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Genome-Wide Analysis of Differentially Expressed miRNA in PLX4720-resistant and Parental Human Thyroid Cancer Cell Lines

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Abstract

Background—Investigating BRAF^(V600E) inhibitors (BRAFi) as a strategy to treat patients with aggressive thyroid tumors harboring BRAF^(V600E) mutant is currently in progress and drug resistance is expected to pose a challenge. MicroRNAs are involved in development of resistance to a variety of drugs in different malignancies.

Methods—miRNA expression profiles in the human anaplastic thyroid cancer cell line (8505c) were compared to its PLX4720-resistant counterpart (8505c-R) using Illumina deep sequencing. We conducted a functional annotation and pathway analysis of the putative and experimentally validated target genes of the significantly altered miRNAs.

Results—We identified 61 known and 2 novel miRNAs whose expression was significantly altered in 8505c-R. qRT-PCR validated altered expression of 7 selected miRNAs in 8505c-R and BCPAP-R (PLX4720-resistant papillary thyroid cancer cell line). We found 14 and 25 miRNAs whose expression levels changed significantly in 8505c and 8505c-R, respectively, following treatment with BRAFi. MAPK and PI3K-AKT pathways were among the prominent targets of many of the deregulated miRNAs.

Discussion—We have identified a number of miRNAs that could be used as biomarkers of resistance to BRAFi in patients with thyroid cancer. In addition, these miRNAs can be explored as potential therapeutic targets in combination with BRAFi to overcome resistance.

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Keywords

BRAF^(V600E) inhibitors; Anaplastic; Papillary thyroid cancer; Resistance; microRNAs

1. Introduction

Identification of aberrant MAPK and PI3K-AKT signaling pathways in many malignancies has led to development of novel targeted therapies (1). One of the well-characterized members of the MAPK pathway is BRAF, a serine/threonine kinase whose constitutively activated mutant (BRAF^(V600E)) has been found to play a critical oncogenic role not only in the development of 45 % of papillary thyroid cancers (PTC), 25 % of anaplastic thyroid cancers (ATC) (2–4), but also in 50–60 % of melanomas and 5–15 % of colon cancers. These discoveries have led to development of a number of selective BRAF^(V600E) inhibitors (BRAFi) that have shown promising outcome in treatment of patients with melanoma (5). Intrinsic and acquired resistance to BRAFi has proven to be a significant clinical obstacle in the treatment of patients with melanoma (4,5). In melanoma, several mechanisms of resistance have been identified, including reactivation of the MAPK pathway, activation of parallel pathways including PI3K-AKT by receptor tyrosine kinases (RTKs) such as IGF-1R and PDGFR- β (6,7). Some thyroid cancer cell lines are intrinsically resistant to BRAF inhibitors due to feedback-triggered activation of HER2/HER3 signaling and subsequent reactivation of the MAPK pathway (8). The process of resistance is heterogeneous, since it can be due to genetic, metabolic, and phenotypic diversity within the same mass of tumor. This heterogeneity poses a significant challenge when attempts are made to overcome resistance. Therefore, identification of additional mechanisms of resistance is crucial for development of fully effective combinatory treatments. While clinical trials using BRAFi in patients with aggressive thyroid cancers are in their infancy, it is clear from the melanoma experience and limited anecdotal experience in ATC patients that resistance will develop (5). In this study, we set out to explore for the first time the effects of long-term exposure of ATC and PTC cell lines to PLX4720 (BRAFi), on micro-RNAs (miRNA) whose deregulated expression may play a role in development of resistance. miRNAs are small non-coding RNAs of 18–25 nucleotides in length that modulate a wide range of biological processes including proliferation, apoptosis and differentiation by post-transcriptionally regulating gene expression (9). Mounting evidence indicates alterations in functions of miRNAs play crucial roles in the process of tumorigenesis and development of drug resistance in tumor cells (10,11). In this study we have identified miRNAs that likely play a critical role in the development of resistance to BRAFi in thyroid cancer. Using Illumina deep sequencing, we identified differentially expressed miRNAs in 8505c-R cell line compared to parental 8505c cells and validated the results for 7 selected differentially expressed miRNAs in both 8505c-R and BCPAP-R using qRT-PCR.

2. Materials and Methods

2.1. Cell lines

The human ATC cell line 8505c(v600E/–) and PTC cell line BCPAP(v600E/wt) were exposed to gradually increasing concentrations of PLX4720 (Gideon Bollag and Paul Lin at

Plexxikon) dissolved in dimethyl sulfoxide (DMSO). The concentration of PLX4720 was gradually increased to levels (30 and 10 μ M for 8505c and BCPAP cells for a total of 7 and 2 months, respectively) above which the cells were no longer proliferating.

2.2. Dose-response curves

Approximately 300 cells/well were treated with PLX4720 (8505c: 0, 10, 20, 30, 40 and 50 μ M and BCPAP cells: 0, 5, 10, 15, 20, 25, 30, and 35 μ M). Cell viability was measured 96 hours later using CellTiter96 AQ assay. The half maximal inhibitory concentration (IC₅₀) was calculated using GraphPad Prism software.

2.3. Immunoblot analysis

Immunoblot analysis was performed according to standard procedures. The antibodies used included p-ERK1/2 (T202/Y204), p-MEK1/2 (Ser217/221), total ERK1/2 and MEK1/2 and β -actin (Cell Signaling Technology).

2.4. Flow Cytometry and BrdU incorporation assays

Cell-cycle profile analysis was performed using propidium iodide (20 μ g/ml, Sigma) in the presence of 1 mg/ml Ribonuclease A (Sigma). The BrdU assays were carried out following manufacturer's instructions (Cell Signaling Technology). All cell cycle analyses were performed at Partners Flow Cytometry Core.

2.5. Growth curve assays

For each drug concentration, approximately 3000 cells were seeded in each well of a 12-well plate in triplicate. Cells remained either untreated or treated with 30 (8505c) or 5 μ M (BCPAP) PLX4720 for 0, 2, 4 and 6 days. Cells were fixed in formalin and stained with 0.1 % crystal violet in 20 % methanol. Absorbance was read at 595 nm.

2.6. Small RNA library construction and sequencing

Total RNA was isolated following manufacturer instructions using the *miRvana* miRNA Isolation Kit (Ambion). Small RNA fragments were isolated from total RNA and Illumina libraries were constructed according the manufacturer instructions (New England Biolabs, Ipswich, MA). The samples were sequenced on an Illumina HiSeq2500 instrument.

2.7. Sequencing data analysis

3' adapters were removed using *cutadapt* followed by read mapping to human genome (hg19) using bowtie (single unique alignment with 1 mismatch allowed, with no alignment ambiguity). To focus on miRNAs, we filtered out reads from repeat regions and non-miRNA RefSeq genes, and reads shorter than 15bp or longer than 30bp. Based on the remaining reads, we (a) identified known miRNAs by overlap with the 2233 known miRNAs of the human genome, and (b) predicted novel miRNAs using Mireap package (<http://sourceforge.net/projects/mireap/>).

2.8. Detection and analysis of differentially expressed miRNAs

Differential expression analyses were performed using Bioconductor packages (<http://www.bioconductor.org>) and in the R scripting environment (<http://www.r-project.org>). For each known and novel miRNA, we calculated read counts using GenomicFeatures package, followed by the analysis of differential expression using EdgeR package (12). Expression levels were normalized using the weighted trimmed-mean at the log-scale. To call differential expression, we used the following criteria: (1) non-zero read coverage in at least one sample, (2) 2-fold ratio of normalized coverage, and (3) p -value < 0.01 and FDR-adjusted p -value < 0.1 . Using FDR cutoff of 0.05 produced similar results. For sample/miRNA clustering, we used the default method of unsupervised hierarchical clustering implemented in R hclust package, which is based on iterative recalculation of distances between clusters by the Lance-Williams dissimilarity update formula according to complete linkage clustering scheme.

2.9. Quantitative RT-PCR (qRT-PCR)

Reverse transcription (RT) reactions were carried out using Taqman® MicroRNA Reverse Transcription Kit together with TaqMan® MicroRNA Assay (Applied Biosystems, Carlsbad, CA, USA) followed by real-time PCR using miRNA-specific TaqMan® Assay and TaqMan® Universal Master Mix II. PCR reactions were carried out in triplicate using LightCycler® 96 (Roche). Relative expressions were normalized to the expression of RNU44 per manufacturer's recommendations and calculated using the comparative CT method.

2.10. Statistical analysis

Statistical analyses were performed with a two-tailed Student's t -test in Microsoft Excel. P -values of < 0.05 were considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The data represent the average $\pm$ standard deviation.

2.11. miRNA target determination and pathway analysis

To predict the targets of the differentially expressed miRNAs, we used TargetScan (13), Targetminer (14) and miRDB (15). In addition, miRTarBase database (16) was used to list experimentally validated targets. DAVID (17) and KEGG (18) databases were used for functional annotation and pathway analyses, respectively.

Results

3.1. Chronic exposure to PLX4720 results in establishment of thyroid cancer cell lines with enhanced resistant to the effects of BRAF^(V600E) inhibition

Two cell lines were chronically exposed to increasing concentrations of PLX4720 until resistant lines 8505c-R and BCPAP-R were established 7 months and 2 months after initial exposure, respectively. The IC₅₀ of 8505c-R and BCPAP-R cell lines were approximately 37 and 13 μ M respectively, approximately 3 and 2 fold higher than of their corresponding parental cells (12 and 6 μ M for 8505c and BCPAP cells, respectively) (Fig. 1A). Resistant cell lines showed reduced phosphorylation of MEK1/2 on Ser217/221 and ERK1/2 on T202/

Y204 a signal pathway targeted by PLX4720 compared to parental cells, (Fig. 1B). Cell cycle profiles showed that 8505c-R cells did not undergo cell cycle arrest even when exposed to 30 μ M PLX4720, whereas the parental 8505c cells demonstrated remarkable cell cycle arrest at this concentration (Fig. 1C). These results were confirmed when number of 8505c cells incorporating BrdU significantly decreased while that of 8505c-R did not change significantly (Supplementary (supp) Fig. S1). Interestingly, 8505c-R cells in general had a higher percentage of S-phase cells regardless of drug treatment compared to exponentially growing 8505c cells as evident in flow cytometry analysis and in BrdU incorporation assays (Fig. 1C and Supp. Fig. S1A and S1C). Treatment of BCPAP cells with 5 μ M PLX4720 led to an increase in number of cells in S phase while number of cells in G2/M phase decreased and BrdU incorporation increased (Fig. 1C and Supp. Fig. S1B and S1C). These results indicate that PLX4720 treatment of BCPAP cells leads to accumulation of cells in S phase most likely due to a delay in exit from S phase. Treatment of BCPAP-R cells did not significantly affect the number of cells in any phase or incorporation of BrdU (Fig. 1C and Supp. Fig. S1B and S1C). Similar to 8505c-R, BCPAP-R cells had a higher percentage of S-phase cells regardless of drug treatment compared to exponentially growing BCPAP cells (Fig. 1C and Supp. Fig. S1B and S1C). Increased drug resistance in 8505c-R and BCPAP-R cells was also evident over longer exposure to PLX4720. In growth curve analyses, a total inhibition of proliferation of 8505c and BCPAP cells was observed early on during 6 days of exposure to 30 and 5 μ M PLX4720, respectively, while proliferation of 8505c-R and BCPAP-R cells was partially inhibited (Fig. 1D). Sequencing of the BRAF gene in the resistant cell lines showed only 1799T>A mutation in exon 15 similar to the parental cells. Both resistant cell lines were found to be tumorigenic when implanted orthotopically in SCID mice; with the 8505-R cell line also showing evidence of numerous lung metastasis (data not shown).

3.2. Whole genome expression of miRNAs in sensitive and resistant cell lines

Genome-wide miRNA expression profiling was performed in 8505c and 8505-R cell lines in the absence of and 24 hours after treatment with 30 μ M PLX4720 using next generation deep sequencing. Supplementary Figure S2A shows the counts of raw reads, total aligned reads, and reads that represent known and novel miRNAs. Sequencing adapters were removed with *cutadapt* and matching was done at no more than 2% error rate. Reads between 15 and 30 bp (with mode at 21–22bp) were selected. Between 23–25% of the reads were uniquely aligned to the human genome, while 38–55% of the reads were aligned to multiple locations in the genome, of which more than half (21–40%) were due to alignment to ribosomal RNA, and therefore were removed from further analysis. Of the uniquely aligned reads, 9–15% passed the three filters outlined in the Methods section and covers either the known or the novel miRNAs. Supplementary Figure S2B shows the size distribution of reads before and after applying the above filters. After filtering, only reads that overlap with known or predicted miRNAs were retained. The read size ranged from 15 to 30 bp, with mode at 21–22 bp. Of all the 2233 known miRNAs, a total of 499 and 439 were found to be expressed in 8505c and 8505c-R cells while 401 and 365 appeared to be expressed in the same cells 24 hours after treatment with 30 μ M PLX4720, respectively. Using Mireap software, 62 and 24 novel miRNAs were predicted in 8505c and 8505c-R cells, respectively (Supp. Table S1, Sheet 1 and 2, respectively) while 25 and 20 were

predicted in the same cells after treatment, respectively (Supp. Table S1, Sheet 3 and 4, respectively).

3.3. Differentially expressed miRNAs in sensitive versus resistant 8505c cell lines

Expression levels of known and novel miRNAs were compared between the cell lines before and after treatment using *edgeR* package. According to criteria (see Methods), we detected differential expression between 8505c-R and parental 8505c cells in 61 known and 2 novel (predicted) miRNAs (Table 1 and Supp. Table S2). Among known miRNAs, 38 (62.3 %) were significantly up-regulated in 8505c-R, with expression fold change between 2 and 63 (for miR-363-3p, Table 1), whereas 23 miRNAs (37.7 %) were down-regulated, with expression fold change between 2 and 11 (for miR-1291, Table 1). The fold change for the two significantly down-regulated novel miRNAs was 11.1 and 6.71 (Supp. Table S2).

To investigate the difference between miRNA expression profiles between the acute effect of PLX4720 and long term exposure to PLX4720, we determined miRNA expression profiles of 8505c and 8505c-R cell lines before and 24 hours after treatment with 30 μ M PLX4720. We identified 14 miRNAs (7 up- and 7 down-regulated) that were differentially expressed significantly in 8505c-30 compared to 8505c (Table 2, left panel and Supp. Fig. S3B). MiR-3651, miR-23b-3p and miR-27b-5p were found to be up-regulated in 8505c-30 and in 8505c-R (Tables 1 and 2). MiR-1291 was at the top of the lists of down-regulated miRNAs in 8505c-30 and 8505c-R (Tables 1 and 2, left panel). Expression of 6 up-regulated and 19 down-regulated miRNAs were significantly altered in 8505c-R-30 compared to 8505c-R (Table 2, right panel and Supp. Fig. S3C). MiR-184 and miR-503-5p, miR-760 and miR-424-3p were down-regulated in 8505c-R but their levels increased in 8505c-R-30 (Tables 1 and 2, right panel). MiR-409-3p, miR-27a-5p, miR-92a-1-5p, miR-1293, miR-660-5p, miR-146a-5p, miR21-5p, miR-23a-3p, miR-99a-5p, miR-155-5p, miR-425-5p and miR-128-3p were up-regulated in 8505c-R but down-regulated in 8505c-R-30 (Tables 1 and 2, right panel). These results indicate that short and long term exposure to PLX4720 alter expression levels of different sets of miRNAs with a few in common (Supp. Fig. S4). Hierarchical clustering of expression ratio between 8505c and 8505c-R before and after drug treatment shows a similar pattern of differential miRNA expression (Fig. 2). Interestingly, however, the difference in miRNA expression for a given cell line is wider before than after treatment

3.4. Quantitative RT-PCR validated differential expression of 7 miRNAs in PTC and ATC cell lines

To validate the deep sequencing results, we selected 7 differentially expressed miRNAs and examined their expression in 8505c-R and BCPAP-R compared to parental cell lines using qRT-PCR. These miRNAs included the top two up-regulated miRNAs: hsa-miR-363-3p and hsa-miR-96-5p, the randomly chosen hsa-miR-21-5p, two of the most down-regulated miRNAs: hsa-miR-1291 and hsa-miR-184 and the 2 novel miRNAs (Table 1 and Supp. Table S2). The qRT-PCR results followed the same pattern of expression changes as observed with the deep sequencing data in 8505c-R cells indicating the reliability of our RNA-Seq data (Fig. 3). Expression levels of these miRNAs except for miR-1291 in BCPAP-

R, correlated with that in 8505c-R, suggesting that these miRNAs can be potentially used as markers of resistance in papillary thyroid cancer as well (Fig. 3).

3.5. Multiple predicted and validated target genes exist for the differentially expressed miRNAs

To predict potential targets of the known differentially expressed miRNAs, we used several Bioinformatics resources. TargetScan (13) predicted more than 30,000 targets for the differentially expressed miRNAs in 8505c-R in the absence of PLX4720. When no distinction was made between 5p- and 3p-arms of a miRNA, targets of the precursor miRNA were taken into consideration. As shown in Fig. 4, one miRNA can target several genes and one gene can be targeted by several miRNAs. To explore the pathways in which these miRNAs could play a role, their predicted targets were subjected to analysis in KEGG database. Among the top 5 enriched KEGG pathways, metabolic pathways were the most prominent (Supp. Table S3).

TargetScan (13) predicted 6214 targets for the miRNAs differentially expressed in 8505c-30 cells. Interestingly, the PI3K-AKT and MAPK pathways were among the top 5 enriched KEGG pathways associated with these targets (Supp. Table S4). The number of targets for the differentially expressed miRNAs in 8505c-R-30 by TargetScan was 12,925. The top enriched pathways for these targets were the same as those identified for the differentially expressed miRNAs in 8505c-30 by KEGG database (Supp. Table S4 and S5). To increase the stringency of target prediction, we used three miRNA target prediction tools (TargetScan (14) and miRDB (15)) and concentrated on the targets that are consistently predicted by all tools for the top 10 differentially expressed miRNAs (5 top up-regulated and 5 top down-regulated, Table 1) in 8505c-R in the absence of PLX4720. The commonly predicted targets by all 3 databases (a total of 361, Supp. Table S6) were subjected to KEGG pathway search as well as functional annotation in DAVID. The MAPK pathway was the most prominent pathway among the top 5 enriched pathways by KEGG analysis (Supp. Table S7). The enriched functional categories predicted by DAVID tool (17) included cell junction proteins, as well as the proteins involved in the regulation of cellular biosynthesis and transcription (Supp. Table S8).

3.6. Validated targets of the differentially expressed mRNAs and their pathways

Given the known high false positive rates in miRNA target prediction, we additionally filtered the predicted targets of differentially expressed miRNAs by using miRTarBase database (16) to select the experimentally validated targets. Of the 61 differentially expressed miRNAs in 8505c-R, 34 appeared to have validated targets in miRTarBase (Supp. Table S9 and S10). KEGG Pathway search of the validated targets revealed that cancer-related pathways were enriched the most, and the PI3K-AKT pathway ranked as number 5 (Supp. Table S11). Validated targets of the differentially expressed miRNAs were also subjected to functional annotation analysis in DAVID tool (17), with the top 3 functional categories being the regulation of apoptosis, cellular biosynthesis and various metabolic processes (Supp. Table S12).

Of the 14 and 25 differentially expressed miRNAs in 8505c-30 and 8505c-R-30 respectively, 7 miRNA from the 8505c-30 (Supp. Table S13) and 15 from 8505cR-30 group (Supp. Table S14) were found to have validated targets in the miRTarBase database. For both groups the KEGG pathway search for validated targets ranked the PI3K-AKT pathway to be highly influenced by these miRNA (Supp. Table S15,S16). Analysis of the 8505c-30 miRNAs targets using DAVID (26) bioinformatics data base, revealed the top 3 pathways to be involved in migration, transcription/gene expression and angiogenesis (Supp. Table 17). While the Same analysis for 8505cR-30 miRNAs targets revealed that the top pathways in which these targets participate are those involved in apoptosis, nuclear lumen and angiogenesis (Supp. Table S18)

Discussion

MiRNAs are being studied for differentiating malignant thyroid nodules from benign nodules and also as markers of aggressive behavior in thyroid cancers (11). Emerging evidence underlines the involvement of miRNAs in development of resistance to various chemotherapeutic drugs and targeted therapies. Since in all likelihood BRAF inhibitors will become an important component of treatment in aggressive thyroid cancer, we undertook the first study to look at the miRNAs during treatment and emergence of resistance in thyroid cancer cells exposed to BRAF inhibitors. For this study, the miRNA expression profile was studied in the PLX4720 resistant cell line that was developed by chronic exposure of 8505c cells to the increasing dose of PLX4720, which is a traditional method employed in the homogenous resistance cell population development, in other cancers like melanoma to study gene expression differences in resistance models. Studying these MiRNAs is important as they may not only play a role in cell resistance but can also potentially serve as markers for predicting resistance and as therapeutic targets. We identified 61 known and 2 novel miRNAs whose expression was significantly altered in PLX4720-resistant 8505c-R cell line compared to the parental 8505c cell line. We further validated differential expression of 7 of these miRNAs (5 known and 2 novels) with qRT-PCR in both PTC and ATC cell lines. Differential expression of these 7 miRNAs except for one (miR-1291) was also observed in BCPAP-R cells and followed the same pattern as in 8505c-R. This is interesting because BCPAP-R cells are papillary thyroid cancer that are heterozygous for the mutant BRAF and are at baseline significantly more sensitive to PLX4720. These results suggest that a specific set of miRNAs are involved in development of resistance regardless of the type of cancer and sensitivity to PLX4720. While differential expression of miRNAs identified in this study will need to be further confirmed in thyroid cancer samples obtained during clinical trials of BRAFi, this is the first step towards identifying the important role, individual miRNAs may play in the development of resistance to BRAFis.

Previous analysis found differences in miRNA expression patterns between normal and malignant thyroid tissue. miRNA-221 and -222 were shown to affect the expression of key cell cycle regulator proteins – KIT and P27^{kip1} while decrease in miRNA-1 affects tumor suppressor gene CCND2, CXCR4 and SDF1 which inhibit thyroid carcinoma cell proliferation and migration [28]. miRNA 17–92 cluster was shown to be overexpressed in ATC cells contributing to the aggressiveness and strong resistance to apoptosis by Takakura et al [29]. In another study Braun et al identified the down regulation of mir –30 and –200 in

ATCs compared to PTCs which affects epithelial-mesenchymal transition contributing to the metastatic potential of ATC [30]. Significant differences in miRNA expression patterns (specifically miR-187,-221,-222,-146b, and -155) were found to be associated with the mutational status of thyroid tumors. miRNA 146b is significantly higher in PTC with BRAF mutation and was shown to be associated with tumor invasiveness [31]. Recently expression of the miRNAs 146-b,-221,-222 and their role in tumor aggressiveness were validated using The Cancer Genome Atlas project results for a cohort of 496 PTC patients samples (32). Interestingly, deregulation of expression of several of the miRNAs identified in this study has been previously observed in the development of drug resistance in other malignancies. Up-regulation of mi-R-96 has been observed in Daunorubicin-resistant cell lines of Ehrlich ascites cancer [33]. Overexpression or downregulation of miR-21 has been shown to play a possible role in development of resistance to Cytarabine and Nogamycin in lung cancer and to Topotecan and Doxorubicin in breast cancer, respectively [34]. This suggests that deregulation of miR-21 expression might be context- and drug dependent. Down-regulation of mi-RNA-1291 has been reported to lead to up-regulation of multidrug resistance-associated protein 1 (MRP1/ABCC1) and subsequent resistance to doxorubicin in pancreatic carcinoma PANC-1 cell line [35]

We have shown that a number of the differentially expressed miRNAs identified here, target multiple members of the MAPK and PI3K-AKT pathways, which is not surprising given previously published results regarding the importance of MAPK pathways to BRAFi resistance. The multiple inputs, including at the miRNA level demonstrated by us here, shows the importance of these pathways in development of resistance to BRAFi. The diversity of experimentally validated as well as the predicted targets and their associated pathways also demonstrate that development of drug resistance similar to the process of tumorigenesis is a complicated process with simultaneous engagement of many biological pathways and processes.

In summary, our results demonstrate that when thyroid cancer cell lines become resistant to the PLX4720, a specific set of miRNAs are deregulated. These deregulated miRNAs affect a number of important cellular pathways such as apoptosis, cellular biosynthesis, various metabolic processes and regulation of transcription. Our study is limited to in vitro and in silico analysis, and ultimately the miRNAs we have identified need to be further studied in collected tissues from thyroid cancer patients treated with BRAFi to see if they prove useful as potential diagnostic biomarkers for predicting both sensitivity and resistance to BRAF inhibitors. Furthermore, these distinct miRNAs once further validated may also have utility in melanoma and colorectal cancer patients whose tumor growth dependent on BRAF^(V600E) mutant. In addition, targeting these miRNAs in combination with BRAFi may help overcome both intrinsic and acquired resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

8505c and BCPAP cell lines chronically exposed to PLX4720 develop resistance. A, Dose-response curves for parental 8505c and BCPAP (solid grey) and resistant 8505c-R and BCPAP-R (solid black) treated with the indicated doses of PLX4720 for 96 hrs. The IC_{50} in 8505c-R (~37) was approximately 3 fold higher than that in 8505c (~12). The IC_{50} in BCPAP-R (~13) was approximately 2 fold higher than that in BCPAP (~6). B, Immunoblot analysis demonstrating that activation of MAPK pathway is resistant to BRAF^(V600E) inhibition by PLX4720 in 8505c-R and BCPAP-R cells. Increasing doses of PLX4720

inhibited phosphorylation of ERK1/2 (on T202/Y204) and MEK1/2 (on Ser217/221) in parental 8505c and BCPAP but had no effect in 8505c-R and BCPAP-R cells up to 30 and 20 μ M, respectively. Total ERK1/2, MEK1/2 and β -actin are loading controls. C, left panel, 24 hrs treatment with 30 μ M PLX4720 reduced 8505c cell population in S phase ($p < .01$) and increased cell population in G0/G1 ($p < .001$). The same treatment had no significant effect on cell cycle profile of 8505c-R cells ($p > .05$). C, right panel, 24 hrs treatment with 5 μ M PLX4720 increased BCPAP cell population in S phase ($p < .001$) and decreased cell population in G2/M ($p < .001$). The same treatment had no significant effect on cell cycle profile of BCPAP-R cells ($p > .05$). Bar graphs represent percentage of average cell numbers in different phases of the cell cycle for each cell line before and 24 hrs after drug treatment. D, Growth curve analysis demonstrating long term effect of PLX4720 on cell proliferation. Drug treatment inhibited proliferation of 8505c cells ($p < .001$) and BCPAP ($p < .001$) cells but did not affect that of 8505c-R and BCPAP-R cells significantly ($p > .05$). Representative plate of one experiment for each cell line is shown in lower panel.

Figure 2.

Hierarchical clustering of miRNA expression levels. The hierarchical clustering heat map of log₂ ratio of miRNA expression levels in the cells lines before and after drug treatment as indicated below each column. Each color bar represents the log₂ ratio between the two conditions being compared from 1/8 (bright green) to 8 (bright red) fold. The most notable is how similar the pattern is between 8505c vs. 8505c-R (4th column) and 8505c vs. 8505c-R after treatment with PLX4720 (2nd column), although after treatment the difference is more mild.

Figure 3.

Comparison of miRNA expression levels obtained by deep sequencing and qRT-PCR. qRT-PCR analysis (grey bar graphs) generally validated alteration in expression levels of the selected up- and downregulated miRNAs obtained by deep sequencing (black bar graphs). Error bar shows the SD of the fold change.

Figure 4.

Many members of the MAPK and PI3K-AKT pathways are targets of the differentially expressed miRNAs. TargetScan was used to predict the putative targets of the differentially expressed miRNAs. KEGG analyses were conducted to identify the pathways in which the putative and validated targets (identified in miRTarBase) operate. MAPK and PI3K-AKT pathways were among the most prominent pathways. Only parts of the pathways are shown. Members of the pathways are displayed in bold double-lined rectangles and bold arrows

indicate the pathways. Upregulated miRNAs are in rectangles with no background and downregulated miRNAs are shown in rectangles with grey background.

Table 1

Differentially expressed miRNAs in 8505c-R cell line compared to parental 8505c line in the absence of PLX4720.

	miRNA	Ratio	DiffPval	DiffQval
1	hsa-miR-363-3p	62.65	1.46E-11	6.93E-10
2	hsa-miR-96-5p	31.69	1.93E-34	2.08E-32
3	hsa-miR-490-3p	30.25	7.63E-06	0.000249
4	hsa-miR-543	25.21	5.68E-14	3.47E-12
5	hsa-miR-335-3p	14.04	0.000145	0.003922
6	hsa-miR-409-3p	12.96	1.55E-06	5.35E-05
7	hsa-miR-4473	11.88	0.000977	0.023022
8	hsa-miR-99a-5p	11.67	1.16E-73	1.84E-71
9	hsa-miR-660-5p	10.64	2.69E-19	2.07E-17
10	hsa-miR-21-5p	8.15	0*	0*
11	hsa-miR-146a-5p	7.23	9.25E-22	7.86E-20
12	hsa-miR-3917	7.10	7.66E-07	2.68E-05
13	hsa-miR-23b-3p	6.76	1.78E-270	1.06E-267
14	hsa-miR-192-5p	6.48	0.000121	0.003341
15	hsa-miR-30b-5p	6.25	1.38E-13	8.20E-12
16	hsa-miR-664a-3p	6.12	8.80E-05	0.002555
17	hsa-miR-3653	5.67	3.21E-14	2.07E-12
18	hsa-miR-27a-5p	5.50	1.17E-75	1.98E-73
19	hsa-let-7a-5p	4.97	1.29E-05	0.000389
20	hsa-miR-1293	4.88	7.03E-11	3.16E-09
21	hsa-miR-128-3p	4.20	4.25E-13	2.20E-11
22	hsa-let-7b-5p	4.02	3.46E-111	8.24E-109
23	hsa-miR-425-5p	3.69	4.69E-135	1.40E-132
24	hsa-let-7c	3.60	1.47E-05	0.000439
25	hsa-miR-3651	3.60	0.000821	0.020162
26	hsa-miR-340-3p	3.34	7.84E-08	2.96E-06
27	hsa-miR-361-5p	3.07	1.01E-05	0.000318
28	hsa-miR-132-5p	2.85	2.15E-12	1.09E-10
29	hsa-miR-24-2-5p	2.60	4.04E-07	1.46E-05
30	hsa-miR-92a-1-5p	2.59	7.04E-21	5.78E-19
31	hsa-miR-155-5p	2.51	0*	0*
32	hsa-miR-320a	2.28	9.73E-50	1.16E-47
33	hsa-let-7e-5p	2.19	1.59E-28	1.65E-26
34	hsa-miR-100-5p	2.11	3.23E-183	1.28E-180
35	hsa-miR-23a-3p	2.10	4.98E-52	6.24E-50

	miRNA	Ratio	DiffPval	DiffQval
36	hsa-miR-331-3p	2.10	4.49E-09	1.88E-07
37	hsa-miR-27b-5p	2.05	7.44E-08	2.86E-06
38	hsa-miR-3607-3p	2.05	0.003372	0.06982
39	hsa-miR-1291	0.09	3.67E-109	7.94E-107
40	hsa-miR-1287	0.12	0.004181	0.085083
41	hsa-miR-184	0.12	5.92E-10	2.56E-08
42	hsa-miR-503-5p	0.15	1.61E-55	2.14E-53
43	hsa-miR-7-5p	0.15	0.000116	0.003293
44	hsa-miR-328	0.16	0.001544	0.034355
45	hsa-miR-504	0.20	1.52E-95	2.79E-93
46	hsa-miR-4745-3p	0.22	0.000156	0.004185
47	hsa-miR-760	0.26	4.60E-14	2.88E-12
48	hsa-miR-4745-5p	0.27	0.001658	0.036219
49	hsa-miR-449c-5p	0.28	0.001658	0.036219
50	hsa-miR-2277-5p	0.30	0.00046	0.011652
51	hsa-miR-4664-3p	0.33	0.000802	0.019899
52	hsa-miR-185-5p	0.34	0.002459	0.053224
53	hsa-miR-424-3p	0.35	5.47E-211	2.60E-208
54	hsa-miR-584-5p	0.39	2.58E-14	1.71E-12
55	hsa-miR-769-5p	0.41	3.45E-06	0.000116
56	hsa-miR-4467	0.41	2.01E-06	6.82E-05
57	hsa-miR-1301	0.45	8.14E-21	6.46E-19
58	hsa-miR-503-5p	0.45	0.001111	0.025933
59	hsa-miR-3200-3p	0.46	0.000445	0.011401
60	hsa-miR-3187-3p	0.47	0.000684	0.017149
61	hsa-miR-30d-5p	0.49	3.26E-128	8.62E-126

* indicates extremely significant P-values and Q-values below the computational precision limit.

Table 2

Differentially expressed miRNAs in 8505c and 8505c-R cell lines (left and right panels, respectively) following treatment with 30 μ M PLX4720.

	miRNA	Ratio	DiffPval	DiffQval
1	hsa-miR-506-3p	7.02	6.60E-05	0.003563
2	hsa-miR-3651	4.49	2.43E-05	0.001372
3	hsa-miR-23b-3p	4.18	3.95E-110	2.50E-107
4	hsa-miR-6087	3.57	3.92E-13	4.14E-11
5	hsa-miR-27b-5p	3.17	5.09E-21	6.79E-19
6	hsa-miR-27b-3p	2.18	2.33E-06	0.000144
7	hsa-miR-935	2.02	3.53E-06	0.000213
8	hsa-miR-1291	0.10	4.69E-98	2.38E-95
9	hsa-miR-22	0.21	5.06E-33	9.87E-31
10	hsa-miR-193a-3p	0.29	0.001641	0.070556
11	hsa-miR-221-5p	0.34	1.94E-77	8.20E-75
12	hsa-miR-31-3p	0.35	0.001563	0.068332
13	hsa-miR-126-3p	0.40	2.75E-12	2.68E-10
14	hsa-miR-31-5p	0.49	1.30E-36	3.29E-34

	miRNA	Ratio	DiffPval	DiffQval
1	hsa-miR-184	8.86	6.68E-08	4.46E-06
2	hsa-miR-504-5p	3.89	5.66E-42	1.03E-39
3	hsa-miR-503-5p	3.16	2.59E-10	1.99E-08
4	hsa-miR-760	2.90	8.58E-06	0.000495
5	hsa-miR-424-3p	2.59	7.62E-112	2.76E-109
6	hsa-miR-1180-3p	2.52	9.26E-251	3.91E-248
7	hsa-miR-96-5p	0.05	1.57E-16	1.89E-14
8	hsa-miR-409-3p	0.08	0.001312	0.051962
9	hsa-miR-27a-5p	0.14	4.17E-47	1.06E-44
10	hsa-miR-31-3p	0.22	1.47E-05	0.000812
11	hsa-miR-92a-1-5p	0.22	1.43E-21	2.13E-19
12	hsa-miR-1293	0.23	5.38E-06	0.000325
13	hsa-miR-660-5p	0.25	1.65E-06	0.000105
14	hsa-miR-146a-5p	0.25	4.25E-08	2.91E-06
15	hsa-miR-221-5p	0.32	7.82E-46	1.80E-43
16	hsa-miR-15b-3p	0.32	0.000307	0.014153
17	hsa-miR-21-5p	0.33	0	0
18	hsa-miR-23a-3p	0.38	1.96E-44	3.82E-42
19	hsa-miR-99a-5p	0.38	3.14E-13	3.06E-11
20	hsa-miR-155-5p	0.39	0	0

	miRNA	Ratio	DiffPval	DiffQval
21	hsa-miR-501-5p	0.40	4.92E-06	0.000304
22	hsa-miR-22-3p	0.45	1.10E-05	0.000621
23	hsa-miR-425-5p	0.47	4.66E-34	7.88E-32
24	hsa-miR-128-3p	0.49	0.002315	0.088937
25	hsa-let-7e-3p	0.50	5.22E-14	5.52E-12