Article

Highly expressed miR-375 is not an intracellular oncogene in Merkel cell polyomavirus-associated Merkel cell carcinoma

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Abstract: miR-375 is a highly abundant miRNA in Merkel cell carcinoma (MCC), in other cancers it acts either as a tumor suppressor or oncogene. While free-circulating miR-375 serves as surrogate marker for tumor burden in patients with advanced MCC, its function within MCC cells has not been established. Nearly complete miR-375 knockdown in MCC cell lines was achieved using antagomiRs via nucleofection. Neither cell viability, growth characteristics nor morphology were altered by this knockdown. miR-375 target genes and related signaling pathways were determined using ENCORI revealing Hippo signaling and EMT-related genes likely to be regulated. Thus, their expression was analyzed by multiplexed qRT-PCR after miR-375 knockdown demonstrating only a limited change in expression. In summary, highly effective miR-375 knockdown in classical MCC cell lines did neither significantly change cell viability, morphology, nor oncogenic signaling pathways. These observations render miR-375 an unlikely intracellular oncogene in MCC cells, thus suggesting to address likely functions of miR-375 for intercellular communication of MCC.

Keywords: miR-375, antagomiRs, Merkel cell carcinoma, Hippo signaling, focal adhesion

1. Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer. Risk factors for MCC include advanced age, ultraviolet (UV) light exposure and immune suppression [1]. About 80% MCC tumors are associated with genomic integration of the Merkel cell polyomavirus (MCPyV) bearing truncating tumor specific large T antigen mutations, while the others are characterized by UV-induced tumor mutational burden [1]. The pathogenesis of these two types of MCC tumors is surmised to be distinct: MCPyV-positive MCC is associated with MCPyV T antigens mediated tumor suppressor genes inhibition and/or oncogenes induction [1-3], while in MCPyV-negative MCC tumors the comparable oncogenic observations are caused by UV-induced DNA mutations [1,4-6]. However, the specific molecular alterations caused by either MCPyV or UV-mutations are just starting to emerge [7,8].

Transcription factor Atonal homolog 1 is characterized as a lineage-dependency oncogene in MCC, which induces miR-375 expression [9]. microRNAs (miRNAs) are small, ~21nt single stranded RNAs, which post-transcriptionally regulate the stability and translation of genes mainly by binding to the 3' UTR of mRNAs [10,11]. Each miRNA can bind a specific set of genes, which are referred to as its

target genes. Dysregulation of miRNAs has been reported in almost all types of human cancer [10,12]. miRNA expression profiling in MCCs revealed miR-375 as one of most abundant miRNAs in classical MCC cell lines and tumor tissues [13-16]. Physiologically, miR-375 acts as a pancreatic-islet miRNA essential for β -cell formation and regulation of insulin secretion [17,18]. Divergent miR-375 expression was described for multiple cancer types, e.g., reduced expression in gastric [19,20], pancreatic [21], colon [22,23] and liver cancer [24]; high expression in medullary thyroid carcinoma [25], prostate cancer [26] and MCC [13-16]. Therefore, miR-375 was assumed as an oncogenic miRNA in the latter group.

However, when the function of miR-375 in MCC was studied by different groups, results were inconsistent. Abraham *et al.* reported that miR-375 was involved in neuroendocrine differentiation and miR-375 knockdown in classical MCC cell lines (MKL-1 and MS-1) did not alter their growth properties [13]. Our preliminary results of miR-375 knockdown experiments were consistent with their report in the tested MCC cell lines [9]. In contrast, Kumar *et al.* reported that miR-375 inhibition in WaGa and MKL-1 cells reduced cell growth and induced apoptosis by targeting lactate dehydrogenase b (*LDHB*) [27]. Recent reports from the same group showed that miR-375 together with other miRNAs - inhibit autophagy thus protecting MCC cells from autophagy associated cell death [28]. To resolve these controversies, we here scrutinize the function of miR-375 in MCC. For this, we established a highly efficient method for miR-375 knockdown in classical MCC cell lines and analyzed the thereby inflected effects with an emphasis on intracellular signaling.

2. Results

2.1. Effective knockdown of miR-375 by nuclear transfection using miR-375 antagomiRs

To explore the function of miRNAs, it is essential to achieve largely complete knockdown. To achieve a highly effective knockdown in classical MCC cell lines is trivial. Thus, we tested different transfection methods, *i.e.*, lipofectamine and nucleofection, in the two classical MCC cell lines WaGa and PeTa using miR-375 antagomiRs.

Transfection of miR-375 antagomiRs by lipofectamine reduced miR-375 expression in a dose dependent manner but was not sufficient for a complete knockdown of the highly expressed miR-375 (Fig. 1a, b). Next, we performed nucleofection and optimized the transfection conditions. Program D23 with 25nM miR-375 antagomiRs was determined as the optimal protocol for knockdown, which rendered dramatically reduced miR-375 expression in both WaGa and PeTa cells (Fig. 1c, d and Suppl. Fig. 1). All further experiments were carried out using these conditions.

Figure 1

Figure 1. Knockdown of miR-375 in classical MCC cell lines

Relative miR-375 expression was determined in triplicates by qRT-PCR in WaGa (a, c) and PeTa (b, d) cells transfected with miR-375 antagomiRs (anta-375) or negative control (anta-NC) using lipofectamine (top row; a, b) or nucleofection (bottom row; c, d). Cq values were normalized to the small nucleolar RNA RNU6B (U6) and calibrated to the untreated WaGa cells. All experiments were independently repeated three times. Error bars represent SD, *** indicates $p < 0.001$.

2.2. miR-375 knockdown neither impacts morphology, proliferative capacity nor apoptosis of MCC cells

We were able to confirm our previous observation that miR-375 knockdown has no major impact on either cell proliferation, survival, growth characteristics or cell morphology (Fig. 2 and Suppl. Fig. 2, 3). Notably, even the highly effective miR-375 knockdown did not alter morphologic appearance as cells still show a neuroendocrine growth pattern as loose spheroids or single cells, which was identical to the growth pattern in cells transfected with unspecific control antagomiRs (Fig. 2a, b). Furthermore, neither the metabolic nor proliferative activity was affected by the miR-375 knockdown (Fig. 2c, d). While the harsh transfection conditions for the highly efficient miR-375 knockdown inhibited the proliferation of the MCC cells *per se* - indeed, we observed around 40% apoptotic cells 24h after nucleofection in both WaGa and PeTa - no difference was observed in MCC cells transfected with miR-375 antagomiRs or negative control (Fig. 2e, f and Suppl. Fig. 3). Sequential analyses on days 3 and 5 after transfection further supported that miR-375 knockdown had no specific impact on cell survival or metabolic activity (Fig. 2c-f and Suppl. Fig. 3).

Figure 2

Figure 2. miR-375 knockdown does not alter cell morphology, viability and apoptosis of MCC cells

(a and b) Morphology of WaGa (a) and PeTa (b) cells, untransfected (untreated) and 120 hours after nucleofection with either anta-NC or anta-375. (c, d) Cell proliferation (metabolic activity) of WaGa (c) and PeTa (d) cells after nucleofection with miR-375 antagomiRs or negative control was measured by MTS Assay at the indicated time points. Absorbance values at 490 nm were presented. (e, f) Apoptotic cells rate of untreated or nuclear transfected WaGa (e) and PeTa (f) cells was determined by flow cytometry using the NucView 488/ MitoView 633 apoptosis assay. Scale bar represents 50µm. All experiments were independently repeated three times, error bars represent SD, * indicates $p < 0.05$ and ** indicates $p < 0.01$.

2.3. miR-375 target genes are involved in Hippo and EMT related signaling pathways

To further investigate the role of miR-375 in MCCs, we predicted target genes of this miRNA using the miRNA target prediction tool ENCORI. This tool has the advantage that the results can be filtered for experimentally validated target genes. Nevertheless, more than 3000 target genes were predicted, thus, top ranking 500 genes were selected for further analysis (Suppl. Table 1). Gene Ontology (GO)

analysis shows that miR-375 target genes contribute to several signaling pathways, including Golgi transport, cell junction assembly, Hippo signaling and neuron differentiation (Fig. 3a). To test the relevance of these predictions in MCC, we reanalyzed previously published transcriptome microarray data of MCC cell lines [29]. Of this data set, four MCC cell lines were selected according to their miR-375 expression level: WaGa and MKL-1 with high and, MCC13 and MCC26 with low miR-375 expression [14]. Gene Set Enrichment Analysis (GSEA) confirmed that particularly genes related in focal adhesion signaling pathway were lower expressed in cell lines with high miR-375 expression (Fig. 3b). Moreover, focal adhesion signaling pathways included most of the experimentally confirmed miR-375 target genes; this notion applies also for miR-375 target genes related to the Hippo signaling pathway. Both pathways regulate epithelial to mesenchymal transition (EMT) [30] (Fig. 3c).

Figure 3

Figure 3. miR-375 target genes are involved in Hippo and EMT signaling pathways in MCC cells

(a) Gene ontology analysis was performed in Metascape using the top 500 predicted miR-375 target genes. (b) Gene set enrichment analysis was performed using previously published transcriptome microarray data of MCC cell lines with high (WaGa, MKL1) and low (MCC13, MCC26) miR-375 expression. Enrichment plot of kegg_focal_adhesion signaling pathway is depicted. (c) miR-375 target genes involved in Hippo and focal adhesion signaling pathways.

2.4. Hippo and EMT signaling pathway related genes are marginally altered by miR-375 knockdown

Since our *in-silico* analysis suggested that miR-375 may regulate Hippo and EMT-related signaling pathways, we tested this hypothesis by miR-375 knockdown experiments together with qRT-PCR based expression arrays for Hippo and EMT signaling related genes. These experiments, however, did not reveal any statistically significant changes in gene expression of compounds of these two signaling pathways in MCC cell lines upon miR-375 knockdown. In detail, miR-375 knockdown only resulted in a non-significant (*i.e.*, less than +/- two-fold changes in expression) upregulation of eleven (11/84, 13.1%) and downregulation of four genes (4/84, 4.8%) related to the Hippo signaling pathway, as well as a non-significant upregulation of eleven (11/84, 13.1%) and downregulation of three genes (3/84, 3.5%) with respect to EMT-signaling pathway (Fig. 4, Suppl. Table 2).

Figure 4

Figure 4. Moderate changes in the expression of Hippo and EMT signaling pathway related genes by miR-375 knockdown

Expression of genes related to Hippo (a) and EMT (b) signaling pathways were determined by multiplexed qRT-PCR expression array in WaGa cells transfected with anta-375 or anta-NC, normalized to the average Cq values of housekeeping genes (*GAPDH*, *HPRT* and *RPLP0*) and calculated to the ΔCq of WaGa cells transfected with anta-NC. Gene names colored in red represent genes upregulated upon miR-375 knockdown, while gene names colored in blue represent downregulated genes. Dotted lines represent +/- two-fold changes. Experiments were independently repeated twice.

3. Discussion

Despite the fact that miR-375 is highly expressed in classical MCC cell lines and MCC tumors, its function in MCC is not clear. To study the relevance of miR-375 on intracellular signaling in detail, we performed a series of knockdown experiments using specific antagomiRs. Surprisingly, even nearly complete knockdown of miR-375 expression neither affected proliferation, growth pattern nor cell morphology. Similarly, the impact of miR-375 knockdown on expression of Hippo and EMT

signaling pathway related genes, *i.e.*, pathways predicted to be regulated by miR-375, were only marginal. These results taken together with our previous observations that miR-375 is present in MCC cell line conditioned medium, in sera of preclinical xenotransplantation animal models and in sera of MCC patients [14] suggests that, miR-375 may rather serve intercellular than intracellular signaling in MCC. Indeed, miR-375 was recently characterized as an exosomal shuttle miRNA [31,32]. In previous reports, miR-375 knockdown or inhibition in MCC cell lines resulted in different consequences. miR-375 knockdown using antagomiRs did not alter growth properties [13], whereas inhibition of miR-375 using a miRNA sponge suppressed cell growth and induced cell death via downregulation of *LDHB* gene [27]. Recently, the same group demonstrated that miR-375 inhibits autophagy to protect MCC cells from cell death [28]. AntagomiRs bind particular miRNAs causing its degradation, while sponge RNAs compete with target mRNAs. Differences in the specificity and/or effectivity of the used methods are likely to explain some of the conflicting results. Quantification of miRNA expression after miR-375 knockdown by antagomiRs might be helpful to better understand this controversy. Notably, miR-375 is lowly expressed in variant MCC cell lines and ectopic expression of miR-375 decreased their cell viability and migratory potential [13,27], suggesting miR-375 to be a tumor suppressor in these cells. However, several reports question if these variant MCC cell lines are indeed representative for MCC tumors [29,33].

Knockdown of abundant miRNAs can be challenging [34]. AntagomiRs have been employed for miRNA silencing *in vitro* and *in vivo* via miRNA degradation for years [34,35]. In our study, we introduced the respective antagomiRs with two different transfection conditions, which revealed that nuclear transfection was much more efficient and only this method succeeded in a nearly complete knockdown up to five days post-transfection. To be noted, we observed a slight increase in miR-375 expression over time after antagomiRs transfection. Thus, the described method is very effective for short-term knockdown, but not for long-term inhibition (Suppl. Fig. S2). Besides miRNA antagomiRs, miRNA sponge is another powerful tool to inhibit miRNA function. Notably, a miRNA sponge was used by Kumar *et al.* to inhibit miR-375 function in MCC cells [27]. To achieve long-term miRNA inhibition, viral vectors based stable miRNA antagomiRs or sponge expression and CRISPR-mediated miRNA knockout are feasible [36-38].

Testing for expression of Hippo and EMT signaling pathway related genes after of miR-375 knockdown, we observed moderate expression changes of only a few genes. Furthermore, even these changes did not clearly reveal any relevant role of miR-375 in regulating these pathways. Indeed, both EMT negative (*CAV2* and *IL1RN*) as well as positive (*BMP2*, *ITGB1* and *TGFB2*) regulators were induced upon miR-375 knockdown. Thus, miR-375 may inhibit or induce EMT in MCC cells. More importantly, none of the changes were greater than two-fold. The gap between the predicted and functional observed effects of miR-375 knockdown are not entirely unexpected. Several reports provide a possible explanation: long non-coding RNAs, such as *TINCR*, *HNGA1* and *CircFAT1* act as a miR-375 sponge [39-41]. Alternatively, other miRNAs in MCC may have redundant functional targets as miR-375 [42].

In summary, we demonstrate that even the highly efficient, almost complete knockdown of the highly abundant miR-375 in classical MCC cells lines, has no relevant impact on cell viability, metabolic activity, morphology or oncogenic signaling pathways targeted by miR-375. These observations render miR-375 unlikely to function as intracellular oncogene in MCC cells.

4. Materials and Methods

Cell culture

The classical, MCPyV positive MCC cell lines WaGa and PeTa were maintained in RPMI-1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Munich, Germany) and 1% penicillin/streptomycin (Biochrome, Berlin, Germany) as previously described [43].

miR-375 knockdown

For miR-375 knockdown specific miR-375 inhibitors (Assay ID: MH10327, Catalog: 4464084, Thermo Fisher Scientific, Frankfurt, Germany) or respective controls (Catalog: 4464076, Thermo Fisher Scientific) were used.

For transfection of MCC cells two methods were compared. Lipofectamine 3000 reagent (Thermo Fisher Scientific) was used according to the instructions of manufacturer, *i.e.*, 2×10^6 cells were seeded into 6-well-plate 24 hours before transfection with 100nM or 250nM of antagomiRs. Alternatively, the Nucleofector™ 2b Device (Lonza, Basel, Switzerland) with the Cell Line Nucleofector® Kit V (Lonza) were used. D-23 was established as the appropriate program to transfect MCC cells (https://bioscience.lonza.com/lonza_bs/CH/en/nucleofector-technology). 100μL of buffer V was mixed with 10μL miRNA antagomiRs (25nM) and 2×10^6 MCC cells before being transferred into an electroporation cuvette. After the pulse, cells were immediately transferred into 6-well-plates containing pre-warmed culture media.

qRT-PCR for miR-375

Applied Biosystems TaqMan MicroRNA Assays (Thermo Fisher Scientific) were performed according to the manufacturer's instructions. Pre-designed TaqMan microRNA assays for miR-375 (ID000564) were used. The quantification cycle threshold (Cq) values of miR-375 were normalized to the small nucleolar RNA RNU6B (ID001093) and relative expression to the respective comparator was calculated using the $2^{-\Delta\Delta Cq}$ method.

MTS assay

Dead cells and cell debris after nucleofection were removed using Ficoll mediated gradient centrifugation (Biochrom, Berlin, Germany) before. For MTS assays, 10^4 living cells per well of each group (untreated, anta-NC and anta-375) were seeded into 96 well plates. CellTiter 96 AQueous One Solution (Promega, Walldorf, United States) was used to determine relative cell proliferation every other day. In brief, 20ul of the CellTiter solution was added to each well and incubated for two hours and then the absorbance was measured using a plate reader at 490nm.

Apoptosis assay

NucView 488/ MitoView 633 apoptosis assay kit (Biotium, Fremont, United States) was used to determine apoptotic cell rate according to the manufacturer's instruction. Viable cells stain red with MitoView 633 (red, APC-A channel), and apoptotic cells stain green with NucView 488 (green, PE-A channel). Twenty-four hours' post nucleofection and subsequently every other day cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Krefeld, Germany).

Prediction of miR-375 targets genes, Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA)

The Encyclopedia of RNA Interactomes (ENCORI, <http://starbase.sysu.edu.cn/index.php>) provides miRNA-target gene interactions, which are based on miRNA target prediction programs, *i.e.*, TargetScan, miRanda, microT, PITA, miRmap, and PicTar. All miRNA target predictions are supported by published Argonaute-crosslinking and immunoprecipitation (AGO-CLIP) data [44]. Predicted target genes are ranked based on the predicted efficacy of targeting as calculated using cumulative weighted context++ scores of the sites and related AGO-CLIP scores (clipExpNum, Suppl. Table 1) [44,45]. The top 500 highest ranking predicted target genes were selected for following analysis.

Metascape (<http://metascape.org>) was applied for GO analysis [46]. Metascape incorporates a core set of default ontologies including GO processes, KEGG pathways, Reactome gene sets, canonical pathways, and CORUM complexes for enrichment analysis.

GSEA the desktop application from the MSigDB of Broad Institute (Cambridge, Massachusetts, United States.) was used for re-analysis of the transcriptome microarray of selected MCC cell lines (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>) [47]. The transcriptome microarray data set GSE50451 was downloaded from Gene Expression Omnibus. Four MCC cell lines were selected and analyzed in GSEA: WaGa and MKL-1 as miR-375_high, MCC13 and MCC26 as miR-375_low.

Pathway finder gene expression arrays

The RT2 Profiler PCR arrays (SABioscience via Qiagen, Hilden, Germany) for epithelial to mesenchymal transition (EMT) (PAHS-090Z) and Hippo signaling (PAHS-172Z) were performed according to manufacturer's instructions. Total RNA was isolated from MCC cell lines three days after nucleofection with miR-375 inhibitors or the respective control. 200ng of total RNA was transcribed into cDNA using RT2 first strand kit (Qiagen). Relative gene expression was determined using the RT² Profiler PCR Array software from Qiagen (<https://dataanalysis.qiagen.com/pcr/arrayanalysis.php>).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 Software (GraphPad Software Inc., San Diego, CA, USA). Experiments containing more than two groups were analyzed using Kruskal–Wallis test, an unpaired nonparametric ANOVA. R studio (version 3.6.0) was used in statistical analysis as indicated: ggpubr R package (version 3.2.0) for dot plot of gene expression in EMT and Hippo signaling. A p-value smaller than 0.05 was considered significant; the respective p-values are indicated in the figures as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

5. Conclusions

Highly efficient knockdown of abundant miR-375 achieved by miR-375 antagomiRs with nucleofection did not cause obvious effects on cell viability, apoptosis, morphology or oncogenic Hippo and EMT related signaling pathways. These observations render miR-375 unlikely to function as intracellular oncogene in MCC cells.

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Conflicts of Interest: J.C. Becker is receiving speaker's bureau honoraria from Amgen, Pfizer, MerckSerono and Sanofi, and is a paid consultant/advisory board member for eTheRNA, MerckSerono, Pfizer, 4SC, REcordati, InProTher, and Sanofi. His group receives research grants from IQVIA, Merck Serono, and Alcedis. None of the activities are related to the submitted work. None of other authors indicated any potential conflicts of interest.

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