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microRNA biogenesis and cellular proliferation

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Abstract

Given the fundamental roles of microRNAs (miRNAs) in physiological, developmental and pathological processes, we hypothesized that genes involved in miRNA biogenesis contribute to human complex traits. For thirteen such genes, we evaluated the relationship between transcription and two classes of complex traits, namely cellular growth and sensitivity to various chemotherapeutic agents in a set of lymphoblastoid cell lines. We found a highly significant correlation between protein argonaute-2 (*AGO2*) expression and cellular growth rate (Bonferroni-adjusted $p < 0.05$), and report additional miRNA biogenesis genes with suggestive associations with either cellular growth rate or chemotherapeutic sensitivity. *AGO2* expression was found to be correlated with multiple drug sensitivity phenotypes. Furthermore, small interfering RNA (siRNA) knockdown of *AGO2* resulted in cellular growth inhibition in an ovarian cancer cell line (OVCAR3), supporting the role of this miRNA biogenesis gene in cell proliferation in cancer cells. Expression quantitative trait loci mapping indicated that genetic variation (in the form of both single nucleotide polymorphisms (SNPs) and copy number variations (CNVs)) that may regulate the expression of *AGO2* can have downstream effects on cellular-growth-dependent complex phenotypes.

Keywords

microRNA biogenesis genes; cellular proliferation; chemotherapeutic sensitivity

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Introduction

MicroRNAs (miRNAs), the non-coding small RNA molecules that have been shown to play important roles in post-transcriptional gene expression, are predicted to target a third of all human mRNAs (1,2). Studies have demonstrated the role of miRNAs in diverse cellular, developmental and pathological processes (3) as well as in drug sensitivity (4,5). Given the important role of miRNAs, we hypothesized that genes affecting miRNA biogenesis and function may broadly influence complex phenotypes, and altering the expression and/or the function of these genes may have substantial downstream phenotypic effects.

Many gene products have been found to be involved in miRNA biogenesis and function (6–9). miRNA biogenesis is a highly complex and finely tuned series of biological processes, including transcription, nuclear processing, export from nucleus, and cytoplasmic processing. We selected thirteen genes that have been shown to be directly involved in converting primary miRNA to pre-miRNA (*DGCR8* and DROSHA *(RNASEN*)), exporting pre-miRNA from nucleus to cytosol (*RAN* and *XPO5*), and regulating post-transcriptional and translational processes (*DICER1*, *TARBP2*, *PRKRA*, *AGO1*, *AGO2*, *AGO3*, *AGO4*, *DDX20* and *GEMIN4*). Furthermore, to explore the functional significance of these genes as well as to evaluate the effect of genetic variation on their expression, we conducted our study in a collection of cell lines from the International HapMap project (10). The primary reason for the choice of such a discovery model is the readily available genomic, transcriptomic and miRNA expression data along with a wide range of phenotypic information (e.g. intrinsic cellular growth rate, cellular sensitivity to different drugs) in these cell lines (11–14).

In this study, we evaluated the expression correlation between each of the thirteen miRNA biogenesis/function related genes and cell growth rate and sensitivity to various drugs. Expression quantitative trait loci (eQTL) mapping was performed to quantify the effect of genetic variation (in the form of SNPs and CNVs) on the expression of these miRNA biogenesis genes. Functional validation experiment was carried out in cancer cell lines.

Materials and Methods

Cell lines

EBV-transformed B-lymphoblastoid cell lines (LCLs) from the International HapMap consortium were purchased from the Coriell Institute for Medical Research (Camden, NJ). Fifty-three unrelated CEU (Utah residents with northern and western European ancestry) and 54 unrelated YRI (Yoruba people from Ibadan, Nigeria) samples were used for this study. These LCLs were maintained as suspension cultures in RPMI 1640 with supplements described previously (15). For functional studies, OVCAR-3, an ovarian cancer cell line, was procured from ATCC (Manassas, VA) and grown as an adherent culture in RPMI-1640 medium with 20% fetal bovine serum (Atlanta Biologicals, GA) and 0.01 mg/mL bovine insulin. OVCAR-3 cells were passaged every three days at a ratio of 1:3.

Genomic, transcriptomic, microRNAomic expression information and intrinsic cellular growth rate, drug sensitivity data

We included in our study a total of 13 genes that have been shown to influence miRNA biogenesis and function (6–9). The expression levels of these genes in the HapMap samples were obtained from Gene Expression Omnibus (GEO, GSE7761) which was quantified using microarray-based gene expression profiling (Affymetrix GeneChip® Human Exon 1.0 ST array).

Genome-wide miRNA expression in these HapMap samples were characterized using the Exiqon miRCURY LNA arrays v.10.0 (Exiqon array), obtained from GEO (GSE34406) (4,16). SNP genotypes were downloaded from International HapMap database (www.hapmap.org, release 27). CNV data were obtained based on the Conrad et al 2010 publication (17).

We have previously generated an intrinsic cellular growth (iGrowth) phenotype in over 500 HapMap LCLs (11). The iGrowth for the CEU and YRI samples were used in this study. Data on sensitivity to chemotherapeutic drugs carboplatin, cisplatin, daunorubicin and etoposide were queried using a publicly available pharmacogenomics resource we developed (www.PACdb.org) (14).

Integrative analysis of miRNA biogenesis genes, miRNA expression, genomic and other cellular phenotypes

We combined the CEU and YRI samples and performed linear regression of gene expression against iGrowth or IC_{50} for each drug independently with the ancestral group as covariate. Linear regression analysis was also performed for each of the cellular phenotypes in the HapMap CEU and YRI samples separately using the R Statistical Software (http://www.r-project.org/) (18). For this study, Bonferroni-adjusted $p < 0.05$ was considered significant, but we report all suggestive associations ($p<0.05$).

SNP and CNV associations with miRNA biogenesis gene expression were identified using SCANdb (www.scandb.org), an online gene expression regulation database we developed (19). SNP and CNV associations with cellular growth rate and drug sensitivity were evaluated by generalized linear regression assuming an additive genetic model. The presence of a negative correlation between expression of a miRNA biogenesis gene and genome-wide miRNA expression was tested through linear regression.

Functional validation

Functional evaluation of the biological role of *AGO2* was subsequently conducted in OVCAR-3 cell line, an ovarian cancer cell line. The rationale for selecting OVCAR-3 cells as a model was the observed common over-expression of *AGO2* in primary ovarian cancers (data obtained through The Cancer Genome Atlas [TCGA] data query (Supplemental Fig 1)). Gene knockdown was conducted through small interfering RNA (siRNA). Specifically, siAGO2 (Cat. No. 1027416, 25nM) and scrambled control (AllStars negative control siRNA, Cat No. 1027292), were purchased from Qiagen. Transfection experiments were conducted using DharmaFECT 1 (Dharmacon™). The effect of transfection was confirmed

by measuring *AGO2* expression at 0, 24 and 48 hours post transfection using quantitative PCR (qPCR). The cellular growth rate was measured using CellTiter-Glo luminescent cell viability assay (Promega) at 0, 24, 48 and 72 hours post transfection. Two-way ANOVA was performed to compare cellular growth rate obtained after siAGO2 and that from scramble control. $P<0.05$ was considered statistically significant for validation.

Results

miRNA biogenesis/function related genes in human complex traits

The expression levels of 13 genes directly involved in miRNA biogenesis and function were compared with iGrowth and sensitivity to each of 4 chemotherapeutic agents (carboplatin, cisplatin, daunorubicin and etoposide) independently. In the pooled CEU and YRI samples, *AGO2* ($p=4\times10^{-6}$) showed a highly significant correlation (Bonferroni-adjusted $p < 0.05$) with iGrowth, and several additional miRNA biogenesis genes showed suggestive associations: *DGCR8* (p=0.0002), *DROSHA* (p=0.075), *PRKRA* (p=0.033) and *TARBP2* (p=0.066). Higher *AGO2* expression was correlated with faster cellular growth in the combined CEU and YRI LCLs (Figure 1A). In each ancestral group (CEU or YRI), 3 genes had expression levels that were correlated with at least one of the four drug IC_{50}s (Table 1 for all nominal associations, $p<0.05$). Notably, *AGO2* expression was correlated with almost all drugs evaluated in both populations with increasing expression level resulting in lower IC_{50}, suggesting greater sensitivity to these agents (Figure 1B and 1C).

Functional validation of *AGO2* in a cancer cell line

To explore the role of miRNA biogenesis genes in cancers, we analyzed The Cancer Genome Atlas (TCGA) dataset, in which a large number of tumors representing over 20 different types of cancers have undergone genomic profiling (http://www.cbioportal.org/public-portal/), for the miRNA biogenesis genes. We found that genetic mutations and altered gene expression are common for *AGO2* in various types of cancers (including ovarian, breast, liver, prostate, uterine, head and neck cancers). More importantly, over 30% of the primary ovarian cancer samples evaluated by TCGA showed *AGO2* over-expression relative to normal, making ovarian cancer a good candidate in evaluating the role of *AGO2* through gene knockdown (Supplemental Figure 1).

We conducted *AGO2* inhibition experiment in an ovarian cancer cell line (OVCAR3) using siRNA. The transfection of siAGO2 resulted in significantly decreased expression of *AGO2* compared to scramble control (quantified through qPCR. Supplemental Figure 2). Subsequently, we observed a significant cellular growth inhibition after siAGO2 transfection when compared to that of control (two-way ANOVA p=0.036, Figure 2). This growth inhibition effect is most pronounced at 72 hours post transfection (t-test p= 0.002).

Genetic variation, miRNA biogenesis genes and downstream miRNA expression

To identify genetic effect on the miRNA biogenesis genes, we performed eQTL mapping for the 13 miRNA processing genes. We found a number of expression-associated SNPs (eSNPs, at nominal $p\leq10^{-4}$) for each of the 13 genes in either the CEU or YRI samples (Supplementary Table 1). In addition, a number of CNVs were found to be associated with 5

of these 13 genes ($p \leq 10^{-4}$, Supplementary Table 1), including CNVR5446_full and *DROSHA* (CEU, p=4.1E-5), CNVR841.1 and *DICER1* (YRI, $p=1.4\times10^{-5}$), CNVR5184_full and *AGO2* (YRI, $p=5.1\times10^{-6}$), CNVR81.1 and *AGO3* (YRI, $p=2.6\times10^{-5}$) and CNVR6423.1 and *GEMIN4* (YRI, $p=2.3\times10^{-5}$).

We tested the identified eSNPs for association with cellular growth. Particularly for *AGO2*, 38 eSNPs were identified in CEU ($p \leq 10^{-4}$, MAF>0.1). An example of such an eSNP (rs10508745) for *AGO2* is shown in Fig 3A (p=0.0001). Notably, this SNP rs10508745 was also associated with cellular growth rate in CEU (Fig 3B, p=0.0006). A CNV (CNVR5184_full) was significantly associated with *AGO2* expression in YRI (Fig 3C, $p=5\times10^{-6}$); CNVR5184_full was also associated with cellular growth rate in this population (Fig 3D, p=0.006).

For 12 of the 13 miRNA biogenesis genes, we found significant negative correlations between gene expression and miRNA expression in the HapMap samples ($p \leq 10^{-4}$, FDR<0.05). The one exception was *AGO2*, for which we did not observe expression correlation between *AGO2* and any of the miRNAs (p>0.05), suggesting that *AGO2* may affect miRNA function rather than expression.

Discussion

In this study, we evaluated the effect of key miRNA biogenesis genes on two human complex traits (cellular proliferation and drug sensitivity) with implications for a range of complex phenotypes and implicated *AGO2* in cellular proliferation and cellular sensitivity to drug. The effect of *AGO2* in cell growth was originally observed in LCLs derived from apparently healthy individuals and subsequently validated in an ovarian cancer cell line.

It has been shown that genes involved in miRNA biogenesis can affect multiple human physiological and pathological functions. For example, *DICER* was found to play an essential role in thyroid function (20), hepatocyte survival, metabolism, tumor suppression (21) and in tumorigenesis (22). *RNASEN* (DROSHA) regulates cell proliferation and affects survival in esophageal cancer patients (23). *RNASEN*, *DGCR8*, *DICER*, *TARBP2*, and *PRKRA* were found to interact with miRNAs and affect liver regeneration (24). Furthermore, factors affecting miRNA biogenesis and function may also affect an individual's susceptibility to pollutants (25) and sensitivity to drugs (26). Kovalchuk et al. observed decreased protein expression of *DICER* and Argonaute 2 (encoded by *AGO2*) in the doxorubicin resistant MCF-7 breast cancer cell line compared to the parent doxorubicin sensitive MCF-7 cells (26).

AGO2, a gene coding Argonaute 2 protein, plays a significant role in the regulation of post-transcriptional and translational processes through miRNA and siRNA (27). It is an important component of RNA induced silencing complex (RISC) catalyzing RNA interference (RNAi) (28) which leads to alteration of many bioprocesses. In our study, the expression of *AGO2* was found to be highly correlated with cellular proliferation in LCLs and with cellular sensitivity to multiple chemotherapeutic agents. Given the mechanism of action for these cytotoxic agents, for which cytotoxicity is highly dependent on cellular

proliferation rate (29), it is plausible that the observed relationship between *AGO2* expression and drug sensitivity may be a consequence of this gene's effect on cell growth. To examine the correlation between *AGO2* and cell growth in cancers, we utilized the comprehensive TCGA data. We found *AGO2* is commonly over-expressed in many cancers when compared to the adjacent normal tissue. This observation finds support in the literature. For example, up-regulation of *AGO2* mRNA and protein was observed in urothelial carcinoma of bladder compared to paired normal bladder (30). Chang et al. reported *AGO2* over-expression in head and neck squamous cell carcinoma (HNSCC) cells (31). Multiple myeloma cell lines showed increased expression of *AGO2* through DNA copy number gain (32) while *AGO2* was overexpressed in pleural effusions when compared to primary ovarian caricinoma in patients with disease that spread beyond the ovary (33). In addition, knocking down *AGO2* was shown to induce apoptosis in HL-60 (myeloid leukemia cells) and was, furthermore, involved in regulation of siRNA mediated RNAi pathways in HEK-293 cells (34). However, to our knowledge, no study to date has reported on the effect of *AGO2* in ovarian cancer. Our *AGO2* knockdown experiment in OVCAR3 resulted in decreased cell proliferation in ovarian cancer. Taken together, we have shown evidence of the effect of *AGO2* on cell proliferation in both normal and cancer cells.

To further examine how *AGO2* affects cell proliferation, we performed expression correlation study between this gene and all expressed miRNAs in the genome. Surprisingly, we did not observe any expression correlation between *AGO2* and any of the miRNA expression in HapMap samples. AGO2 is one of the 4 argonaute proteins (AGO1-AGO4) that play major roles in guiding siRNAs or miRNAs to perform post-transcriptional gene silencing or activation (35). *AGO1, AGO3* and *AGO4* are present as tandem copies on chromosome 1 whereas *AGO2* is present on its own on chromosome 8. Differing from the other 3 AGO subfamily proteins, AGO2 can carry out both site-specific cleavage and non-cleavage mediated inhibition while the other AGO proteins are restricted to non-cleavage mediated inhibition (36). Based on these, we speculated that the variable *AGO2* expression may result in differential miRNA-target gene binding, with downstream regulation of various cellular processes, rather than having a direct effect on miRNA expression.

Lastly, we hypothesized that genetic polymorphisms (in the form of SNPs and CNVs) that regulate the expression of genes in the miRNA biogenesis pathway may influence cellular growth. Indeed, we identified several SNPs/CNVs that were associated with the transcription of miRNA biogenesis genes. Both, the SNP rs10508745 and a CNV (CNVR5184_full) were associated with *AGO2* expression and with cellular growth. These findings support a highly complex network of relationships among genetic variants, miRNA biogenesis and biological function.

In summary, through a comprehensive evaluation of 13 miRNA biogenesis genes, we found that *AGO2* expression was associated with both cellular growth rate and sensitivity to multiple chemotherapeutic drugs. *AGO2* inhibition results in cellular growth inhibition in an ovarian cancer cell line. Finally, eQTLs for *AGO2* may provide genetic effects on cellular proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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All authors have read the journal's authorship agreement and the manuscript has been reviewed and approved by all named authors.

Abbreviations

AGO1	Argonaute RISC catalytic component 1
AGO2	Argonaute RISC catalytic component 2
AGO3	Argonaute RISC catalytic component 3
AGO4	Argonaute RISC catalytic component 4
CEU	Centre d'Etude du Polymorphisme Humain (CEPH) people from Utah, USA
CNV	Copy Number Variation
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20
DGCR8	DiGeorge syndrome critical region gene 8
DICER1	Drosophila, Homolog of, 1
eQTL	expression Quantitative trait Locus
FDR	False Discovery Rate
GEMIN4	Gem (nuclear organelle) associated protein 4
GEO	Gene Expression Omnibus
HNSCC	Head and Neck Squamous Cell Carcinoma
iGrowth	Intrinsic cellular growth
LCLs	Lymphoblastoid Cell Lines
miRNA	microRNA
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator
RAN	Ras-related nuclear protein
RNAi	RNA interference

RNASEN	Ribonuclease type III, nuclear
siRNA	small interfering RNA
SNP	Single Nucleotide Polymorphism
TARBP2	TAR RNA-binding protein 2
TCGA	The Cancer Genome Atlas
XPO5	Exportin-5
YRI	Yoruba people from Ibadan, Nigeria

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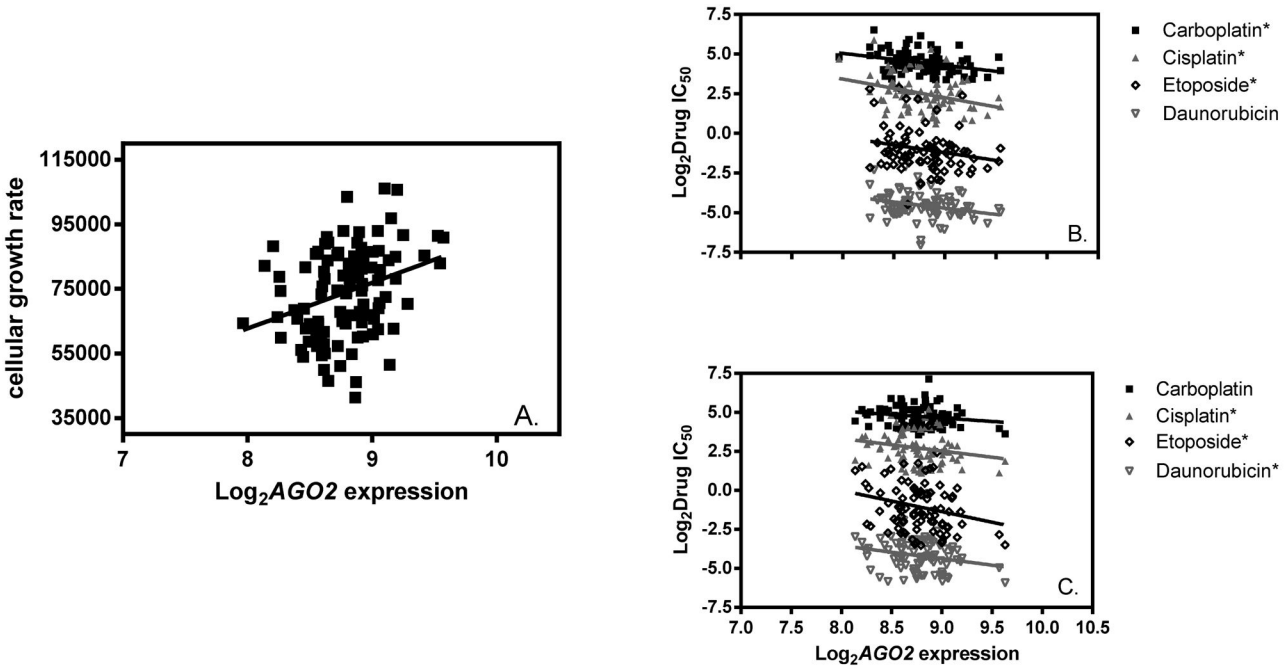

Figure 1. Relationships among *AGO2* expression, cellular growth rate and drug sensitivity in the HapMap LCLs

A) Correlation between *AGO2* expression and cellular growth rate in HapMap CEU and YRI samples (n=107); B) Correlation between *AGO2* expression and each of four drug sensitivity phenotypes in HapMap CEU samples (n=53); and C) Correlation between *AGO2* expression and each of four drug sensitivity phenotypes in HapMap YRI samples (n=54). A "*****" next to the drug name represents suggestively significant correlation between *AGO2* expression and drug IC_{50} ($p<0.05$). All drug IC_{50} values are in μmol unit.

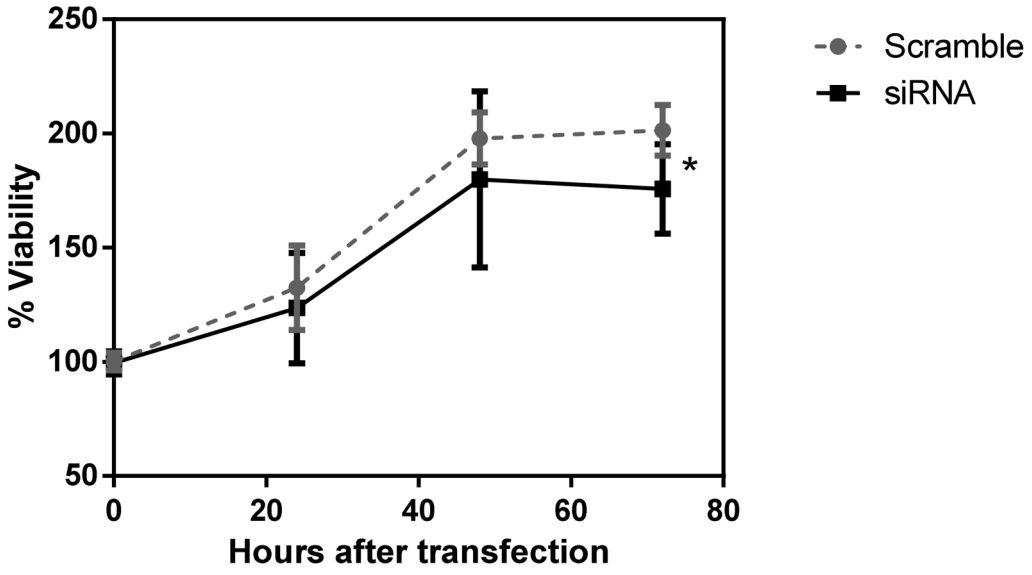

Figure 2. The effect of *AGO2* inhibition on OVCAR-3 cellular growth

Significant inhibition of cell growth observed in OVCAR3 at 72 hours post-transfection (t test p=0.002, two-way ANOVA, p=0.036). Cellular growth was determined using CellTiter Glo reagent. “si” represents siRNA treatment while “scr” represents the control experiment.

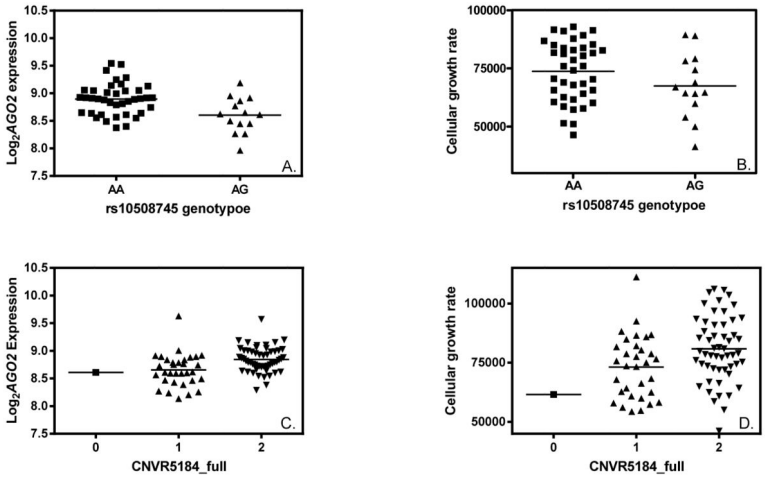

Figure 3. Genetic variations associated with *AGO2* expression are also associated with cellular proliferation

A) rs10508745 and *AGO2* expression association in HapMap CEU samples (p=0.0001); B) rs10508745 and cellular growth rate association in CEU (p=0.0006); C) CNVR5184_full and *AGO2* expression association in YRI ($p=5\times10^{-6}$); D) CNVR5184_full and cellular growth rate association in YRI (p=0.006).

Table 1

miRNA biogenesis genes whose expression levels correlated with a drug IC_{50} ($P<0.05$).

Genes	Drugs	Population	Beta value	P-value
DGCR8	daunorubicin	YRI	0.079935	0.0027
DGCR8	etoposide	YRI	0.055369	0.0003
AGO2	carboplatin	CEU	−0.13163	0.0023
AGO2	cisplatin	CEU	−0.08967	0.0003
AGO2	daunorubicin	CEU	−0.086	0.0043
AGO2	etoposide	CEU	−0.03606	0.0123
AGO2	cisplatin	YRI	−0.06142	0.0244
AGO2	daunorubicin	YRI	−0.05969	0.0181
AGO2	etoposide	YRI	−0.03971	0.0259
AGO3	daunorubicin	CEU	−0.0772	0.0337
GEMIN4	cisplatin	CEU	−0.05419	0.0407
GEMIN4	daunorubicin	CEU	−0.09949	0.0030
GEMIN4	etoposide	CEU	−0.044	0.0301
PRKRA	etoposide	YRI	−0.04417	0.0366