Abstract. Breast cancer ranks first in incidence and mortality in working age women. Cancer initiation and progression relies on accumulation of genetic and epigenetic aberrations that alter cellular processes, among them, epithelial to mesenchymal transition (EMT) denotes particularly aggressive neoplasias given its capacity to invade and metastasize. Several microRNAs (miRNA) have been found able to regulate gene expression at the core of EMT. In this study, the Affymetrix CytoScan HD array was used to analyze three different primary tumor cell isolates from Mexican breast cancer patients. We found an amplification in band 22q11.2 shared by the three samples, in the region that encodes miRNA-650. Overexpression of this miRNA has been associated with downregulation of tumor suppressors ING4 and NDRG2, which have been implicated in cancer progression. Using the Pathway Linker platform the ING4 and NDRG2 interaction networks showed a significant association with signaling pathways commonly deregulated in cancer. Also, several studies support their participation in the EMT. Supporting the latter, we found that the three primary isolates were E-cadherin negative, vimentin positive, presented a cancer stem cell-like phenotype CD44$^+$CD24$^-$/low and were invasive in Transwell invasion assays. This evidence suggests that the gain of region 22q11.2 contributes to trigger EMT. This is the first evidence linking miR-650 and breast cancer.

Introduction

Cancer is one of the most important challenges of human health today. Breast cancer (BrC) is the most common malignancy in women with more than 500,000 deaths globally per year (1). Despite advances in diagnosis and treatment, most cases are still diagnosed at advanced stages, particularly in developing countries in which more than 60% of the fatalities occur (2). Genetic modifications in tumor suppressor genes and proto-oncogenes, together with epigenetic changes in DNA methylation, histone modifications and altered miRNA expression drive cancer evolution from a pre-malignant cell to a highly aggressive cancer cell (3,4).

In aggressive tumors, cells acquire the ability to detach from neighboring cells, migrate and invade the surrounding tissue, access the blood or lymphatic vessels and colonize distant organs (5-7). These processes of invasion and metastasis are facilitated by epithelial to mesenchymal transition (EMT). During EMT tumor cells undergo a transcriptional re-programing, losing expression of epithelial genes, such as adhesion molecules that sustain cell to cell contacts, and instead express mesenchymal genes (8). Invasive cells are characterized by lack of expression of E-cadherin and gain of expression of vimentin and N-cadherin, which are commonly used to identify EMT cells. EMT cells also switch from a cobblestone- to spindle (fibroblast-like) morphology. Expression of EMT markers often correlates with expression of a stem-like phenotype (for instance CD44$^+$/CD24$^-$low) (9-11). Therefore, it is thought that EMT is a mechanism that also facilitates stemness (12-14).

miRNAs are evolutionarily conserved noncoding RNA molecules that regulate gene expression. Today, more than 1800 miRNAs have been shown to regulate cellular processes such as cell differentiation, cell cycle and cell death, and thus their abnormal function also impacts human diseases. In cancer,
different miRNAs have been classified as tumor suppressors when they regulate the expression of cellular oncogenes and as oncomiRs when they target tumor suppressor genes (15). For instance, recent studies have shown that miRNAs are important regulators of EMT (16). In the mammary gland for instance, recent studies have shown that miRNAs are downregulating expression of zEB1 and zEB2, the master transcriptional activators of the EMT program (17). ZEB1 and ZEB2 are also transcriptional repressors of Cdh1, the gene that encodes E-cadherin (18,19). On the other hand, the miR-221 family is frequently expressed in poorly differenti- ated aggressive breast tumors with EMT characteristics (20). miR-650 is a novel oncomiR also implicated in EMT. miR-650 is often expressed in prostate, colorectal, hepatocellular, skin and gastric cancers (21-25), with evidence that it targets tumor suppressors ING4 and NDRG2 (23,26-28). ING4 and NDRG2 expression is often lost in cancer and different lines of evidence support their participation in EMT (29-33).

In this study, we examined the chromosomal abnormalities of three primary cultures isolated from Mexican patients with BrC using the microarray CytoScan HD and Chromosome Analysis Suite 3.0 software (Affymetrix, Santa Clara, CA, USA). We found a common genetic lesion in the three samples, an amplification of band q11.2 in chromosome 22, in the region that encodes miR-650. We also found that the three primary isolates were negative to E-cadherin, positive to vimentin and to the stem cell marker CD44, and were invasive in Transwell migration assays, supporting their acquisition of EMT-related properties.

Materials and methods

Patient description and ethics statement. Patient samples were obtained from the tissue bank of the Unidad de Investigación en Virología y Cáncer, Hospital Infantil de México Federico Gómez. All patients included in the study signed an informed consent to participate. The study was approved by the Scientific, Ethics and Biosafety Institutional Review Boards of the participating hospitals. Patients included were diagnosed with invasive ductal carcinoma, histological grade 2 and clinical stage II, with no previous neoadjuvant therapy before tissue resection. Patients were all female aged 42, 64 and 55 years.

Tissue processing for primary cell culture isolation. Tumor tissues were rinsed with sterile PBS and mechanically disaggregated with a scalpel in 1-2-mm fragments, which were subsequently digested for 2 h at room temperature (RT) with a mixture of 1 mg/ml collagenase type I (C0130-100 MG, Sigma, St. Louis, MO, USA) and 100 U/ml hyaluronidase (H3506-100MG, Sigma) in DMEM/F12 (Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12) (11039-047, Gibco-Invitrogen Cell Culture, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (15140-122, Gibco-Invitrogen Cell Culture) in constant stir- ring. The resulting suspension was filtered through a wide pore membrane and a 100 µm membrane. The cells were pelleted and washed twice with sterile PBS and plated in an enriched medium for epithelial cells [DMEM/F12 supplemented with 5% horse serum (26050088, Gibco, Auckland, New Zealand), 10 ng/ml cholera toxin (C8052), 0.5 µg/ml hydrcocortisone (H8088), 5 µg/ml insulin (91077C), all from Sigma Chemical Co. (St. Louis, MO, USA), 5 ng/ml epidermal growth factor (EGF) (AF-100-15, PeproTech, Rocky Hill, NJ, USA)] and 1% penicillin/streptomycin 100X, and then incubated at 37°C in a 5% CO2 atmosphere. To assure the epithelial origin of the isolated cells, three epithelial markers were tested by immunocytochemistry: mouse monoclonal anti-human anti-PanCytokeratin (CM011A, clone: AE1/AE3, Biocare Medical, Concord, CA, USA), mouse monoclonal anti-human anti-pan-cytokeratin 1 (MUC-1) [Epithelial Membrane Antigen (EMA) (559774, clone: Mc-5, Biocare Medical),] and mouse monoclonal anti-human anti-epithelial cell adhesion molecule (EpCAM) (ab20160, clone: AU01, Biocare Medical) all in a working dilution 1:50. The epithelial cell isolates were named as UIVC-IDC-6, -9 and -10.

Analysis of the primary cultures using the microarray CytoScan HD and Chromosome Analysis Suite 3.0 software. Total DNA from an early passage (e6) of the three primary BrC cultures was isolated using the QIAamp® DNA Micro kit (56304, Qiagen, Gaithersburg, MD, USA) according to the manufacturer's protocol. To verify the quality of DNA, the endogenous β-actin gene was amplified by PCR. The Affymetrix CytoScan HD array (901835, Affymetrix) was used to evaluate copy number and loss of heterozygosity (LOH). This array contains more than 2.6 million copy number markers, of which 750,000 are ‘genotype-able’ single nucleotide polymorphisms (SNPs) and 1.9 million are non-polymorphic probes. Chromosome Analysis Suite (ChAS) software v3.0 (Affymetrix) was used for data analysis; for gains and losses we considered a minimum length of 100,000 base pairs (bp), and for LOH a minimum length of 3 Mbp. We compared all resulting alterations in the three BrC primary samples against female and male non-cancer control array data. Data from 1038 phenotypically healthy individuals (Affymetrix) and from Database of Genomic Variants were used as reference. Alterations found only in <1% (rare) or never described (new rare) of the reference population were considered.

Bioinformatic analysis. The data were first classified as gains, losses and LOH for each individual sample, and then as shared by more than one sample. An amplification in the region 22q11.2 was found common to all the primary cultures, harboring the gene for miR-650. We searched for interaction networks between miR-650 and cancer, and more specifically with BrC, using the Cytoscape® Analysis platform (http://www. cytoscape.org). The target genes of miR-650 were confirmed with three databases: Target Scan® (http://www.targetscan.org/), miRDB® (http://mirdb.org/miRDB/) and microRNA.org (http://www.microrna.org/). We searched for the proteins encoded by the target genes identified. The online platform Pathway Linker® (http://pathwaylinker.org) was used to determine protein-protein interaction networks; this platform uses three different databases: KEGG (Kyoto Encyclopedia of Genes and Genomes), Reactome and SignaLink database.

Immunofluorescence assay. Cells (3x104) of each of the primary isolates were seeded on coverslips for 24 h, fixed with paraformaldehyde 4% for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 20 min (both from Sigma-Aldrich Co., St. Louis, MO, USA, ref. P6418-500G and T8787-100 ML, respectively). Cells were blocked with
blocking buffer [10% goat serum (Sigma, ref. G9023-10ML), 0.2% Triton X-100 and 1% BSA (bovine serum albumin, Sigma, ref. A1933), in PBS 1X] for 1 h, after which, cells were stained with mouse monoclonal anti-E-cadherin antibody (1:100, 610181, BD Biosciences, San Jose, CA, USA) and with rabbit monoclonal anti-vimentin antibody-Alexa Fluor-594 (1:2000, ab154207, Abcam, Cambridge, UK) overnight at 4˚C. Cells were then incubated with a goat anti-mouse-IgG-FITC antibody for 30 min (1:50, F0257, Sigma-Aldrich Co.). Finally, nuclei were stained with DAPI (H1200, vector Laboratories, Youngstown, OH, USA) for 25 min. Cells were observed using a fluorescence microscope Olympus BX51 and images were acquired with a digital camera (Camedia C4040, Olympus, Segrate, Milan, Italy).

Invasion assay. Cells (2x10^4) were resuspended in 200 µl DMEM/F12 medium and placed in the upper chamber of a Transwell [6.5-mm diameter, 8-µm pore size (3422, Corning Inc., Corning, NY, USA)] covered with Matrigel (356237, Corning Inc.). Finally, nuclei were stained with DAPI (H1200, Vector Laboratories, Youngstown, OH, USA) for 25 min. Cells were observed using a fluorescence microscope Olympus BX51 and images were acquired with a digital camera (Camedia C4040, Olympus, Segrate, Milan, Italy).

Flow cytometry. Cells (3x10^5) were blocked with PBS 1x supplemented with 50% FBS and incubated for 30 min with mouse anti human CD44-Phycoerythrin (555479) and CD24-PE-Cy7 (561646) both from BD Biosciences. Cells were then incubated with 7AAD (559925, BD Biosciences). All acquisitions were performed in a FACS Canto II flow cytometer (Becton Dickinson, Frankin Lakes, NJ, USA). Finally, the analysis of flow cytometry data was performed on 7-AAD
negative cells using FlowJo V10 software (Tree Star Inc., Ashland, OR, USA).

**Results**

**Confirmation of the isolation of epithelial cells of breast tumors.** We obtained three primary cultures of Mexican BrC patients and identified them as UIVC-IDC-6, -9 and -10. Cultures were chosen in early passages (<6) to reduce the risk of genetic changes induced during culture. All primary cultures were positive for epithelial markers PAN-CK, EpCAM and MUC-1 (data not shown). Expression of these markers is known to be maintained even after EMT.

**Gain of miR-650 in the three BrC primary cultures.** Using the microarray CytoScan HD and the software ChAS 3.0, we detected many chromosomal alterations (gains, losses and LOH) per sample and in common in at least two samples. There were 103 LOH in common between the sample UIVC-IDC-9 and UIVC-IDC-6, and 40 between the samples UIVC-IDC-10 and UIVC-IDC-6 (Fig. 1A). There were no LOH alterations in common in the three BrC samples. For gains and losses, UIVC-IDC-9 and UIVC-IDC-10 shared 11 alterations, and samples UIVC-IDC-10 and UIVC-IDC-6 presented 4 common alterations. We found an amplification on the region 22q11.2 common to all samples (Fig. 1B), harboring the gene encoding miR-650 present in all the amplifications.

**ING4 and NDRG2, target genes of miR-650.** We first searched for the interaction networks of miR-650 using the platform CytoScape, no relation with other miRNAs was found. We found that ING4 and NDRG2 were two potential miR-650 targets by reviewing preview reports and using the platforms Target Scan, miRDB, and MicroRNA.org (Fig. 2A and B). Then we used Pathway Linker platform to find interaction networks of these proteins observing an association with cell cycle and motility regulators (Fig. 3A and B). However, only for ING4 the platform found significant associations with signaling pathways and cellular processes (Table I).

**Characterization of the EMT profile in BrC primary cultures.** Several studies have shown that ING4 and NDRG2 suppress EMT (29-33). To determine if the gain of the region 22q11.2 was associated with EMT, we analyzed cell morphology and the presence of EMT markers E-cadherin and vimentin by immunofluorescence. Noteworthy, all three primary cultures displayed a very homogeneous profile, the cells shared mesenchymal characteristics such as spindle-like morphology, negative expression of E-cadherin and high expression of vimentin (Fig. 4A). EMT identifies aggressive tumors because of the capacity of invasion of tumor cells. Transwell invasion assays confirmed that all samples were highly invasive (Fig. 4B). Moreover, the three primary isolates contained a high proportion of a population with a BrC stem cell-like phenotype CD44high CD24low, UIVC-IDC-6 = 76.8%, UIVC-IDC-9 = 67.8% and UIVC-IDC-10 = 72.3% (Fig. 4C). The non-invasive BrC cell line MCF-7 was used as a negative control.

| Cellular process or signaling pathway | P-value |
|--------------------------------------|---------|
| Cancer                               | 2.5e-10 |
| Pancreatic cancer                     | 4.2e-09 |
| Chronic myeloid leukemia              | 4.4e-09 |
| Cell cycle                            | 2.5e-08 |
| Bladder cancer                        | 1.7e-07 |
| Non-small cell carcinoma              | 3.9e-07 |
| P53                                  | 6.2e-07 |
| Melanoma                             | 6.6e-07 |
| Glioma                               | 7.0e-07 |
| Prostate cancer                       | 1.6e-06 |
| Renal cancer                          | 0.00015 |
| Systemic lupus erythematosus          | 0.00017 |
| Small cell lung cancer                | 0.00024 |
| Apoptosis                             | 0.00027 |
| Neurotrophin signaling pathway        | 0.00052 |
| Huntington                            | 0.00066 |
| Wnt signaling                         | 0.00077 |
| MAPK signaling                        | 0.0022  |

P-values ≥0.01 denotes significant associations. WNT, Wingless-related integration site; MAPK, mitogen-activated protein kinase.
control, and the highly aggressive BrC cell line MDA-MB-231 was used as positive control (data not shown). These results are in agreement with a model in which miR-650 amplification results in downregulation of ING4 and NDRG4 tumor suppressors and EMT (Fig. 5).

Discussion

We performed a cytogenetic analysis of three BrC primary cell cultures, using the CytoScan HD platform and ChAS 3.0 software of Affymetrix in order to search for genetic abnormalities associated with breast cancer (BrC). We found a large number of gene copy number alterations, and LOH in each individual sample, but interestingly, we also observed a previously undescribed alteration in BrC studies that was common in the three primary isolates, pointing out a possible new highly represented feature in Mexican BrC patients.

Although, *in vitro* and *in vivo* evidence supports the association of miR-650 with cancer, to the best of our knowledge miR-650 has not been previously documented to be involved in BrC. Furthermore, increased expression of miR-650 often denotes cancers with aggressive features, which is also in support of our observations. Levels of miR-650 expression in glioma and hepatocellular carcinoma correlated with the grade of the tumor (23,34). In gastric cancer the expression level of miR-650 was significantly associated with metastasis (25). Overexpression of miR-650 in gastric cancer cell lines increased the size and number of tumors in xenografted nude mice, and inhibition of miR-650 neutralized this effect (25). Other human cancers in which miR-650 has been implicated are prostate cancer, chronic lymphocytic leukemia, osteosarcoma and lung adenocarcinoma (21,35-37). miR-650 has been associated with downregulation of tumor suppressors ING4 and NDRG2 (22,23,25,26). Because of
these associations, it has been proposed that miR-650 is an oncomiR that regulates, apoptosis, cell cycle, DNA repair, EMT and metastasis (22,23,34,35). Thus, miR-650 has also been proposed as a potential target for cancer treatment.

EMT is a normal de-differentiation process during embryogenesis. In the inner mass of the blastocyst, the pluripotent embryonic stem cells have epithelial characteristics and during gastrulation, the pluripotent epithelial epiblast ingress to form the primary mesoderm trough EMT (38,39). In adults EMT has been associated with wound healing, tissue fibrosis and tissue regeneration, also named as Type 2 EMT (40). It is believed that in cancer, cellular changes associated with EMT facilitate cell invasion and metastasis (41). Tumor suppressors are important regulators of EMT. Wang and collaborators found that ING4 inhibits migration of a thyroid cancer cell line by reversing the Wnt/β-catenin pathway induced-EMT (30). Downregulation of ING4 has also been associated with progression of lung cancer and head and neck carcinomas (42,43), brain tumors and in some cases, ING4 expression also correlated with tumor grade (44). Using comparative genomic hybridization (CGH), Kim and colleagues found a deletion of the ING4 loci in 10-20% of primary breast tumors and BrC cell lines (26). In a more mechanistic study, Bayron and collaborators found that ING4 inhibited NF-κB activity in the BrC cell line T47D (45). On the contrary, upregulation of ING4 in osteosarcoma cells induced cell apoptosis and suppressed cell invasion through downregulation of MMP-2, which also correlated with inhibition of NF-κB activity (46). Other studies support that ING4 negatively regulates NF-κB, and that ING4 downregulation associates with tumor progression and poor patient outcome (27,45-48).

There is also evidence that NDRG2 supports EMT-induced aggressive features of different types of cancer cells, such as invasion and migration. Hong and collaborators found that epigenetic silencing of NDRG2 induced proliferation and invasion of primary colorectal cancer cells, and that this could be associated with advanced stages of the disease (49). Similarly, Lee and collaborators found that in patients with gallbladder carcinoma loss of NDRG2 expression was an independent predictor of decreased patient survival and was significantly associated with a more advanced tumor stage (32). In addition, loss of NDRG2 expression in gallbladder carcinoma cells resulted in enhanced proliferation, migration and invasiveness in vitro, and enhanced tumor growth and metastasis in mice (32). Using immunohistochemistry of tissues from patients with colon carcinoma, Kim and collaborators found that NDRG2 and E-cadherin were highly expressed in normal mucosa and in areas with well differentiated tumor cells, while areas of poorly differentiated carcinoma there was low to no expression of both proteins (50).

In the above study, NDRG2 knockdown induced downregulation of CDH1 (encodes E-cadherin) promoter activity and upregulation of Snail-1, a master transcription factor regulator of EMT. In the aggressive BrC cell line MDA-MB-231, overexpression of NDRG2 results in downregulation of Snail-1 and upregulation of CDH1 (33). TGF-β is perhaps the most used inducer of EMT in in vitro studies, and Shen and collaborators showed that NDRG2 abrogates the TGF-β-induced EMT (31).

In another study in gallbladder carcinoma cells, loss of NDRG2 created an EMT positive feedback loop by increasing the expression of the tyrosine kinase receptor Axl, that regulated epigenetic silencing of the tumor suppressor genes NDRG2 and Snail-1. This created a positive feedback loop that promoted cell invasion and metastasis (32). In addition, in vitro studies, and Shen and collaborators showed that NDRG2 abrogates the TGF-β-induced EMT (31).

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Figure 5. Model of miR-650 functions in breast cancer. A 22q11.2 amplification harboring the miR-650 coding sequence, leads to miR-650 overexpression and silencing of ING4 and NDRG2 tumor suppressor genes. Lack of expression of ING4 and NDRG2 contributes with the acquisition of aggressive features related to the epithelial to mesenchymal transition and to stemness.
In solid tumors, a scarce population with capacity of self-renew has been described, this population seeds new tumors upon serial passages in mice and reconstitutes all types of tumor cells both in vivo and in culture. These cells are thought to be cancer stem cells (CSC) and are considered the most clinical relevant population because this may be the population that is responsible for metastases, resistance to chemotherapy and disease relapse (54-57). There is experimental evidence of a direct molecular link between EMT and stemness. In 2003, Mani et al demonstrated that the induction of EMT resulted in acquisition of both mesenchymal and stem characteristics, for instance a fibroblastoid morphology together with a BrC stem cell (BrCSC) phenotype CD44hi CD24low (9). Since EMT is a prerequisite for the invasion-metastasis cascade (58), evidence shows that metastatic CD44hi CD24low BrCSCs strongly express the TGF-β-induced signature associated with EMT (59). Of clinical significance, Santisteban et al demonstrated that induction of EMT increased the density of BrCSCs, which correlated with high tumorigenicity in mice, and resistance to pharmacological and radiation treatment (60). Thus, there is an important association between EMT, CSCs, relapse and treatment failure (61-63). Although, there is no direct evidence linking expression of miR-650, ING4 and NDRG2 with CSCs, various evidence supports that high miR-650 and/or low ING4 and NDRG2 correlates with advanced cancers and poor patient outcome (22,35,43,48,53,64). Furthermore, NDRG2 has been found to downregulate CD24 expression in hepatocellular carcinoma cell lines and tissues and miR-650 has been found in microvesicles released by CD105 positive renal carcinoma stem cells (65,66).

In this study, we found a common amplification in the 22q11.2 band in primary isolates of three Mexican patients with BrC. In all the cases this amplification included the region encoding miR-650, an oncomiRNA previously associated with invasive cancers, although never before described in BrC. Two of the most studied targets of miR-650 are tumor suppressors NDRG2 and ING4, which have been documented to negatively regulate EMT. Our data support a model in which miR-650 amplification results in NDRG2 and ING4 downregulation that then triggers EMT, acquisition of cancer stem cell markers and invasion. A limitation of this study is the small number of samples analyzed to have a better idea of how common this genetic lesion is in Mexican patients with BrC. Of note, our data also suggest that these patients harbor aggressive tumors. However, these tumors were classified with invasive ductal carcinoma, histological grade 2 and clinical stage II, considered of intermediate prognosis. In future studies, it is critical to follow up BrC patients with the 22q11.2 amplification.

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