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Underexpression of miR-224 in methotrexate resistant human colon cancer cells

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs involved in RNA silencing that play a role in many biological processes. They are involved in the development of many diseases, including cancer. Extensive experimental data show that they play a role in the pathogenesis of cancer as well as the development of drug resistance during treatments. The aim of this work was to detect differentially expressed miRNAs in MTX-resistant cells. Thus, miRNA microarrays of sensitive and MTX-resistant HT29 colon cancer cells were performed. The results were analyzed using the GeneSpring GX11.5 software. Differentially expressed miRNAs in resistant cells were identified and miR-224, which was one of the most differentially expressed miRNAs and with high raw signal values, was selected for further studies. The underexpression of miR-224 was also observed in CaCo-2 and K562 cells resistant to MTX. Putative targets were predicted using TargetScan 5.1 software and integrated with the data from expression microarrays previously performed. This approach allowed us to identify miR-224 targets that were differentially expressed more than 2-fold in resistant cells. Among them CDS2, DCP2, HSPC159, MYST3 and SLC4A4 were validated at the mRNA level by qRT-PCR. Functional assays using an anti-miR against miR-224 desensitized the cells towards MTX, mimicking the resistant phenotype. On the other hand, siRNA treatment against SLC4A4 or incubation of Poly Purine Reverse Hoogsteen (PPRH) hairpins against CDS2 or HSPC159 increased sensitivity to MTX. These results revealed a role for miR-224 and its targets in MTX resistance in HT29 colon cancer cells.

1. Introduction

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related deaths (610 000 worldwide per year) among adults in the Western world [1]. Therefore, new effective targets and better approaches are still needed.

The antimetabolite and antifolate methotrexate (MTX) is used alone or in combination for the treatment of many cancer types and autoimmune diseases [2]. Nowadays, the efficiency of combinational therapy using MTX is being tested in clinical trials on a wide range of cancer types, including colorectal carcinoma [2].

Drug resistance is usually observed upon treatment with MTX. Combination treatments of MTX with other drugs that could modulate the expression of genes involved in MTX resistance would be an adequate strategy to prevent the development of resistance. In this direction, we have previously identified and validated genes associated to MTX resistance such as *AKR1C1*, *UGT1A6*, *DKK1*, *E-Cadherin*, *Caveolin 1* and *S100A4* [3–6].

MicroRNAs (miRNAs) are a new class of small, non-protein-encoding RNAs [7,8]. Imperfect base pairing between miRNAs and their target mRNAs leads to repression of translation and/or deadenylation, followed by destabilization of the target [9]. Many cellular pathways are regulated by miRNAs such as development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction, organ development, host–viral interactions and tumorigenesis [10]. MiRNAs play key roles in the pathogenesis of cancer. Many miRNAs have been shown to function as either oncomirs or tumor suppressors [11–17] (for review see [18]). Furthermore, miRNAs have an important role in the development of chemosensitivity or chemoresistance in different types of cancer (summarized in [19]). Blower et al. [20] studied miRNA expression patterns in the NCI-60 cancer cell line panel and assessed their potential role in chemotherapy response. These authors demonstrated that changes in let-7i, miR-16 and miR-21 expression levels affected the potencies of several anticancer drugs.

Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; miR and miRNA, microRNA; CRC, colorectal carcinoma; PDAC, pancreatic ductal adenocarcinoma; APRT, adenine phosphoribosyltransferase; CDS2, CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2; DCP2, DCP2 decapping enzyme homolog; HSPC159, galectin-related protein; MYST3, MYST histone acetyltransferase (monocytic leukemia) 3; SLC4A4, Na/bicarbonate cotransporter 1; ENO2, enolase 2; CLU, clusterin; PDCD4, programmed cell death 4; S100A4, S100 calcium binding protein A4; TOP1, topoisomerase1; TERT, telomerase reverse transcriptase; XRCC4, X-ray repair complementing defective repair in Chinese hamster cells 4; HAPLN1, hyaluronan and proteoglycan link protein 1.

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Regarding colon cancer, miRNA expression is strongly implicated in tumorigenesis and progression [21]. Many studies showed that miR-15b, miR-21, miR-181b, miR-191, miR-200c, miR-17 and miR-92 may play a role in CRC development and progression and they could be considered good markers for the prognosis [22,23] and diagnosis of non-invasive CRC [24]. With respect to drug resistance, specific miRNA changes, such as underexpression of let-7b and let-7e and upregulation of miR-17*, were observed in HCT116 colon cancer cells irresponsive to cetuximab, due to mutated k-ras, upon treatment with this drug [25].

In the present work, miRNA microarrays were performed with the aim to identify relevant miRNAs affecting the sensitivity of cells to MTX treatment to provide a more comprehensive study into the insights of drug resistance. We found that MTX-resistant colon cancer cells have different miRNA expression patterns when compared to their sensitive counterparts. miR-224 was very underexpressed in MTX-resistant cells. Functional validation assays revealed the role of miR-224 and its targets in MTX-resistance.

2. Materials and methods

2.1. Cell culture

Human colon cancer cells (HT29) were routinely maintained in Ham's F12 medium (GIBCO, Barcelona, Spain) supplemented with 7% fetal bovine serum (FBS, GIBCO, Barcelona, Spain) at 37 °C in a 5% CO_2 humidified atmosphere. Resistant cells to 10^{-5} M methotrexate (MTX, Almirall, Barcelona, Spain) were previously generated in the laboratory [4] and were incubated in selective dihydrofolate reductase (DHFR) medium (–GHT medium, GIBCO, Barcelona, Spain) lacking glycine, hypoxanthine and thymidine, the final products of DHFR activity, supplemented with 7% dialyzed FBS. At the time of performing all experimental procedures, microarrays included (miRNA and expression), both sensitive and resistant cells were cultured in –GHT selective medium (7% FBS) exclusively, since HT29 sensitive cells grow perfectly in –GHT medium.

2.2. Microarrays

Total RNA for microarray analyses was prepared from triplicate of sensitive and resistant cell lines. For gene expression analyses, GeneChip® Human Genome U133 PLUS 2.0 microarrays from Affymetrix containing over 54 000 transcripts and variants were used (GEO series accession number GSE11440) [5]. MiRNA expression was analyzed in RNA samples obtained by Real Total microRNA kit (Real, Valencia, Spain) using Human miRNA Microarray Kit (V2) from Agilent (Madrid Spain) containing probes for 723 human and 76 viral microRNAs from the Sanger database v 10.1 (GEO Series number GSE28547). Labeling, hybridization and detection were carried out in each case following the recommendations of the manufacturer.

2.3. Microarray data analyses

Data files from miRNA and mRNA microarrays were analyzed with GeneSpring GX11.5 software (Agilent Technologies, Madrid, Spain) to find differentially expressed miRNAs and their cellular target genes in the resistant cell lines compared to their sensitive counterparts. Triplicate samples for each condition, sensitive and resistant, for both miRNA and mRNA, were imported into one single experiment. Average values of the replicate spots of each mRNA and miRNA were background subtracted, and normalized against the median of the control samples. Gene and miRNA expression was calculated as the ratio of the normalized values obtained for the resistant and sensitive conditions, after normalization of the data. Differentially expressed miRNAs which expression was within the 75–100th percentile, with a p-value of less than 0.05 and a fold change (FC) ≥ 2 were selected (Volcano plot). Multiple testing correction was applied (Benjamini–Hochberg *false discovery rate*, FDR). Then, putative targets were identified using TargetScan 5.1 software. This TargetScan list was merged with a list of differentially expressed cellular genes (FC ≥ 2, $p < 0.05$, Benjamini–Hochberg FDR) using Venn diagrams, to find those differentially expressed genes within the putative targets of the chosen miRNA differentially expressed in MTX-resistant cells.

2.4. RT-real time PCR

2.4.1. MicroRNA expression levels

Expression levels of selected miRNAs were confirmed by real time miRNA reverse transcription-PCR (RT-PCR) analysis using TaqMan MicroRNA Assay Kit (Applied Biosystems, Barcelona, Spain) following the manufacturer's protocol. Total RNA was extracted using Ultraspec™ (Biotecx, Houston, Texas), according to the recommendations of the manufacturer. Specific RT and real time primers for the mature sequence of miR-224 were used (Assay ID 002099 from Applied Biosystems, Barcelona, Spain). MicroRNA expression levels were quantified with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Barcelona, Spain). Relative quantification was measured using the $2^{-\Delta\Delta C_t}$ method and normalized against RNU6B (Assay ID 001093 from Applied Biosystems, Barcelona, Spain) in each sample.

2.4.2. Gene expression levels

Total RNA was extracted using Ultraspec™ (Biotecx) in accordance with the manufacturer's protocol. Each RT reaction (final volume 20 μl) was performed mixing 1 μg of total RNA, 125 ng of Random Hexamers (Roche, Mannheim, Germany), in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units of RNAsin (Promega Biotech Ibérica, Madrid, Spain), 0.5 mM dNTPs (AppliChem, Ecogen, Barcelona, Spain), 200 units of M-MLV reverse transcriptase (Invitrogen, Barcelona, Spain) and 50 mM Tris–HCl buffer, pH 8.3. The reaction mixture was incubated at 37 °C for 60 min. Real time-PCR was used for subsequent amplification of cDNA using 3 μl of RT product. Gene expression levels were quantified with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Barcelona, Spain). Relative quantification was measured using the $2^{-\Delta\Delta C_t}$ method and normalized against APRT in each sample. All primers used are listed in Table 1.

2.5. Transfection of anti-miR and PPRH-hairpins

Inhibition of miR-224 was carried out using an antisense oligodeoxynucleotide (aODN) bearing the phosphothioate modification (at the 3′ end of the bases) directed against the mature miRNA sequence (anti-miR-224). Two kind of negative controls were used: (i) anti-miRs bearing either 7 mismatches corresponding to the seed sequence of miR-224 (anti-miR-7MIS) or 13

Table 1
Primers sequences.

Gene	Sequence FOR (5′ to 3′)	Sequence REV (5′ to 3′)
APRT	GCAGCTGGTTGAGCAGCGGAT	AGAGTGGGGCCTGGCAGCTTC
CDS2	GTTGCGCCACCCGAGGACAA	AGACTGGCAGGGGTGCGGAT
DCP2	CCTTCTCGTCTCCGTTGGAGTCG	CCGGGAATCTCCACCCGTTTGG
HSPC 159	CGGCAAGCACCTTCGCCCTC	TCGGCCACTGATCCCGCCAT
MYST3	GTTTGGGGCATCTCCGCGGT	TCCGCAATCTCTGCTCACCAGTC
SLC4A4	GCCCGGAGCTCCACTTTCC	GTGGCCACATGGGGCTTCC

List of sequences of all primers used for mRNA determination. Primers were designed using the Primer-Blast tool.

Table 2
PPRHs, anti-miR and siRNA sequences.

	Sequence (5′ to 3′)
anti-miR7-MIS	A*A*C*GGAAC*CAC*T*ACAC*T*G*A*A*G
anti-miR13-MIS	T*T*G*CCT AC*CAC*T*ACAC*T*G*A*A*G
anti-miR-NR	T*C*AC*AGGT*T*AAAGGGT*C*T*CAG*G*G*A
anti-miR-224	A*A*C*GGAAC*C*AC*T*AGT*GAC*T*T*G
hpSC1	AAGAGAAAAAGAGAAAGAAGAGAGGGTTTTTGGGAGAGAAGAAAGAGAAAAAGAGAA
hpSC2	AGAGAAGAGGAAGAGAGGAAAGAGAGGAAGAGGATTTTTAGGAGAAGGAGAGAAAGGAGAGAAAGAAGAGA
hpMYST3	GGAGAGGGAAGGGAAAGAGAGAGTTTTTGAGAGAGAAAGGGAAGGGAGAGG
hpDCP2	GAAGGAGAGAGAAGGGAAGGGGTTTTTGGGGAAGGGAAGAGAGAGGAAG
hpHSPC159	GGGAGGAGGGGAGGGGAGGGAGGTTTTTGGAGGGAGGGGAGGGGAGGAGGG
hpCDS2	GGAAAGAGAGAGGAAGAAGGAGGGAAGTTTTT GAAGGGAGGAAGAAGGAGAGAGAAAGG
siNR	UAAGGCUAUGAAGAGAUACUU
siSLC4A4	AGUGGGCUACAACACUCUCUU

Sequences of inhibitory molecules used including negative controls. Phosphorotioate-modified aODNs were designed according to the minimal modification criteria [58] and are indicated as *.

mismatches (anti-miR-13MIS); and (ii) a non-related anti-miR against the mature sequence of miRNA-125a-5p (anti-miR-NR) (Table 2).

Transfection was performed as follows. For viability assays HT29 cells (30 000) were plated 6 h prior to transfection. aODNs were lipofected using DOTAP (Roche, Mannheim, Germany) in a molar ratio 1:10 (ODN:DOTAP). MTX was added 24 h later and cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, Madrid, Spain) assay 6 days later [26]. Absorbance was measured at 570 nm and survival expressed as a percentage of the control in each case.

Inhibition of miR224 targets was performed with PPRH-hairpins [27] (hpCDS2, hpHSPC159 and hpMYST3) which were transfected at a 1:100 molar ratio hairpin:DOTAP. Two negative controls were used, PPRH-scrambled 1 and 2 (hpSC1 and hpSC2) (Table 2). For viability assays cells were plated the night before transfection and MTX was added 48 h later. Cell survival was determined 6 days later using the MTT assay.

For RNA determination, HT29 cells were plated and transfected after overnight incubation. Transfection was performed as described above and RNA extracted 48 h later.

All DNA-oligonucleotides (aODNs and PPRHs) were purchased from Sigma–Aldrich (Madrid, Spain).

2.6. Transfection of siRNAs

siSLC4A4 (ThermoFisher Scientific, Barcelona, Spain) was transfected at a concentration of 100 nM (except noted otherwise) using Metafectene®PRO (Biontex, Martinsried/Planegg, Germany) following the specifications of the manufacturer. Total RNA was obtained 48 h after transfection of siSLC4A4, and mRNA levels were determined as described in Section 2.4.2. For viability assays cells were treated with MTX 48 h after transfection for 6 days. A siRNA against a non-related gene (Luciferase) was used as a negative control (siNR) (ThermoFisher Scientific, Barcelona, Spain) (Table 2).

2.7. Binding assays

For binding assays, 20-mer RNA sequences for each 3′UTR corresponding to CDS2, HSPC159 or SLC4A4, containing the target site for miR-224 were used. The sequence corresponding to the mature form of miR-224 was used in the binding analyses. Radiolabelled probes (20 000 cpm [^{32}P]) were incubated for 30 min at 37 °C with increasing concentrations of miR-224 in the presence of 10 mM $MgCl_2$, 100 mM NaCl, 50 mM HEPES pH 7.2 (AppliChem, Ecogen, Barcelona, Spain) and 5% glycerol (Sigma–Aldrich, Madrid, Spain). Binding reactions were run in a 12% polyacrylamide gel (10 mM $MgCl_2$, 50 mM HEPES pH 7.2 and 5% glycerol) for 4 h at 190 V (4 °C). Gels were exposed to europium plates and scanned in a Storm 840 scanner (Molecular Dynamics, GE Healthcare, Barcelona, Spain) 18 h later. As negative controls 3′UTR sequences for each target gene bearing 6 mismatches in the target site for miR-224 were used (3′UTR-MM). All RNA oligonucleotides were purchased from Sigma–Aldrich (Madrid, Spain) and their sequences are listed in Fig. 7.

2.8. Apoptosis assay

Effect of miR-224 inhibition on apoptosis was determined by flow cytometry using rhodamine 123/propidium iodide (PI) (Sigma–Aldrich, Madrid, Spain). HT29 cells (60 000) were plated in 1 ml –GHT medium and transfected with 1 μM anti-miR-224 18 h later. MTX was added 24 h later and apoptosis determined 6 h upon MTX treatment. Cells were incubated with rhodamine 123 for 30 min. The supernatant was collected, and added to harvested cells and centrifuged together for 5 min at 800 × *g* at 4 °C. The pellet was washed with 1 ml of cold PBS-1% BSA and centrifuged for 5 min at 800 × *g* (4 °C). The supernatant was discarded and cells resuspended in 500 μl cold PBS-1% BSA in the presence of 5 ng/μl of PI.

2.9. Statistical analysis

Data are presented as the mean ± S.E.M. Statistical analysis was performed using Student's *t*-test using PASW Statistics v18.0.0 software for Mac OS X. Differences were considered significant if $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

3. Results

3.1. Underexpression of miR-224 in MTX-resistant cells

MiRNA microarrays were performed with the aim to find differentially expressed miRNAs in HT29 resistant cells compared to their sensitive counterparts. Microarray data were obtained using the Feature Extraction software and further analyzed with GeneSpring GX11.5 as described in Section 2. Fig. 1A shows a profile plot of all miRNAs which expression was within 75th percentile (126 out of 723 miRNAs, Fig. 1A). These miRNAs were used for a Volcano plot analysis (Fig. 1B) where a filtering of $p < 0.05$ and a fold change ≥2, including multiple testing correction was applied. As a result, 10 miRNAs fulfilled these criteria (Table 3).

We selected miR-224 as our target to study, as it was one of the most differentially expressed miRNAs, with higher raw signal values and the only underexpressed among the miRNAs in Table 3.

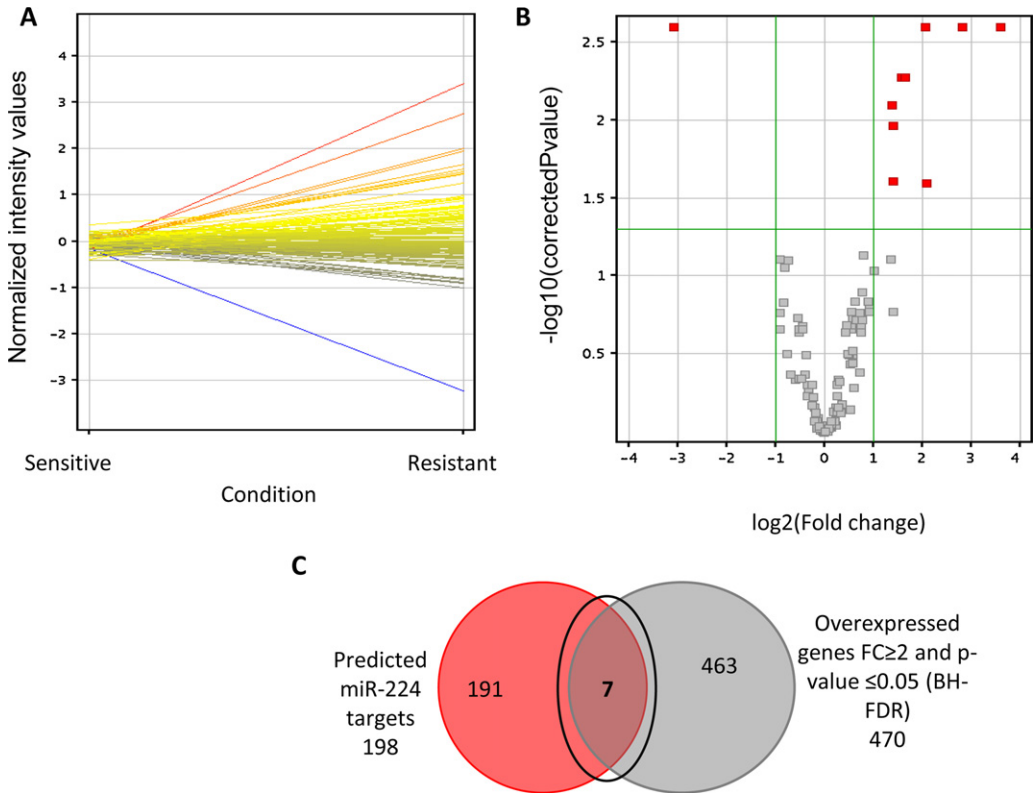

Fig. 1. Microarray analysis. (A) Profile plot image of miRNAs with expression raw signal values within the 75–100th percentile in HT29 sensitive and resistant cells (126 out of 723 miRNAs). Normalized intensity values for each miRNA in both conditions, sensitive (left) and resistant (right), are shown. (B) Volcano plot representation of differentially expressed miRNAs in HT29 resistant cells with a FC ≥ 2, p-value <0.05 (Benjamini–Hochberg FDR). The 126 miRNAs initially filtered on expression were analyzed by Volcano plot filtering. The image shows the corrected p-value (y axis) and the fold change (x axis) (both in log scale) for each miRNA represented by single squares. Those miRNAs that appear significantly overexpressed are found in the upper right corner, and those that are significantly underexpressed are found in the upper left corner. (C) Venn diagram representation of the combination of miRNA profiling with previous transcriptomic studies in HT29 cells with the aim to find miRNA putative targets. miR-224 predicted targets using the TargetScan software are represented in the left circle. The right circle shows those overexpressed genes (FC ≥ 2, p-value <0.05 and Benjamini–Hochberg FDR) in HT29 resistant cells. The intersection of both circles corresponds to those miR-224 predicted targets that were overexpressed in HT29 MTX-resistant cells (listed in Table 4).

miR-224 was also underexpressed in other MTX-resistant cell lines (CaCo-2 and K562). Additionally, in breast cancer cells (MCF-7 and MDA-MB-468) and pancreatic carcinoma cells (MiaPaca-2) down-regulation of miR-224 was also observed, although the basal levels were very low (data not shown).

Underexpression of miR-224 was confirmed by qRT-PCR in HT29 (Fig. 2), K562 and CaCo-2 cells (with a fold change of −11.6, −2.6 and −1.6, respectively).

Table 3
Differentially expressed miRNAs in MTX-resistant cells.

miRNA	Ratio	Raw values	
		Sensitive	Resistant
miR-149	7.06	81.60	395.73
miR-193b	4.22	515.18	1535.77
miR-210	12.02	73.54	583.74
miR-224	−8.47	1526.97	112.33
miR-27b	2.95	492.61	987.12
miR-320	2.65	309.36	547.94
miR-361-5p	2.60	237.71	416.95
miR-365	4.2	512.11	1351.27
miR-455-3p	2.65	666.0	1152.56
miR-615-3p	3.14	20.80	43.12

Differentially expressed miRNAs by more than 2-fold, t-test p-value <0.05 and Benjamini–Hochberg FDR. The ratio column corresponds to the fold change in expression for each miRNA relative to the control, calculated from the normalized values. The mean of the raw values in sensitive and resistant cells obtained in the microarray are shown.

3.2. Underexpression of miR-224 decreases MTX chemosensitivity

Since miR-224 was particularly underexpressed in MTX-resistant HT29 cells, we assessed the possible role for this miRNA associated to MTX resistance. To do so, miR-224 levels were decreased using an anti-miR-224 in HT29 sensitive cells and the resulting viability upon incubation with MTX was determined.

As it can be observed in Fig. 3A, miR-224 levels were effectively decreased upon transfection with the anti-miR-224 at all concentrations tested, achieving in the case of 1 μM anti-miR-224, levels of miR-224 similar to those present in the resistant cells. Two negative controls were used (anti-miR-NR and anti-miR-13MIS) which did not affect the levels of miR-224 (Fig. 3A).

Cells treated with anti-miR-224 plus MTX displayed a marked decrease in sensitivity against MTX when compared to the treatment with the chemotherapeutic agent alone (Fig. 3B). This effect was dose dependent (supplementary Fig. 1). Anti-miR-7MIS and anti-miR-13MIS were used as negative controls. As shown in Fig. 3B, they did not affect the viability of cells, either alone or in combination with MTX. These results indicate that deregulation of miR-224 contributes to MTX resistance in HT29 colon cancer cells. The effect of miR-224 on apoptosis was also evaluated. miR-224 inhibition led to a decrease of 50% in the levels of apoptosis determined in the presence of 100 nM MTX.

To investigate the relationship between the reduced expression of miR-224 in CaCo-2 and K562 cells, we determined the sensitivity

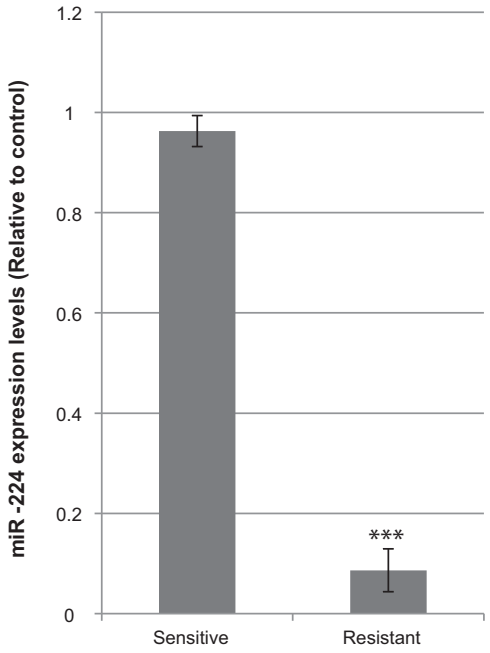

Fig. 2. Validation of miR-224 downregulation. Levels of miR-224 were determined in HT29 sensitive and resistant to 10^{-5} M MTX cells by quantitative RT-PCR as described in Section 2. Results are shown as the mean of the ratio compared to the control ± S.E.M. for triplicate samples. $^{***}p < 0.001$.

to MTX upon miR-224 inhibition by anti-miR-224 in those cells. The decreased levels of miR-224 in the two cell lines led to a decrease in sensitivity towards 50 nM MTX of 25 and 42% for CaCo-2 and K562 cells, respectively.

3.3. miR-224 differentially expressed targets

TargetScan software predicted 198 putative target genes for miR-224, 10 of which were, in turn, differentially expressed in resistant cells (FC > 2, $p < 0.05$; Benjamini–Hochberg FDR). Since miR-224 is underexpressed in the resistant cells, loss of its regulatory effect would allow its target mRNAs to increase. Therefore, only upregulated genes among all predicted putative targets for miR-224 were selected for further analyses. Table 4 summarizes the seven overexpressed putative targets for miR-224 found using a Venn diagram (Fig. 1C).

As can be observed in Fig. 4A, overexpression of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 in HT29 resistant cells was confirmed by qRT-PCR as described in Section 2. Supplementary Table 1 summarizes the potential targets for the overexpressed miRNAs listed in Table 3.

3.4. mRNA levels of predicted miR-224 targets are increased upon inhibition of miR-224

To further analyze the mechanism by which miR-224 was modulating the response to MTX, cells were transfected with anti-miR-224 and the mRNA levels of its putative targets were determined.

Transfection with 1 μM anti-miR-224 significantly increased the mRNA levels of its predicted targets SLC4A4, MYST3, HSPC159 and CDS2; with the exception of DCP2 (Fig. 4B). The expression levels of other genes not predicted as putative targets for miR-224 such as S100A4, ENO2, XRCC4, HAPLN1 and CLU were also determined. Those levels were not significantly changed upon anti-miR-224 transfection (Table 5).

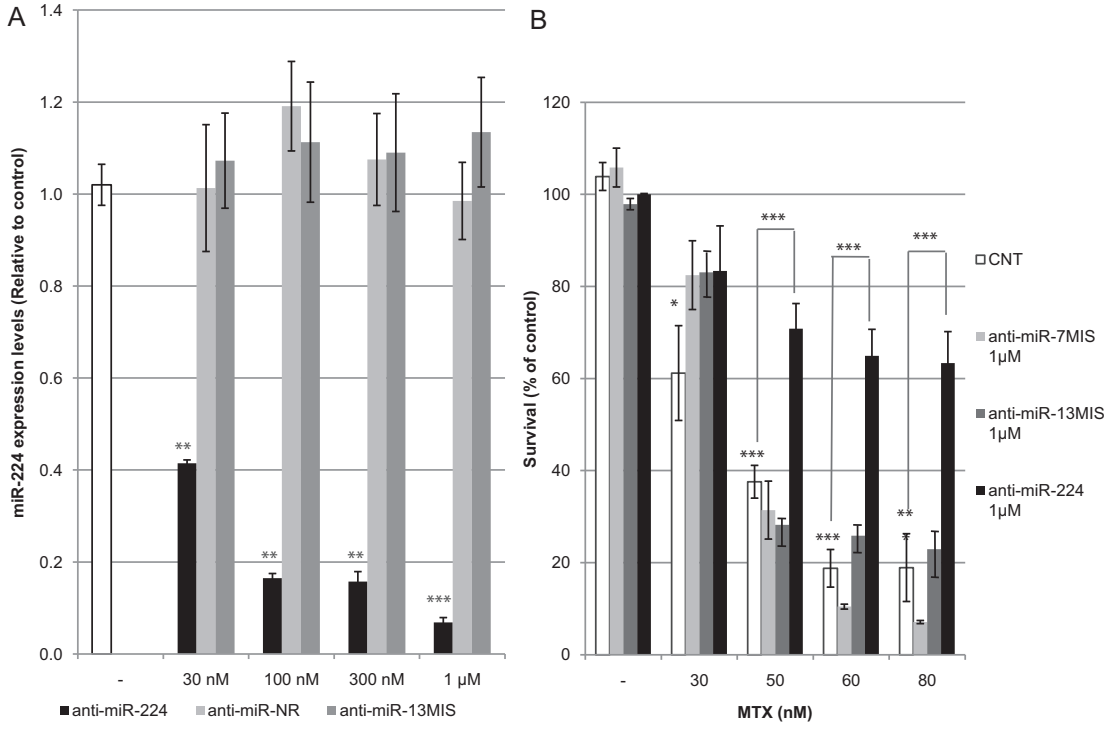

Fig. 3. Effect of anti-miR-224 on miR-224 levels and sensitivity towards MTX. (A) Inhibition of miR-224 levels upon transfection with anti-miR-224. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium, with different concentrations of anti-miR-224 (black bars), anti-miR-NR (light grey bars) or anti-miR-13MIS (dark grey bars). White bars correspond to the control of non-transfected cells. Total RNA was extracted after 48 h and miR-224 levels were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples. $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Response to MTX upon inhibition of miR-224 levels in HT29 sensitive cells. Cells (30 000) were transfected in 1 ml of –GHT medium as described in Section 2. After 24 h cells were treated with MTX and viability assessed 6 days later. White bars correspond to the control non-transfected cells; grey bars correspond to cells transfected with either 1 μM anti-miR-7MIS (light) or anti-miR-13MIS (dark); and black bars indicate the survival percentage of cells transfected with 1 μM anti-miR-224. Results are expressed as the mean of the percentage of survival compared to the control ± S.E.M. for triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 4
miR-224 targets overexpressed in HT29 resistant cells.

Gene symbol	Gene description	Ratio	Raw values	
			Sensitive	Resistant
CDS2	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	2.11	61.65	129.37
DCP2	DCP2 decapping enzyme homolog	2.00	111.76	223.59
HGSNAT	Heparan-alpha-glucosaminide N-acetyltransferase	2.95	25.91	76.66
HSPC159	Galectin-related protein	2.09	86.59	182.55
KATNAL1	Katanin p60 subunit A-like 1	2.90	23.95	69.22
MYST3	MYST histone acetyltransferase (monocytic leukemia) 3	2.29	117.71	269.90
SLC4A4	Na/bicarbonate cotransporter 1	2.11	72.58	153.35

Overexpressed predicted targets for miR-224. Target prediction was performed using TargetScan software. A list of differentially expressed (more than 2-fold, *t*-test *p*-value < 0.05 and Benjamini–Hochberg FDR) putative targets for miR-224 was obtained. The ratio column corresponds to the fold change in expression for each gene relative to the control, calculated from the normalized values. The mean of the raw values in sensitive and resistant cells obtained in the microarray are shown.

Furthermore, DHFR mRNA levels were also determined. No changes were observed (Table 5), suggesting that changes in MTX sensitivity upon miR-224 inhibition were not due to the modulation of DHFR, the direct target of MTX, but rather to miR-224 targets.

3.5. Inhibition of miR-224 targets leads to increased sensitivity towards MTX

To assess whether the effect of miR-224 on cell sensitivity to MTX could be mediated by one of its validated targets (CDS2, HSPC159, MYST3 and SLC4A4) the mRNA levels of these genes were decreased using as tools either PPRHs or siRNAs. The sequences of the PPRHs and siRNAs are listed in Table 2. Transfection with the specific siRNA or PPRHs effectively reduced the mRNA levels of their target genes (Figs. 5A and 6A, respectively). Then, the inhibitory effect of these molecules was assayed on MTX sensitivity. Inhibition of SLC4A4, when combined to MTX, reduced the viability of HT29 sensitive cells by 50% as compared to cells treated with MTX alone (Fig. 5B). Although less pronounced, inhibition of CDS2 and HSPC159 had also an effect on cell survival in the presence of MTX. Cells transfected with hpCDS2 or hpHSPC159 and treated with MTX presented a higher sensitivity towards MTX than that of cells treated with MTX alone (20% and 13%, respectively; Fig. 6B). No changes in MTX sensitivity were observed in cells transfected with hpMYST3. SLC4A4, CDS2 and HSPC159 triple KO was also performed in HT29 cells. In this case the sensitivity to MTX was increased by 30% compared to the summatory effect produced by the inhibition of each individual gene.

To confirm if the MTX-sensitivity effects of miR-224 were mediated, at least in part, by its identified target, we co-transfected HT29 cells (30 000) with siSLC4A4 (100 nM) and 24 h later with anti-miR-224 (1 μM) in a functional assay. MTX was added 24 h after anti-miR-224 and viability assessed 5 days later. The increase

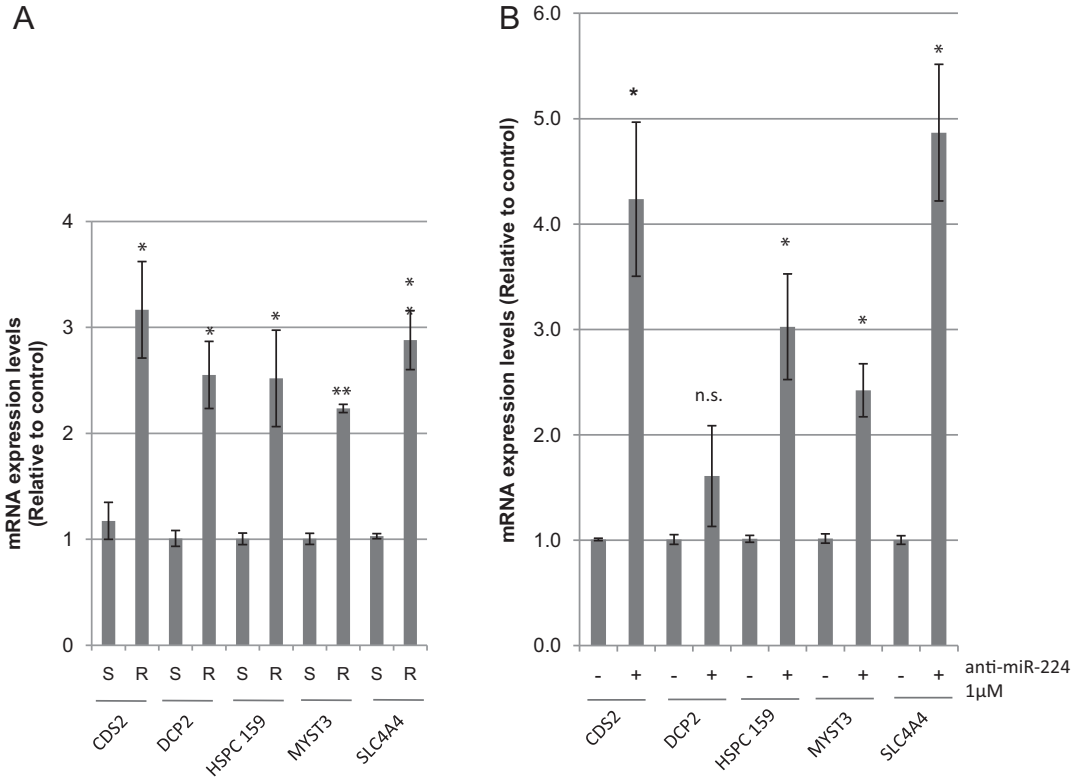

Fig. 4. Effect of anti-miR-224 on miR-224 targets mRNA levels. (A) Endogenous levels of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 (miR-224 putative targets) in HT29 sensitive and 10^{-5} M MTX-resistant cells. Total RNA was extracted and mRNA levels were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) Determination of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 mRNA levels upon inhibition of miR-224 levels in HT29 sensitive cells. Cells (30 000) were transfected in 1 ml of –GHT medium as described in Section 2. Total RNA was extracted after 48 h and miR-224 levels were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples. $^{*}p < 0.05$, n.s.: non-significant.

Table 5
Specificity of anti-miR-224.

	S100A4	ENO2	CLU	XRCC4	HAPLN1	DHFR
CNT	1.03 ± 0.04	1.01 ± 0.10	1.00 ± 0.09	1.01 ± 0.16	1.15 ± 0.15	0.97 ± 0.10
anti-miR-224 1 μM	1.12 ± 0.09	1.11 ± 0.10	1.30 ± 0.30	1.24 ± 0.12	1.00 ± 0.08	1.30 ± 0.32

mRNA levels of 6 genes not related with miR-224 were determined to assess the specificity of anti-miR-224 effect on miR-224 putative targets. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium with anti-miR-224 1 μM. Total RNA was extracted after 48 h and the mRNA levels of S100A4, ENO2, CLU, XRCC4, HAPLN1 and DHFR were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples.

in MTX-sensitivity produced upon siSLC4A4 transfection was reversed by 75% upon anti-miR-224 transfection.

As negative controls, a non-related siRNA (siNR) or scrambled PPRHs (hpSC1 and hpSC2) were used. siSLC4A4 off-target effect was assessed by determining the expression levels of ENO2, CLU, S100A4, CDH1, PDCD4, TOP1 and TERT. Transfection of siSLC4A4 did not cause any changes in the expression levels of such genes (Table 6). The effect of scrambled PPRHs 1 and 2 on miR-224 targets was also assessed. Transfection with either hpSC1 or hpSC2 had no effect on mRNA levels of miR-224 target genes (Table 7).

Altogether, the presented results indicate that SLC4A4, CDS2 and HSPC159 overexpression due to reduced miR-224 levels decreases the sensitivity of HT29 cells towards MTX.

3.6. miR-224 binds to CDS2, HSPC159 and SLC4A4 3′UTR

Once established the functional link between miR-224 and CDS2, HSPC159 and SLC4A4 (Fig. 4B) and their role in MTX resistance (Figs. 5B and 6B), we wanted to confirm the effective interaction between miR-224 and its targets. For that purpose, binding assays (electrophoretic mobility shift assays, EMSA) between miR-224 and each of its targets involved in MTX resistance were performed. As can be observed in Fig. 7, a shifted band appeared when radiolabeled SLC4A4-3′-UTR (A), CDS2-3′-UTR (B) and HSPC159-3′-UTR (C) were incubated with increasing concentrations of miR-224. Importantly, the binding was not observed when using the 3′-UTR-MM sequences (Fig. 7D). These results prove that miR-224 binds specifically to the 3′-UTR sequences of its targets CDS2, HSPC159 and SLC4A4.

4. Discussion

The efficiency of MTX-based therapy is reduced by the appearance of resistance. This resistant phenotype can be achieved by different mechanisms [28], including *dhfr* gene overexpression, which arose as one of the most important features in our MTX-resistant cancer cells [3]. However, other important genes, besides *dhfr*, have been identified and proved to contribute to the resistant phenotype, such as *AKR1C1*, *UGT1A6*, *DKK1*, *E-Cadherin*, *Caveolin 1* and *S100A4* [3–6,29]. Drug resistance is a complex multistep

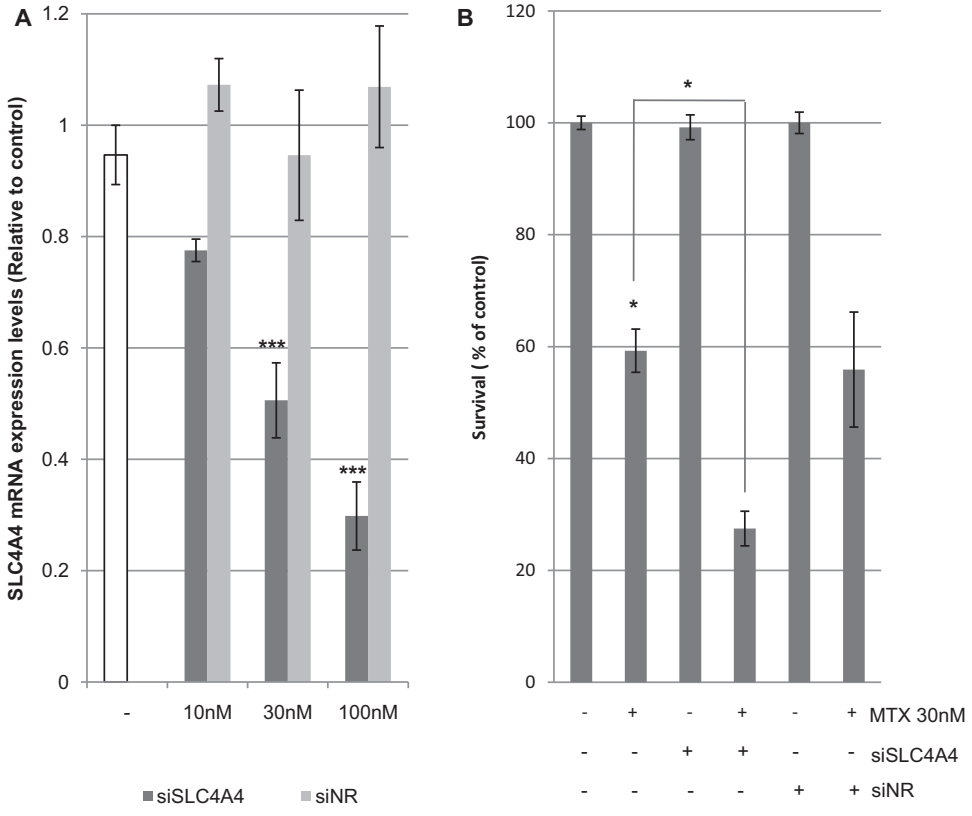

Fig. 5. Effect of SLC4A4 inhibition on MTX sensitivity. (A) Inhibition of SLC4A4 levels upon transfection with siSLC4A4. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium, either with siSLC4A4 (dark grey bars) or siNR (light grey bars). White bars correspond to the control of non-transfected cells. Total RNA was extracted after 48 h and SLC4A4 mRNA levels were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples. ***$p < 0.001$. (B) Response to MTX upon inhibition of SLC4A4 levels in HT29 sensitive cells. Cells (30 000) were transfected in 1 ml of –GHT medium with either siSLC4A4 or siNR 100 nM as described in Section 2. After 48 h cells were treated with MTX and viability was assessed 6 days later. Results are expressed as the mean of the percentage of survival compared to the control ± S.E.M. for triplicate samples. *$p < 0.05$.

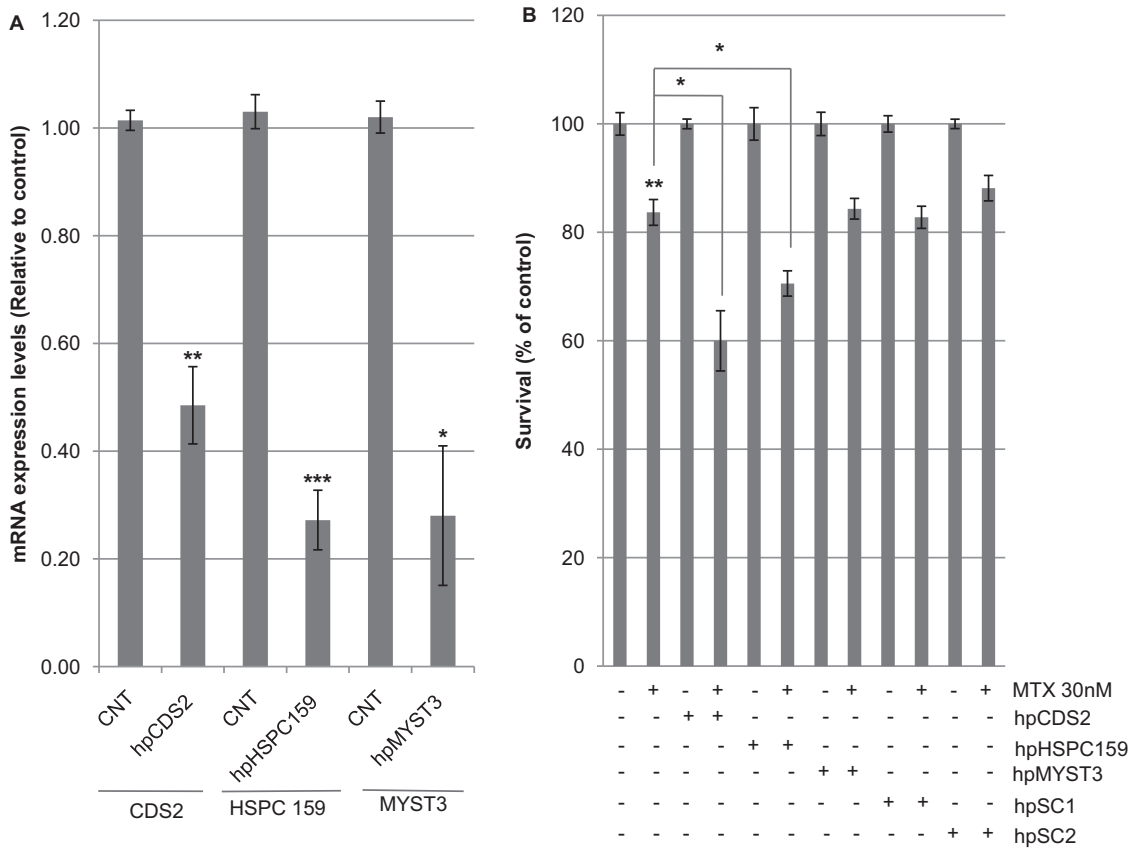

Fig. 6. Effect of CDS2, HSPC159 and MYST3 inhibition on MTX sensitivity. Inhibition of CDS2, DCP2, HSPC159 and MYST3 mRNA levels upon transfection with hpCDS2, hpHSPC159 and hpMYST3, respectively, at a concentration of 100 nM. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium with the specific PPRHs. Total RNA was extracted after 48 h and mRNA levels were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Response to MTX upon inhibition of CDS2, HSPC159 or MYST3 levels in HT29 sensitive cells. Cells were plated (30 000) in 1 ml of –GHT medium and transfected with 100 nM of each PPRH as described in Section 2. After 48 h cells were treated with MTX and viability assessed 6 days later. Scrambled PPRH (hpSC1 and hpSC2) were used as negative controls. Results are expressed as the mean of the percentage of survival compared to the control ± S.E.M. for triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$.

process in which many different pathways are involved. To obtain a more comprehensive view, in the present study we assessed the implication of miRNAs in the development of MTX resistance. In this sense, few references link DHFR, MTX and miRNAs. The group of Bertino showed that the presence of a SNP near the target site for miR-24 interferes with the inhibitory effect of miR-24 on the 3′-UTR of *dhfr* mRNA. This leads to overexpression of the protein and allows the cell to overcome MTX-induced cytotoxic effect, which would lead to MTX resistance [30–32]. On the other hand, Ju and co-workers described that overexpression of miR-140 [33] or miR-215 [34] correlates with resistance to MTX. These authors showed that in both cases, the effect on MTX sensitivity was not due to specific changes in the MTX pathway but rather to a cell cycle regulatory effect. MiR-215 and miR-140 overexpression lead to G_1 and G_2 phase arrest, which caused a reduced proliferation rate. The lack of active DNA synthesis made these cells less sensitive to MTX-induced DNA damage. However, this overexpression was not found in our MTX-resistant HT29 cells. This difference might be due to the fact that the previous studies were performed not only in different cell lines, but also in cells not resistant to MTX, since the incubations were performed only for short periods of time.

A first conclusion of this work is that miR-224 is greatly underexpressed in MTX-resistant colon cancer cells. This effect was validated in both HT29 and CaCo-2 cell lines as well as in K562 leukemia cells. Functional validation of this underexpression by means of an anti-miR causes a decrease in MTX cytotoxicity in a dose-dependent manner, thus reproducing the resistant phenotype. Furthermore, inhibition of miR-224 reversed MTX-induced apoptosis in HT29 cells. miR-224 has also been described to be deregulated in different types of cancer such as prostate [35], thyroid [36], pancreatic ductal adenocarcinoma (PDAC) [37], hepatocellular carcinoma [38–40], as well as acute myeloid leukemia [41]. miR-224 has been linked to PDAC progression, invasion promotion and metastasis; and in HCC, evidence of a link between miR-224 and PAK4 (p21 protein (Cdc42/Rac)-activated kinase 4), a regulator of cell invasion in tumor cells, has been

Table 6
siSLC4A4 specificity (off-target effect).

	S100A4	ENO2	CLU	CDH1	PDCD4	TOP1	TERT
CNT	1.01 ± 0.11	1.02 ± 0.13	1.02 ± 0.11	1.00 ± 0.01	1.02 ± 0.09	1.02 ± 0.16	1.03 ± 0.24
siSLC4A4 100 nM	0.70 ± 0.08	0.88 ± 0.13	1.05 ± 0.11	1.21 ± 0.09	1.56 ± 0.15	0.91 ± 0.13	0.81 ± 0.01

Possible off-target effects of siSLC4A4 were analyzed by determining the mRNA levels of S100A4, ENO2, CLU, CDH1, PDCD4, TOP1 and TERT. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium with 100 nM siSLC4A4. Total RNA was extracted after 48 h and mRNA were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples.

Table 7
Effect of scrambled PPRHs, hpSC1 and hpSC2, on mRNA levels.

	CDS2	DCP2	HSPC 159	MYST3	SLC4A4
CNT	1.06 ± 0.05	0.98 ± 0.01	1.00 ± 0.01	1.09 ± 0.07	0.96 ± 0.03
hpSC1 100 nM	0.88 ± 0.01	1.46 ± 0.01	1.10 ± 0.08	1.21 ± 0.03	1.53 ± 0.10
hpSC2 100 nM	1.07 ± 0.05	1.13 ± 0.09	0.84 ± 0.07	1.07 ± 0.01	1.30 ± 0.09

The mRNA levels of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 were determined upon incubation with the negative controls PPRHs SC1 and SC2. HT29 sensitive cells (30 000) were transfected in 1 ml of –GHT medium with either 100 nM hpSC1 or 100 nM hpSC2. Total RNA was extracted after 48 h and mRNA levels of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 were determined by qRT-PCR as described in Section 2. Results are the mean of the ratio compared to the control ± S.E.M. for triplicate samples.

provided [39]. Interestingly, Wang et al. showed that miR-224 has a dual effect on cell survival in HCC: it is able to increase both apoptosis, through inhibition of Apoptosis inhibitor-5 (API-5) and proliferation although the target was not identified) [40]. Regarding drug sensitivity, Gmeiner et al. showed a significant negative correlation among miR-224 and FdUMP[10], using the NCI-60 panel cancer cell lines [42].

Then, we searched for miR-224 targets that might have a functional effect on MTX resistance. Among all predicted targets for miR-224, we validated the endogenous overexpression of CDS2, DCP2, HSPC159, MYST3 and SLC4A4 in MTX-resistant HT29 cells. We also confirmed that inhibition of miR-224 with anti-miR224 produced an increase in the mRNA levels of CDS2, HSPC159, MYST3 and SLC4A4. This indicates a regulatory effect of miR-224 on these genes and suggests that a decreased inhibition by miR-224 on its targets could make cells less sensitive to MTX. Further proof of the involvement of miR-224 targets on MTX resistance was obtained using inhibitory molecules against them, either PPRHs or siRNAs. Decreased mRNA levels of SLC4A4, CDS2 and HSPC159 cause an increase in MTX sensitivity in HT29 cells. Additionally, the effective interaction between miR-224 and each of its functional targets (SLC4A4, CDS2 and HSPC159) was confirmed by binding analyses to the specific binding site for miR-224 present in the corresponding 3′-UTRs. Analysis of the potential target sites for other miRNAs in the 3′-UTR of miR-224 targets was performed. Fifteen miRNAs presented potential target sites within the SLC4A4-3′-UTR. Nevertheless, such miRNAs were not found differentially expressed in the resistant cells with exception of miR-224 and miR-149. Since both, miR-149 and SLC4A4 are upregulated in the resistant cells it is unlikely that miR-149 might be regulating SLC4A4 in this scenario. CDS2 and HSPC159 3′-UTRs contain 2 and 8 predicted target sites for miRNAs. However, aside from miR-224 none of them were differentially expressed in the resistant cells. Therefore, we think

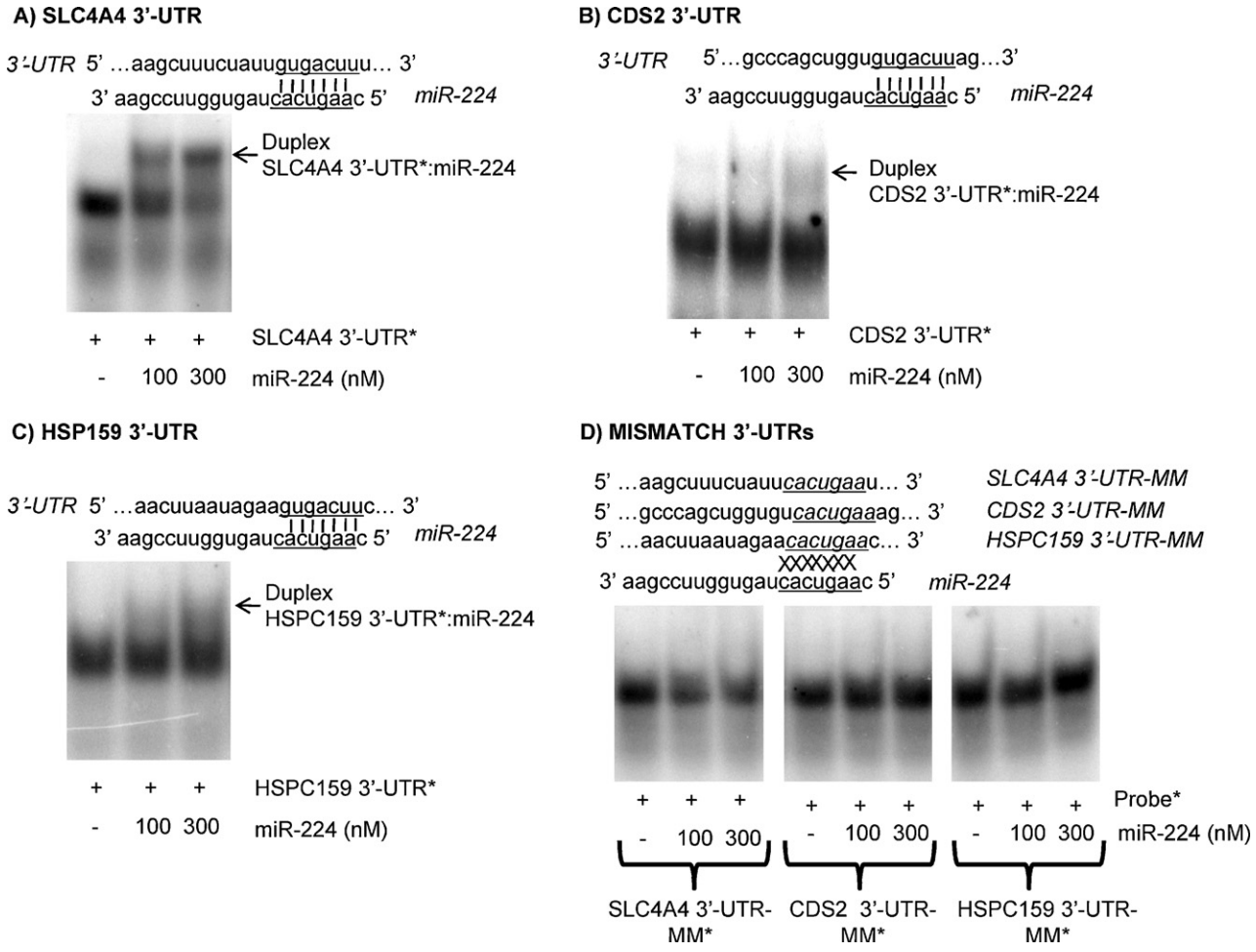

Fig. 7. Binding assays between miR-224 and its targets CDS2, HSPC159 and SLC4A4. Binding assays were performed as described in Section 3.6 between miR-224 and SLC4A4-3′-UTR (A), CDS2-3′-UTR (B) or HSPC159-3′-UTR (C) using 20 000 cpm of each radiolabelled probe and increasing concentrations of miR-224. Part (D) corresponds to the incubation with miR-224 in the presence of radiolabelled 3′-UTR-MM sequences specific for CDS2, HSPC159 or SLC4A4.

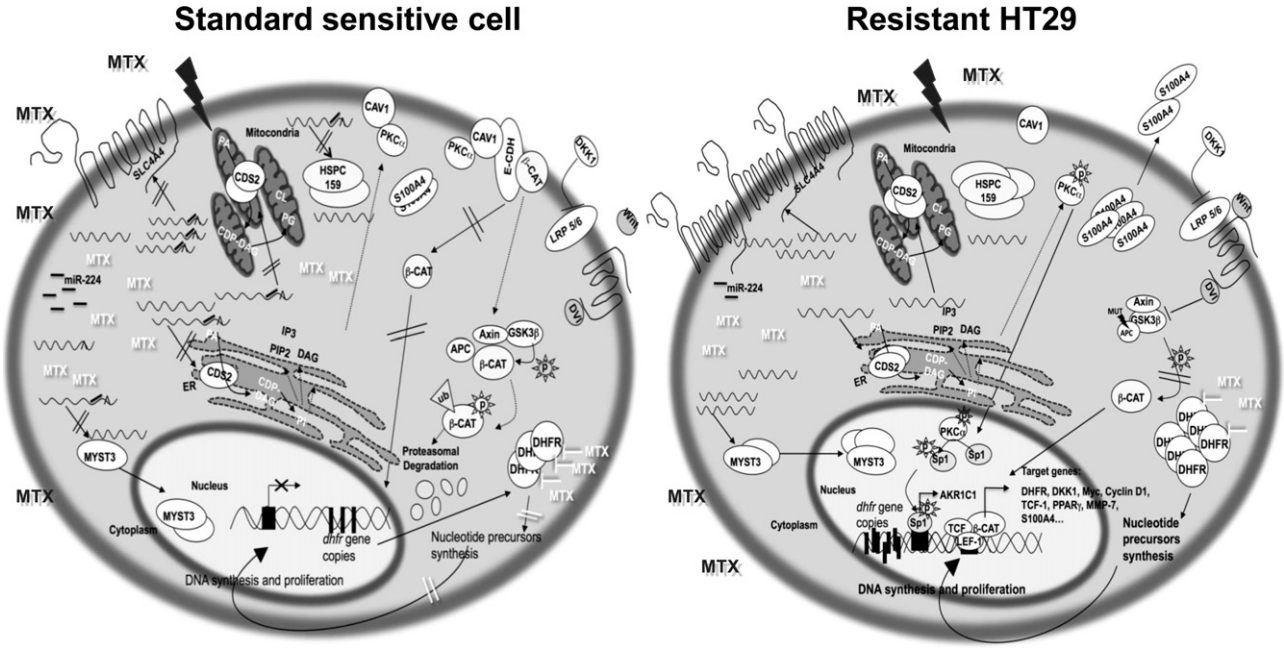

Fig. 8. Main features involved in the development of MTX-resistance in HT29 cells. Important factors leading to MTX-resistance are represented: *dhfr* gene amplification; overexpression of *AKR1C1*, *PK-C*, *Caveolin*, *β-catenin*, *S100A4* and *Sp1*; and underexpression of *E-cadherin* and miR-224. *SLC4A4*, *CDS2*, *HSPC159* genes are also overexpressed as targets of miR224.

that, altogether, our results prove that miR-224 is responsible for the regulation of SLC4A4, CDS2 and HSPC159 expression levels in HT29 MTX-resistant cells.

SLC4A4 or NBC1e (electrogenic Na/bicarbonate cotransporter 1) is an electrogenic transmembrane transporter responsible for the transport of sodium and bicarbonate across the membrane in epithelial cells [43]. All members of the SLC4 family are responsible for base transport in a cell type- and cell membrane-specific manner. They play important roles in the carriage of CO_2 by erythrocytes, the absorption or secretion of acid base equivalents by numerous epithelia, as well as the regulation of cell volume and intracellular pH in nearly every cell of the body [44]. Three different tissue specific isoforms have been identified for SLC4A4, 2 or A in the kidney [43], 1 or B more widespread but mainly in pancreas [45] and 3 or C in brain [46]. SLC4A4-A is the most overexpressed isoform in HT29 MTX-resistant cells, and the most responsive to miR-224 inhibition (data not shown). Although mainly found in the kidney, different studies have demonstrated that SLC4A4-2 can be also found in different cell types in the pancreas, as well as the eye and other epithelia [47]. Alterations in membrane transport have been shown to be a determinant factor for MTX activity and a frequent determinant of tumor cell resistance to this drug in both model systems and humans [28]. In fact, one of the first steps in MTX resistance is the decreased uptake of MTX by the cell [48]. Deregulation of folate/antifolate specific transporters such as the reduced folate carrier (RFC) or the proton-coupled folates transporter (PCFT) and unspecific transporters such as the MDR protein [49] is the main cause of impaired transport of MTX. MTX is mainly transported by means of the RFC, with optimal activity at pH 7.4. Folates can also be transported by PCFT with an optimal activity at pH 5.5, responsible for the uptake of oxidized and reduced folates [50–52] and important for the absorbance of MTX in the gout and the incorporation of MTX to the acidic lumen of solid tumors [53,54]. In our model, RFC was underexpressed in resistant cells, and no overexpression of MDR was observed. Changes in pH caused by SLC4A4 differential expression could affect the transport activity of RFC and/or PCFT, thus decreasing the amount of MTX inside the cell and favoring drug resistance.

CDS2 (CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2) encodes for an enzyme that regulates the amount of phosphatidylinositol available for signaling by catalyzing the conversion of phosphatidic acid to CDP-diacylglycerol [55]. Breakdown products of phosphoinositides are ubiquitous second messengers that regulate important processes such as cell growth, calcium metabolism, and protein kinase C activity. In a previous study the role for PKCα in MTX resistance in colon cancer cells was already elucidated [5]. A combined strategy was used to inhibit the mRNA levels of PKCα, CAV1 and ENO2 in HT29 sensitive cells. Inhibition of these genes caused a chemosensitization towards MTX. These previous results are in agreement with the results presented here, supporting the idea that PKCα, possibly regulated by products of CDS2 activity, plays an important role in MTX resistance in colon cancer cells.

Finally, HSPC159 (galectin-related protein) belongs to the galectin family, a family of lectins that bind β-galactosides [56]. However, unlike other lectins, HSPC159 lacks β-galactosides binding capacity [57], and its biological function still remains unclear.

In summary, aside from the amplification of the *dhfr locus,* and the increased expression of detoxifying activities and proliferative genes we previously reported in MTX-resistant HT29 cells, we have now identified the underexpression of miR-224 in such cells. Reduction of miR224 levels, through an increase in the expression of its targets SLC4A4, CDS2 and HSPC159, leads to insensitivity towards MTX, favoring the resistant phenotype (Fig. 8).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.08.009.

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