MicroRNA profiling in human breast cancer cell lines exposed to the anti-neoplastic drug cediranib

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Abstract. Cediranib, a pan-tyrosine kinase inhibitor is showing promising results for the treatment of several solid tumours. In breast cancer, its effects remain unclear, and there are no predictive biomarkers. Several studies have examined the expression profiles of microRNAs (miRNAs) in response to different chemotherapy treatments and found that the expression patterns may be associated with the treatment response. Therefore, our aim was to evaluate the cellular behaviour and differential expression profiles of miRNAs in breast cancer cell lines exposed to cediranib. The biological effect of this drug was measured by viability, migration, invasion and cell death in *in vitro* assays. Signaling pathways were assessed using a human phospho-receptor tyrosine kinase array. Furthermore, using a miRNA array and quantitative real-time PCR (qRT-PCR), we assessed the relative expression of miRNAs following cediranib treatment. The breast cancer cell lines exhibited a distinct cytotoxic response to cediranib treatment. Cediranib exposure resulted in a decrease in the cell migration and invasion of all the breast cancer cell lines. Treatment with cediranib appeared to be able to modulate the activation of several RTKs that are targets of cediranib such as EGFR and a new potential target ROR2. Furthermore, this drug was able to modulate the expression profile of different microRNAs such as miR-494, miR-923, miR-449a, miR-449b and miR-886-3 in breast cancer cell lines. These miRNAs are reported to regulate genes involved in important molecular processes, according to bioinformatics prediction tools.

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Introduction

Breast cancer is the second most prevalent neoplastic disease among women and is characterised by a complex aetiology and chemoresistant behaviour (1,2). Although most women are treated with tamoxifen, the current standard adjuvant therapy in women with estrogen receptor (ER)-positive breast cancer, they develop resistance to the drug (3). Cediranib (AZD2171, Recentin, AstraZeneca) is a potent inhibitor of several receptor tyrosine kinases (RTKs), such as VEGFR, KIT and PDGFRA (4,5). Significant results have been obtained for patients with advanced solid tumours, such as glioblastoma, lung and prostate tumours, after cediranib treatment (6-9). Recently, novel targets of cediranib have been identified by *in vitro* and *in vivo* studies with glioblastoma cell lines (10). Furthermore, it was shown that cediranib alone or in combination with temozolomide is an effective drug in anti-angiogenic therapies due to its greater antitumour activity (10). Animal models of breast cancer revealed that cediranib affected vessel density and cell proliferation, but not with chronic administration (11,12). Phase I/II studies have been conducted with cediranib for breast cancer treatment, especially in combination with other drugs, such as fulvestrant, olaparib and RO4929097, a γ-secretase inhibitor (13-15). The promise of cediranib treatment is an improvement in overall survival of up to six months in several tumour types (6,7), and while it has not yet been approved by the FDA, cediranib, alone or in combination with other drugs, represents a new potential therapy for breast cancer.

Despite these observations, in breast cancer cell lines, the effects of cediranib on cellular processes such as proliferation, migration and invasion are unknown. Therefore, exploring the biological effect of cediranib on breast cancer cell lines and revealing the pathways that can be predictive biomarkers of cediranib response are necessary. MicroRNAs (miRNAs) are small noncoding RNAs approximately 22-26 nucleotides (nt) long that are involved in post-transcriptional repression or mRNA degradation in a sequence-specific manner (16,17). The role of miRNAs as new regulatory molecules that are involved in different cancer processes have recently emerged (18). Several studies on miRNA profiling have shown that these

molecules can act as oncogenes and tumour suppressors (19). Accordingly, strategies to correct the deficiencies associated with miRNA dysregulation have been proposed as future clinical interventions for cancer patients (20). In breast cancer, several miRNAs have been described in several steps of tumorigenesis, angiogenesis and metastasis (21-23). Furthermore, miRNAs have been considered as potential biomarkers in breast cancer metastasis (24).

miRNAs also play a role in anticancer drug resistance (25). Several miRNAs have been associated with chemoresistance in breast cancer, including miR-34a with docetaxel, miR-125b with anthracycline, miR-451 and miR-27 with doxorubicin and miR-326 with doxorubicin and VP-16 resistance (26-31). Several reports have shown that deletions of miRNA regions can affect the regulation of RTKs and the response to gefitinib or imatinib (32,33), reinforcing a possible role for miRNAs in the mechanism of the response and/or resistance to RTK inhibitors.

Overall, miRNAs can affect breast cancer drug sensitivity with implications in clinical management and in understanding the molecular mechanisms that may contribute to drug susceptibility and resistance (17). Therefore, in the present study, we aimed to evaluate the miRNA expression profiles of breast cancer cell lines exposed to cediranib.

Materials and methods

Cell lines and determination of the half-maximal inhibitory concentration (IC$_{50}$). In the present study, the breast carcinoma cell lines Hs578T, MDA-MB-231 and T47D were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM 1X, high glucose), supplemented with 10% FBS (both from Gibco, Invitrogen Life Technologies Grand Island, NY, USA) and 1% penicillin and streptomycin solution (Sigma-Aldrich) at 37°C and 5% CO$_2$.

Authentication of cell lines was performed by short tandem repeat (STR) DNA typing according to the International Reference Standard for Authentication of Human Cell Lines using a panel of eight (D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX and CSF1P0) STR loci plus gender determination (AMEL), using the fluorescent labeling primers as reported by Dirks *et al* (34). Briefly, 50 ng of DNA was amplified in multiplex PCR reaction carried out in a total volume of 10 μl with Qiagen Multiplex PCR kit (Qiagen) comprising 0.5 μM of all fluorescent primer pairs plus 1 μM of TH01 primer reinforcement, performed in a Veriti® 96-well Thermal Cycler with an initial denaturation at 95°C for 15 min, amplified for 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min 30 sec, extension at 72°C for 1 sec and a final cycle at 72°C for 30 min. The DNA amplification products were diluted 1:100 in Nuclease-Free Water Ultrapure (USB, Cleveland, OH, USA) and combined with 0.3 μl of internal size standard GeneScan 500™ ROX™ (Applied Biosystems, Foster City, CA, USA) in 8.7 μl formamide and loaded automatically in a capillary electrophoresis using an Genetic Analyzer ABI PRISM 3500 (Applied Biosystems). The analysis was performed in GeneMapper software version 4.1 (Applied Biosystems). Genotyping confirmed the identity of all three cell lines.

The drug used, cediranib (Selleck Chemicals, Houston, TX, USA), was diluted in 1% DMSO. To determine the IC$_{50}$, the cells were plated in increasing concentrations of the drug from 0.1 to 100 μM, and an MTS proliferation assay was performed. The IC$_{50}$ concentration was calculated using the drc package in R (35,36), and the best model for each cell type was selected according to the Akaike information Criterion (AIC).

Invasion and migration assays. The invasion assays were performed using BD BioCoat Matrigel invasion chambers (BD Biosciences) according to the manufacturer's instructions and as previously described (10,37). Briefly, 2.5×10^4 cells were plated in the Matrigel-coated 24-well Transwell inserts in DMEM-0.5% containing fixed concentrations of the drug. DMEM-10% was used as a chemoattractant, and the cells that attached to the inserts were fixed with methanol and stained with haematoxylin. The cells were photographed at a x40 magnification level and counted on a pixel-by-pixel basis using ImageJ software. The invasiveness of the cells exposed to cediranib (IC$_{50}$ dose at 72 h) was expressed in relation to the DMSO control (considered 100% invasion) as the mean percentage of invasion ± SD. The assays were performed in triplicate.

The migration capacity of the cells after cediranib exposure was assessed using a wound-healing assay as previously described (10,38). The cells were seeded in 6-well plates and cultured until reaching 95% confluency. The monolayer of cells was washed with PBS; a wound was created, and the cells were incubated with the IC$_{50}$ concentrations of cediranib for varying times. The selected areas were photographed at x40 magnification at 0 to 24 h for the Hs578T and MDA-MB-231 cells and at 0 to 72 h for T47D cells. The relative migration distance was calculated using the following formula: Percentage of wound closure (%) = 100(A − B)/A, where A is the width of the cell wound before incubation and B is the width of the cell wound after different lengths of cediranib exposure. The assay was performed in triplicate with four measurements for each well.

Western blot analysis and human phospho-RTK array. To evaluate the inhibition of the intracellular signalling pathways, Hs578T, MDA-MB-231 and T47D cells were cultured in T25 culture flasks in DMEM 10% FBS. After reaching 90% confluency, the cell lines were starved and exposed to cediranib (IC$_{50}$ values) for 12 h. Analysis of apoptosis was conducted in 6-well culture plates at 70% confluency and incubated for 12, 24 and 72 h. For all experiments, after time points, the cell lines were washed and scraped in cold PBS and lysed in buffer, containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na$_3$VO$_4$, 10 mM NaF, 10 mM sodium pyrophosphate, 1% NP-40 and protease cocktail inhibitors. Western blot analysis was performed using standard 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading 30 μg protein per line. To assess apoptosis and the activation of intracellular signaling pathways, the antibodies used were the following: primary antibody PARP total/cleaved [Cell Signaling Technology (CST), no. 9532]; total p44/42 MAPK (CST, no. 137F5) and phospho-p44/42 MAPK (CST, no. 8544); pan AKT (CST, no. C67E7) and phospho-Akt (Ser473) (CST, no. 4060); β-actin (CST, no. 12262). Secondary antibodies were used according to the manufacturer's instructions. Immune blots were performed using ECL western

blotting detection reagent (GE Healthcare) and finally the bands were detected and images were captured using an Automatic ImageQuant Mini LAS 4000 (GE Healthcare).

Human phospho-RTK array (PN no. 894042, R&D Systems) was used according to the manufacturer's instructions. In brief, after the blocking step, 750 μg of protein were incubated overnight at 4°C with nitrocellulose membranes containing 49 different anti-RTK Abs spots in duplicate. Next they were incubated with anti-phospho-tyrosine-HRP for 2 h and detection was performed with a Chemi Reagent Mix in Automatic ImageQuant Mini LAS 4000 (GE Healthcare).

RNA isolation and quality control. Total RNA was isolated using an miRNeasy kit (Qiagen) according to the manufacturer's instructions. Quantification was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA), and the RNA quality was assessed using an Agilent Nano RNA chip with a bioanalyser device (Agilent Technologies), as previously described (39).

miRNA microarrays. To assess the expression of miRNAs after 24 h of treatment with an IC_{50} dose of cediranib in Hs578T, MDA-MB-231 and T47D cells and the same cell lines without treatment were considered as controls, the Agilent Human miRNA Microarray (8x15K-G4471A, Agilent Technologies) was used. Total RNA samples (200 ng) were hybridised using an miRNA complete labelling and a Hyb kit (Agilent Technologies) according to the manufacturer's instructions. The reactions followed a two-step preparation; an initial dephosphorylation and denaturation of the total RNA was performed, and the Cy3 fluorochrome was then incorporated with T4 ligase. The next steps included standard washing procedures and hybridisation with the microarray slides. Images were scanned with an Agilent DNA microarray scanner with SureScan technology (Agilent Technologies), as previously described (40).

miRNA microarray data analysis. The raw data were obtained using the Feature Extraction software v.11.0 (Agilent Technologies) and submitted to R environment version 3.0.1 (36) for additional analyses. Median signals (*gMedianSignal* and *gBGMedianSignal*) were used as intensity values. Normalisation was performed using the quantile method with the Bioconductor 'aroma.light' package (41). miRNAs differentially expressed between the cediranib-treated and control cells were obtained by rank product analysis considering a P-value and pfp (positive false predictions) ≤0.05 using the RankProd package (42). Heatmaps of the differentially expressed miRNAs were constructed using hclust R and the distance and average Pearson linkage were determined using the gplots R package (43).

Target prediction and functional analysis. Target prediction was performed using the mirDIP interface (44). At least 3 of the 12 available algorithms were selected for prediction. To perform a functional enrichment analysis, all the targets were separated according to up- or downregulation and submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (45). This approach was used to identify significant biological processes in Gene Ontology level 2 that

Table I. Half-maximal inhibitory concentration (IC_{50}) at 24, 48 and 72 h of cediranib exposure in the Hs578T, MDA-MB-231 and T47D cells.

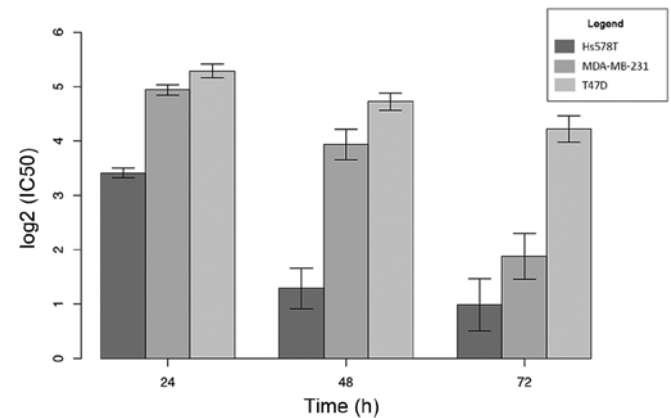| Cell line	IC_{50} (μM)		
	24 h	48 h	72 h
Hs578T	10.66±0.66	2.51±0.62	2.08±0.77
MDA-MB-231	30.77±2.01	15.57±3.03	2.52±0.81
T47D	38.69±2.90	26.54±2.79	18.85±3.21

Figure 1. Half-maximal inhibitory concentration (IC_{50}) in log2 scale in μM at 24, 48 and 72 h of cediranib exposure in the Hs578T, MDA-MB-231 and T47D cells.

may be shared among the targets of the miRNAs of interest. A biological process or pathway was considered significant if it contained a minimum of 3 genes per category that had score values <0.05 after a Benjamini-Hochberg correction. A compilation of the categories according to a GoSlim summarisation was performed using the REVIGO tool (46).

Confirmation by quantitative real-time PCR (qRT-PCR). qRT-PCR using TaqMan miRNA assays (Life Technologies, Foster City, CA, USA) was used to confirm the expression of five miRNAs from the microarray data that were differentially expressed between cells exposed to cediranib compared with the control group. The criterion for selecting the miRNAs for confirmation was the level of expression. All of the samples used in the microarray experiments were performed in technical triplicates for the RT-PCR reactions. A total of 10 ng of RNA was used in the reverse transcription reaction with miRNA-specific primers in an Eppendorf Mastercycler (Eppendorf). The real-time reactions were performed in a 7900 HT Fast Real-time PCR system (Applied Biosystems).

All of the analysis procedures and graphs were constructed using the R environment v.3.0.1. The normalisation step was performed according to the $2^{-\Delta\Delta Ct}$ method (47) using the minimum value of expression of the untreated group as a calibrator. The cycle threshold (Ct) values from the selected miRNA targets were subtracted from the Ct values of the endogenous small noncoding RNA controls RNU44
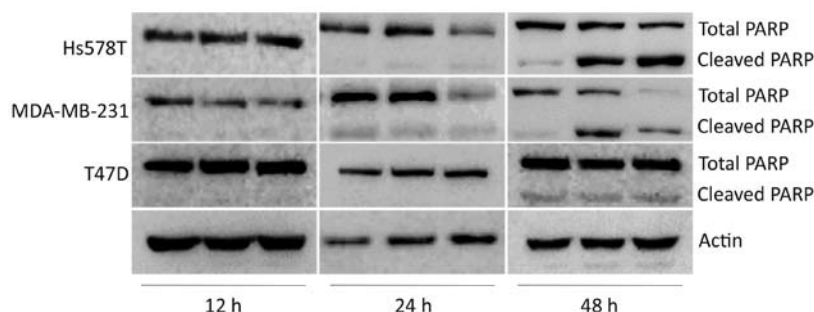
Figure 2. Apoptosis was evaluated by western blot analysis for poly(ADP ribose) polymerase (PARP) cleavage. Breast cancer cell lines were exposed to cediranib (IC_{50} values) at 12, 24 and 48 h.
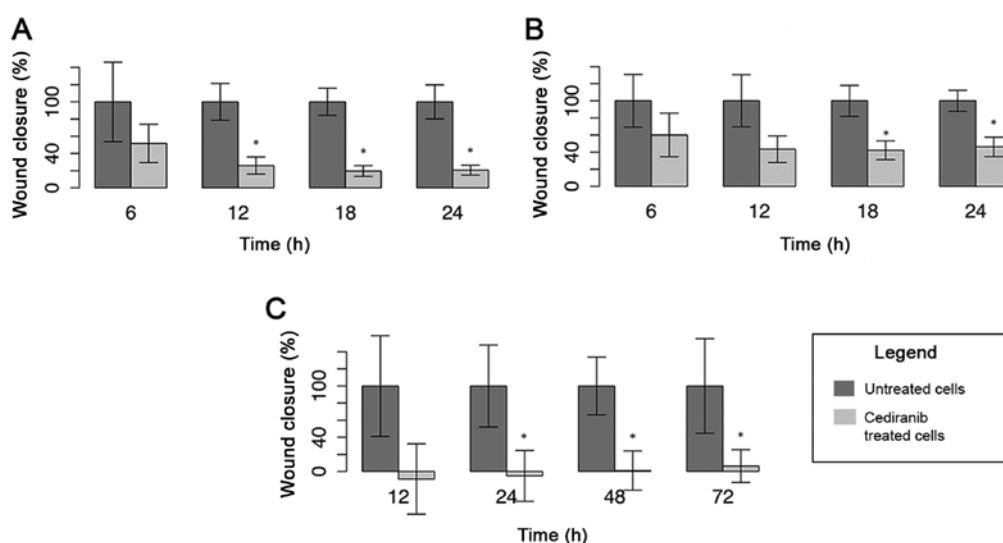

Figure 3. Effect of cediranib on cell migration in the (A) Hs578T cells evaluated from 6 to 24 h, (B) MDA-MB-231 cells from 6 to 24 h and (C) T47D cells from 12 to 72 h. The relative distance of migration was measured with a wound-healing migration assay. The data are represented as the means ± SD, and differences with *P<0.05 according to Student's t-test were considered statistically significant.

and RNU48 (control miRNA assay; Applied Biosystems). The criterion for selecting the best endogenous RNA was the stability of expression according to the NormFinder software (48). Differences in miRNA expression between the treated and non-treated cell lines (Hs578T, MDA-MB-231 and T47D) were evaluated using Mann-Whitney U test, and differences with a P≤0.05 were considered significant.

Results

Following an initial assessment of the basal viability conditions for all the three breast cell lines, a total density of $4x10^3$ cells/well for Hs578T, $6x10^3$ cells/well for MDA-MB-231 and $1x10^4$ cells/well for the T47D cell line was used. The IC_{50} was then determined for the breast cancer cells at 24, 48 and 72 h, as shown in Table I and Fig. 1. Our results showed that the most sensitive breast cancer cell line was Hs578T, followed by MDA-MB-231. The T47D cell line was the most resistant, with an IC_{50} value 4-fold higher than that of the Hs578T cells and almost 2-fold higher than that of the MDA-MB-231 cells after a 24-h treatment.

To determine whether decreased viability was due to cytotoxic effects, we analyzed poly(ADP ribose) poly-

merase (PARP) cleavage. We observed a striking effect on PARP cleavage using low doses (0.5 and 2.0 μM) of cediranib in the Hs578T cell line. Similar effects were demonstrated in the MDA-MB-231 cell line that exhibited higher cleaved PARP levels. In contrast, the T47D cell line showed lower PARP cleaved levels (8 and 18 μM) using a high dose of cediranib drug, representing a resistant profile. Cytotoxic effects demonstrated by cleaved PARP were not found at times less than 24 h for both cell lines (Fig. 2).

In order to assess the functional impact of cediranib in migration, we used the wound-healing migration assay, and observed the inhibition of cell migration in all of the cell lines. After 24 h of cediranib exposure, the Hs578T cells exhibited the most inhibition (80%), followed by the T47D cells (70%) and MDA-MB-231 cells (54%) (Fig. 3). Even the adhesion of this cell type appeared to be affected by the drug (data not shown), leading to negative values, i.e., the wound was extended because of the detachment of these cells. Due to this behaviour, we studied the T47D migration ability for 72 h. Similarly, cediranib treatment significantly inhibited cell invasion in all breast cancer cell lines. The reduction in the percentage of cell invasion at 24 h was ~70% in T47D, 60% in Hs578T and 30% in the MDA-MB-231 cells (Fig. 4).
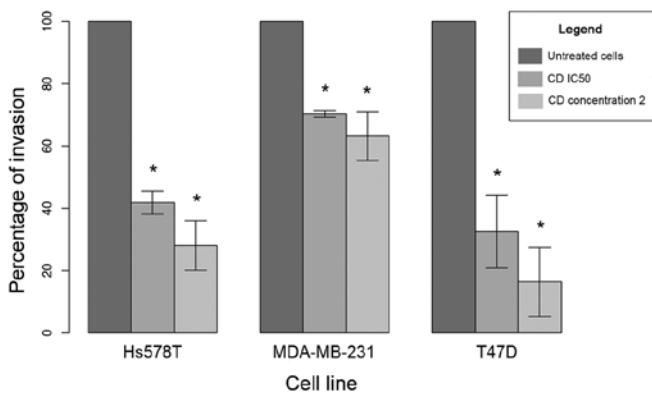
Figure 4. Invasion was assessed using a Matrigel invasion assay in the Hs578T, MDA-MB-231 and T47D cells. The invasion of cells exposed to cediranib (IC_{50} at 72 h) was expressed in relation to the DMSO control (100%). The additional dose (dose 2) represents the average of the IC_{50} doses at 24 and 72 h. The data are represented as the means ± SD, and differences with *$P<0.05$ according to the Student's t-test were considered statistically significant.

To identify the RTKs that are targets of cediranib, we used a phospho-RTK array that assesses the levels of 49 RTKs, in the most sensitive cell line, Hs578T, which was exposed to

2 μM cediranib. Under basal levels, we observed the presence of the active forms of EGFR, Tie-2, RYK, FGFR2α, m-CSFR, c-RET, AXL and ROR2 (RTK-like orphan receptor 2) tyrosine kinase receptors (Fig. 5A). Following cediranib treatment, we found a slight increase in EGFR and Tie-2 phosphorylation levels (Fig. 5A-C) and diminished phosphorylation levels of FGFR2α and ROR2. We did not detect any significant changes in the phosphorylation of the other RTKs after treatment with cediranib (Fig. 5C).

We further addressed the inhibitory effect of cediranib in intracellular pathways, using higher concentrations of the drug for 12 h in both cell lines (Fig. 5D). We observed a decrease in ERK phosphorylation at the concentrations analyzed in the Hs578T cells. However, this cell line showed that the inhibition of AKT seemed to be dose-dependent (2 μM) after cediranib treatment. Both cell lines, HS578T and MDA-MB-231, were exposed to equal cediranib concentrations (0.5 and 2 μM), and only the MDA-MB-231 cell line did not exhibit decreased ERK phosphorylation levels. The absence of AKT phosphorylation was detected in the MDA-MB-231 cell line. The phosphorylation levels of ERK and AKT were unchanged in the T47D cell line after exposure to cediranib (Fig. 5D).

To further identify potential miRNAs in response to cediranib, we performed miRNA expression profiles in each
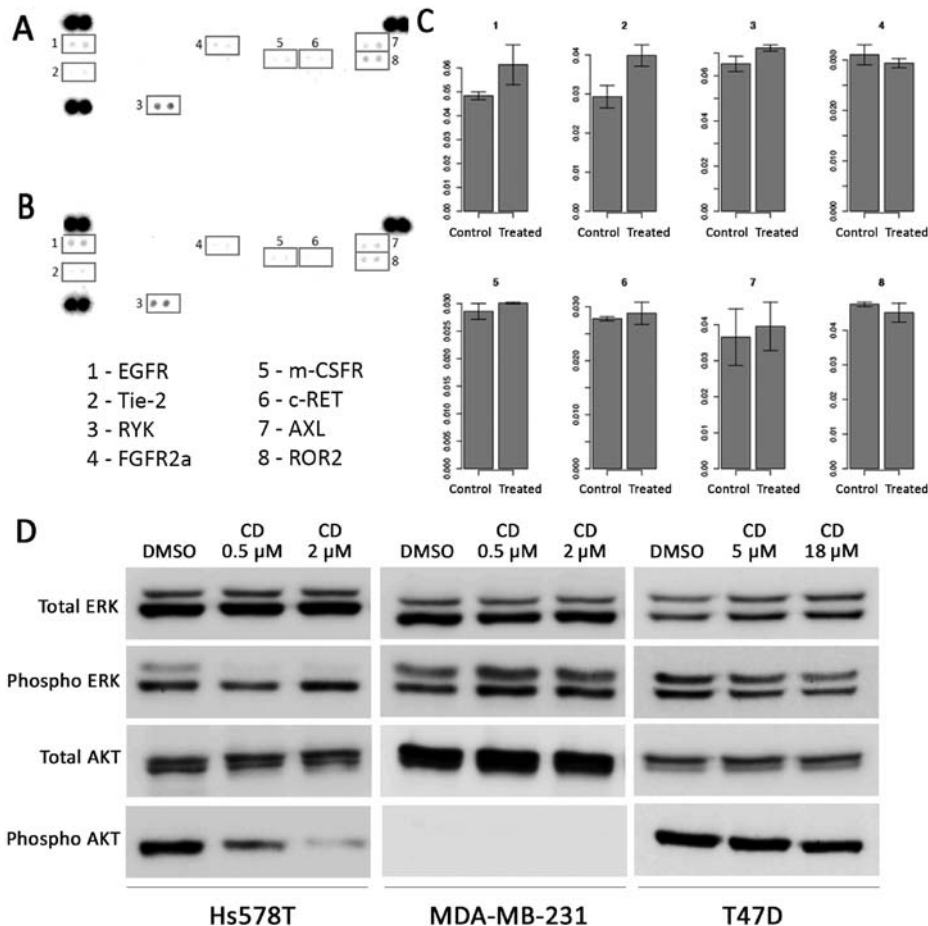

Figure 5. Effects of cediranib on activation of the intracellular pathway and receptor tyrosine kinases (RTKs) in the Hs578T cell line. (A) Profile of the human phospho-RTK array of untreated Hs5678T cell line. (B) Profile of the human phospho-RTK array of the Hs578T cell line treated with 2 μM of cediranib for 4 h. (C) Densitometric analysis of proteins (spots). (D) Activation of the intracellular pathways ERK and AKT. All cell lines were incubated with increasing concentrations of cediranib for 2 h and the phosphorylation levels were detected by western blot analysis.
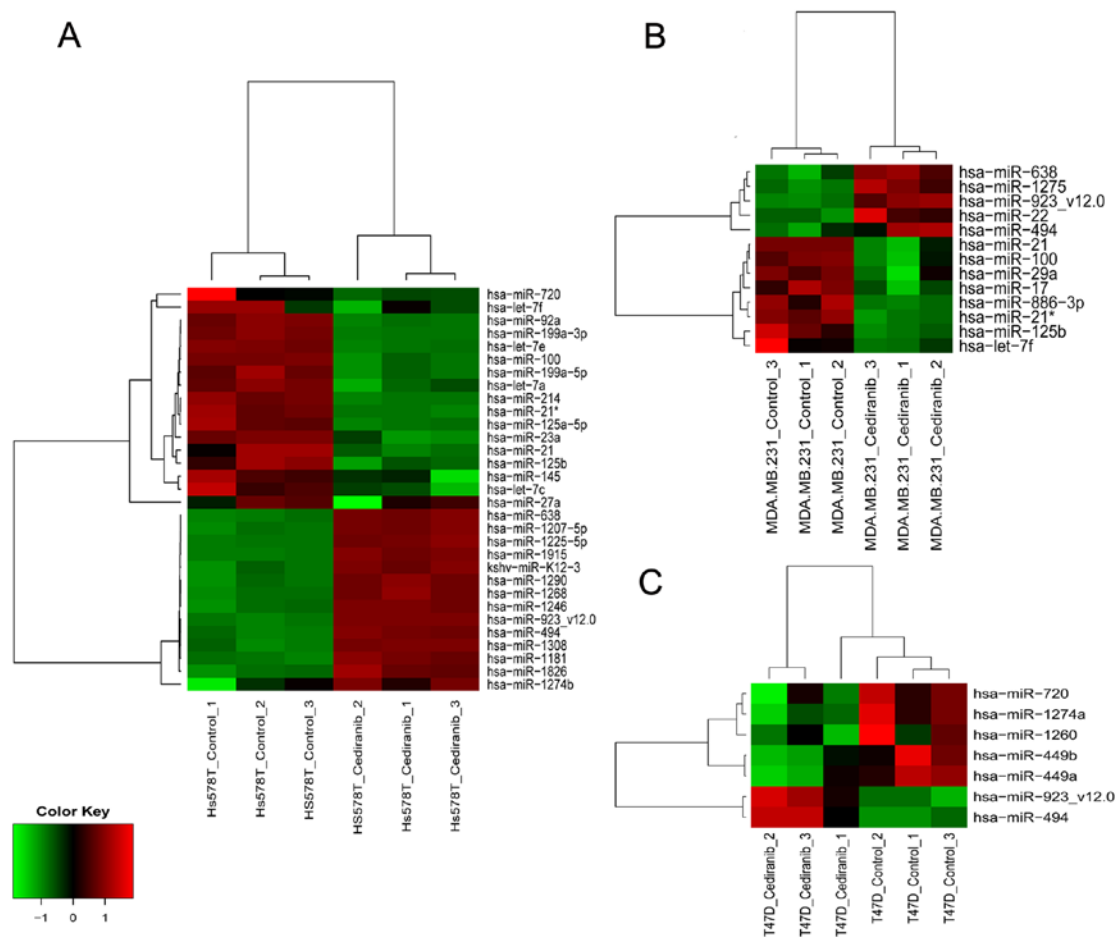
Figure 6. Heatmaps of all of the differentially expressed miRNAs compared with the controls after 24 h of cediranib exposure in the (A) Hs578T, (B) MDA-MB-231 and (C) T47D cells.
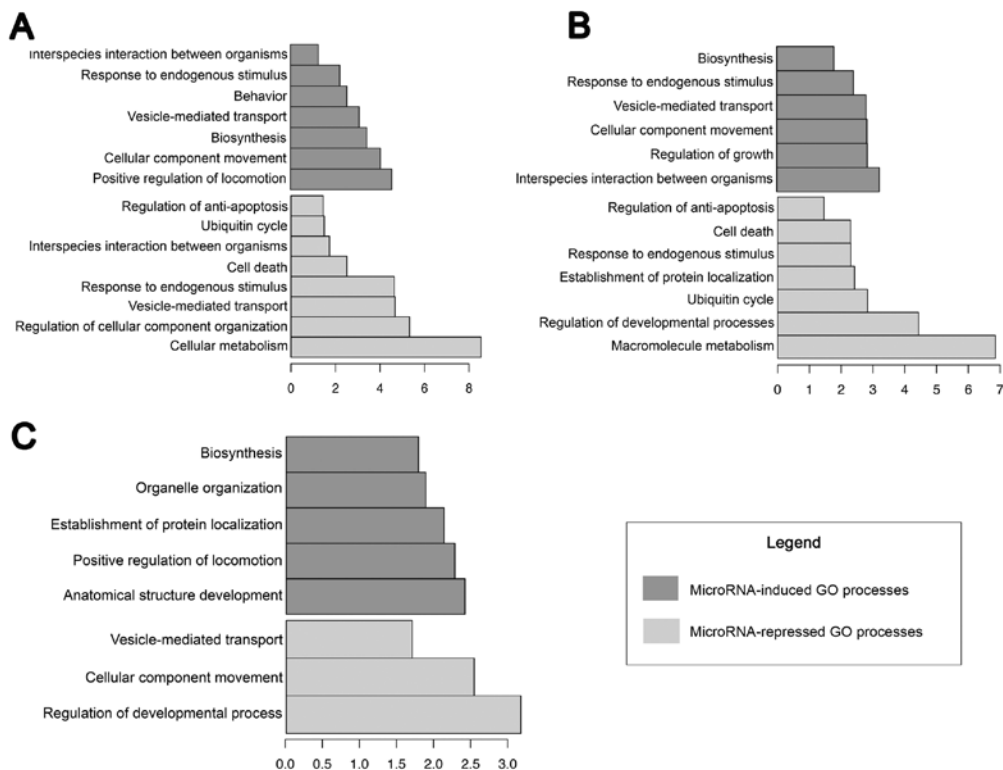

Figure 7. Summary of significant functional categories (Gene Ontology level 2) among the targets of differentially expressed miRNAs in (A) Hs578T, (B) MDA-MB-231 and (C) T47D cells. P-values are represented using a -log10 scale.
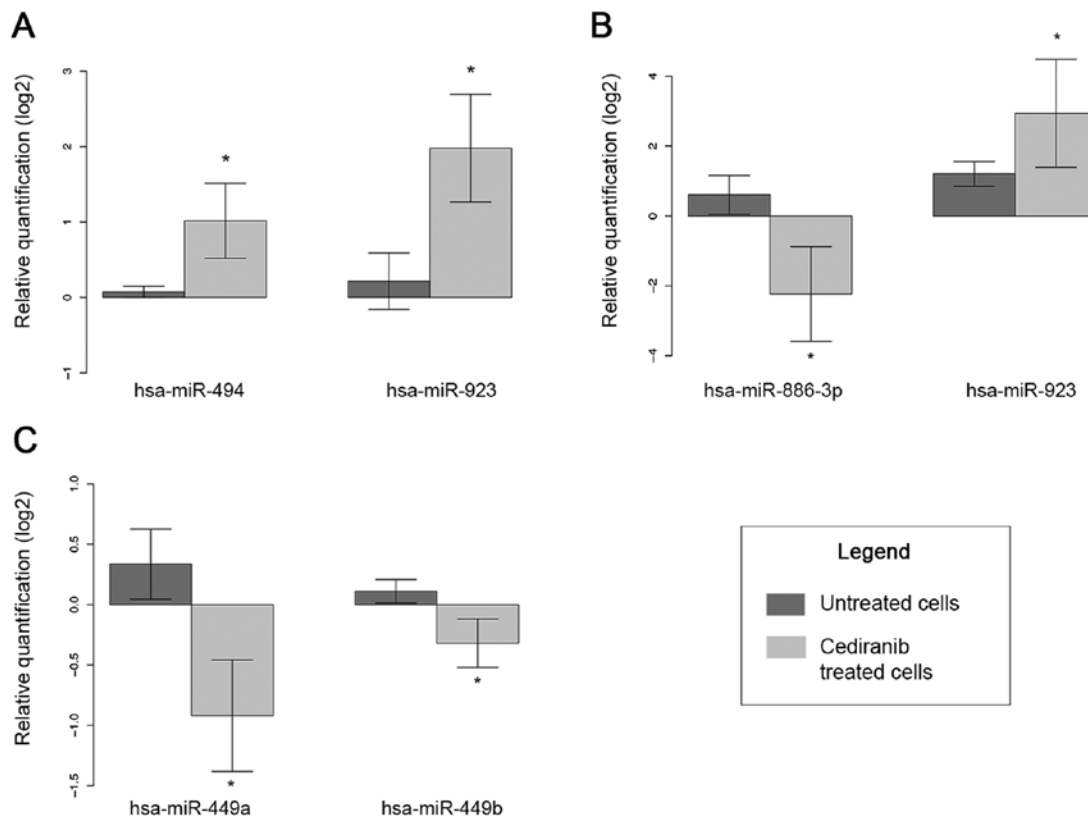
Figure 8. qRT-PCR confirmation of the miRNAs that were differentially expressed according to the microarray analysis in the (A) Hs578T, (B) MDA-MB-231 and (C) T47D cells. The data are represented as the means ± SD, and differences with *P<0.05 according to Student's t-test were considered statistically significant.

breast cancer cell line and compared them with the controls. The results revealed 31 differentially expressed miRNAs in the Hs578T cells, 13 miRNAs in the MDA-MB-231 cells and 7 miRNAs in the T74D cell line (Fig. 6). The targets of these identified miRNAs are summarised according to their biological processes. The Hs578T cell line shared several processes with the MDA-MB-231 cells, including the induction of a response to endogenous/chemical stimuli, biosynthesis, vesicle-mediated transport, the movement of cellular components, the repression of the ubiquitin cycle, and the regulation of anti-apoptosis and cell death (Fig. 7). The T74D cell line presented specific biological processes; only biosynthesis was upregulated in the other cell lines in a similar manner, while vesicle-mediated transport and cellular component movement were repressed (Fig. 7).

Among the miRNAs identified, five miRNAs, miR-494, miR-923, miR-449a, miR-449b and miR-886-3p, were selected for further confirmation of expression due to the greater change in expression and the biological relevance of these miRNAs (Fig. 8). The endogenous miRNA RNU-48 was the most stable in our results, and this miRNA was thus used for all the analyses. The results confirmed the overexpression of miR-494 and miR-923 in the Hs578T cells exposed to cediranib compared to the control cells. In the MDA-MB-231 cells, the overexpression of miR-923 and the decreased expression of miR-886-3p after exposure to cediranib were confirmed. Both miR-449a and miR-449b were downregulated in the T47D cell line after cediranib treatment.

Discussion

Cediranib is a tyrosine kinase inhibitor that represents promise in the treatment of several tumours (6). The main targets of this pharmacological drug are the VEGFR proteins, which regulate blood vessel formation in tumours and are associated with the anti-angiogenic effects of cediranib (4). In the present study, we showed that cediranib exhibited an effect on breast cancer cells, affecting cell migration and invasion, and the molecular processes associated with the miRNA expression altered in response to treatment was also studied.

Regarding the sensitivity to the agent, the IC_{50} value varied according to the cell line used. The most sensitive cell line (Hs578T) had IC_{50} values (~2 μM) similar to those of glioma cells after 72 h of cediranib exposure (10). Notably, PARP is an abundant, chromatin-associated enzyme which responds to DNA damage (49). Our results showed that when exposed to cediranib (according to the IC_{50} values) the cell line Hs578T displayed cleavage of PARP at 48 h of exposure, followed by MDA-MB-231 and T47D cell lines. Based on this result, we demonstrated that the Hs578T cell line had greater sensitivity to cediranib. ERK inhibition was detected only in sensitive cell line Hs578T, showing the efficient inhibition in the conserved RAS-mitogen activated protein kinase (MAPK) signalling pathway which may affect the cellular growth, survival, and differentiation (50). In the MDA-MB-231 and T47D cell lines, the phosphorylation of ERK was not significantly blocked. A recent study showed similar results in another breast cancer

cell line, which presented a resistance profile when exposed to cediranib at 1-10 μM/l (51). The persistent AKT activation in some solid cancers, such as non-small cell lung carcinomas (NSCLCs), is associated with TKI response (52). We observed a total blockade of the AKT phosphorylation in the Hs578T cell line when exposed to different cediranib concentrations. However the MDA-MB-231 cell line showed absence of phosphorylated proteins and T47D displayed no significant changes. Therefore, we may hypothesized that AKT inhibition may be related with cediranib response in breast cancer cell lines.

The proteome profiler of phospho-RTKs showed that although Hs578T cells did not express the described cediranib targets under baseline conditions, such as VGFRs, KIT or PDGFRA, this cell line exhibited other important RTKs, such as EGFR, Tie-2, RYK, FGFR2α, m-CSFR, c-RET, AXL and ROR2, and upon cediranib exposure we observed reduction of the ROR2 and FGFR2α RTKs. ROR2 is a novel Wnt receptor recently discovered and is associated with progression of solid tumours such as melanoma, osteosarcoma and prostate cancer (53). An immunohistochemistry study conducted in patients with breast cancer showed ROR2 overexpression in 87% of the cases, and its association with a worse outcome (54). Further studies are needed to validate our findings, and more importantly to determine whether the expression levels of ROR2 and FGFR2α in human breast cancer tissue may predict the response to cediranib.

Regarding the expression of miRNAs, the Hs578T cell line presented a greater number of modulated miRNAs than the MDA-MB-231 and T47D cell lines, as an indicator of sensitivity to the drug. Although the Hs578T cell line presented 22 specific miRNAs, the most significant biological processes, such as biological regulation, development, metabolic processes, cell motility and homeostasis, were related to targets that were shared with the MDA-MB-231 cell line, including *VEGFA*, *VEGFC*, and *PDGFRA*. Several studies support the roles of these molecules in these processes, especially roles in tissue metabolism and homeostasis in breast cancer (55,56).

Of the targets shared between the Hs578T and MDA-MB-231 cell lines, miR-923 upregulation was observed. Moreover, few miRNAs were upregulated in our findings. miR-923 dysregulation was highly associated with drug resistance. The upregulation of this miRNA was also observed in breast cancer cells that are resistant to Taxol (57). In the same study, miR-125b was upregulated, which was associated with Bcl-2 antagonist killer 1 (Bak1) expression. In our findings, miR-125b was also modulated but was downregulated. The interaction between specific targets may further be explored. miR-923 and miR-886-3p, which were also validated in the present study, were shown to be involved in cisplatin resistance in bladder cancer; changes in expression were also correlated with survival (58). The upregulation of miR-923 was also associated with multidrug resistance in the HEp-2 cell line (59).

Among the specific miRNAs found in the MDA-MB-231 cell line, miR-886 has been recently proposed as a vault RNA (vtRNA2-1), which may form a complex that is implicated in cancer drug resistance. Recently, Lee *et al* (60) identified pre-miR-886 as a 102-nt, abundant cytoplasmic RNA that is neither a pre-miRNA nor a vault RNA but is a noncoding molecule (nc886). Some evidence indicates that nc886 in an immature state is physically associated with PKR (protein kinase RNA-activated), a double-stranded RNA-dependent kinase (61).

The involvement of miR-886 in the drug response and the modulation of this molecule by cediranib in the sensitive cell line MDA-MB-231 supports evidence that some mechanisms related to apoptosis could be activated in this context and affect the sensitivity of these cells, as observed in the present study. In addition, miR-886-3p repression was recently described as being mediated by methylation processes in lung cancer cell lines (62) and can affect cell proliferation, migration, and invasion in lung and non-medullary thyroid cancer (63,64). This fact is in concordance with our findings because we determined that migration and invasion processes were highly affected by cediranib in MDA-MB-231 cells.

In contrast to the other cell lines studied, few miRNAs were modulated in the T47D cell line. The T47D-specific miRNAs miR-449a and miR-449b were highly downregulated, and these results were confirmed via qRT-PCR. The miRNA miR-449 has been identified in a wide range of tumours (65-69) and has several identified targets, including *c-Myc* and *c-Met* (66,67). This miRNA induces cell cycle progression and apoptosis by regulating CDK6 and CDC25A, direct targets that lead to the phosphorylation of E2F1 (70-72). In contrast to the Hs578T and MDA-MB-231 cells, no target associated with *VEGFR* or *PDGFRA* was identified in the T47D cells with the target prediction. Considering the difference in the T47D resistance to cediranib, this finding suggests that other RTK targets are possibly affected by cediranib, in addition to VEGFR, the most potent RTK affected, and these other targets may activate other downstream pathways that affect the invasion and migration processes, instead of cell death. Further studies are necessary to confirm this hypothesis.

The crosstalk of the EGFR pathways and certain miRNAs has been described; for example, *EGFR* mutations can regulate miR-21 in lung cancer and glioblastoma cell lines (73). Similarly, miR-145 was found to inhibit cell proliferation by targeting EGFR in lung adenocarcinoma and in a model of colon cancer (74,75). In the present study, both miRNAs were modulated in the Hs578T cells, and only miR-21 was downregulated in MDA-MB-231 cells. However, some different processes seem to occur in the T47D cells. Considering that miR-449 (specific to T47D) can also modulate *EGFR*, the ambiguities of this process should be further explored.

The present study assessed for the first time the antineoplastic effects of cediranib in breast cancer cell lines. We observed a distinct degree of response in all the cell lines. Cediranib impaired mechanisms leading to cancer cell invasion and migration. The present study revealed an anti-neoplastic role of cediranib in human breast cancer cell lines. Importantly, we identified ROR2 and FGFR2α as novel RTK targets of cediranib. The miRNA expression profiling revealed the effect of this drug on several miRNAs involved in several molecular processes. Finally, more studies are necessary to validate these miRNA signatures as indicators of the therapeutic response in human breast cancer, and these signatures may lead to the selection of patients who would be most effectively treated with cediranib.

Acknowledgements

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