MicroRNAs affect complement regulator expression and mitochondrial activity to modulate cell resistance to complement-dependent cytotoxicity

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**Disclosure of Potential Conflicts of Interest**

The authors declare no conflict of interest.
Abstract

MicroRNAs (miRs) are small RNA molecules that shape the cell transcriptome and proteome through regulation of mRNA stability and translation. Here, we examined their function as determinants of cell resistance to complement-dependent cytotoxicity (CDC). To achieve this goal, we compared the expression of microRNAs between complement-resistant and -sensitive K562 leukemia, Raji lymphoma, and HCT-116 colorectal carcinoma cells. Global microRNA array analysis identified miR-150, miR-328, and miR-616 as regulators of CDC resistance. Inhibition of miR-150 reduced resistance, whereas inhibition of miR-328 or miR-616 enhanced cell resistance. Treatment of K562 cells with a sublytic dose of complement was shown to rapidly increase miR-150, miR-328, and miR-616 expression. Protein targets of these microRNAs were analyzed in K562 cells by mass spectrometry–based proteomics. Expression of the complement membrane regulatory proteins CD46 and CD59 was significantly enhanced after inhibition of miR-328 and miR-616. Enrichment of proteins of mitochondria, known target organelles in CDC, was observed after miR-150, miR-328, and miR-616 inhibition. In conclusion, miR-150, miR-328, and miR-616 regulate cell resistance to CDC by modifying the expression of the membrane complement regulators CD46 and CD59 and the response of the mitochondria to complement lytic attack. These microRNAs may be considered as targets for intervention in complement-associated diseases and in anti-cancer, complement-based therapy.
Introduction

In our body, cells constantly interact with proteins of the complement system. For protection from the potentially noxious effects of complement, cells block or regulate the activation of the complement initiation process and terminal cascades through specific inhibitors expressed on their surface, mainly CD46, CD55, and CD59 (1,2). CD46, the membrane cofactor protein, and CD55, the decay-accelerating factor, inhibit the activation of C3b deposition on cells at an early stage (3,4), whereas CD59 blocks the formation of C5b-9 or the membrane attack complex (MAC) at the terminal stage (5). Malignant transformation and postulated sculpting interactions with the complement system yield cancer cells with elevated expression of complement membrane regulators and, thus, are more resistant to complement-dependent cytotoxicity (CDC)(6). Cancer cells are also known to overexpress several claimed intracellular inhibitors of CDC, mortalin/GRP75 (7), Hsp90 (8), PKC, and ERK (9). This study examined the hypothesis that global changes at the transcription/translation level, in particular, at the microRNA level, occurring during the malignant transformation process and possibly involving cell encounters with the innate immune system, generate a complement-resistant cancer cell phenotype.

MicroRNAs (miRs) can potentially halt the expression of multiple protein-coding genes (10,11), and typically, each microRNA binds at its specific “seed” sequence region at the 3’ UTR of mRNAs (12). This promotes mRNA degradation or impairs mRNA translation (13), leading to a decline in protein expression (11). The human genome encodes thousands of microRNA genes, several of which are expressed in each cell (14) and body fluids (15), that regulate at least half of all human gene transcripts (16). MicroRNAs are implicated in multiple diseases,
including cancer (17-19). Hence, miR-based therapy is now emerging as a novel approach in treating cancer and various other diseases (20-23).

There is almost no data on the association between microRNAs and the cells’ sensitivity to CDC. We previously identified the contribution of miR-200 and miR-217 to mortalin function and also to the extent of cell resistance to CDC (24). Here, we first performed a systemic microRNA array analysis in which the microRNAs’ expression was compared between complement-resistant and complement-sensitive tumor cells. Validated differential expression analysis identified miR-150-5p, miR-328-3p, and miR-616-3p as regulators of CDC resistance. Next, the differential expression of proteins whose expression was affected by these microRNAs was characterized by comparing the proteome in extracts of microRNA-inhibited versus control cells. The results suggest that miR-150-5p, miR-328-3p, and miR-616-3p may be therapeutic targets in cancer immunotherapy and complement-associated diseases.

Materials and Methods

Cells, sera, and antibodies

Cells were cultured in RPMI-1640 (Sigma, Rehovot, Israel) (K562 and Raji cells) or DMEM (HCT-116 cells) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY), 1% glutamine, 2% pyruvate, 100 μg/ml penicillin, and 400 μg/mL streptomycin (Bio-Lab, Jerusalem, Israel) at 37°C and 5% CO₂. Fresh cells were thawed every 2 months. Cells were purchased from ATCC (Manassas, VA) and were mycoplasma free. Cell line authentication was performed by STR DNA profiling analysis. Complement-sensitive and -resistant cell lines were generated as indicated in the Results.
Normal human serum (NHS) prepared from the peripheral blood of healthy individuals (3H Biomedical AB, Uppsala, Sweden, 3H1001-100-P, <https://www.3hbiomedical.com/>) served as a source for complement. Its use was approved by the Ethics Committee of Tel Aviv University. Heat-inactivated serum (HIS) was prepared by heating NHS at 56°C for 45 minutes. NHS and HIS were kept frozen at -7°C in small aliquots. A polyclonal anti-serum, directed to human K562 or carcinoma cells, was prepared in rabbits in the animal facilities of Tel Aviv University (approved by the Animal Ethics Committee of Tel Aviv University)(25). The collected antiserum was kept frozen at -70°C in small aliquots. A polyclonal goat anti-human C3 antibody was purchased from Complement Technology, Inc. (Tyler, Texas). The mouse monoclonal antibody directed to a neoepitope in human C5b-9 (clone aE11), was purchased from Hycult Biotech (Uden, The Netherlands). Mouse monoclonal anti-human CD46 (clone MEM-258), anti-human CD55 (clone 67) and anti-human CD59 (clone MEM-43) antibodies were purchased from AbD Serotec (Oxford, UK). FITC-conjugated goat anti-mouse IgG, FITC-conjugated donkey anti-goat IgG, Cy3-conjugated goat anti-mouse IgG, and Cy3-conjugated donkey anti-goat IgG were purchased from Jackson Immunoresearch (West Grove, PA).

**Plasmids and transient transfection**

Precursor microRNA expression clones and microRNA inhibitor expression clones for miR-150, miR-328, miR-616, and control plasmids were purchased from GeneCopoeia (Rockville, MD, USA). MicroRNA inhibitors specific for miR-328-3p (MIMAT0000752: 5'CUUGCCCUUCUGCCUUCGCCGU), miR-25-5p (MIMAT0004498: 5'AGGCAGGAGACUUGGGCAUUG), miR-126-3p (MIMAT0000445: 5'UCGUACCUAGAGAUUAUAUGCG), miR-500a-5p (MIMAT0004773: 5'UAAUCUCUUGCUACCUGGGUGAGA), and miR-150-5p (MIMAT0001144: 5'CUUCCUUGCUACCUGGGUGAGA) were purchased from GeneCopoeia.
(MIMAT0000451: 5' UCUCCCAACCCUUGUACCAGUG), miR-532-3p
(MIMAT0004780: 5'CCUCCCACACCCAAGGCUUGCA), miR-199a-3p
(MIMAT0000232: 5'ACAGUAGUCUGCACAUUGGUUA), and a non-specific oligonucleotide (negative control A: YI00199006; TAACACGTCTATACGCCCA) were purchased from Exiqon (Vedbaek, Denmark). The non-specific oligonucleotide was designed to have no known microRNA targets. K562 cells (4x10^6) were resuspended in electroporation buffer (20 mM PIPES, 128 mM glutamate, 10 µM calcium acetate, 2 mM magnesium acetate in RPMI-1640). They were then mixed with: (a) 15µg of precursor microRNA expression plasmid or a microRNA inhibitor expression plasmid, or control plasmids; or (b) 5nM of miRNA inhibitor or negative control. Finally, they were subjected to electroporation (300 mV, 15 msec) in ECM-830 (BTX Harvard Apparatus, Holliston, MA) in 4 mm electroporation cuvette. The cells were then suspended in culture medium and cultured at 37°C for 24 hours (miRNA overexpression) or 48 hours (miRNA inhibition).

Complement-dependent cytotoxicity and flow cytometry

To investigate complement-dependent cytotoxicity (CDC), parental and variant K562, Raji, and HCT-116 cells were first treated with diluted antibodies in Hank's Balanced Salt Solution (Sigma-Aldrich) for 30 minutes at 4°C and then with complement for 60 minutes at 37°C. Rabbit antibodies directed to K562 or carcinoma cells were used. NHS (final: 50%) was used as the source for complement and HIS (final: 50%) as a negative control. The antibody dose used was determined based on a cytotoxicity titration curve in which the antibody dose was varied in the presence of 50% NHS. When treatment inhibited CDC, the percentage of control cell death was set to be >50%, and when treatment enhanced CDC, the percentage of control cell death was set to be <50%. Specific details are given in legends of figures.
The percentage of cell death was determined by propidium iodide or DAPI inclusion in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The PI and DAPI concentrations used did not label intact viable cells. Flow cytometry data were analyzed with Flowing Software 2.5.1. To calculate the inhibition percentage of cell death, the cytotoxicity percentage was converted into \( y/1-y \) in which \( 100y = \) the percentage of cell death (26). Thus, a percentage cytotoxicity of 50\%, \( y=0.5 \) and \( y/1-y = 0.5/(1-0.5) = 1 \).

To measure C3b or C5b-9 deposition, K562 cells (1x10^6) were incubated with a sublytic dose of antibodies (yielding 10–20% dead cells, as determined by titration curve), diluted in in Hank's Balanced Salt Solution (HBSS), for 30 minutes at 4°C and then with NHS or HIS (final: 50%) for 10 minutes at 37 °C. These conditions were chosen in order to minimize noise from non-specific binding of C3 and C5b-9 to dying, necrotic cells. Under lytic antibody and complement conditions (yielding >30% dead cells), many cells were necrotic within 10 minutes treatment. The cells were then labeled with goat anti-C3 (diluted 1:500 in HBSS) or mouse anti-neo C5b-9 (clone aE11; diluted 1:100 in HBSS) for 30 minutes at 4°C, followed by FITC- or Cy3-conjugated secondary antibodies (diluted 1:100 in HBSS) for 30 minutes at 4°C. To quantify complement regulator expression, cells were treated with mouse anti-CD46, anti-CD55, or anti-CD59 (10 μg/ml in HBSS) for 30 minutes at 4°C and then with FITC-conjugated secondary antibodies (diluted 1:100 in HBSS) for 30 minutes at 4°C. Finally, the cells were analyzed by flow cytometry as described above.

RNA extraction and real-time PCR

RNA was extracted from K562, Raji, or HCT-116 cells using TRI Reagent (Sigma), and the RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE). First-strand complementary DNA (cDNA) was synthesized from total RNA (10
ng) using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). This reaction contained a specific stem–loop primer for each mature target microRNA. Each stem–loop primer was designed to hybridize to only the fully mature microRNA, and not to the precursor forms of its target. PCR amplification was carried out using a Step One Plus Real-Time PCR System (Applied Biosystems) under the following thermal cycler conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C and 1 min at 60 °C. Results in triplicates were analyzed with SDS software (Applied Biosystems) and RQ (relative quantity) Manager Software (Applied Biosystems), for automated data analysis. MicroRNA relative expression was calculated based on the comparative threshold cycle (Ct) method. The Ct for each microRNA and endogenous control U6 small nuclear (sn)RNA (used for normalization) in each sample was used to create ΔCt values (Ct\text{microRNA} – Ct\text{U6 snRNA}). Next, ΔΔCt values were calculated by subtracting the ΔCt value of the control group from the ΔCt value of the tested group. The RQs were calculated using the equation: $RQ = 2^{-\Delta\Delta ct}$. TaqMan miRNA Assays for hsa-miR-500a-5p (Assay ID 478309_mir), hsa-miR-532-3p (Assay ID 478336_mir), hsa-miR-199a-3p (Assay ID 477961_mir), hsa-miR-328-3p (Assay ID 478028_mir), hsa-miR-616-3p (Assay ID 478177_mir), hsa-miR-486-3p (Assay ID 478422_mir), hsa-miR-548b-5p (Assay ID 478589_mir), hsa-mir-1254 (Assay ID 478660_mir), hsa-miR-25-5p (Assay ID 478786_mir), hsa-miR-597-5p (Assay ID 478339_mir), hsa-miR-509-5p (Assay ID 478965_mir), hsa-miR-150-5p (Assay ID 477918_mir), hsa-miR-126-3p (Assay ID 477887_mir) and hsa-miR-1225-3p (Assay ID 477875_mir) were purchased from Applied Biosystems.

**MicroRNA arrays**
Complementary DNA (cDNA) was synthesized from total RNA extracted from K562, Raji, or HCT-116 cells using the Megaplex reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). The TaqMan Low-Density Arrays (TLDAs) are quantitative real-time PCR assays (Applied Biosystems technology, catalog number 4342265) that enable accurate quantitation of 754 human microRNAs. Each array includes three TaqMan MicroRNA Assay endogenous controls to aid in data normalization. For each sample, cDNA (500 ng), the TaqMan Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems), and RNase-free water were mixed and loaded onto human TLDA cards A and B (according to the manufacturer’s instructions). The card was centrifuged and sealed, and PCR amplification was performed using an ABI Prism 7900HT Sequence Detection System (Supplementary Array S1) and analyzed as described above for real-time PCR.

Proteomic analysis

K562 cells subjected to inhibition of miR-150, miR-328, or miR-616 expression or treated with control, non-specific miR inhibitor (see above) were lysed using 6 M urea and 2 M thiourea in 0.1 M Tris, pH 8.5, then reduced with 1 mM dithiothreitol and alkylated with 5 mM iodoacetamide for 30 minutes each, followed by overnight digestion using LysC-trypsin mix (Promega, Madison, WI). The resulting peptides were fractioned using strong cation exchange fractionation in StageTip format and then fractionated by high performance liquid chromatography (Easy nLC 1000 HPLC system; Thermo Fisher Scientific) coupled online to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) using the EASY-Spray ionization source (NSI with spray voltage of 2.10). Peptides from each fraction were separated with a flow rate of 0.3 μl/min for 140-minute linear gradient of water-
acetonitrile using a PepMap 50 cm long C_{18} column. All the measurements were done in positive mode. Raw MS files were analyzed by MaxQuant (version 1.5.3.36) with the integrated Andromeda search engine. MS/MS searches were performed against the human Uniprot database (published September 2015). Protein sequences were reversed to form a decoy database for FDR calculation. Then, a 1% false discovery rate was applied to both protein and peptide identification. To obtain quantitative data, the label-free quantification (LFQ) algorithm in Maxquant was used. Predictions of the miRNA targets were made by using TargetScan (release 7.2) [http://www.targetscan.org]).

**Bioinformatics and statistical analysis**

Bioinformatics analysis was performed using Partek Genomics Suite v 6.6 (http://www.partek.com/pgs). Because the results are based on two separate experiments, batch removal was performed. A total of 20 samples were analyzed (five replicates for each treatment). Student’s t-test was performed to compare the control group (cells transfected with control plasmid) and cells transfected with a microRNA inhibitor plasmid (anti-miR-150, anti-miR-328, or anti-miR-616) with a fold change cutoff of 1.5. Results are expressed as the arithmetic mean±S.D. An ANOVA test was performed on LFQ intensities (\(P<0.05\)) to compare the groups. Hierarchical clustering was performed after z-score normalization of the rows (proteins) using Euclidean distances between averages. For gene ontology enrichment analysis, the bioinformatics software DAVID (The Database for Annotation, Visualization and Integrated Discovery) was used (27). Predicted functional associations between proteins were analyzed with STRING software (https://string-db.org) (28). In the functional cell analyses, statistical significance (two-sided unpaired Student’s t-test) was determined with a cutoff of \(P<0.05\).
Results

Complement-sensitive and -resistant cells: MicroRNA array analysis

In order to identify microRNAs involved in complement-dependent cytotoxicity (CDC), we generated complement-resistant cell variants. K562 leukemia, Raji lymphoma, and HCT-116 colorectal carcinoma cells were exposed to several cycles of CDC over 2-3 months. After each cycle, the surviving cells were further grown and subjected to another cycle of CDC. Briefly, in each exposure cycle, cells (1x10^6) were treated at 4°C for 30 minutes with a rabbit anti-cancer antibody and then with NHS (50%) for 60 minutes at 37°C, under conditions yielding 70-90% cell death, washed into culture medium, and were grown in the incubator until disappearance of most dead cells from cell culture (3-10 days). The cells were supplemented with fresh culture medium every 48 hours. The number of exposure cycles required to gain resistance to CDC was 5 for HCT-116 cells, 8 for Raji and 10 for K562 cells. Parental cells (non-treated) were maintained during the selection period under continuous growth at 37°C and were divided in fresh culture medium every 48 hours. Consequently, the surviving cells became significantly more resistant to CDC than parental cells, which were kept during the selection period under continuous growth (Figs. 1A-C). To identify miRNA-mediated resistance, RNA samples were extracted from parental and resistant variant cells, analyzed on microRNA array cards, and parental (sensitive) and variant (resistant) cells were compared (Supplementary Array S1). The expression of 19 microRNAs was found to be higher in all resistant K562, Raji, and HCT-116 cells relative to their parental cells, and the expression of five microRNAs was lower (Student's t-test, P<0.01)(Fig. 1D, Supplementary Table S1). The expression fold-change of 14
microRNAs, calculated from the microRNA array data (Fig. 1E), was confirmed by real-time PCR of the same RNA samples (Fig. 1F).

**MicroRNA expression and complement-dependent cytotoxicity**

The interaction of cancer cells with complement has been investigated in great detail in K562 cells (1,29-31). Hence, these cells were chosen for an in-depth analysis of the function of the microRNAs in CDC. To determine whether the observed upregulation or downregulation of microRNA expression observed above was causative for abnormal CDC responses or incidental to the selection process, the effect of microRNA inhibition on CDC was examined. For this purpose, seven microRNAs were tested, and two of them were found to have a statistically significant impact on CDC (Fig. 2A). Inhibition of miR-328 resulted in reduced sensitivity to CDC, whereas inhibition of miR-150 increased it. Therefore, miR-328, miR-150, and miR-616 were chosen for further investigation. MiR-616 was included in the extended analysis for two reasons: (i) its expression was elevated in complement-resistant cells (Supplementary Table S1), and (ii) it was predicted (according to TargetScan.org) to target CD46 and CD59, two important complement regulatory proteins.

The specific effect of these three microRNAs on the cell sensitivity to CDC was then examined in HCT-116 cells transfected with a miR-150, miR-328, or miR-616 expression plasmid or a control plasmid for 24 hours. Upon treatment with rabbit anti-carcinoma antibody and complement (as described in the Methods), the death of cells overexpressing miR-150 was significantly (half-fold change, \( P=0.006 \)) than that of cells overexpressing miR-328 or miR-616 (1.35-fold change, \( P=0.0008 \) or 1.69-fold change, \( P=0.022 \), respectively) relative to the death of the control cells (Supplementary Fig. S1). K562 cells were transfected with a specific microRNA inhibitory plasmid (48 hours) or with a miR-150, miR-328, or miR-616 expression
plasmid (24 hours). The cells were then treated with rabbit anti-K562 antibody and complement and cell death was measured. Inhibition of miR-150 increased cell sensitivity (Fig. 2B), whereas overexpression of miR-150 resulted in reduced sensitivity (Fig. 2E). Inhibition of miR-328 or miR-616 resulted in reduced cell death (Figs. 2C-D), whereas overexpression of miR-328 or miR-616 increased cell death (Figs. 2F-G). These observed effects were not consistent with the findings of the global profiling of miRNAs in complement-selected cells (Fig. 1). Because expression of miR-328 and miR-616 was upregulated in the selected, relatively resistant cells, we expected that their inhibition would lead to increased sensitivity to CDC and not to protection from CDC. An explanation addressing this discrepancy is offered in the Discussion.

**Effect of sublytic complement on expression of miR-150, miR-328, and miR-616**

Sublytic and non-lytic doses of complement do not cause cell death but instead activate a variety of cellular responses (32,33), including elevation of cell resistance to CDC (34). Therefore, the impact of sublytic complement on the basal expression of miR-150, miR-328, and miR-616 was also investigated. K562 or HCT-116 cells were incubated with a sublytic dose of rabbit anti-K562 or -carcinoma antibody, respectively, and complement for 1 hour. RNA was extracted from half of the cells, while the other half was grown in culture medium for 3 hours at 37°C and then RNA was extracted from these cells. RT-PCR showed that treatment for 1 hour with sublytic complement triggered in K562 cells enhanced expression of miR-150 (3.5-fold), miR-328 (2.5-fold), and miR-616 (9.4-fold)(Fig. 2H). After 4 hours, microRNA expression declined. HCT-116 cells also responded, albeit to a lower extent, to sublytic complement with enhanced microRNA expression (Fig. 2I). However, the
response in HCT-116 cells was more pronounced 4 hours after treatment (miR-150: 1.9-fold; miR-328, and miR-616: 1.5-fold).

Effect of modified microRNA expression on C3 and C5b-9 deposition

The ability of microRNAs to regulate cell resistance to complement-dependent cytotoxicity raises the possibility that these microRNAs, through their target genes, affect the extent of C3 and C5b-9 binding to the cells. To test this hypothesis, K562 cells were transfected with a microRNA inhibitor or with a microRNA expression plasmid, followed by rabbit anti-K562 antibody and complement treatment. Inhibition of miR-150 increased C3 deposition (Fig. 3A), and overexpression of miR-150 reduced C3 deposition (Fig. 3B). Inhibition of miR-328 did not have an appreciable effect on C3 deposition (Fig. 3C), and overexpression increased C3 deposition (Fig. 3D). MiR-616 inhibition or overexpression had no appreciable effect on the amount of bound C3 (Figs. 3E-F). The extent of C5b-9 deposition was then analyzed. Inhibition of miR-150 increased C5b-9 deposition (Fig. 4A), and its overexpression reduced C5b-9 deposition (Fig. 4B). Inhibition of miR-328 reduced C5b-9 deposition (Fig. 4C), whereas its overexpression increased C5b-9 deposition (Fig. 4D). C5b-9 deposition was significantly reduced after miR-616 inhibition and enhanced after overexpression (Figs. 4E-F).

Proteomic analysis of the differentially expressed proteins

To identify the proteins regulated by miR-150, miR-328, or miR-616 that are involved in complement resistance, a proteomic analysis was performed on K562 cell lysates following inhibition of each of these specific microRNAs. The intensity values of the expressed proteins (7987 proteins, expressed in log2) in K562 cells transfected with miR-150, miR-328, or miR-616 inhibitor and the control group (five repeats for
each treatment group) were compared. T-test analysis showed that considerably more proteins were up- or downregulated by miR-616 inhibition (285 proteins) than by miR-150 (125 proteins) or the miR-328 inhibition (162 proteins), and several proteins were affected by two or three of the microRNA inhibitors (Fig. 5, All differentially expressed proteins). Mostly, the influence of each microRNA inhibitor on the K562 proteome was unique. Hierarchical clustering of the proteins differentially expressed after inhibition of miR-150, miR-328, or miR-616 (each relative to the control) demonstrated that inhibition of each of the microRNAs resulted in a similar number of up- and downregulated proteins and that the five experimental replicates of each treatment were homogenous (Supplementary Fig. S2).

**Effect of the microRNAs on the expression of membrane complement regulators**

The observed impact of miR-150, miR-328, and miR-616 on complement deposition and cell sensitivity to CDC suggested that one or more of the proteins regulated by them may modulate cell-complement interactions and resistance to CDC. The first candidates tested were the complement membrane regulatory proteins CD46, CD55, and CD59. Both CD46 and CD59 were predicted to be direct targets of miR-616 (TargetScan). However, a secondary effect through transcription or translation regulatory factors cannot be ruled out. The proteomic data revealed a significant increase in CD46 and CD59 expression, but not in CD55, after inhibition of miR-328 (CD46: FC=1.78, P=0.009; CD59: FC=1.47, P=0.01) or miR-616 (CD46: FC=1.97, P=0.003; CD59: FC=1.67, P=0.001)(Fig. 6A-C). To validate the proteomic findings, the effect of miR-616 inhibition on complement regulatory protein expression was further examined. To this end, K562 cells were transfected with a miR-616 inhibitor or a control plasmid and after 48 hours, were labeled with mouse anti-CD46, anti-CD55, or anti-CD59 and FITC-conjugated secondary antibody and then analyzed. A
significant CD46 and CD59 upregulation in cells expressing a miR-616 inhibitor was confirmed by flow cytometry (Fig. 6D). MiR-150 overexpression in K562 cells significantly increased CD46 and CD55 expression on the cell surface after 24 hours (Figs. 6E-F), but had no effect on CD59 expression (Fig. 6G).

**Enrichment analysis of the differentially expressed mitochondrial proteins**

Analysis of the biological pathways, significantly affected in the list of proteins up- or downregulated by each microRNA inhibitor was performed by using DAVID gene ontology enrichment software. Several biological processes (BP) and molecular functions (MF) were enriched after each microRNA inhibition (Supplementary Table S2). The three microRNA inhibitors significantly affected the mitochondrial proteome (GOTERM_CC_ALL: GO:0005739–mitochondrion) (anti-miR-150: \( P=0.0099 \); anti-miR-328: \( P=0.010 \); anti-miR-616: \( P=0.0009 \)). The proteins involved in the enriched mitochondrial processes identified by DAVID for each microRNA inhibitor treatment group were combined with additional proteins identified as mitochondrial in the STRING analysis (https://string-db.org) of the differentially expressed proteins. The combined lists of postulated mitochondrial proteins were confirmed as presenting mitochondrial proteins in the GeneCards database (https://www.genecards.org)(35). Thus, all together, the number of mitochondrial proteins found differentially expressed was 15 by anti–miR-150, 20 by anti–miR-328, and 70 by anti–miR-616 (Fig. 5). Of the upregulated proteins, only three predicted miR-150 targets, no predicted miR-328 targets, and 23 predicted miR-616 targets were identified (TargetScan Human; Supplementary Fig. S3). The other differentially expressed proteins were probably incidental targets of these microRNAs.
We then analyzed the mitochondrial proteins affected by each microRNA inhibitor by heatmap and by STRING. Heatmap diagrams (Figs. 7A,C,E) show the segregation of the differentially expressed mitochondrial proteins into two groups based on treatment (control vs. microRNA inhibitor). The corresponding STRING diagrams (Figs. 7B,D,F, respectively) provide insight into protein-protein interactions and the top enriched functions of the differentially expressed mitochondrial proteins. MiR-150 inhibition affected proteins involved in catalytic activity, organonitrogen compound metabolic processes and oxidoreductase activity. MiR-328 inhibition affected proteins involved in small-molecule metabolic processes, substrate-specific transmembrane transporter activity and organonitrogen compound metabolic processes. MiR-616 inhibitions affected proteins involved in catalytic activity, oxidation-reduction, and organonitrogen compound metabolic processes.

Discussion

MicroRNAs have been studied extensively and have been shown to regulate various cell functions and to be involved in pathogenesis (36,37). Alterations in microRNA expression are detected in many cancer types and are implicated in cancer progression (17-19). This study demonstrated the extensive involvement of microRNAs specifically in cell resistance to complement-dependent cytotoxicity. We postulated that alterations in microRNA expression, which arise during tumor progression, may also account for the complement resistance of cancer cells. The results shown here indicated an association between the basal complement resistance of cancer cells and their global microRNA expression profile. Comparison of microRNA profiles between parental K562, Raji, and HCT-116 cells and their selected variant cells, which express increased resistance to CDC, indicated an altered
expression of multiple microRNAs. Of those, 24 microRNAs were common to the three cell types, and 19 microRNAs were upregulated and five were downregulated compared to control. Based on the validation results, the anticipated target genes for each microRNA, and the initial testing of their relevance to CDC, we focused on three of the 24 common microRNAs: miR-150, miR-328, and miR-616.

Deregulated expression of miR-150 is seen in juvenile myelomonocytic leukemia and may play a role in the pathogenesis of this disorder (38). Reduced miR-150-5p expression may contribute to cholangiocarcinoma development and progression (39). MiR-150 is also suggested to regulate ovarian cancer cell malignancy (40), and it suppresses colorectal cancer and hepatoma cell migration and invasion (41,42) and glioma cell proliferation and migration (43). Upregulation of miR-328 sensitizes non-small cell lung cancer to radiotherapy (44). MiR-328 is also found to suppress the survival of esophageal cancer cells (45), promote glioma cell invasion (46), and regulate cancer stem cell-like cells in colorectal cancer (47). MiR-616 is upregulated in hepatocellular carcinoma (HCC) and is associated with tumor recurrence and metastasis (48). miR-616 also can potentiate the migration, invasion, and the epithelial-mesenchymal transition (EMT) phenotype of HCC cells in addition to also inducing androgen-independent growth of prostate cancer cells (49).

Sensitivity to CDC was examined in cells where microRNA activity was blocked with specific microRNA inhibitors, as well as with cells overexpressing the microRNAs. Our results indicated that inhibition of miR-150 increases cell sensitivity to CDC, whereas inhibition of miR-328 or miR-616 inhibits it. Inversely, overexpression of miR-150 inhibits cell sensitivity to CDC, whereas overexpression of miR-328 or miR-616 increases it. Thus, we claim that miR-150 exerts a net protective impact on CDC, whereas the net impact of miR-328 and miR-616 is the
promotion of CDC. However, the findings of the miRNA array analysis of the selected, relatively resistant cells challenged this claim. An explanation to this discrepancy is that the overall miRNome landscape of the complement selected/resistant cells was vastly modified relative to that of the parental/sensitive cells. This had developed following prolonged exposure to CDC, once the cells underwent significant modifications and adaptations to this selective stress. In contrast to cells undergoing a prolonged selection, cells transfected with a miRNA inhibitor or an overexpression plasmid underwent microRNA-specific and transient changes, lasting 24-48 hours, before they were examined for CDC sensitivity. Thus, the microRNA array analysis showed the net effect of sensitivity-conferring and resistance-conferring microRNAs, whereas the transient transfection experiments showed the specific effect of miR-150, miR-328, and miR-616.

Upon treatment with a sublytic dose of complement, cells also increased their expression of miR-150, miR-328, and miR-616. These effects were observed shortly after treatment (within 1 hour), suggesting a rapid induction of microRNA synthesis upon triggering by sublytic complement. Sublytic complement can also upregulate the expression of miR-200b and miR-200c (24). The downstream effects of the modulated expression of microRNAs awaits further investigation. However, sublytic complement is known to modify cells in various ways. At non-lytic or sublytic doses, the C5b-9 complexes are known to activate several intracellular signals and cascades, such as PKC, ERK, NF-κB, RIPK1, and RIPK3, in K562 cells (50-53). Sublytic complement induces the secretion of pro-inflammatory cytokines (54), the expression of adhesion molecules (55), and activation of the inflammasome (56), and is also shown to enhance the resistance of K562 cells to CDC (34), to perforin toxicity (57), and to TNF-induced apoptosis (51). It remains to be determined whether these
microRNAs, upon their upregulation by sublytic complement treatment, further regulate the various cellular activities through their specific target genes.

Two essential steps in complement-dependent cytotoxicity are (a) deposition of C3 on the surface of the activating cells, and (b) the assembly and insertion of C5b-9 complexes into the plasma membrane of the target cells (58). The observed effects of microRNA inhibition and microRNA overexpression on CDC may reflect modified activation of C3 and/or C5b-9. This was found to be partly correct. MiR-150 inhibition upregulated C3 and C5b-9 deposition, whereas miR-150 overexpression reduced it. MiR-328 inhibition reduced C5b-9 deposition but not C3 deposition, whereas miR-328 overexpression enhanced C3 and C5b-9 deposition. MiR-616 inhibition reduced C5b-9 deposition but had no apparent effect on C3 deposition, whereas miR-616 overexpression enhanced C5b-9 deposition but not C3 deposition.

Because each microRNA can affect, directly and indirectly, the expression of hundreds or thousands of proteins (10,11), we analyzed which proteins were regulated in K562 cells by miR-150, miR-328, and miR-616 and determined whether they affected cell sensitivity to CDC. The proteomic analysis revealed that the expression of numerous proteins was modified in K562 cells after inhibition of the microRNAs. Many differentially expressed proteins resulted from inhibiting two of the three microRNAs and 15 proteins were affected by three microRNA inhibitors.

Most cancer cells overexpress membrane complement regulatory proteins (6). The membrane complement regulatory proteins CD46, CD55, and CD59 represent the first line of protection from CDC and by far, they are the most studied cellular inhibitors of CDC (1,2). CD46 and CD59 were indicated by TargetScan as potential targets of miR-616. Examination of the proteomic profile of K562 cells subjected to microRNA inhibition revealed an increase in CD46 and CD59 expression after
inhibition of miR-328 or miR-616, but no effect on CD55. This was confirmed for miR-616-inhibited cells by flow cytometry but not for miR-328-inhibited cells. Further analysis by flow cytometry revealed that overexpression of miR-150 increased CD46 and CD55 expression but not CD59 expression. Thus, to some extent, these microRNA-induced changes in the expression of CD46 and CD59, especially when both were affected, may account for the observed changes in C3 and C5b-9 deposition and in cell death (6). PIGA (phosphatidylinositol N-acetylgalactosaminyltransferase subunit A), a predicted target of miR-616 was also significantly upregulated after miR-616 inhibition. PIGA is an essential enzyme for synthesis at the endoplasmic reticulum of the glycosylphosphatidylinositol (GPI) required for anchoring CD59 and CD55 to the cell surface (59). Hence, upregulation of PIGA expression may contribute, at least to some extent, to the upregulated expression of CD59 recorded in cells with inhibited miR-616. Based on our findings, overexpression of complement regulatory proteins may result from reduced expression of miR-616 or elevated expression of miR-150. Previously, we showed that miR-200b/c upregulates CD55 expression (24). In breast cancer cells, CD46 is elevated, and C3 deposition is inhibited upon reduced expression of miR520b and miR520e (60). MiR-19a and miR-20a regulate CD46 expression in endothelial cells (61). It is therefore envisaged that microRNA-targeted therapy, in cancer or other diseases, is expected to have an impact on the expression of membrane complement regulators and, thus, on the resistance of diseased cells to CDC.

Enrichment and functional analyses of the proteins differentially expressed after microRNA inhibition indicated alterations in several organellar and functional pathways that could be relevant in cell sensitivity to CDC. Two mitochondrial proteins, MSRA (methionine sulfoxide reductase A) and MT-CO3 (mitochondrially
encoded cytochrome C oxidase III), were affected by the three microRNA inhibitors. MSRA functions in the repair of oxidatively damaged proteins and protects cells from reactive oxygen species (ROS) (62). It also enhances the activity of complex IV of the respiratory chain and increases mitochondrial ATP synthesis (63). MT-CO3 is a subunit of the mitochondrial respiratory chain complex IV that catalyzes electron transport from cytochrome c to oxygen and the pumping of protons, thus, supporting oxidative phosphorylation and ATP synthase activity (64). The mitochondrion is considered to be the primary target of the death signal inflicted by the complement C5b-9 complexes (65). Hence, modulation of mitochondrial protein expression and mitochondrial metabolic activities are expected to affect cell sensitivity to CDC. It remains to be further investigated which of the numerous differentially expressed mitochondrial proteins are indeed protective from CDC.

Whether or not the CDC-regulating microRNAs miR-150, miR-328, and miR-616 participate in cancer cell resistance to other non-complement stresses awaits further investigation. However, based on their observed targets in the mitochondria, we hypothesize that they will significantly affect other cell responses to oxidative stress and toxicity. As discussed above, the mitochondrial proteins MSRA and MT-CO3 were upregulated upon inhibition of miR-150, miR-328 and miR-616. Based on their functions (62-64), together with the other mitochondrial proteins identified by STRING, they are expected to protect cells from oxidative stress. Cytotoxic lymphocytes damage the mitochondrial outer membrane via granzyme B, causing cytochrome C release and apoptosis (66). Granule-mediated cytotoxicity by cytotoxic T lymphocytes (CTLs) and NK cells induces mitochondrial damage via caspase-dependent and caspase-independent mechanisms, causing disruption of the mitochondrial transmembrane potential and the generation of ROS, which are
required for the induction of cell death (67,68). NK cells are shown to induce apoptosis in eosinophils through ROS generation, a process inhibited by mitochondrial inhibitors (69). Regulated necrosis induced by TNF also targets the mitochondria and causes ROS accumulation (70). Based on the above, it is reasonable to assume that the observed modulation by miR-150, miR-328, and miR-616 on the expression of mitochondrial resilient proteins will also affect CTL and NK cell-mediated cytotoxicity. Two complement membrane regulators, CD55 (71,72) and CD59 (73), inhibit NK-mediated cytotoxicity. As shown here, miR-150 upregulated CD55 expression, and miR-616 downregulated CD59 expression. MiR-200b/c was also shown to upregulate CD55 expression (24). Thus, miR-150, miR-200, and miR-616 are expected to affect NK-mediated cytotoxicity via CD55 and/or CD59 regulation.

In conclusion, through reorganization of the cell proteome, miR-150, miR-328, and miR-616 were shown to regulate the assembly and/or stability of the C5b-9 complex and the durability of cells attacked by C5b-9. The influence of microRNAs on CDC was reciprocal. Cell triggering with a sublytic dose of complement induced upregulation of miR-150, miR-328, and miR-616 expression. Thus, following membrane insertion of C5b-9, the balance between cell survival and death was shifted according to the relative expression of miR-150, miR-328, and miR-616. Enforced modulation of the expression of these microRNAs may be considered as a means to increase cancer cells’ sensitivity to CDC during cancer therapy or to reduce tissue sensitivity to CDC before transplantation or during autoimmune and inflammatory reactions.
Authors Contributions

YH and ZF designed the experiments and wrote the paper; YH and MM conducted the experiments; LZ assisted in performing several of the experiments; MP-C performed the bioinformatic analysis; TG supervised the proteomic analysis; NS supervised the microRNA array analysis; MP-C, TG, MM and NS critically reviewed the paper.
References


Legends to Figures

Figure 1. Analysis of microRNAs in complement-resistant and complement-sensitive cells. (A) K562, (B) Raji, and (C) HCT-116 cells were treated with rabbit polyclonal antibody for 30 minutes at 4°C and then with complement (normal human serum, NHS) [50%] for 1 hour at 37°C, and the surviving cells were re-grown and further subjected to several cycles of these treatments (10 cycles for K562 cells, 8 cycles for Raji cells and 5 cycles for HCT-116 cells). Sensitivity of the selected, relatively resistant cells and control non-treated cells (NT) to antibody and complement was compared. Percentage cell death (Mean± SD) was determined by propidium iodide inclusion. **P <0.01 relative to NT cells (student's t-test). (D) Identification of differentially expressed microRNAs via a microRNA array. The quantity of microRNAs in complement-resistant and non-treated K562, Raji, and HCT-116 cells was examined, as described in Methods. The number of microRNAs either upregulated or downregulated (resistant/NT) is presented in a Venn diagram; cutoff: 0.67>fold change>1.5 and P<0.05 (student's t-test). The 19 microRNAs that were upregulated and the five microRNAs that were downregulated in the three types of resistant cells are listed. (E) The fold-change in the expression of 14 microRNAs up- or downregulated in the three resistant cell types, relative to parental cells, is shown. (F) Validation of the microRNA data. The DNA samples used for the microRNA array analysis were further subjected to quantitative RT-PCR. The fold-change in the expression of the same 14 microRNAs in resistant/NT cells is shown.

Figure 2. Modulation of microRNA expression affects cell sensitivity to CDC. (A) K562 cells were transfected with microRNA inhibitors specific for the indicated microRNAs or with a non-specific oligonucleotide (C) as a negative control. After 48 hours, the cells were treated with rabbit polyclonal antibody for 30 minutes at 4°C and
then with NHS (50%) for 1 hour at 37°C and the percentage of cell death (Mean±SD) was determined by PI inclusion. *P<0.05, relative to control (student's t-test). (B-G) K562 cells were transfected either with a microRNA inhibitor plasmid (expressing mCherry) for (B-D) 48 hours or (E-G) a microRNA expression plasmid (expressing GFP) for 24 hours or (B-G) a control (C) plasmid. The cells were then treated with antibody and NHS, as described above, and the percentage of cell death (Mean±SD) was measured by (B-D) DAPI inclusion or (E-G) by PI inclusion. *, P<0.05, **, P<0.01 relative to the control (student's t-test). (H-I) Sublytic complement effects on expression of miR-150, miR-328, and miR-616. (H) K562 or (I) HCT-116 cells were incubated with a sublytic dose of antibody for 30 minutes at 4°C and then with HIS or NHS for 1 hour at 37°C, and the cells were cultured further at 37°C. RNA was extracted from the cells at 1 hour or 4 hours after treatment, reverse transcribed, and subjected to quantitative RT-PCR. The fold-change in the expression of miR-150, miR-328, and miR-616 in cells treated with NHS in comparison with their expression in cells treated with HIS were calculated ((Mean±SD; representative of 3 independent experiments; Line drawn at Relative Quantification=1).

Figure 3. Modulation of miR-150, miR-328, and miR-616 and complement C3 deposition. (A,C,E) K562 cells were transfected with a microRNA inhibitor plasmid for 48 hours, (B,D,F) a microRNA expression plasmid for 24 hours, or (A-F) a control plasmid. Then, the cells were treated with a sublytic dose of rabbit anti-K562 antibody for 30 minutes at 4°C followed by NHS (50%) for 10 minutes at 37°C. The cells were then labeled with goat anti-C3 antibody and a fluorescently labeled secondary antibody and subsequently analyzed by flow cytometry. Mean fluorescence intensity (MFI) values of cell-bound C3, representative of three independent experiments, are shown. *P<0.05 relative to control (Mean±SD, student's t-test). MFI values of cells
labeled with secondary antibody only or control cells treated with antibody and HIS and labeled for C3 were below 100 and did not differ between the two cell types.

**Figure 4. Modulation of miR-150, miR-328, and miR-616 and complement C5b-9 deposition.** K562 cells were transfected with (A,C,E) a microRNA inhibitor plasmid for 48 hours, (B,D,F) a microRNA expression plasmid for 24 hours, or (A-F) a control (C) plasmid. Then, the cells were treated with a sublytic dose of antibody for 30 minutes at 4°C, followed by NHS for 10 minutes at 37°C. The cells were then labeled with anti-C5b-9 (aE-11) and with a fluorescently labeled secondary antibody and analyzed by flow cytometry. Mean fluorescence intensity (MFI) values of cell-bound C5b-9, representative of three independent experiments, are shown. *P<0.05, **P<0.01 relative to control (Mean±SD).

**Figure 5. Proteomic analysis of K562 cells transfected with a microRNA inhibitor.** (Upper Venn diagram) Comparison of all differentially expressed proteins resulting from each microRNA inhibition vs. control. K562 cells were transfected with a microRNA inhibitor plasmid or a control plasmid. After 48 hours, cells lysates were prepared and analyzed as described in Methods. The resulting peptides were fractioned by HPLC and analyzed in a mass spectrometer. To obtain quantitative data, a label-free quantification algorithm, integrated into Maxquant, was used. Proteins whose expression was either upregulated or downregulated upon inhibition of miR-150, miR-328, or miR-616 compared to control are shown in a Venn diagram; fold-change >1.5 and P<0.05 (student's t-test). Names of differentially expressed proteins shared by the three microRNAs are listed. Shared mitochondrial differentially expressed proteins appear in **Bold.** (Lower Venn diagram) Focus on mitochondrial proteins. The differentially expressed proteins that
reside and function in the mitochondria are presented in a Venn diagram; fold-change difference $>1.5$ and $P<0.05$ (student's $t$-test).

**Figure 6. Modulation of the membrane complement regulatory proteins by microRNAs.** (A-C) The expression of (A) CD46, (B) CD55, and (C) CD59 in lysates of K562 cells transfected with microRNA inhibitors or with control, determined by the proteomic analysis described in Methods, is shown as the mean±SD of Log2 of intensity value units of five independent experiments. *$P<0.05$; **$P<0.01$, relative to control (student's $t$-test). (D) K562 cells were transfected with miR-616 inhibitor plasmid or with control (C) plasmid as a negative control. After 48 hours, the cells were labeled with anti-CD46, anti-CD55, or anti-CD59 and then with fluorescently labeled secondary antibody. Cells were then analyzed by flow cytometry, and the mean fluorescence intensity (MFI) values, representative of 3 independent experiments, were determined. The expression of each regulator in anti-miR-616-treated cells were normalized to their levels in control cells (set as 100). *$P<0.05$; **$P<0.01$ relative to control (student's $t$-test). (E-G) K562 cells were transfected with a miR-150 expression plasmid or a control plasmid. After 24 hours, the cells were labeled with (E) mouse anti-CD46, (F) anti-CD55, or (G) anti-CD59 and fluorescently labeled secondary antibody. Cells were then analyzed by flow cytometry and MFI values, representative of 3 independent experiments, were determined. *$P<0.05$ relative to control (student's $t$-test).

**Figure 7. Proteomic analysis of K562 cells transfected with a microRNA inhibitor: Focus is on mitochondrial proteins.** K562 cells were transfected with a microRNA inhibitor plasmid or a control plasmid. After 48 hours, cells lysates were prepared and analyzed as described in Methods. The resulting peptides were fractioned by HPLC and analyzed in a mass spectrometer. To obtain quantitative data,
a label-free quantification algorithm, integrated into Maxquant, was used. Mitochondrial proteins whose expression was either upregulated or downregulated after inhibition of (A-B) miR-150, (C-D) miR-328, or (E-F) miR-616 are shown as Heatmaps and STRING interactomes, respectively. The color-coded legend for each STRING interactome represents the major location or function of each protein. Fold-change >1.5 and $P<0.05$ (student's t-test).
Figure 2

A

Cell Death (%)

anti-328  anti-25  anti-126  anti-500  anti-150  anti-532  anti-199

B

Cell Death (%)

C  anti-150

miR inhibition

C  anti-328

miR inhibition

C  anti-616

miR inhibition

E

Cell Death (%)

C  miR-150

miR overexpression

C  miR-328

miR overexpression

C  miR-616

miR overexpression

H

K562 cells

Relative Quantification

miR-150-5p  miR-328-3p  miR-616-3p

1h  4h

I

HCT-116 cells

Relative Quantification

anti-150  anti-328  anti-616

1h  4h
Figure 3

A

C3 (MFI) vs. miR inhibition

B

C3 (MFI) vs. miR overexpression

C

C3 (MFI) vs. miR inhibition

D

C3 (MFI) vs. miR overexpression

E

C3 (MFI) vs. miR inhibition

F

C3 (MFI) vs. miR overexpression
Figure 4

A

B

C

D

E

F

C5b-9 (MFI)

C5b-9 (MFI)

C5b-9 (MFI)

C5b-9 (MFI)

C5b-9 (MFI)

C5b-9 (MFI)

C

anti-150

miR inhibition

anti-328

miR inhibition

anti-616

miR inhibition

miR overexpression

miR-150

anti-328

miR-328

miR overexpression

miR-616

miR overexpression

* Significant difference

** Highly significant difference

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Figure 5

All differentially expressed proteins

- miR-150
- miR-328
- miR-616

Differentially expressed proteins:

- CERS6
- CHMP1B
- CLEC16A
- CUTA
- EBP
- FAM173B
- HBA1;HBA2
- LIPA
- MSRA
- MT-CO3
- PATZ1
- PLS1
- PTTG1IP
- RPUSD1
- YIF1A

Mitochondrial differentially expressed proteins

- miR-150
- miR-328
- miR-616

Differentially expressed proteins:

- FXN
- MRPS28
- TOMM6
- MT-CO3
- MSRA
MicroRNAs affect complement regulator expression and mitochondrial activity to modulate cell resistance to complement-dependent cytotoxicity

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