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Exposure to *Moringa oleifera* microRNAs induces proteomic changes linked to tumorigenesis and epithelial-mesenchymal transition in HeLa cells

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ABSTRACT

Cervical cancer (CC) is one of the most frequent cancers in women worldwide. The epithelial-mesenchymal transition (EMT) and the extracellular release of TGF-β are phenomena typically associated with different tumorigenic processes, including tumour cell proliferation and metastatization. Specific human microRNAs (miRNAs; miRs) involved in these tumorigenic processes have been identified, becoming important diagnostic and prognostic markers, and even potential therapeutic targets. In parallel, different studies have also shown that plant miRNAs can mediate a cross-kingdom regulation (CKR) of mammalian genes and modulate host's gene expression under pathological conditions, restoring the regulatory activity of endogenous miRNAs lost in cancer. In our previous studies, the miRNome from Moringa oleifera Lam. (henceforth moringa or mol) has been sequenced, showing the presence of several conserved miRNAs in the plant kingdom, whose ability to differentially regulate proliferation and apoptosis in healthy and cancer cells has been demonstrated. Furthermore, the effects of mol-miR treatment on tumorigenesis and EMT have been proved in liver tumour cells. According to these premises, we here investigated the proteomic profile of CC-derived HeLa cells exposed to a mol-miRNA pool, demonstrating the down-representation of specific factors involved in tumorigenesis. The treatment with plant miRs was able to modulate proteins involved in several biological processes linked to EMT. Furthermore, it reduced the expression of TGF-β and significantly inhibited cell motility, as observed following Scratch test and cell viability measurements, with a significant increase of apoptotic events. In conclusion, our results suggest and pave the way for the development of new potential therapeutic approaches based on CKR mediated by plant miRNAs for contrasting human cervical cancer, even in the form of adjuvants to classic treatments for limiting their side effects.

1. Introduction

Cervical cancer (CC), which is almost exclusively associated with human papillomavirus infection [1–3], is one of the most frequent tumours in women worldwide [4]. A decline in incidence and death related to CC has been documented in the last years in high-income countries, due to more effective screening and vaccination procedures; by contrast, this trend appears opposite in low-income countries, where the prognosis for patients with advanced or recurrent CC is poor [5,6]. Tumour cells exhibit typical morphological transformations, acquiring a

Abbreviations: ALB, Albumin; CC, Cervical cancer; CKR, cross-kingdom regulation; EMT, epithelial-mesenchymal transition; HNC, head and neck cancer; HPV, Human Papilloma Virus; KRTs, keratins; MFI, mean fluorescence intensity; MicroRNAs (miRNAs miRs), Moringa oleifera Lam. (henceforth moringa or mol); PEA-15, Phosphoprotein enriched in astrocytes 15, isoform CRA_a; PSAP, Prosaposin; TCGA, the Cancer Genome Atlas; TCF7, transcription factor 7; TGF-β, Transforming growth factor β; TEAB, triethylammonium bicarbonate buffer; TNFR1, Tumor Necrosis Factor Receptor 1; ACTB, β-Actin.

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mesenchymal phenotype that leads to the loss of apical-basal polarity and contact with adjacent junctions. This phenomenon is known as epithelial-mesenchymal transition (EMT) and is characterized by the activation of several tumorigenic processes, including unregulated proliferation, migration, invasiveness, and resistance to apoptosis and therapeutic treatments [7–9].

Transforming growth factor β (TGF-β) is a secreted cytokine that regulates many cell mechanisms, such as differentiation, proliferation, and migration. The dysregulation of this cytokine has been documented in cancer, where it would stimulate tumour cells to undergo EMT, evading immune response [10,11]. In particular, increased levels of TGF-β in the extracellular matrix have been registered in the late stages of CC and have been associated with morphological changes, invasiveness, and metastatisation, which are favoured by the expression of Snail and other transcription factors able to promote EMT [9,12,13].

MicroRNAs (miRNAs; miRs) are a group of small non-coding single-strand RNAs that play crucial roles in the gene regulation of all living organisms by binding specific mRNA targets and influencing their stability or translational rate [14]. In general, they are directly or indirectly involved in all biological processes and a loss of their activity has been correlated to malignancy [15,16]. Indeed, specific mammalian miRNAs involved in tumorigenesis have been identified [17–19] and classified as important diagnostic and prognostic markers, and even potential therapeutic targets in cancer [20,21]. This post-transcriptional regulatory mechanism mediated by miRNAs is conserved across species belonging to all life kingdoms and, surprisingly, in the last decades, many studies have shown that miRNAs can even exert a cross-kingdom regulation (CKR) [22,23]. In this regard, different papers have demonstrated the capacity of plant miRNAs to modulate animal host's gene expression [22,24–26]. Among all, worthy of note is the evidence about the role of plant miRNAs in restoring the regulatory activity of endogenous miRNAs lost in cancer [27]. Thus, plant miRNAs could be used to restore disrupted mammalian pathways and reverse tumorigenic processes, such as EMT. In support of this phenomenon, we can mention several studies describing the effect of some plant miRNAs on cancer. For instance, plant miR159a has been found to reduce the expression of the transcription factor 7 (TCF7) and the oncogene c-Myc in a breast cancer model [24], similarly, conserved plant miR171 was shown to down-regulate mRNA levels of the alpha 12 subunit of the G protein, modulating the mTOR pathway in HEK293 cells [28]. A similar work has showed that miR159a and miR156c contained in dried nuts exert an anti-inflammatory role in mammal adipose tissue by targeting tumor necrosis factor receptor 1 (TNFR1) [29]; similarly, miR34a from Olea europaea drupes was demonstrated to inhibit the expression of Snail and to promote that of E-cadherin in HepG2 cells [27]. In the present study, we further investigated this general phenomenon by evaluating possible molecular processes induced following treatment of mammalian cells with plant miRNAs.

Moringa oleifera Lam. (henceforth moringa or mol) is a plant species widely cultivated throughout the equatorial belt and known for its nutritional and nutraceutic properties [30–32]. Used in Asian and African traditional medicine, moringa extracts have been scientifically proved to possess anti-inflammatory, immunomodulatory, and anti-tumor properties [33–37]. In our previous studies, the moringa miRNome has been sequenced and the CKR carried out by some of its components (e.g., miRNAs) has been demonstrated [25,37,38], i.e. the ability of these polynucleotide to regulate proliferation and apoptosis in human healthy and cancer cells. We have also proved that the treatment with moringa miRs (mol-miRs) inhibits tumorigenesis and EMT in human liver tumour cells [26]. According to these premises, we here investigated the effects of mol-miRs in counteracting the metastatisation process in a cellular model of CC through the use of integrated experimental approaches.

2. Materials and methods

2.1. Plant material and isolation of miRNAs

M. oleifera Lam. mature seeds were collected in the Dschang district (West Cameroon, Africa) by the Cooperative of Medical Plant Producers SOCOPOMO. They were sun dried and powdered. The miR pool was isolated from the powder of moringa seeds (mol-miRs pool) using the NucleoSpin miRNA kit (MACHEREY-NAGEL, Germany) and following the manufacturer's instructions. The presence of the most conserved miRs of moringa, listed in our previous works [25,37,38], was evaluated by qPCR in the pool of mol-miRs and MOE, as described in Refs. [27,38].

2.2. Cell propagation and treatment

The human cervix epithelioid carcinoma cell line (HeLa) was propagated as an adherent monolayer at a density of 0.15×10^6 cells per mL of Dulbecco's Modified Eagle Medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen, Germany), 2 mM glutamine (HyClone, UK), 50 U/mL penicillin and 50 U/mL streptomycin (HyClone, UK). Cells were transfected with mol-miRs, for 72 h, using a polynucleotide pool corresponding to that contained in 1 mg of plant material and lipofectamine (Hi-Fect, Qiagen; HF; vehicle control HF CNT), according to our previous investigations [25–27].

2.3. Proteomic analysis

After washing in PBS, treated or untreated HeLa cells (10×10^6) were pelleted and lysed in the RIPA buffer added with protease inhibitors. Samples were sonicated (Labsonic U sonicator) five times at 45 W per 30 s, preserving cells for 30 s on ice after each event. Then, they were vortexed and centrifuged at 11.000 g for 20 min, at 4 °C. The protein concentration was quantified by using a BCA protein assay kit (Thermo Fisher Scientific). One hundred μg of each protein sample was adjusted to a final volume of 100 μL using 100 mM triethylammonium bicarbonate buffer (TEAB) and subsequently mixed with 5 μL of 200 mM tris (2-carboxyethylphosphine). The samples were stored for 60 min at 55 °C, alkylated by 5 μL of 375 mM iodoacetamide, incubated for 30 min at 25 °C, in the dark, and precipitated with 6 vol of cold acetone. After precipitation, the proteins were centrifuged at 8.000 g for 10 min, at 4 °C. The pellets were air dried, solved in 100 mM TEAB and digested with trypsin (enzyme: protein, 1:50) at 37 °C, overnight. The resulting peptides from each protein sample were labelled with TMT label reagent set (Thermo-Fisher Scientific, USA), following the matching CTR-TMT2-126 and mol-miRs-TMT2-127, at 25 °C, and according to manufacturer's instructions. To stop the peptide derivatization reaction, 8 μL of 5% hydroxylamine was added to each sample. Labelled peptides from mol-miRs pool-treated and control cells were mixed in equal molar ratios (1:1) and dried by vacuum rotation. Then, the pooled peptides were suspended in 0.1% trifluoroacetic acid and resolved in 8 fractions using the Pierce™ High pH Reversed-Phase Peptide fractionation kit (Thermo-Fisher Scientific), according to manufacturer's instructions. Each fraction was vacuum dried, resuspended in 0.1% formic acid, and subjected to mass spectrometric analysis, which was carried out in duplicate with a nanoLC-ESI-Q-Orbitrap-MS/MS platform consisting of an UltiMate 3000 HPLC RSLC nanosystem (Dionex, USA) coupled to a Q-ExactivePlus mass spectrometer through a Nanoflex ion source (Thermo Fisher Scientific). The system was equipped with an Acclaim PepMap TM RSLC C18 column (150 mm × 75 μm ID, 2 μm particles, 100 Å pore size) (Thermo-Fisher Scientific). The run was carried out at a flow rate of 300 nl/min, using water/formic acid (99.9/0.1; v/v) as solvent A and water/acetonitrile/formic acid (19.92/80/0.08; v/v/v) as solvent B. The gradient of solvent B started at 5%, increased to 60% over 125 min, increased to 95% over 1 min, remained at 95% for 8 min, and finally returned to 5% in 1 min, with a column equilibrating step of 20 min before the subsequent chromatographic run. Mass spectrometer

operated with a full scan (m/z range 375–1500; nominal resolution of 70.000), followed by MS/MS scans of the 10 most abundant ions. MS/MS spectra were acquired in a scan m/z range of 110–2000 (normalized collision energy: 32%; automatic gain control target: 100.000; maximum ion target: 120 msec; resolution: 17.500; dynamic exclusion value: 30 s). The raw MS and MS/MS data per each sample were merged for protein identification and relative quantitation by Proteome Discoverer v. 2.2 software (Thermo Scientific) and Mascot algorithm v. 2.4.2 (Matrix Science, UK). In particular, the latter was used as a database searcher in UniProtKB protein database (Homo sapiens, 159615 protein sequences; accessed on 07/2017 and including the most common protein contaminants). Searching set up parameters were: carbamidomethylation at Cys and TMT2-plex modification at lysine and peptide N-terminus as fixed modifications; oxidation at Met, deamidation at Asn and Gln, and pyroglutamate formation at N-terminal Gln as variable modifications. The peptide mass tolerance was set to ±10 ppm and the fragment mass tolerance to ±0.02 Da. Proteolytic enzyme and maximum number of missed cleavages were set to trypsin and 2, respectively. Protein candidates assigned based on at least two sequenced peptides and an individual Mascot score greater or equal to 30 were considered confidently classified. The results were filtered to a 1% false discovery rate. For quantitation, ratios of TMT reporter ion intensities in the MS/MS spectra from raw datasets were used for calculating the fold changes between samples. Differentially represented were considered proteins that showed a fold change of abundance $\geq \pm 1.5$, with a p-value ≤ 0.05. Proteomic data were deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD039475.

2.4. Bioinformatic analysis

To identify predominant biological processes and networks regulated by the mol-miR pool in HeLa tumor cells, enrichment analysis of protein representation data was performed using the Metascape online tool (http://metascape.org) according to Zhou and coworkers [39]. The DAVID online tool (https://david.ncifcrf.gov/) was employed to perform functional annotation clustering, using the reference database of the Genetic Association Database (GAD_DISEASE) and a p value < 0.05. To predict the human mRNA targets of mol-miRs involved in the TGF- β pathway and EMT, a bioinformatic analysis was performed taking into consideration the list of miRNAs found in the moringa miRNome, as detected in MOE [32], and belonging to the most conserved plant families [38]. A support vector machine (SVM) classifier was used as a prediction tool, after training it with an experimentally validated set of miRNA-mRNA interactions. The detailed description of the implementation process of the sklearn.SVM.SVC classifier was already reported by our group [37].

2.5. TGF-β evaluation

Intracellular TGF-β expression was measured by flow cytometry, after staining cells with FITC-conjugated anti-human TGF-β1 (BD Biosciences, USA). Labelling occurred on cells treated for 72 h, fixed, and permeabilized by 70% v/v ethanol. The analysis was performed with Cytoflex (Beckman Coulter, USA), using Cytexpert 1.2 software (Beckman Coulter, USA).

2.6. Scratch test

An in vitro scratch test was performed on HeLa cells in the presence or absence of treatments. In detail, cells were seeded and left to proliferate for 24 h. Subsequently, a 10 μL pipette tip was used to make a scratch in the middle of the plate and the treatments were applied. To allow the cells to migrate, the experiment was carried out for 72 h. Scratching widths were taken under optical microscopy for each sample at 0 and 72 h.

2.7. Cell viability and apoptosis assay

Alive and dead cells were counted at optic microscopy at 72 h of treatment, after staining with 10% Trypan Blue (EuroClone S.p.A., Italy). The percentage of apoptotic cells was estimated by flow cytometry (CytoFLEX; Beckman Coulter, Inc.), measuring the number of hypodiploid nuclei in 50,000 events. In detail, cells were harvested, washed three times in PBS, and incubated for 20 min in 70% v/v ethanol, at −20 °C. Then, they were washed with PBS and stained with propidium iodide (1.25 μg/mL). Data acquisition and analysis were performed by CytExpert 2.0 (Beckman Coulter, Inc.).

2.8. Statistics

The results were reported as means ± standard deviation (SD) of 3 independent measurements. Data were subjected to a one-way analysis of variance (ANOVA) and a post-hoc lowest standard deviations (LSD) test (Excel software); p values were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Results

3.1. Computational prediction of mol-miRs putative human targets

Starting from the dataset relative to the moringa miRNome that we profiled in our previous work [38], a bioinformatic tool obtained by combining different RNA-RNA interaction prediction algorithms was used to test the probability that the most conserved mol-miRs interacted with human transcripts involved in tumorigenesis, EMT, TGF- β signalling, and metastatisation. All selected mol-miRs were predicted to bind with a high probability at least three genes, confirming our published evidence [37]. Among all, six mol-miRs (i.e., mol-miR393, mol-miR159, mol-miR166, mol-miR6478, mol-miR6300, and mol-miR398) were found to target genes involved in tumorigenesis, metastatisation, TGF-β and Wnt β-catenin pathways, all exhibiting a high affinity (Fig. 1 and Table S1). The presence of these miRNAs was checked by qPCR analysis in the mol-miR pool, confirming our previous evidence (data not shown).

3.2. Proteomics analysis

To evaluate the global changes in protein representation induced by the mol-miR pool in HeLa cells, a tandem mass tag (TMT)-based quantitative proteomic procedure was used. A total of 5959 proteins were

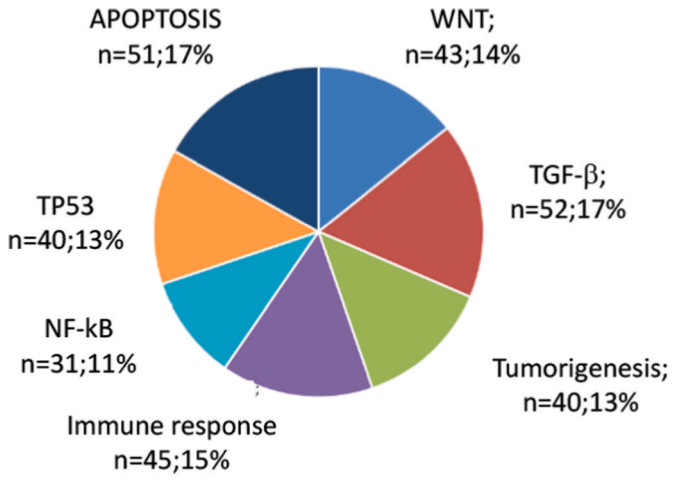


Fig. 1. Mol-miRs putative human targets. Number and percentage distribution of all mol-miRs putative human mRNA targets with respect to the principal pathways in which they are involved.

identified according to the criteria reported in the experimental section. Quantitative data on HeLa cell proteins were filtered according to a fold change value $\geq \pm 1.5$ between treated and control groups, considering only significant changes of the abundance ratios (p-value ≤ 0.05). This setup allowed quantitation of 5901 proteins, and the recognition of 26 differentially represented proteins (DRPs) (Table 1). In detail, compared to untreated HeLa cells, mol-miR induced the over-representation of 1 protein and the down-representation of 25 proteins (Fig. 2A).

To better understand the role of the proteins whose levels appeared influenced by mol-miR and the cell pathways in which they are involved, a protein-protein interaction (PPI) network was constructed using the STRING database (Fig. 2B). Above-mentioned DRPs appeared interestingly correlated, forming two complex interaction networks with 13 nodes and 2 nodes strictly joined, respectively. In the whole, the STING output described a total of 20 edges, with an average degree of 1.54 and a clustering coefficient equal to 0.395. The expected number of edges was 17, substantially lower than the observed one, while the p-value for PPI enrichment was 0.254.

3.3. Analysis of the biological processes modulated by mol-miRs in HeLa cells

To better identify the biological processes associated with the proteins whose quantitative levels are modulated by mol-miR pool and to amplify the knowledge about the connections among these proteins, the Metascape online tool was exploited to carry out an in-depth bioinformatic analysis [39]. Also in this case, the results highlighted that moringa miRs treatment was capable of modulating proteins associated with several biological processes, including metabolic and immune system ones; in particular, the enrichment analysis showed that mol-miRs were potentially able to regulate cellular processes associated with EMT. Overall, HeLa cells exposed to mol-miRs were subjected to protein changes that might influence their development, multicellular organization, and epithelial cell differentiation (Fig. 3A and B).

In addition, the DisGeNet tool allowed us to point out that mol-miR pool treatment might regulate the synthesis of a range of factors dysregulated in several diseases. The wide range of disorders linked to the proteins modulated by moringa miRs is listed in Fig. 3C through the application of a disease enrichment analysis. Notably, the top-level identified diseases were 'undifferentiated carcinoma', such as gastrointestinal and mammary metaplasia-associated diseases.

3.4. Effects of mol-miR treatment on EMT and cell death in HeLa cells

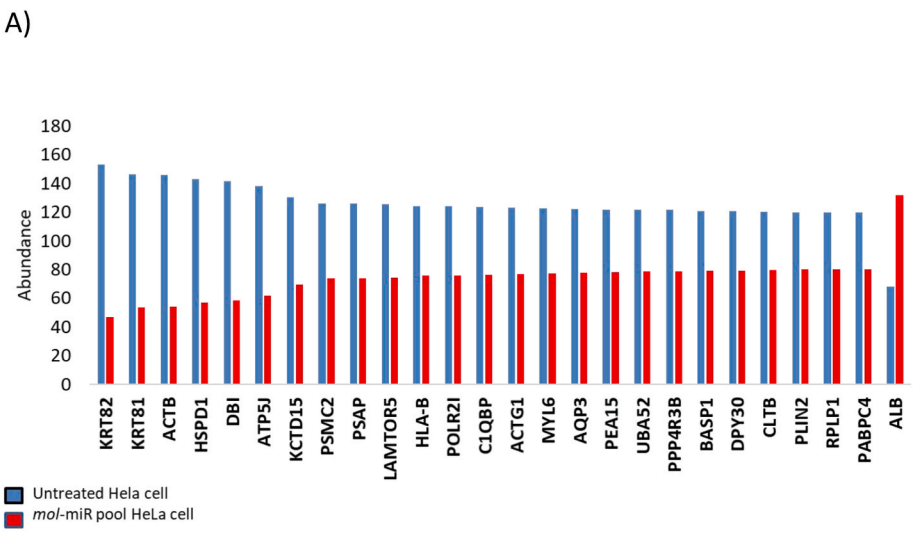
Above-reported findings indicated that the treatment of HeLa cells with mol-miR pool induces a modulation of factors involved in tumorigenesis, differentiation, and TGF-β. In order to validate these suggestions, HeLa cells were subjected to specific, confirmative in vitro experiments. First, we decided to quantify TGF-β1 levels, a cytokine involved in multiple cellular processes, including cell growth, differentiation, tumor development, and activation of EMT. Thus, HeLa cells were transfected with the mol-miR pool, stained with TGF-β1 antibody, and analysed by flow cytometry. A significant decrease in the mean fluorescence intensity (MFI) and percentage of TGF-β1-positive cells was observed in treated cells, compared to the control (Fig. 4A and B), indicating the ability of the mol-miR pool to reduce intracellular TGF-β1 expression.

Then, a scratch test was carried out to evaluate HeLa cell migration rate after 72 h of treatment with the mol-miR pool. As shown in the photos and graphs reported in Fig. 4C, while control cells (treated only with the vehicle, HF) presented a normal cell growth, proliferation rate, and migration activity, HeLa cells exposed to the pool of moringa miRNAs exhibited a significant reduction of cell motility, suggesting that the observed reduction of TGF-β1 levels could be hypothetically associated with the inhibition of EMT.

Lastly, the number of alive and dead cells was estimated by direct

Table 1
Differentially represented proteins in HeLa cells following treatment or not with mol-miRs. UniProKB accession code, gene name, fold change value, abundance ratio adjusted p-value, and protein description are shown.

UniProtKB accession	Gene Name	Fold change value (mol-miRs-treated versus control)	Abundance ratio adjusted p-value	Description2
Q701L7	KRT82	0.307	4.68069E-15	Type II hair keratin 2
Q14533	KRT81	0.367	4.68069E-15	Keratin, type II cuticular Hb1
Q53GK6	ACTB	0.369	4.68069E-15	Beta actin variant
B7Z4F6	HSPD1	0.4	4.68069E-15	Highly similar to 60 kDa heat shock protein
B8ZWD9	DBI	0.414	4.68069E-15	Diazepam binding inhibitor, splice form 1D (2)
P18859	ATP5J	0.445	4.68069E-15	ATP synthase-coupling factor 6, mitochondrial
B4DGD9	KCTD15	0.534	5.43645E-12	Highly similar to the BTB/POZ domain-containing protein KCTD15
P35998	PSMC2	0.584	1.16487E-06	26S proteasome regulatory subunit 7
A0A024QZQ2	PSAP	0.586	4.68069E-15	Prosaposin (Variant Gaucher disease and variant metachromatic leukodystrophy)
A0A0C4DGV4	LAMTOR5	0.59	9.00687E-11	Hepatitis B virus x interacting protein
A0A186VN52	HLA-B	0.609	1.46803E-06	MHC class I antigen
P36954	POLR2I	0.612	2.65027E-11	DNA-directed RNA polymerase II subunit RPB9
I3L3B0	C1QBP	0.618	4.93589E-06	Complement component 1 Q subcomponent-binding protein
V9HVZ7	ACTG1	0.624	4.26331E-10	Epididymis luminal protein 176
G3V1V0	MYL6	0.629	4.68069E-15	Myosin light polypeptide 6
Q92482	AQP3	0.633	0.007739358	Aquaporin-3
B1AKZ4	PEA15	0.643	1.87854E-07	Phosphoprotein enriched in astrocytes 15, isoform CRA_a
Q3MIH3	UBA52	0.646	7.76545E-05	Ubiquitin A-52 residue ribosomal protein fusion product 1
Q5MIZ7	PPP4R3B	0.646	3.89343E-07	Serine/threonine protein phosphatase 4 regulatory subunit 3B
P80723	BASP1	0.653	1.94515E-13	Brain acid soluble protein 1
Q9C005	DPY30	0.654	1.50553E-11	Protein dpy-30 homolog
A0A024R7S3	CLTB	0.66	4.70135E-12	Clathrin light chain
Q99541	PLIN2	0.667	1.54503E-07	Perilipin-2
Q6FG99	RPLP1	0.668	0.000338201	RPLP1 protein
Q15164	PABPC4	0.669	2.06864E-06	Polyadenylate binding protein II
B2RBS8	ALB	1.947	4.68069E-15	Homo sapiens albumin (ALB), mRNA



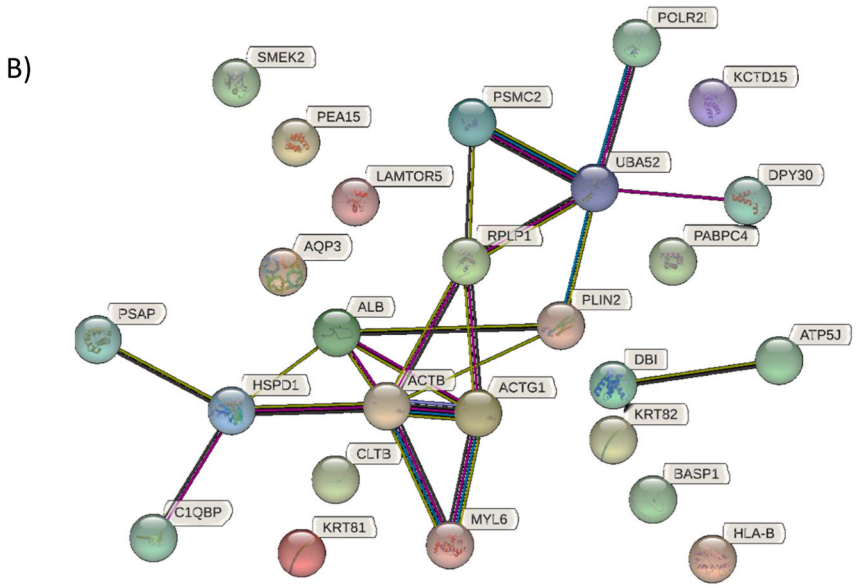


Fig. 2. Differentially represented proteins in HeLa cells after treatment with mol-miRs. A) Quantitative changes observed in mol-miRs treated HeLa cells (red columns) with respect to untreated ones (blue columns). Results are reported in arbitrary, absolute abundance values; proteins are indicated using the corresponding gene names. B) STRING analysis of the differentially represented proteins. The associations are based on data recorded for H. sapiens. Only medium-confidence interactions (0.4) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

count, after Trypan blue staining, and through indirect count of hypodiploid nuclei using flow-cytofluorimetry. The treatment with moringa miRNAs determined a significant reduction in HeLa cell viability, which was associated with a substantial increase in the number of apoptotic events (Fig. 5).

4. Discussion

M. oleifera is a plant widely used for folk remedies, and the benefits of its consumption have been scientifically documented [40,41]. In 2016, the miRNome of moringa has been sequenced, showing the presence of several miRNAs conserved in the plant kingdom [38]. For several years, our research group has studied the effects of natural substances on human health, especially the function of the bioactive components isolated from the aqueous extracts of moringa seeds, which were obtained according to the traditional African procedure [36]. Therefore, we have demonstrated the antiproliferative and pro-apoptotic properties of moringa phytocomplex against tumour cell lines, suggesting the evidence that not only secondary metabolites but also mol-miRs exert specific CKRs on in vitro and in vivo mammalian systems, such as the regulation of tumorigenesis and EMT [25,27,28,36].

Cancer is widely acknowledged to pose the highest clinical, social, and economic burden, in terms of cause-specific disability adjusted life years [42], and CC is ranked as the fourth for incidence and mortality in women worldwide [4]. Invasion and metastatisation of CC cells are phenomena associated with a poor prognosis, making this pathology the most prevalent cause of cancer-associated deaths [43–45]. Among all, EMT is considered one of the most crucial steps responsible for migratory, invasive, and metastatic properties of the tumour cells [46–48]. Thus, given the complexity of tumorigenesis, it is fundamental to analyse the expression of any gene related to onset and development of neoplasia and to evaluate their potential correlations with all existing molecular mediators and protein factors involved in the still poorly known mechanisms that induce cancer.

The proteomic analysis conducted in this study highlighted the efficacy of mol-miR pool treatment in inducing the down-representation of specific proteins involved in tumorigenesis. Using the public database of the Cancer Genome Atlas (TCGA), which contains numerous functional genomics datasets relative to different tumours, we decided to perform a protein-cancer analysis [49]. Most of the modulated proteins appeared to be up-regulated in all cancer tissues and, accordingly, they have a low cancer specificity (Table 1; i.e., ACTB, ACTG1, HSPD1, DBI, PSMC2, PSAP, LAMTOR5, HLA-B, POLR2I, C1QBP, MYL6, PEA15,

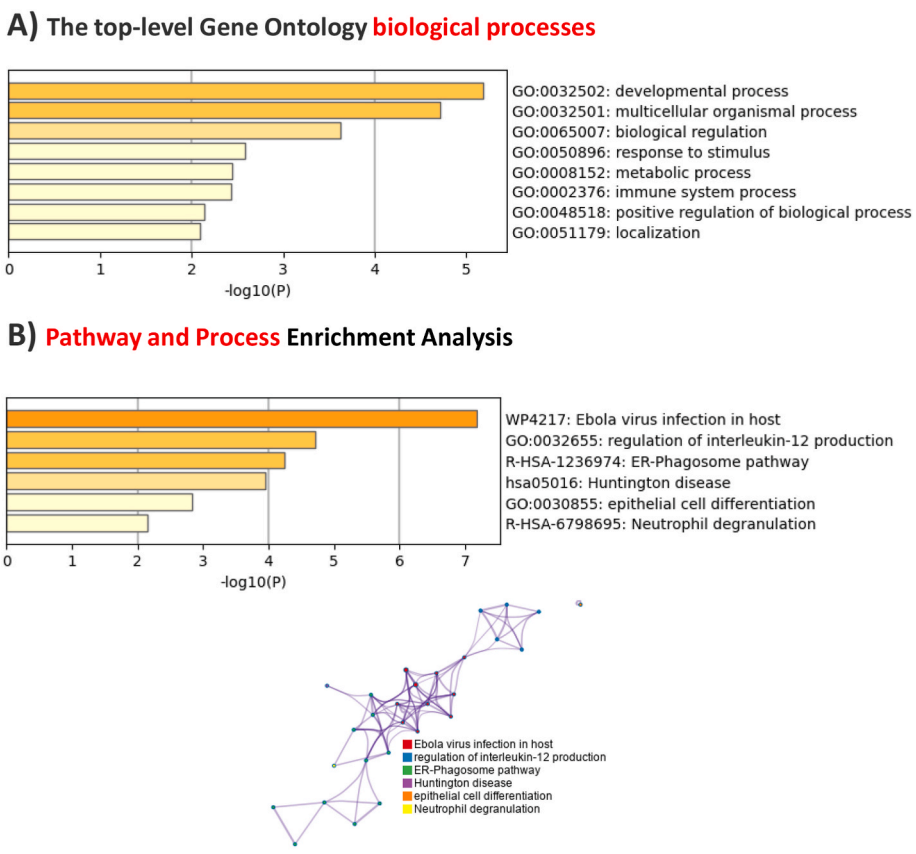


Fig. 3. Biological process, pathway, and disease enrichment analysis. Biological processes (A) and pathways (B) enrichment analysis of the genes modulated by mol-miRs in HeLa cells. All statistically enriched terms (GO/KEGG terms, canonical pathways, hallmark genes, etc.) were identified and accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Then, a 0.3 kappa score was applied as the threshold to divide the output into term groups. In the network layout of the clusters generated using the list of genes regulated by mol-miRs, each circle node represents one enriched term, where its size is proportional to the number of input genes falling into that term, while the colour represents its cluster identity. All similar terms with a Kappa similarity score >0.3 are connected by edges (the thicker the edge, the higher the similarity). C) Disease-enriched analysis associated with mol-miRs modulated proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

UBA52, PPP4R3B, BASP1, CLTB, PABPC4) [49].

The mol-miR treatment was capable of modulating proteins associated with several biological processes, and the enrichment analysis underlined that moringa miRNAs were potentially able to influence significantly important cellular pathways, also associated with EMT. In addition, the DisGeNet tool allowed us to put in prominence the fact that mol-miR pool could also potentially modify the expression of a range of proteins dysregulated in several diseases. Regarding this, it should be noted that the most identified diseases were undifferentiated carcinoma, proposing for moringa miRNAs a potential antineoplastic effect. In particular, among the proteins modulated by the mol-miR treatment, we found those overexpressed in many tumours, but especially in cervical (i.e., KRT81, AQP3), neck, and head cancer (i.e., KRT82) ones [50,51].

Among all proteins that appeared down-regulated by mol-miRs, it is interestingly to note that seven of them were direct targets of the conserved mol-miRs, as predicted by our bioinformatics analysis (Table S1; i.e., KRT81, KRT82, ACTB, PSAP, HLA-B, PEA15, and UBA52). As these proteins are involved in EMT, TGF-β1/Smad, and Wnt/β-catenin signalling pathways, it is possible to suppose that the reduction of their levels, mediated by the mol-miR pool, might be hypothetically linked to the counteraction of the metastatisation process in cancer cells. Particular attention should be paid to keratins (KRTs), which were found to be among the most negatively modulated components by mol-miR treatment. Indeed, KRTs are considered widely detectable tumour markers [50], and some of them have been also considered typical expression of EMT, via the PI3K/Akt/Nf-κB axis [51].

ACTB (i.e., β-actin), one the most down-represented protein affected by treatment with moringa miRNAs, plays a critical role in cell growth and migration [52,53], and it is significantly overexpressed in different tumour cell lines that show highly invasive capacity [54]. Moreover, its overexpression has been associated with EMT-related signalling pathways; more specifically, it has been correlated to metastatic and invasive

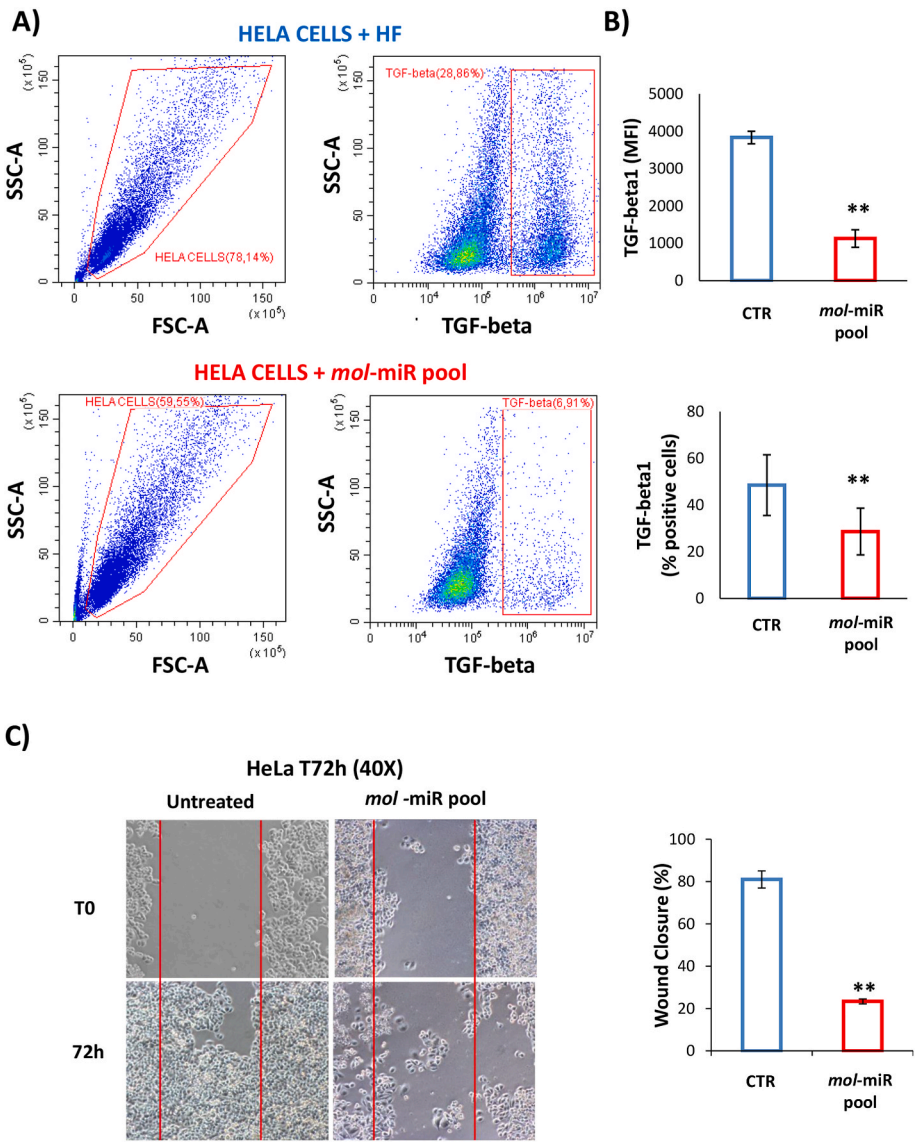


Fig. 4. Effects of mol-miR treatment on EMT in HeLa cells. A) Representative dot plots of FSC-A/SSC-A dimensions (left panels) and of SSC-A/TGF-β1 (right panels) of HeLa cells treated (lower panels) or not (upper panels) with mol-miRs; B) Histograms of the TGF-β1 mean fluorescence intensity (MFI) and positive cells (%) reflecting the cytofluorimetric results shown in A. C) Representative microscopy images (40X) showing the scratch test at times 0 and after 72 h of treatment with the vehicle (CTR HF) and mol-miRs; on the right, the percentage of wound closure of cells quantified by ImageJ software (**p<0.01).

properties by NF-κB and Wnt/β-catenin pathways in squamous cell carcinomas and other cancers [55]. However, ACTB accumulation has been also observed in cervical, head, and neck cancer, leukaemia, lymphoma, and pancreatic tumours, compared to normal tissues, while a decrease of its levels has been registered in breast, lung, ovarian, and prostate cancer [55].

Another important factor involved in the regulation of tumorigenesis is PSAP (i.e., prosaposin), here found as down-represented after moringa miRs treatment; this protein plays a critical role in cancer and sphingolipid metabolism. Indeed, its overexpression has been associated with a poor prognosis, as it would promote the proliferation of cancer stem cells in subcutaneous and orthotopic models of glioma [56], and would regulate invasion and migration in glioblastoma, through the TGF-β1/Smad signalling pathway [57].

In cervical cancer, HLA-B molecules (MHC class I antigens) carry out an important function: the presentation of human papilloma virus (HPV)-derived peptides in HPV-integrated cells. As the immune cells can be activated differently by the presented peptides, the role of these mediators is essential to favour the clearance of infected cells [58]. Furthermore, the risk of HPV-positive head and neck cancer (HNC) is associated with distinct HLA variants, and some of them are also shared by both CC and HPV-positive HNC [59].

Another protein we detected to be modulated was PEA-15 (i.e., phosphoprotein enriched in astrocytes 15, isoform CRAa). This protein is widely expressed in human tissues and participates in the interaction between various proteins. It can regulate cell apoptosis, proliferation, and glucose metabolism, by acting on key functions of cellular effectors in vivo [60,61]. In vitro and in vivo experiments have confirmed that PEA15 triggers cell growth, invasion, migration, and EMT of colorectal cancer cells [62]; consequently, the effect of mol-miRs on this protein in HeLa cells would seem to be potentially antineoplastic.

Surprisingly, we found a significant increase in ALB (i.e., albumin) levels due to the plant treatment. Its role in cancer regulation is controversial; in general, it is used to assess the nutritional status, the severity of the disease, and the progression and the prognosis of colorectal and liver cancers [63]. In some cancers, albumin is also considered as a prognostic factor; low albumin concentrations predicate poor survival of cancer patients, while high levels are associated with a better survival [64,65].

In most tumorigenic and metastatisation processes, multifunctional cytokine transforming growth factor-beta (1) (TGF-β1) is involved in immunosuppression, and stroma and extracellular matrix formation. Moreover, TGF-β controls (epithelial) cell growth and works as a tumour suppressor in healthy tissues. However, in tumour cells this suppressive

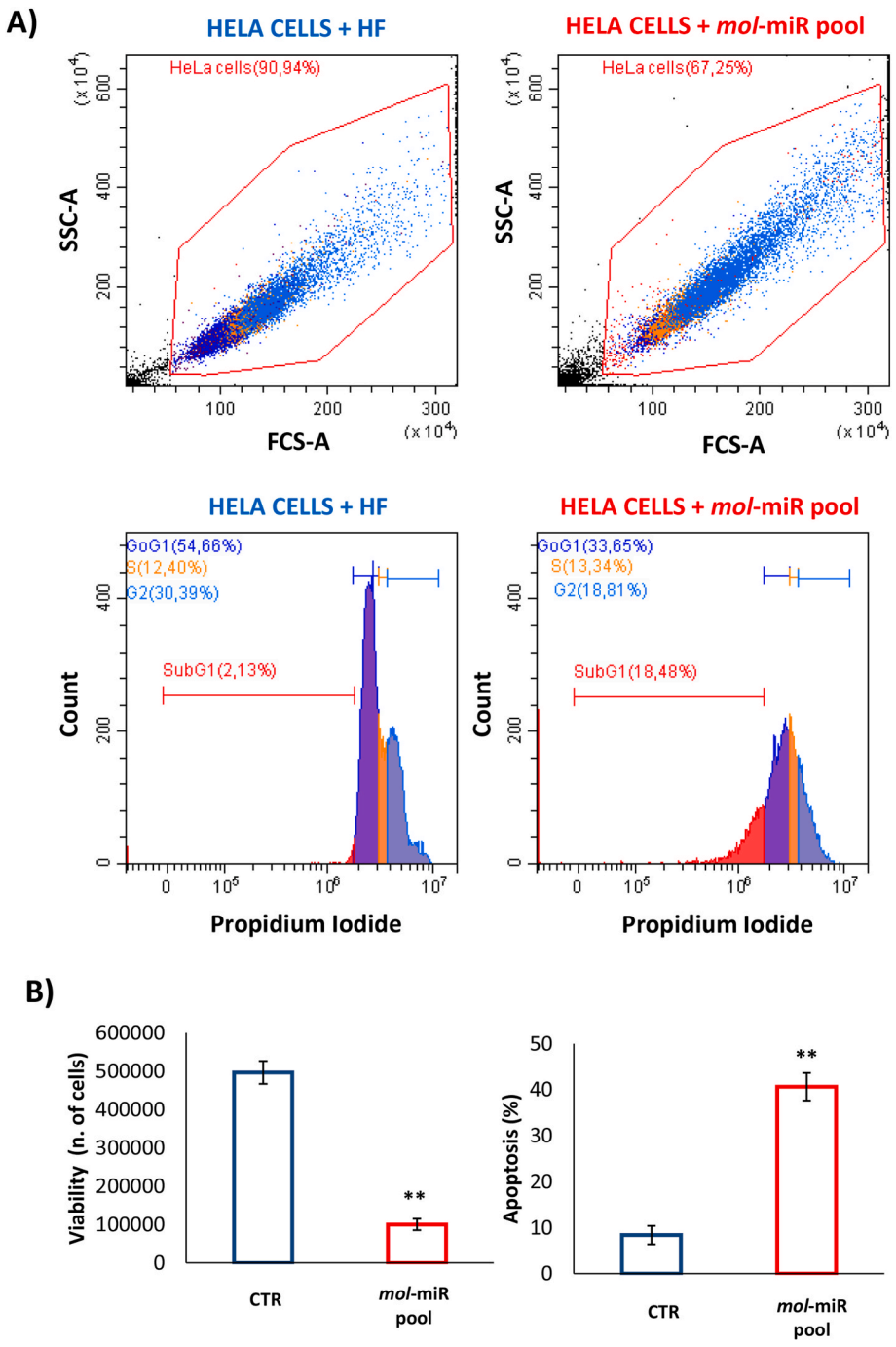


Fig. 5. Effects of mol-miRs treatment on cell viability and apoptosis. A) Representative dot plots of cells dimension and complexity (FSC-A vs SSC-A) in presence or not of mol-miR pool (upper line), histogram represented the cell cycle analysis (lower line). B) In the left panel, the viability of HeLa cells after 72 h of treatment with the vehicle (CTR HF) or mol-miR pool estimated by Trypan blue exclusion test. In the right panel, the percentage of hypodiploid nuclei (apoptotic cells) of HeLa cells treated as reported for Trypan blue assay was shown (**p<0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

function can be overcome, and in various cancers, such as squamous cell carcinoma, TGF-β promotes heterogeneity and drug resistance [66,67]. Therefore, there is increasing interest in using TGF-β inhibitors for the treatment of cancer. For example, TGF β-signalling inhibitors have been shown to block hepatocellular carcinoma growth and progression by modulating EMT in different experimental models, leading to clinical investigation on the TGF-β inhibitor LY2157299 monohydrate in patients showing the same type of carcinoma. The preliminary results of a phase II clinical trial have shown improved clinical outcomes and changes consistent with a reduction in EMT [68]. In our study, we appreciated the down-modulation of TGF-β expression in HeLa cells treated with mol-miR, and a significant regression of EMT analysed by Scratch test. As previously reported by Chin and co-workers in an in vitro model, plant miR159 would be able to suppress breast cancer cell proliferation, by binding to and inhibiting its specific human transcripts, namely TCF7 (i.e., transcription factor 7), which is involved in the Wnt signaling pathway [24]. Our data demonstrated that in CC cells, like HeLa ones, mol-miR exposure determined similar effects, probably acting on the same or parallel pathways. In addition, our treatment significantly reduced the viability of the cells and induced a high level of apoptosis.

In conclusion, our results provide preliminary information about the possible antineoplastic effect of the pool of miRNAs from moringa seed against HeLa cells, paving the way for the development of new therapeutic approaches based on the CKR mediated by plant miRNAs potentially involved in tumorigenesis and EMT of cervical cancer. These miRNAs could be also used as adjuvants to complement the actual antitumoral therapies and/or limit their side effects.

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Authors contribution

Conceptualization: AM, MP, AG, AC, CM;
Data curation, AM, MP, MM, VR, CDA, AS, SA;
Formal analysis, AM, VR, MP, CDA, SA, CDA;
Investigation, AM, MP, AG, LC, VR, CDA, SA;
Supervision, AM and AG; Validation, MP, VR, CDA, SA;
Visualization, VR, CDA, SA, and AM;
Writing: original draft, AM, MP, VR;
Writing: Review and editing, AG, MM, LC, AS, SA, CDA, CM, AC, AM, MP, VR.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.adcanc.2023.100097.

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