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MicroRNA-20a-3p and microRNA-20a-5p exhibit anti-proliferative activities in a melanoma in vitro model

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Research

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ABSTRACT

MicroRNAs control numerous cancer-related signaling pathways and play pivotal role in cancer initiation and progression. Recent studies have indicated variable and cancer-specific expression patterns of microRNA-20a (miR-20a), which have been attended by varying and sometimes contrary tumor biological functions. This is the first study regarding to the characterization of miR-20a's functionality in melanoma cells.

miR-20a expression was examined by reverse transcriptase and quantitative polymerase chain reaction in an in vitro melanoma model containing HaCat keratinocytes and B16 melanoma cells. For cell growth analysis, miR-20a vectors were cloned and transfected into B16 cells. Cell growth kinetics were performed utilizing a Cell Counter and Analyzer Model TT (Roche Applied Science). The expression

of both the 3p and the 5p strand processed from the miR-20a precursor was suppressed in melanoma cells B16 compared to the expression in non-malignant HaCat keratinocytes. Recombinant restoration of miR-20a levels in malignant B16 cells attenuated cellular growth. Our data suggest that miR-20a bears biological functions in melanoma cells and thus represents an anti-oncogenic factor which is suppressed during cancer progression.

Key Words: microRNA-20a, cancer, melanoma cells, keratinocytes, skin cancer model, tumor suppressor

INTRODUCTION

Each microRNA controls the expression of up to hundreds of genes thereby modulating pivotal signaling cascades including cancer-related

pathways. Compared to other malignancies, little is known about microRNA's dysregulation and biological efficacy in melanoma cells^[1]. MicroRNA-21, for instance, represents a well-characterized tumor promoter and belongs to the few microRNAs which have been examined in skin cancer^[2-5]. In case of microRNA-20a (miR-20a), little is known about its role in melanoma progression. In other cancer cells, recent studies have indicated highly variable and cancer-specific expression patterns of both the 3 prime (3p) as well as the 5 prime (5p) strand of the miR-20a precursor molecule (miR-20a-3p and miR-20a-5p). Certainly, the differentiation in the two miR-20a forms 3p and 5p is oftentimes being missed in molecular analysis thereby hindering analysis of the biological efficacy. In astrocytoma, miR-20a-5p expression is increased whereas the expression in glioblastoma cells is decreased^[6,7]. In breast cancer, miR-20a is significantly downregulated compared with healthy tissue and the overexpression of miR-20a inhibits cellular proliferation^[8]. Another study, however, demonstrated a 4-fold increase of miR-20a-5p in triple-negative breast cancer compared with luminal A breast invasive ductal carcinoma^[9]. Furthermore, restoration of miR-20a leads to an attenuation of hepatocellular carcinoma growth but in contrast to the promotion of cervical cancer growth^[10,11]. Beside proliferation control, miR-20a is also involved in chemoresistance mechanisms. The microRNA appears as an inhibitor of multi-drug resistance in osteosarcoma as well as an inducer of cisplatin resistance during gastric cancer therapy^[12,13]. Interestingly, in case of hepatocellular and gastric cancer, miR-20a correlates with clinical parameter and thus is discussed as a promising biomarker^[14,15].

The study presented here started to characterize miR-20a's proliferative properties applying an established melanoma model comprising non-malignant HaCat keratinocytes and B16 melanoma cells^[16-19].

MATERIALS AND METHODS

Cell Culture

Human keratinocytes HaCat (German Cancer Research Center (DKFZ), Heidelberg, Germany) and murine melanoma cells B16 (Cell Line Service, Ep-

pelheim, Germany) were propagated in DMEM medium (PAN Biotech, Aidenbach, Germany) supplemented with 2 mM glutamine, 1% penicilline/streptomycine (Biochrom, Berlin, Germany) and 8% fetal bovine serum (Sigma-Aldrich, Deisenhofen, Germany) and DMEM medium (PAN Biotech) containing 4.5 g/l glucose, 2 mM glutamine, 1% penicilline/streptomycine (Biochrom), and 10% fetal bovine serum (Sigma-Aldrich), respectively. Both cell lines were cultivated in a humidified atmosphere at 37°C with 5% CO₂.

Proliferation Assay

Cell growth was determined by cell counting (CASY Cell Counter and Analyzer Model TT, Roche Applied Science, Mannheim, Germany). Therefore, adherent cells were treated with trypsin/ethylenediaminetetraacetic acid (EDTA) and 1:100 diluted in CASYton (Roche Applied Science). Subsequently, 400 µl of the cell dilution was analyzed in triplicates. Measurement was performed applying a capillary of 150 µm in diameter and cell line-specific gate settings to discriminate between living cells, dead cells, and cellular debris: 6.6 µm/10.95 µm (HaCat), 7.8 µm/12.0 µm (B16).

RNA preparation and cDNA synthesis

Total RNA preparation was done using peqGOLDTrifast Reagent (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer's instructions. RNA concentration was determined utilizing a Nanodrop 2000c UV/vis spectrophotometer (Peqlab Biotechnology) and RNA was stored at -80°C. To perform the cDNA synthesis, 100 ng of total RNA were used with Superscript III Reverse Transcriptase (Life Technologies) according to the protocol of Chen *et al.*^[20] and primers as follows: miR-20a-3p stem-loop: 5'-GTCGTATC CAGTGCAGGGTCCGAGGTATTTCGCACTGGAT ACGACCTTTAA-3', miR-20a-5p stem-loop: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCG CACTGGATACGACCTACCT-3', U6: 5'-GTCATC CTTGCGCAGG-3'.

Quantification of miR-20a-3p and miR-20a-5p by polymerase chain reaction (PCR)

Quantification of microRNAs was performed on a CFX96 Real-Time System (Bio-Rad, München, Germany) with SensiMix SYBR hi-ROX Kit (Bioline, Luckenwalde, Germany). Sequences of primers specific for human and murine sequences were as follows: miR-20a-3p forward: 5'-GCCCGCACTGCATTATGAGCACTTAAAG-3'; miR-20a-5p forward: 5'-GCCCGCTAAAGTGCTTATAGTGCAG-3'; universal reverse primer (used for miR-20a-3p and miR-20a-5p): 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-CGCTTCGGCAGCACATATAC-3'; U6 reverse: 5'-AGGGGCCATGCTAATCTTCT-3'. After initial denaturation (95°C for 5 min) 45 amplification cycles were performed (95°C for 10 s, 60°C for 20 s, and 72°C for 10 s), followed by a melting-curve analysis. microRNA signals were standardized to U6 RNA as reference.

Cloning of DNA plasmide pmiR-20a-3p and pmiR-20a-5p

cDNA sequences encoding for mature miR-20a-3p and miR-20a-5p mimicking small hairpin RNA (pmiR-20a-3p, pmiR-20a-5p) were cloned into the pSUPERIORpuro vector (OligoEngine, Seattle, WA, USA). The oligonucleotides miR-20a-3p oligonucleotide 1: 5'-gatccccACTGCATTATGAGCACTTAAAGTtcaagagaCTTTAAGTGCTCATAATGCAGTtttta-3' and miR-20a-3p oligonucleotide 2: 5'-tcgataaaaaACTGCATTATGAGCACTTAAAGTctctttaaCTTTAAGTGCTCATAATGCAGTggg-3' and the oligonucleotides miR-20a-5p oligonucleotide 1: 5'-gatccccTAAAGTGCTTATAGTGCAGGTA GttcaagagaCTACCTGCACTATAAGCACTTTAtttt a-3' and miR-20a-5p oligonucleotide 2: 5'-tcgataaaaaTAAAGTGCTTATAGTGCAGGTAgctct ttaaCTACCTGCACTATAAGCACTTTAagg-3', respectively, were hybridized by a temperature gradient (95°C to 4°C in 40 min). Due to the asymmetrical design of both complementary oligonucleotides, the hybridization products formed defined single stranded 5' overhangs for ligation into the BglII/XhoI (Thermo Scientific, Waltham, MA,

USA) digested pSUPERIORpuro vector. After following ligation (T4 DNA Ligase; Thermo Scientific), positively selected clones were verified by restriction analysis and sequencing.

Transfection experiments

HaCat and B16 cells were transiently transfected with the microRNA mimicking vectors miR-20a-3p and miR-20a-5p, respectively. Transfection experiments were performed using Lipofectamine2000 reagent (Life Technologies) according to the manufacturer's protocol.

Statistics

For data evaluation, the graphics and statistics software Graph Pad Prism V 5.01 (GraphPad Software, La Jolla, CA, USA) was used. Results of at least four experiments were statistically analyzed, using the unpaired Student's t-test, and expressed as the mean \pm SD compared to control cells. Results of $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) were given as significance.

RESULTS

Suppressed expression of miR-20a-3p and miR-20a-5p correlates with with enhanced growth rate of skin tumor cells

Cellular proliferation analysis over a period of 144 h (Figure 1) showed reduced cell growth characteristics of non-malignant HaCat cells (24 h: $3.9 \times 10^4 \pm 1.2 \times 10^4$; 48 h: $4.5 \times 10^4 \pm 3.5 \times 10^4$; 72 h: $1.1 \times 10^5 \pm 2.4 \times 10^4$; 96 h: $2.9 \times 10^5 \pm 9.2 \times 10^4$; 120 h: $4.7 \times 10^5 \pm 1.9 \times 10^5$; 144 h: $7.9 \times 10^5 \pm 9.3 \times 10^4$) compared to malignant B16 cells (24 h: $2.4 \times 10^4 \pm 4.3 \times 10^3$, $p=0.0593$; 48 h: $1.0 \times 10^5 \pm 3.8 \times 10^4$, $p=0.0815$; 72 h: $4.0 \times 10^5 \pm 9.0 \times 10^4$, $p=0.0009$; 96 h: $8.8 \times 10^5 \pm 1.8 \times 10^5$, $p=0.0012$; 120 h: $1.6 \times 10^6 \pm 2.3 \times 10^5$, $p=0.0002$; 144 h: $1.8 \times 10^6 \pm 7.7 \times 10^4$, $p < 0.0001$).

Subsequent detection and quantification of miR-20a-3p and miR-20a-5p demonstrated the reduced expression of both miRs in B16 cancer cells (miR-20a-3p: 1.7-fold \pm 0.3-fold reduction, $p=0.0381$; miR-20a-5p:

1.7-fold \pm 0.2-fold reduction, $p=0.0015$) compared to non-malignant HaCat cells (Figures. 2A and 2B). In solid tumors, microRNA's downregulation frequently point to tumor suppressive and anti-oncogenic properties of these miR species^[21].

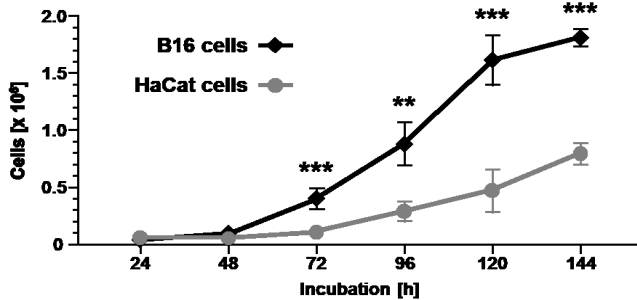


Figure 1. Cell growth of the melanoma cell line B16 compared to the non-malignant keratinocyte cell line HaCat. Cells were counted using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science) at indicated time points. Data are given as the mean \pm SD with $p \leq 0.01$ (**), and $p \leq 0.001$ (***) as determined by Student's t-test.

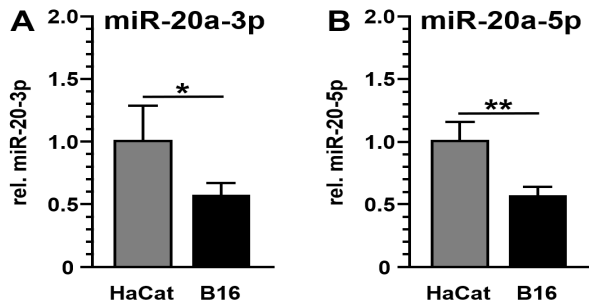


Figure 2. Expression of miR-20a-3p and miR-20a-5p in B16 and HaCat cells. MicroRNA levels of miR-20a-3p (A) and miR-20a-5p (B) were analyzed by quantitative reverse transcription and polymerase chain reaction (qRT-PCR) and standardized to U6 RNA expression levels. Data were given as the mean \pm SD and expressed as fold change (HaCat = 1.0).

Overexpression of miR-20a-3p and miR-20a-5p inhibits cellular growth of skin tumor cells

miR-20a-3p and miR-20a-5p were cloned into the eukaryotic RNA expression vector pSUPERIORpuro (Figures 3A and 3B) and applied in transfection experiments. Transfection of the empty control vector led to slightly reduced cell growth of B16 cells (24 h:

$8.4 \times 10^3 \pm 7.9 \times 10^3$; 48 h: $1.6 \times 10^4 \pm 8.2 \times 10^3$; 72 h: $6.6 \times 10^4 \pm 3.5 \times 10^4$; 96 h: $3.1 \times 10^5 \pm 8.2 \times 10^4$; 120 h: $6.0 \times 10^5 \pm 2.5 \times 10^5$; 144 h: $9.0 \times 10^5 \pm 1.8 \times 10^5$; Fig. 4) compared to untransfected cells (Figure 1). Transient overexpression of recombinant miR-20a-3p and miR-20a-5p revealed a minor but measurable attenuation of B16 cell growth in tendency (pmiR-20a-3p: 24 h: $7.1 \times 10^3 \pm 6.8 \times 10^3$, $p=0.7773$; 48 h: $2.4 \times 10^4 \pm 1.6 \times 10^4$, $p=0.2554$; 72 h: $6.8 \times 10^4 \pm 5.4 \times 10^4$, $p=0.9315$; 96 h: $2.0 \times 10^5 \pm 1.7 \times 10^5$, $p=0.2942$; 120 h: $3.5 \times 10^5 \pm 3.1 \times 10^5$, $p=0.2328$; 144 h: $5.9 \times 10^5 \pm 3.6 \times 10^5$, $p=0.1529$; pmiR-20a-5p: 24 h: $7.5 \times 10^3 \pm 5.1 \times 10^3$, $p=0.8260$; 48 h: $1.7 \times 10^4 \pm 9.2 \times 10^3$, $p=0.8283$; 72 h: $6.5 \times 10^4 \pm 4.1 \times 10^4$, $p=0.9648$; 96 h: $2.4 \times 10^5 \pm 1.7 \times 10^5$, $p=0.4824$; 120 h: $4.5 \times 10^5 \pm 2.4 \times 10^5$, $p=0.8239$; 144 h: $6.7 \times 10^5 \pm 1.4 \times 10^5$, $p=0.9837$; Figure 4).

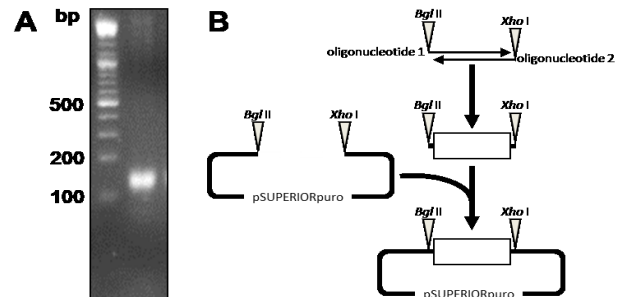


Figure 3. Cloning of miR20a-3p and miR-20a-5p encoding plasmid vector. (A) Oligonucleotides encoding for the miR20a-3p and miR-20a-5p small hairpin RNAs were hybridized forming single-stranded overhangs similar to BglII and XhoI endonuclease digestion. (B) The hybridization product of 160 base pairs (bp) was ligated into the BglII/XhoI digested vector pSUPERIORpuro (OligoEngine) and verified by sequencing.

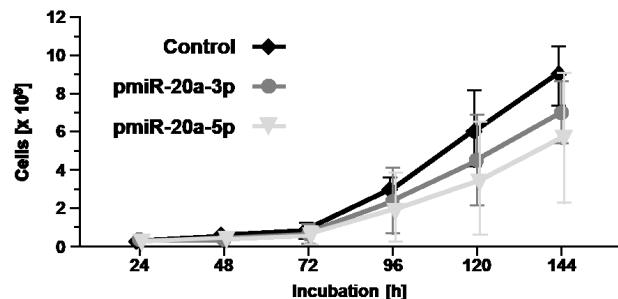


Figure 4. Overexpression of miR20a-3p and miR-20a-5p inhibits cellular growth of B16 tumor cells.

Cell growth of B16 cells overexpressing miR20a-3p and miR-20a-5p compared to mock transfected B16 cells (control). Cells were counted using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science) at indicated time points. Data are given as the mean \pm SD.

DISCUSSION

Cells have to satisfy several requirements to become malignant, elegantly grouped by Hanahan and Weinberg as: enhanced cell motility, escalated angiogenesis, suppressed apoptosis, limitless replication, independence in growth signals, and resistance to anti-growth signals^[22]. Dysregulation in the cellular safeguard machinery has a broad impact on cancer initiation and subsequent progression. In tumor biology, many miRs have been identified as crucial modulators of cancer malignancy usually classified in anti-oncogenic microRNAs (tumor suppressors) and pro-oncogenic microRNAs (oncomirs)^[23]. Although miR-20a-3p's and miR-20a-5p's regulatory properties have been characterized in various tumor entities, surprisingly, nothing is known about their role in skin cancer development.

In the study presented here, expression and functionality of both the 3p and the 5p strand processed from the miR-20a precursor have been evaluated in an established skin cancer model for the first time. Both miR-20a forms were determined to fulfill a biological function. The expression of miR-20a-3p/miR-20a-5p was suppressed in melanoma cells B16 compared to the expression in non-malignant HaCat keratinocytes. Moreover, restoration of miR-20a-3p/miR-20a-5p levels in malignant B16 cells clearly demonstrated anti-proliferative properties of both and thus specifying miR-20a-3p/miR-20a-5p as tumor suppressors. Due to plasmid DNA transfer by electroporation is highly variable^[24], however, transfection experiments overexpressing miR20a-3p/miR-20a-5p failed to become statistically significant.

Our findings are very similar to a study of Ottman et al., in which they showed downregulation and anti-

proliferative properties of miR-20a in prostate cancer cells^[25]. On the other hand, several studies demonstrated an upregulated expression of oncogenic miR-20a in other malignancies, highlighting miR-20a's heterogeneity in expression and functionality in a tissue and cell type-specific manner^[26]. These conflicting data may be explained by the common mode of microRNA synthesis. Numerous microRNA genes are organized in gene clusters in which multiple microRNAs are expressed from a single promoter^[27]. The resulting primary polycistronic transcript is subsequently processed into several individual microRNAs. The microRNA cluster miR-17 ~ 92 represents a microRNA cluster encoding for the microRNAs miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a^[28]. Abasi et al. examined the precursor and mature levels of miR-17, miR-20a, and miR-92a from the miR-17 ~ 92 cluster in various cancer cell lines and found cancer cell-specific variations of the three microRNAs compared to the level of unprocessed polycistronic precursor RNA^[29].

CONCLUSION

In conclusion, our data suggest that melanoma cells may belong to the group of cancer cells in which miR-20a-3p/miR-20a-5p inhibit cell growth. Furthermore, during characterization of miR-20a-3p's/miR-20a-5p's functionality in cancer cells the role of other microRNAs encoded by the miR-17 ~ 92 microRNA cluster should not be ignored.

Abbreviations

microRNA-20a (miR-20a), ethylenediaminetetraacetic acid (EDTA), polymerase chain reaction (PCR).

Author Contributions

HA, GD, EG, and MP conceived and designed the experiments. HA, EG and MP performed all experiments and GD, AM, MB, and MBS participated in analysis of the data. AM, and MBS wrote the paper; all authors read and approved the final manuscript.

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