Published in final edited form as:

Exp Cell Res. 2013 February 15; 319(4): 566–574. doi:10.1016/j.yexcr.2012.10.012.

Contributions of microRNA Dysregulation to Cisplatin Resistance in Adenocarcinoma Cells

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Abstract

Cisplatin resistance in cancer cells is due to a pleiotropic phenotype transition that allows cells to resist cell death. miRNAs have been shown to be reliable markers of phenotype, critical in cell differentiation, and dysregulated in cancer and other pathologies. Here we investigate the influence of miRNA on cisplatin resistance in KB adenocarcinoma cells. Silencing both DICER and TRBP2 in the miRNA biosynthesis pathway in KB-3-1 (sensitive parental), KB-CP.5 (cisplatin-resistant), and KB-CP20 (highly cisplatin-resistant) cells resulted in the reversal of cisplatin resistance, with no effect on cell viability in the absence of cisplatin. We found miR-181 expression differences in the cell lines using RT-PCR, with several members of the miR-181 family overexpressed in two KB cisplatin-resistant lines and in two cisplatin-resistant lung cancer lines, compared to their respective parental cells. Functional assays showed minimal effects of miR-181 on cisplatin resistance. We conclude that the miRNA biosynthesis pathway is critical for maintaining the cisplatin-resistant phenotype, but that it is difficult to determine the precise miRNAs involved in cisplatin resistance simply using expression profiles of individual miRNA species. Functional assays are needed to determine the influence of a specific miRNA and different members of the same miRNA family may have opposite effects.

Keywords

Drug resistance; cisplatin; microRNA; chemotherapy; drug selection

Introduction

A major challenge in the successful treatment of cancer is the ability of cancer cells to acquire a multidrug-resistant phenotype, preventing response to chemotherapeutic agents. Cisplatin [*cis*-diamminedichloroplatinum(II)] is a chemotherapeutic drug commonly used against a range of malignancies in combination therapy [1-5]. The cisplatin-resistant

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Conflicts of interest: None

pleiotropic phenotype can be induced by selection in cultured cancer cells in a single step[6]. While Pt drugs do not occur naturally in the environment, cisplatin resistance is most likely due to a cellular defense mechanism that evolved to protect against other metals and xenobiotics found in nature [2]. Many mechanisms are involved in cisplatin resistance including; i) Alteration of cell cycle checkpoints, ii) Up-regulation of DNA damage repair mechanisms, and iii) Disruption of the cytoskeleton, thereby redirecting cell surface transporters from the surface to the cytoplasm. This produces cells that are resistant to cisplatin and also show cross-resistance to multiple toxic compounds that enter cells *via* existing transporters [1-5, 7, 8]. The primary underlying alteration or alterations that cause cells to resist cisplatin insult have yet to be identified.

MicroRNAs (miRNAs) are functional small RNAs involved in phenotypic stabilization, and cell differentiation [9-12]. They allow cells to set normal parameters for maintaining mRNA and its translation, by binding to the 3' UTR of mRNA and sequestering or degrading the message, preventing translation [9-12]. Dysregulated miRNAs are involved in tumor growth and other pathologies [13-15]. miRNAs have been linked to patient response to chemotherapy [15-18]. Studies in cultured cells have also linked miRNA regulation to chemo-resistance [15, 19-21]. miRNAs are highly regulated. From transcription through maturation they are chaperoned by multi-protein complexes that select, determine strand bias, and determine target. The protein complex RISC, consisting of DICER, TRBP2, and Argonaute, selects, matures, and directs the synthesis of a pre-miRNA precursor to the mature functional miRNA[10, 12].

Changes in expression of miRNAs have been implicated in cellular sensitivity to a range of chemotherapeutic agents, including cisplatin. A preliminary analysis of expression differences in parental and cisplatin-resistant cells conducted by us indicated that miR-181 showed increased expression in cisplatin-resistant cells. Most of the work on the miRNA-181 family has linked members to regulation of differentiation pathways in immune cells including T-cells, B-cells, and Natural Killer cells [21-23], and in myoblast differentiation [24]. Upregulation of miR-181 family members was recently shown to be associated with chemotherapy response in gastric cancer patients (which is treated with a combination of agents including cisplatin) [25].

We hypothesized that miRNAs play a role in orchestrating the underlying phenotypic changes associated with a pleiotropic cisplatin resistance mechanism. In order to investigate miRNA involvement, we examined the parental KB-3-1 human adenocarcinoma cell line and two independently generated cisplatin-resistant sublines KB-CP.5 (resistant) and KB-CP20 (highly-resistant). We first targeted the miRNA biosynthesis pathway using RNAi to knockdown DICER and TRBP2, two critical members of the RISC complex involved in maturation of miRNA and subsequent targeting of mRNA. We next assessed the parental and cisplatin-resistant cells for differences in expression of miR-181 between the cell lines. We performed loss-of-function/gain-of-function experiments to demonstrate the involvement of specific miR-181 family members in the cisplatin resistance phenotype.

Most studies examining miRNA choose a specific miRNA and search for a target, rather than investigating the behavior of an entire family of miRNAs in regards to cisplatin-resistant (CP-r) cells. Historically it was believed that members of a family, having matching seed sequences, all have the same targets, and therefore should have the same effect on a cell mechanism. This is why the available algorithm target program searches are based on a seed sequence which complements a mRNA 3' UTR. As the field advances, the story of miRNA has become more complicated than a stoichiometry match between miRNA and mRNA. Although different miR-18 family members are important for different cell differentiation pathways, we felt that, in light of recent advances in the field, the

examination of individual members of an entire family could provide valuable insight into how miRNAs function based on seed sequence.

Materials and methods

Cell lines and cell culture

The parental human adenocarcinoma cell line KB-3-1 (a HeLa subclone), and its CP-r sub-lines, KB-CP.5 (resistant) and KB-CP20 (highly resistant) were studied. KB-CP.5 cells were originally selected from a single-step clone selected in 0.5 μg cisplatin/mL (1.6 μM), as described previously [6, 26]. KB-CP20 cells were selected by stepwise increases to 20 μg of cisplatin/mL of medium (66.7 μM), as described previously [27, 28]. The KB CP-r cells were maintained in the presence of cisplatin (1.6 μM and 16.7 μM respectively). The PC-9 and PC-14 human lung cancer cell lines were used to generate the CDDP lines PC-9/CDDP and PC-14/CDDP (selected in 1.6 μM cisplatin) [29, 30]. Cisplatin was removed from growth medium three days prior to all experiments. Cell lines were grown as monolayer cultures at 37°C in 5% CO₂, using Dulbecco's modified Eagle medium (DMEM) (KB cells) or Roswell Park Memorial Institute (RPMI) medium (PC cells) with 4.5 g/L glucose (Invitrogen, Carlsbad, CA), supplemented with L-glutamine, penicillin, streptomycin and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). Cisplatin was purchased from Sigma (St. Louis, MO).

Preparation of cell lysates, quantification of protein, and Western blot analysis

Cell samples were obtained by trypsin digestion followed by two washes in PBS. Protein was extracted using Cell Extraction Buffer (Invitrogen) with 50 μL /mL Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using Bio-Rad Protein Assay based on the Bradford method as per the manufacturer's instructions. 50 μg protein samples were loaded onto a NuPage 3-8% TA gel (Invitrogen) and run at 125V. Transfer to nitrocellulose membranes was performed using the iBlot Gel Transfer Device (Invitrogen) at 20 V for 7 min. Membranes were blocked in 5% non-fat milk for 1 h, exposed to primary antibody overnight at 4 °C, and to HRP-tagged secondary antibody for 1 h at room temperature. Blots were incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and developed on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

RNA extraction, cDNA preparation, and quantitative real-time RT-PCR

Cisplatin was removed 72 hrs prior to experiments. RNA was harvested from KB-3-1 and KB-CP.5 cells using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA). RNA integrity and concentration were measured using a NP-1000 Spectrometer to measure absorbance (Thermo Scientific, Wilmington, DE). cDNA was prepared from 20 ng total RNA following the manufacturer's instructions. qRT-PCR was run using Exiqon probes on 7500 fast real-time PCR system (Applied Biosystems). The miRNA data was normalized to U6 RNA. Relative expression was calculated by the Ct method [31]. RT-PCR data represents expression from three separate RNA isolates per condition, and each isolate was run three times (n = 9).

Probe sequences:

hsa-miR-181a: AACAUUCAACGCGUGCGGAGU,

hsamiR-181*: ACCAUCGACCGUUGAUUGUACC,

hsa-miR-181b: AACAUUCAUUGCGUGCGGGU,

hsa-miR-181c: AACAUUCAACCUGUCGGUGAGU,

hsa-miR-181c*: AACCAUCGACCGUUGAGUGGAC,

hsa-miR-181d: AACAUUCAUUGUUGUCGGUGGGU

Target prediction

The number of mRNAs predicted to be targets of miRNA over- or under-expressed in cisplatin-resistant lines compared to sensitive KB-3-1 cells were obtained by using miRanda (<http://www.microrna.org/microrna/home.do>) and TargetScanHuman (<http://www.targetscan.org/>) algorithms.

Transfection of siRNA, miRNA mimics, and miRNA inhibitors

Transfection was performed with RNAiMax (Invitrogen, Carlsbad, CA) (2.5 L/mL). siRNA of DICER and TRBP2 were plated at 10 nM on day 1. The pre-designed miRIDIAN miRNA mimics and inhibitors and AllStars Negative Control (siNegative) (Qiagen) were plated with cells on day 1 at 10 nM. The transfection was performed as described by the manufacturer.

Target sequences:

hsa-miR-181a/a-2: AACAUUCAACGCUGUCGGUGAGU,

hsa-miR-181a*/a*-2: ACCAUCGACCGUUGAUUGUACC,

hsa-miR-181b-1/b-2: AACAUUCAUUGCUGUCGGUGGGU,

hsa-miR-181c: AACAUUCAACCUGUCGGUGAGU,

hsa-miR-181c*: AACCAUCGACCGUUGAGUGGAC,

hsa-miR-181d: AACAUUCAUUGUUGUCGGUGGGU

MTT cytotoxicity assay

Cytotoxicity was measured with a colorimetric viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes, Eugene, OR) as previously described. Cells (5000 cells per well of a 96-well plate) were allowed to attach for 24 h. Stock solutions of compounds (3M) were prepared in PBS and then two-fold serially diluted in media to give a range of final tissue culture concentrations of 100 μ M to 0 μ M. After 72 h, cell viability was examined. Cytotoxicity (IC₅₀) was defined as the drug concentration that reduced cell viability to 50% of the untreated control. Data reported are the mean of at least three independent experiments, with three data points per concentration in each experiment.

Statistical Analysis

GraphPad Prism 5 software was used for graphs and statistics. All data are presented as mean $\pm$ SD. Comparisons among all samples were analyzed using Analysis of Variance (ANOVA). Comparisons between miRNA family members were analyzed using Two-way ANOVA. P values <0.05 were considered significant differences.

Results

Silencing Dicer and Trbp2 using RNAi sensitizes KB-3-1, KB-CP.5, and KB-CP20 cells to cisplatin

In order to investigate the role of miRNA in cisplatin resistance we used siRNA against two critical members of the miRNA biosynthesis pathway. DICER and TRBP2 were selected for knockdown based on their roles in selection, maturation, and binding in the RISC complex. KB-3-1 (parental) adenocarcinoma cells and their derivative cisplatin-resistant lines KB-CP.5 (resistant) and KB-CP20 (highly resistant) were transfected with control siRNA, siDICER, siTRBP2, or both together. Cells were harvested for protein extraction at 48 h. Western blots were performed to confirm knockdown of DICER and TRBP2 protein, with GAPDH used as a control protein to confirm equal loading (Fig. 1). Silencing of DICER by siRNA reduced expression to undetectable levels (Fig. 1A), and silencing of TRBP2 showed decreased expression (Fig. 1B, confirmed with densitometry quantification shown in Supplementary Fig. 4). TRBP2 has an estimated half-life of 4 days [32], and as such only a partial knockdown in the time frame of our experiment is to be expected.

To assess the effect of disrupting the miRNA maturation pathway on the cisplatin-resistant phenotype, cytotoxicity assays were performed with cisplatin following silencing of the two genes. Cells were transfected with siRNA against DICER, TRBP2, or both as before and seeded at 5000 cells per well in a 96-well plate. After 24 h cells were challenged with serially-diluted cisplatin to give the intended final concentrations (100 μ M to 0 μ M). Viability was determined at 96 h by MTT as described in Materials & Methods (Fig. 2).

siRNA against DICER increased sensitivity to cisplatin by 32 % (KB-3-1), 63 % (KB-CP.5), or 93% in KB-CP20 (highly resistant) cells (Table 1). siRNA against TRBP2 alone had a minimal effect on cisplatin cytotoxicity compared with cells transfected with control siRNA, likely due to the partial effect of TRBP2 silencing described above. When cell lines were transfected with siDICER and siTRBP2 in combination and dosed with cisplatin, resistant lines became more sensitive than KB-3-1 cells treated with cisplatin alone (Table 1). These results indicate that the presence of DICER and TRBP2, proteins critical for the maturation of miRNA, are necessary for cells to maintain resistance in response to cisplatin treatment.

miR-181 family expression in cisplatin-resistant cell lines

The miR-181a/a*/b/c/c*/d family was selected for further study due to a preliminary indication from microarray studies that showed increased expression of miR-181 family members in both KB-CP.5 and KB-CP20 cells (data not shown). Members of the miR-181 family are shown in Table 2. While nine miR-181 members are listed, three pairs produce identical mature sequences: miR-181a (miR-181a-1-5p and miR-181a-2-5p), miR-181a* (miR-181a-1-3p and miR-181a-2-3p), and miR-181b (miR-181b-1 and miR-181b-2). As such, six mature miR-181 members are produced, and were examined in this work.

To investigate the miR-181 family further, resistant cells were cultured in the absence of cisplatin for 72 h and RNA was isolated. qRT-PCR was run for each individual family member (probes described in Methods) and normalization was performed using expression of U6 (Fig. 3A). All miR-181 family members were overexpressed in cisplatin-resistant cells relative to parental cells. miR-181a/c/c*/d had statistically equal expression in KB-3-1 cells and were all overexpressed in KB-CP.5 and KB-CP20 lines. miR-181a/c/c*/d showed lower expression in KB-CP20 compared to KB-CP.5 cells. However, these two lines were generated independently, so this result does not reflect a direct loss of expression with the acquisition of higher-level resistance. miR-181a*/b was more highly expressed in KB-3-1 cells compared to other family members. In addition, miR-181a*/b were more highly expressed in KB-CP20 than in KB-CP.5 cells (Fig. 3A). Expression of miR-181 family

members was also assessed in two other cell line pairs, PC-9 and PC-14 human non-small cell lung cancer cell lines, and their respective sub-lines PC-9/CDDP and PC-14/CDDP. Individual miR-181 family members were also found by qRT-PCR to be over-expressed in the cisplatin-resistant cells (Supplementary Figs. 1 and 2). PC-9/CDDP cells showed a large increase in expression of miR-181c, and PC-14/CDDP cells had an increase in miR-181a/b/c/d. Conversely, a loss of expression was noted for miR-181a and miR-181c* in PC-9/CDDP cells (Supplementary Fig. 1), and miR-181a* and miR-181c* were undetectable in PC-9/CDDP cells (Supplementary Fig. 2).

Changes in expression of miR-181 family members shows small functional effects on cisplatin resistance

In order to examine the influence of the miR-181 family members on cisplatin resistance, KB-3-1, KB-CP.5 and KB-CP20 cells were transfected with miRNA mimics (20 nM) and miRNA inhibitors (siRNA directed against miRNA, 20 nM) for each individual member of the miR-181 family. After 24 h, transfected cells were challenged with serial dilutions of cisplatin (100 μ M to 0 μ M). Changes in miRNA levels for each condition were confirmed by qRT-PCR (Fig. 3B), and no off-target effects were observed for other family members (Supplementary Fig. 3). Loss-of-function/gain-of function mimic and inhibitor transfections had no effect on KB-CP20 cells (data not shown).

The influence of individual family members on cisplatin cytotoxicity was examined (Fig. 4 and Table 3). miR-181b had no effect on cell viability (Fig. 4B, Table 3). Inhibition of miR-181a and miR-181d conferred an increase in resistance (for example, for KB-3-1 cells, cisplatin with negative control $IC_{50} = 2.51 \pm 0.39$, cisplatin with miR-181a inhibitor $IC_{50} = 6.35 \pm 0.28$, 2.5-fold resistance), but conferred only a minimal sensitization when mimicked (Fig. 4A,D). Conversely, miR-181a*/c/c*, tested individually, increased resistance to cisplatin when mimicked and increased sensitivity when inhibited (Fig. 4C, E-F). The strongest effect observed was for KB-CP.5 cells co-treated with miR-181c* mimic (cisplatin $IC_{50} = 33.13 \pm 8.25$), compared with negative control (cisplatin $IC_{50} = 10.99 \pm 1.09$), a ~3-fold reduction in sensitivity. However, most effects were not statistically significant, indicating that changes in expression of individual miR-181 family members do not correlate with functional alterations in cisplatin response.

Discussion

We have shown that decreasing the expression of DICER and TRBP2 through specific RNAi's sensitizes resistant KB-CP.5 and KB-CP20 cells to cisplatin. Silencing DICER alone has a less dramatic but significant effect. DICER has been shown to play a role in increasing the toxicity of cisplatin in MCF-7 human breast carcinoma cells [33], so we did expect to see changes in cisplatin resistance when DICER was silenced in our cells. However, because cisplatin resistance results in a pleiotropic phenotype, experiments targeting one gene usually result in small 2- to 3-fold changes in the cisplatin resistance profile, rather than eliminating the full resistance phenotype [28, 34, 35]. We have shown that when the miRNA biosynthesis mechanism is targeted in KB-CP20 cells by silencing both DICER and TRBP2, cells show a reversal of resistance to an IC_{50} below that seen in sensitive KB-3-1 cells, with no effect on the viability of the cells in the absence of cisplatin. This result suggests that an intact miRNA biosynthesis pathway, and the continued production of mature miRNA, is critical in mediating cisplatin resistance.

All members of the miR-181 family show an increase in expression in the resistant cell lines KB-CP.5 and KB-CP20, compared with the parental KB-3-1 cells. When individual miRNA mimics are transfected into KB-3-1 and KB-CP.5 cells, only miR-181a*, miR-181c, and miR-181c* confer a small increase in cell resistance to cisplatin, as might be expected given

the increased expression seen in the resistant cell lines. It is interesting that miR-181c also shows the most overexpression in cisplatin-resistant cells compared to KB-3-1 of all the family members, but our data would suggest that its overexpression alone is not responsible for the full effect of miR-181 family members on cisplatin resistance. What is clear is that the overexpression of miR-181 family members in KB-CP.5 and KB-CP20 cells does not play a large role in conferring the resistance phenotype.

These studies indicate that individual miR-181 family members may play a small role in response to chemotherapy, but the extent to which dysregulation of miR-181 is a cause, or consequence of, cisplatin drug resistance is unclear. In preliminary experiments in which fixed, equal amounts of all miR-181 family member mimics or miR-181 inhibitors were transfected into KB cells (data not shown), overexpression of mimics resulted in some decreased resistance, and inhibitors resulted in increased resistance, suggesting that changes in miRNA-181 family members may be the result, rather than the cause of cisplatin resistance. However, it is also possible that the relative amounts of each miR-181 family member are critical to the development of this cisplatin resistance. In two other cell line pairs (PC-9 and PC-14 human non-small cell lung cancer cell lines, and their respective sub-lines PC-9/CDDP and PC-14/CDDP), some but not all miR-181 family members were found to be over-expressed in the cisplatin-resistant cells (Supplementary Figures 1 and 2), suggesting that these consequential increases in miRNA-181 family members are not cell-line specific.

Individual miR-181 family members have previously been shown to play a role in response to cisplatin. Galluzziet al. found that miR-181a was upregulated in response to cisplatin in non-small cell lung cancer (NSCLC) A549 cells [36]. While we also observed increased miR-181a levels, the PC-14/CDDP cells actually expressed less miR-181a than their parental line. Galluzziet al. also demonstrated that when cells were treated with a pre-miR-181a mimic the same cells were protected from cell death elicited by cisplatin and other toxins [36]. This initially seems contrary to our findings (miR-181a mimic had a minimal effect on cell sensitivity), but Galluzziet al. found that pre-miR-181a and not miR-181a mimic exerted an effect on the cells, and the authors also noted that HeLa cells (KB-3-1 cells are a sub-clone of HeLa) showed less response than A549 cells. Zhu *et al.* examined a cisplatin-resistant A549 cell line (both studies in the literature linking miR-181 with cisplatin response have been conducted in A549 cells) and found that miR-181a/b/c/d were down-regulated (~3-fold) in their cisplatin-resistant cells [37]. The authors showed that miR-181b targeted the anti-apoptotic gene BCL2, and miR-181b mimic (which silenced BCL2 expression) sensitized the cells to cisplatin. Again, this study is contrary to our observations that miR-181b was over-expressed in three of four cell lines examined (CP.5, CP20 and PC-14/CDDP) and unchanged in the other. miR-181 has also been measured in patient samples and identified as a possible marker for poor outcome [17, 18]. The differences between these studies [36, 37] and ours reinforce our conclusion that miRNA-181 family members do not play a critical role in the development of cisplatin resistance.

The TargetScanHuman prediction program indicates that the miRNA-181 family members have over 1100 potential gene transcript targets. Identifying which targets are of importance in cisplatin resistance mechanisms is beyond the scope of this study, but further investigation should help in understanding not only cisplatin resistance, but miR-181 family behavior.

As interference in the biosynthesis pathway of miRNA confers a drastic change in a cell's ability to resist killing by cisplatin, and multiple miRNA expression changes are observed in cisplatin-resistant cells, understanding the role miRNA have in regulating cisplatin

resistance is a critical area of study. In other work, we have conducted a functional screen for miRNA mimics that have an effect on cisplatin resistance to identify miRNAs that control the expression of cell cycle-related kinases[38]. Here, we show that the miR-181 family members are increased in expression in cisplatin-resistant cell lines, but that the members of this family have a minimal effect on the cisplatin-resistance phenotype. While expression profiles of miRNA species can be valuable in biomarker studies, it appears that assessing the functional contribution of a miRNA to the phenotype may provide a more reliable strategy than simply searching for large changes in miRNA expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Carlo Croce, Timothy Wise and Stefano Volinia for the preliminary analyses that led to our decision to study the miR-181 family in more detail. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. We thank George Leiman for editorial assistance.

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Highlights for Pouliot et al

- miRNA maturation is critical for cisplatin resistance
- The miR-181 family is over-expressed in cisplatin-resistant cells
- miR-181 silencing produced minimal effects on resistance

Fig. 1. siRNA interference against DICER and TRBP2 demonstrated by Western blot. A. KB-3-1, KB-CP.5, and KB-CP20 cells were transfected with siRNA against DICER or DICER + TRBP2. Knockdown of DICER protein was assessed using DICER 1° antibody at a 1:1000 dilution. Goat anti-rabbit HRP linked 2° antibody was used at a 1:2000 dilution. B. Cell lines were transfected with siRNA against TRBP2 or TRBP2 +DICER. TRBP2 protein expression was assessed using mouse 1° antibody at a 1:1000 dilution, followed by a goat anti-mouse 1:10000 HRP-linked 2° antibody.

Fig. 2. siRNAs directed to silence DICER, TRBP2, or a combination of both confers sensitivity to cisplatin in all cell lines. Transfection with siNegative in KB-3-1 or in KB-CP.5 showed no significant difference compared to cells treated with cisplatin alone, or cisplatin and lipofectamine (data not shown). Data reported are the mean of at least three independent experiments, with three data points per concentration in each experiment. In some cases, error bars are obscured by the data points due to the low experimental error.

Fig. 3.

Expression of miR-181 family members in KB-3-1, KB-CP.5, and KB-CP20 cell lines. A. RT-PCR was performed as described in methods. Samples were normalized to U6 and presented as $2^{-\Delta\Delta C_t}$. Using ANOVA analysis all p-values < 0.05. B. RT-PCR showing miRNA mimics increased expression of individual family members while inhibitors showed a decrease in expression. In most cases, miRNA could not be detected in miRNA inhibitor (siRNA)-treated cells (signified by Ø). RT-PCR data represents expression from three separate RNA isolates (rather than three PCR experiments using a single RNA isolate). All miR-181a-5p miRNA family members have identical mature sequences; therefore RT-PCR data listed as miR-181a represents the effect of both miR-181a-1 and miR-181a-2. The same is true for miR-181b family members.

Fig. 4.

Response of KB-3-1 and KB-CP.5 cell lines to miRNA inhibitor and miRNA mimic data in the presence of cisplatin. Cells were transfected with a mimic or inhibitor against one member of the miR-181 family. After 24 h samples were challenged with cisplatin in concentrations from 100 μ M to 0 μ M. KB-3-1 and KB-CP.5 cells had no significant difference between treatments of drug only, RNAiMax alone, or siNegative transfection (data not shown). Data reported are the mean of at least three independent experiments, with three data points per concentration in each experiment. In some cases, error bars are obscured by the data points due to the low experimental error. Mimic and inhibitor for miR-181a-1-5p and miR-181a-2-5p were both used despite having identical mature sequences, and gave similar results. As such, data for miR-181a-2-5p is not shown. The same is true for miR-181b-1 and miR-181b-2.

Table 1

Cytotoxicity values ($IC_{50} \pm SD$) for cisplatin-treated KB-3-1, KB-CP.5, and KB-CP20 cells transfected with siRNA against DICER and TRBP2

Cell line	IC_{50} (μM) cisplatin + siNegative	IC_{50} (μM) cisplatin + siDicer	IC_{50} (μM) cisplatin + siTrbp2	IC_{50} (μM) cisplatin + siDicer + siTrbp2
KB-3-1	$4.4 \pm 0.02 \mu M$	$3.0 \pm 0.1 \mu M$	$3.6 \pm 0.2 \mu M$	$0.9 \pm 0.01 \mu M$
KB-CP.5	$10.0 \pm 0.1 \mu M$	$3.7 \pm 0.2 \mu M$	$9.0 \pm 0.1 \mu M$	$2.3 \pm 0.3 \mu M$
KB-CP20	$> 100 \mu M$	$6.6 \pm 0.2 \mu M$	$> 100 \mu M$	$2.5 \pm 0.2 \mu M$

NOTE: Cells were treated with cisplatin on day 2 ($50 \mu M - 0 \mu M$) and IC_{50} s were calculated as the amount of cisplatin needed to kill 50% of the cell population compared to control at day 5 ($0 \mu M$ cisplatin). Data reported are the mean of at least three independent experiments, with three data points per concentration in each experiment. Error values reported are standard deviations (SD).

Table 2

Members of the miR-181 family sequences, with chromosomal and gene location

miRNA	Alternative name	Chromosome	Start	End	Sequence
miR-181a	miR-181a-1-5p	1	198828173	198828282	AACAUUC AACGUGUCGGUGAGU
miR-181a	miR-181a-2-5p	9	127454721	127454830	AACAUUC AACGUGUCGGUGAGU
miR-181a*	miR-181a-1-3p	1	198828173	198828282	ACCAUCG ACCGUUGAUUGUACC
miR-181a*	miR-181a-2-3p	9	127454721	127454830	ACCAUCG ACCGUUGAUUGUACC
miR-181b	miR-181b-1	1	198828002	198828111	AACAUUC AUUGCUGUCGGUGGGU
miR-181b	miR-181b-2	9	127455989	127456077	AACAUUC AUUGCUGUCGGUGGGU
miR-181c	miR-181c-5p	19	13985513	13985622	AACAUUC AACCGUGUCGGUGAGUX
miR-181c*	miR-181c-3p	19	13985513	13985622	AACCAUCG ACCGUUGAGUGGAC
miR-181d	N/A	19	13985689	13985825	AACAUUC AUUGUCGGUGGGU
miR-4262 [†]	N/A	2	11977059	11977112	GACAUUC AGACUACCUG

NOTE: Changes in nucleotide sequence compared to miR-181a are shown in red.

* miRNA denoted with an identifies the minor strand when both strands of the pre-miRNA hairpin mature to functional miRNA.

[†] miR-4262 is a provisional family member predicted in human embryonic stem cells and neural precursors. Therefore it was not included further in this study, as it is outside the scope of adenocarcinoma function. The seed sequence for 5p miR family members is shown in bold.

Table 3

Cytotoxicity values ($IC_{50} \pm SD$) for cisplatin-treated KB-3-1 and KB-CP.5 cells transfected with miRNA mimics and inhibitors.

microRNA	KB-3-1		KB-CP.5	
	IC_{50} (μM) (cisplatin + mimic)	IC_{50} (μM) (cisplatin + inhibitor)	IC_{50} (μM) (cisplatin + mimic)	IC_{50} (μM) (cisplatin + inhibitor)
negative control	2.51 ± 0.39		10.99 ± 1.09	
miR-181a	1.41 ± 0.17	6.35 ± 0.28	9.29 ± 0.16	15.56 ± 0.30
miR-181a*	5.18 ± 0.05	1.22 ± 0.21	15.64 ± 2.99	10.27 ± 1.20
miR-181b	3.92 ± 2.50	3.51 ± 0.25	10.07 ± 0.66	11.08 ± 0.71
miR-181c	7.32 ± 0.89	1.25 ± 0.27	13.67 ± 3.25	8.93 ± 0.48
miR-181c*	6.12 ± 0.23	2.0 ± 0.30	33.13 ± 8.25	10.92 ± 0.47
miR-181d	1.93 ± 0.53	2.55 ± 0.99	9.84 ± 0.05	14.63 ± 0.57

NOTE: Cells were treated with cisplatin on day 2 ($50 \mu M - 0 \mu M$) and IC_{50} s were calculated as the amount of cisplatin needed to kill 50% of the cell population compared to control at day 5 ($0 \mu M$ cisplatin). Data reported are the mean of at least three independent experiments, with three data points per concentration in each experiment. Error values reported are standard deviations (SD).