysis

buffer suitable for isoelectric focusing (IEF) (urea 7 M, thio-urea 2 M, CHAPS 2%, DTT 40 mM and 0.4% ampholytes 3-10) (GE Healthcare Europe GmbH, Vélizy-Villacoublay, France) containing 1% ASB-14 (Sigma-Aldrich), and stored at -70°C.

2-DE samples. A total of 100 μg of membrane proteins prepared as described above were lysed with 50 μl of lysis buffer as described above and processed for IEF by incubation for 1 h in 2 μl of 200 mM tributylphosphine (TBP) (Sigma-Aldrich) followed by incubation for 1.5 h with 5 μl 200 mM iodoacetamide (IAM) (GE Healthcare). At this stage, samples were loaded for IEF. Three independent experiments were carried out in duplicate.

IEF and SDS-PAGE. IEF was performed using Ettan IPGphor 3 apparatus using 7 cm strips with pH 3.0-10.0 (both from GE Healthcare Europe GmbH). Strips were rehydrated overnight at room temperature according to the manufacturer's instructions with DeStreak Rehydration Solution containing 0.4% ampholytes pH 3.0-10.0 (GE Healthcare). The samples (100 μg) were cup-loaded near the anode of the IPG strips and three drops of mineral oil were introduced in the cups. Then, the tray was filled with mineral oil. The run was defined as follows: step at 500 V for 500 Vh, gradient to reach 3,000 V for 5,000 Vh, step at 3,000 V for 12,000 Vh, step 1,000 V for 1,000 Vh. Once the IEF was completed, the strips were processed for SDS-PAGE after equilibration in urea 6 M, PlusOne Glycerol 30% w/v, SDS 2% w/v (Bio-Rad), 0.125 M Tris, 0.1 M HCl containing 50 mM DTT (first equilibration step; Sigma-Aldrich) and 150 mM IAM (second equilibration step; GE Healthcare), and consisting in two baths of 20 min each. The strips were placed at the top of 12% acrylamide-bisacrylamide gels and maintained in position with 2 ml of stacking gel. The run was performed with a PROTEAN 3 apparatus (Bio-Rad) at a constant power of 8 W until the Bromophenol Blue (Merck S.A., Lyon, France) reached the bottom of the gels. Gels were washed twice for 5 min and stained with Imperial Blue® (Fisher Scientific, Illkirch-Graffenstaden, France) according to manufacturer's instructions. Three independent experiments were performed in duplicate.

Spot detection and quantification. The 2-D gels were scanned with a GS-800 densitometer (Bio-Rad). Spot detection, quantification and analysis were performed with the SameSpots® v4.1 analysis software (Nonlinear Dynamics, Ltd., Newcastle upon Tyne, UK). Following linearization towards a reference gel chosen among the experimental gels, they were grouped either as control or treated. Each group was the result of three independent experiments performed in duplicate. Spot detection and quantification were determined and a difference was considered to be significant, due to the staining method used, when a 1.5-fold increase or decrease at least was reached. Statistics using ANOVA were given with the in-built statistical software.

In-gel digestion of protein. The protein spots differentially expressed were excised manually and washed five times for 6 min with 100 μl water. Then the gel spots were soaked in

acetonitrile and dried under vacuum. The gel pieces were rehydrated in a reduction buffer [ammonium bicarbonate 100 mM (Sigma-Aldrich), DTT 10 mM (GE Healthcare)] for 1 h at 56°C and 5 min at room temperature. After removing this buffer, they were incubated with an alkylation buffer (ammonium bicarbonate 100 mM, IAM 55 mM) for 45 min at room temperature and protected from light. Then, they were washed in a 25-mM ammonium bicarbonate buffer followed by acetonitrile (Merck S.A.) and finally dried under vacuum. The gel pieces were rehydrated in 100 μ l of 25 mM ammonium bicarbonate and incubated with 125 ng of Trypsin Gold (Mass Spectrometry Grade; Promega France, Charbonnières-les-Bains, France) for 1 h on ice. The trypsin digestion was performed for 12 h at 37°C after addition of 30 μ l of 25 mM ammonium bicarbonate.

Mass spectrometry analysis. Mass spectrometry analyses were performed using an Ultraflex™ II MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). MALDI target plate (AnchorChip™; Bruker Daltonics) was covered with extracted peptides mixed-up with α -cyano-4-hydroxycinnamic acid matrix (0.3 mg/ml in acetone:ethanol, 3:6 v/v). The molecular mass measurements were obtained as previously described (26). Database searches, through Mascot v.2.2.1 (Matrix Science, Ltd., London, UK), using combined PMF and PFF datasets were performed against the UnitProt 2013-06 database (2013-06-17) via ProteinScape 2.1 (Bruker Daltonics). A mass tolerance of 75 ppm and one missing cleavage site for PMF and MS/MS tolerance of 0.5 Da and one missing cleavage site for MS/MS search were allowed. Carbamidomethylation of cysteine and oxidation of methionine residues were also considered. Relevance of protein identities was judged according to the probability-based MOWSE score calculated with a P-value of 0.05 ($P \leq 0.05$).

Preparation of siRNA and cell transfection. A siRNA (Eurogentec S.A., Seraing, Belgium) directed against KRT1 was used and defined by (GGA-UGU-GGA-UGG-UGC-UUA-U55) for the forward strand and (AUA-AGC-ACC-AUC-CAC-AUC-C55) for the reverse. In addition a control siRNA (NEG) provided by the manufacturer was used. The different siRNA were rehydrated with ultrapure water to obtain a concentration of 20 μ M. In a 24-well plate, 4×10^5 living cells/well were seeded. After 12 h, the medium was removed and rinsed twice with 1 ml/well of Opti-MEM. Then 950 μ l of Opti-MEM were added with 50 μ l of a mixture containing Lipofectamine (Invitrogen) with or without the appropriate siRNA. To form the mixture, 2.5 μ l siRNA at 20 μ M were mixed with 22.5 μ l Opti-MEM, and apart, 8.32 μ l Lipofectamine were homogenized with 16.68 μ l Opti-MEM. The two solutions were then mixed. After 10 min of incubation at room temperature the mixture was transferred to culture wells. Incubation was for 4 h at 37°C with 5% CO₂. The medium was discarded and replaced with 1 ml Opti-MEM containing 5% FCS with or without 100 μ M DHA. After 24 h, cells were harvested for invasion assay.

Invasion assays and Hoechst staining. Invasion assays were done in 12-well Boyden microchambers (Transwell®, Fisher

Scientific) with 8- μ m pore membranes. Matrigel® (100 μ l; BD Biosciences, Le Pont de Claix, France) at 10% in MEM were introduced in the upper chamber and dried overnight at 37°C. Cells treated or not for 24 h as described in the previous section were dissociated with Versene and counted by using a Malassez hemocytometer. Living cells (2×10^5) treated or not in 400 μ l MEM supplemented with 0.5% FCS and 1% BSA were then loaded into the upper chamber. A volume of 800 μ l of MEM with 0.5% FCS, and 1% BSA was introduced into the lower chamber. After incubating for 24 h, the Transwell® was rinsed with PBS, and the Matrigel® was scraped off the upper surfaces of the membranes. The cells remaining on the underside of the membrane were fixed for 30 min at -20°C in methanol, then stained with Hoechst stain (H6024; Sigma-Aldrich), and mounted on glass slides with glycerol for fluorescence microscopy (Merck S.A.) before counting (15 fields/membrane) under a UV microscope (Biomed with fluorescence equipment; Leica, Rueil-Malmaison, France). Light and fluorescent micrographs were taken with the Lasez software (Leica). Three independent experiments were performed in duplicate.

Immunocytochemistry. Experiments were performed with 5×10^4 cells/chamber on a 16-chamber slide (Fisher Scientific) overnight. Then 200 μ l of the Lipofectamine mixture with or without siRNA were added for 4 h after medium withdrawal and rinsing with 500 μ l Opti-MEM. Then 200 μ l Opti-MEM containing 5% SVF with or without 100 μ M DHA were added for 24 h. Chambers were rinsed with PBS and cells fixed with ethanol-methanol-ultrapure water (1:1:2) for 1 h at -20°C. Cells were treated for endogenous peroxidase with 100 μ l PBS containing 3% H₂O₂ 20 vol, for 10 min at room temperature. Then the medium was discarded and 200 μ l of PBS containing 5% BSA was added for 1 h followed by an incubation of 2 h with 100 μ l mouse monoclonal anti-KRT1 antibody (Mab 191-05; Diagnostic BioSystems, Inc., Hague, The Netherlands) at 1/500 diluted in PBS with 0.5% BSA. Cells were rinsed three times with 200 μ l PBS 0.5% BSA. HRP anti-mouse antibody (Sigma-Aldrich) at 1/200 in PBS 0.5% BSA was added for 1 h. Cells were rinsed three times with PBS 0.5% BSA and three times with PBS prior HRP revelation by the adjunction of 100 μ l diaminobenzidine (DAB) prepared in 10 ml water with 20 μ l H₂O₂ 20 vol and counterstained with Hoechst staining. Slides were washed with water and mounted with glycerol for fluorescence microscopy. For each chamber, 10 randomized fields were photographed and analyzed with the Quantity One software (Bio-Rad). Staining intensity in one field was divided by the number of nuclei observed by Hoechst staining in the same field in order to have the average value of KRT1 immunoreactivity. Three independent experiments were performed in duplicate.

Statistics. Statistical analyses for cell culture were performed using KyPlot® (KyensLab, Inc., Tokyo, Japan) for a one-way ANOVA followed by a Dunnett's test to compare untreated or control cells with the treated one. P-value of <0.01 and <0.001 respectively, indicates statistically significant result. In the figures shown as ** $P < 0.01$ and *** $P < 0.001$ Statistics for 2-DE are described in the corresponding paragraph.

Table I. Upregulated proteins in DHA-treated MDA-MB-231 cells. Cells were treated for 24 h with 100 μ M DHA before membrane protein preparation, 2-DE and identification in mass spectrometry.

Spot identifier	Fold	Regulation	ANOVA P-value	Protein name	UniProt ID
70	2.0	Up	0.017	KRT1	P04264
110	1.7	Up	0.008	Catalase	P04040
219	1.9	Up	0.022	Lamin-A/C	P02545

DHA, docosahexaenoic acid; 2-DE, two-dimensional electrophoresis; KRT1, keratin, type II cytoskeletal 1.

Table II. Characteristics of the different peptides for each protein identified in mass spectrometry.

UniProt ID	MW (kDa)	pI	Score mascot	MS1 ^a coverage (%)	Peptide sequence MS2 ^b	Score MS2 ^b	MS2 ^b coverage (%)
P04264	65.9	8.1	65.8	31.7	WELLQQVDTSTR THNLEPYFESFINNLR	62.4	4.4
P04040	59.7	6.9	110	44.8	LFAYPDTHR LGPNYLHIPVNCYPYR AFYVNVLNNEEQR	116.4	6.8
P02545	74.1	6.6	107	37.5	LQEKEDLQELNDR NSNLVGAAHEELQQSR	62	4.4

^aMALDI-TOF mass spectrometry. ^bMALDI-TOF/TOF mass spectrometry.

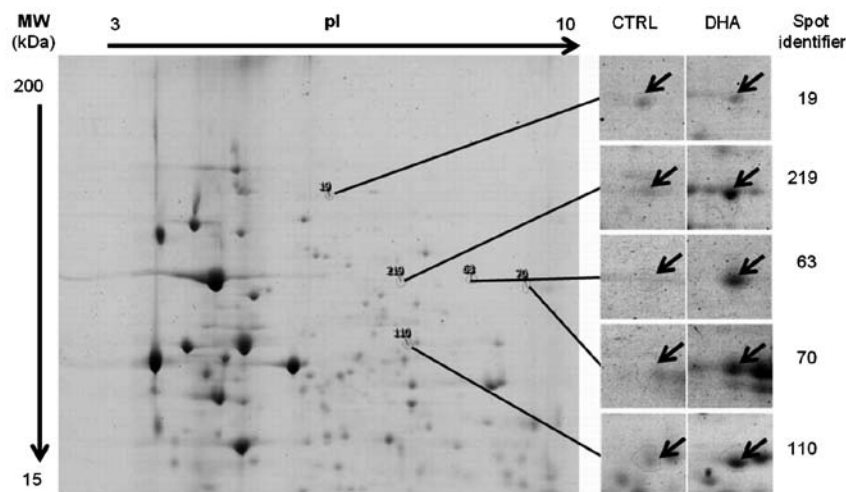


Figure 1. Docosahexaenoic acid (DHA)-treated MDA-MB-231 cells induced a differential membrane protein expression. Two-dimensional electrophoresis (2-DE) was performed with membrane proteins of MDA-MB-231 cells treated or not for 24 h with 100 μ M DHA, using 7 cm isoelectric focusing (IEF) strips pH 3.0-10.0 and 12% SDS-PAGE. The left panel shows the location of the different spots on the gel of MDA-MB-231 membrane proteins. The right panel shows the details of five spots having at least a 1.5-fold down- or upregulation between control and DHA-treatment. Results correspond to the compilation of three independent experiments performed in duplicate. Other spots were still statistically different but were not taken into consideration because their respective regulation fold was <1.5.

Results

Identification of differentially expressed proteins in DHA-treated cells. MDA-MB-231 cells treated or not with 100 μ M DHA for 24 h were processed to obtain membrane extracts. After 2-DE of membrane proteins (from both DHA-treated and control cells), it appears that only a few membrane

proteins displayed at least a 1.5-fold differential expression pattern (Fig. 1). Four proteins were found upregulated by DHA treatment compared to the control, and one protein was down-regulated (Table I). Mass spectrometry for spot no. 19 was not possible due to insufficient protein quantity and for spot no. 63 it was a mixture of proteins. Spots nos. 70, 110 and 219 were identified (Fig. 2; Table II) by microsequencing using coupled

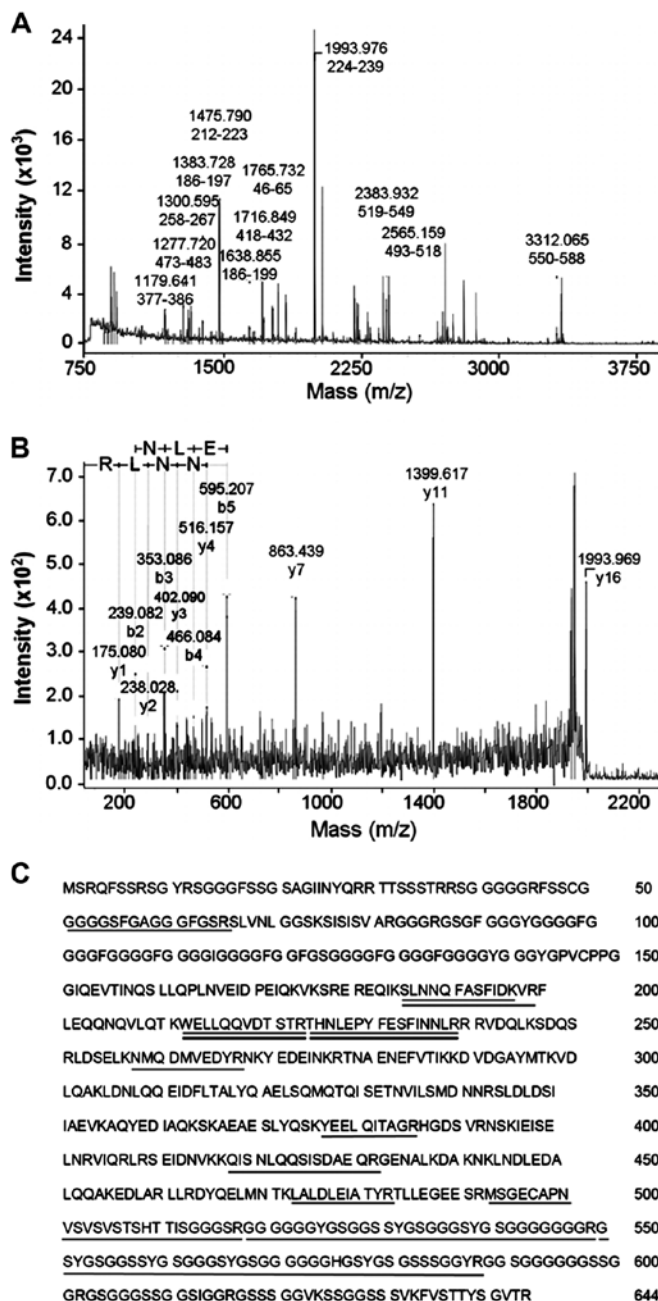


Figure 2. MALDI-TOF and MS-MS spectra corresponding to keratin, type II cytoskeletal 1 (KRT1). (A) MALDI-TOF and (B) an example of MS-MS spectra obtained for spot no. 70 (Fig. 1). The spot was excised from the gel and trypsin-digested. After MALDI-TOF and database searching, 12 peptides matched with theoretical masses, leading to a sequence coverage of 31.7%. This identification of KRT1 was confirmed by the sequencing of two peptides in MS-MS. (C) The amino acid sequences underlined with a thin grey line correspond to the 12 peptides obtained in MS and the sequences underlined twice with a thin grey line and a thick dark line correspond to the two sequences obtained in MS-MS.

mass spectrometry as being KRT1 (UniProt ID P04264), catalase (UniProt ID P04040) and lamin-A/C (UniProt ID P02545), respectively.

DHA-induced KRT1 protein upregulation and inhibition by siRNA. The increase of KRT1 in DHA-treated cells was confirmed by immunocytochemistry (Fig. 3). KRT1 was present in the cytoplasm of untreated (Fig. 3A) and

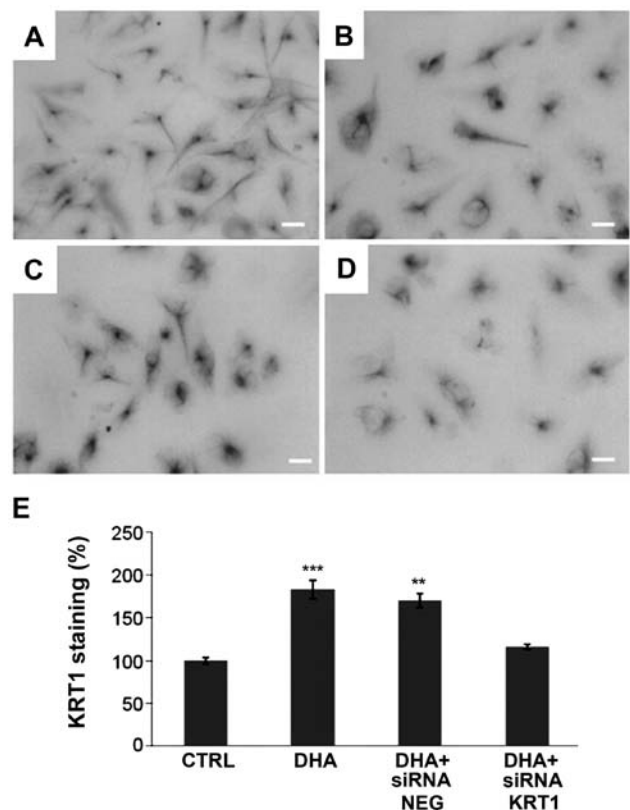


Figure 3. Keratin, type II cytoskeletal 1 (KRT1) detection and expression in MDA-MB-231 cells. The microphotographs show the presence of KRT1 in (A) untreated control cells and (B-D) docosahexaenoic acid (DHA)-treated cells. (C) Negative control siRNA and (D) KRT1-directed siRNA were added at a concentration of 50 nM and Lipofectamine was added to all conditions as detailed in Materials and methods section. Cells were incubated with mouse monoclonal anti-KRT1 antibodies and anti-mouse HRP secondary antibody and stained using diaminobenzidine (DAB). A counterstain of nuclei was made with Hoechst staining. (E) Bars correspond to 25 μ m. KRT1 expression in DHA-treated cells is inhibited by siRNA against KRT1. After immunocytochemistry, micrograph was performed by using a light microscope with a 40x objective lens for KRT1 location. Micrograph of the same field was also done with a fluorescence microscope to obtain the number of nuclei. The quantification of KRT1 among the different treatments was obtained for a given field after micrograph by calculation of the density within the field minus the background obtained by the density present on a slide without cells and then divided by the number of cells present in the field. Results are the mean \pm SD of three independent experiments. Statistics were obtained by using KyPlot[®] software with a Dunnett's test and comparison with the control. ** $P \leq 0.01$ and *** $P \leq 0.001$.

treated (Fig. 3B-D) cells. KRT1 labeling was quantified by using Quantity One software (Bio-Rad). The result was then subtracted by the blank of a similar surface without cells and divided by the number of nucleus present in the field. Thus, the average KRT1 quantification corresponded to a single cell expressed as a percentage (Fig. 3E). The result indicated that control siRNA (NEG) had no effect on the level of KRT1 in the DHA-treated cells and in the control. In the cells treated with DHA and siRNA against KRT1, we observed a level of KRT1 that was reduced nearly to what was observed in the control (Fig. 3E).

DHA-induced decrease of breast cancer cell invasiveness is reversed by siRNA against KRT1. Using Matrigel[®] in Boyden chambers, DHA was shown to reduce the invasive potential of MDA-MB-231 cells (Fig. 4). In order to investigate the role of

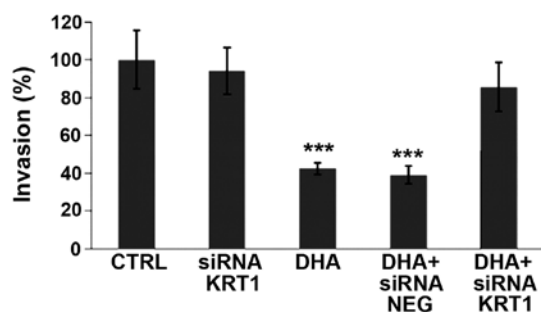


Figure 4. Docosahexaenoic acid (DHA)-induced decrease of the invasive potential of MDA-MB-231 cells is reversed by keratin, type II cytoskeletal 1 (KRT1) siRNA. The invasive potential of control and DHA-treated cells was assayed using Matrigel[®] in Boyden chambers. Control siRNA (NEG) and siRNA directed against KRT1 were added at a concentration of 50 nM to DHA-treated cells and non-treated cells. Results are the mean \pm SD of three independent experiments performed in duplicate. Statistics were obtained by using KyPlot[®] software with a Dunnett's test and comparison with the control. *** $P \leq 0.001$.

the differentially expressed KRT1 protein, siRNAs were used in the invasion assay in presence or absence of DHA. siRNA against KRT1 was able to restore a percentage of invasive cells similar to the control level while the control siRNA NEG was inefficient (Fig. 4). This indicated the involvement of KRT1 in the DHA-induced decrease of MDA-MB-231 cell invasiveness.

Discussion

The pro-apoptotic effect of DHA on cancer cells and especially in breast cancer is well known (11). However, few studies have also shown the inhibitory effect of DHA on the metastatic and invasive potential of cancer cells (9,27,28). In order to identify membrane proteins from MDA-MB-231 that could be involved, a 2-DE-based proteomic analysis was performed with crude membrane preparations. This allowed the identification of three differentially expressed proteins after DHA treatment. Besides, it appeared that these proteins are well expressed in differentiated cells and it is possible that the effects observed could be due to the DHA effect on cell differentiation as reported by Siddiqui *et al.* (29). Interestingly, lamin-A/C, a protein of the nuclear envelope, was present and upregulated in the crude membrane extract from DHA-treated cells. In stage II and III colon cancer patients, low expression of lamin-A/C was associated with an increased disease recurrence (30). In breast cancer, it has been shown that higher lamin-A/C expression is associated with: i) early clinical stage; ii) a better clinical outcomes; and iii) a better overall and disease-free survival, suggesting a significant role for nuclear and chromosomal stability in this pathology (31). Therefore, an increased in lamin-A/C expression appears to be related to a less aggressive phenotype of breast cancer cells, and our results are well in range with this notion by showing that the inhibition of MDA-MB-231 invasiveness induced by DHA is accompanied by an increase in lamin-A/C.

The enzyme catalase was also upregulated after DHA treatment, which is in agreement with its protective role against reactive oxygen species (ROS) and the induction of apoptosis by DHA (32,33). Moreover, ROS can induce cell migration and invasion (34,35), and their impact is well established in

the migration process triggered by growth factors able to activate tyrosine kinase receptors and MAPK (36). It has been shown that the lysyl oxidase (LOX) facilitates the invasion of MDA-MB-231 cells and that the removal of hydrogen peroxide leads to a dose-dependent loss in Src activation (37). Consequently, LOX was shown to facilitate migration and cell-matrix adhesion in invasive breast cancer cells through a hydrogen peroxide-mediated mechanism involving the FAK/Src signaling pathway (37). It has been shown that an increase in catalase results in a decreased ROS level close to the plasma membrane and leads to a reduction of migration and invasion (38). Consequently, the increased level of catalase observed in MDA-MB-231 crude membranes is well in range with other studies and is related to a decreased invasiveness.

Our study reports that KRT1 is induced upon stimulation of cancer cells by DHA. siRNA against KRT1 was able to reduce the *de novo* expression of KRT1 induced by DHA treatment in MDA-MB-231 cells, then leading to the reacquisition of an invasive potential. It has been shown that DHA is able to selectively alter the subcellular distribution of lipidated cytosolic proteins, including Ras isoforms, by modifying membrane lipid composition (39), indicating that KRT1 can be associated with membrane proteins. In addition, KRT1 was shown to interact with the tyrosine kinase Src through binding to integrin $\beta 1$ (23) and therefore the presence of KRT1 in a crude membrane preparation is not surprising. It has been shown that KRT1 level is strongly decreased in breast cancer cells reaching a metastatic phenotype (40). In addition, a recent study has shown that KRT1 is decreased in breast tumors (41). In the same study, KRT1 was also found to be released in sera concomitantly with a 130 kDa epithelial membrane antigen (EMA) and the EMA/CK1 ratio was correlated with more aggressive tumor types. Therefore, KRT1 expression is associated with a less aggressive phenotype of breast cancer and our results suggest a mechanism involving the inhibition of cancer cell invasiveness.

An indirect interaction between KRT1 and Src was previously reported (23) as well as the interaction between Src and the membrane protein $\text{Na}_v 1.5$, a sodium ion channel protein encoded by the *SCN5A* in humans (24,42). Inactivation of $\text{Na}_v 1.5$ is known to induce a loss of invasion capacity in triple-negative highly metastatic breast cancer cells (18,43). Then it is conceivable that the overexpression of KRT1 observed following DHA treatment may lead to KRT1 interaction with Src and then to $\text{Na}_v 1.5$, but further experiments are needed to elucidate this hypothesis and define the precise mechanisms linking KRT1 and tumor cell invasion.

In conclusion, this proteomics-based study provides new mechanistic insights into the activity of DHA in breast cancer cells and in particular identifies KRT1 upregulation as being involved in the DHA-induced inhibition of breast cancer cell invasion.

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