MicroRNA-1 and MicroRNA-21 Individually Regulate Cellular Growth of Non-malignant and Malignant Renal Cells

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Abstract. Background/Aim: Due to its poor prognosis, it is increasingly necessary to understand the biology of renal cell cancer (RCC). Therefore, we investigated the role of microRNAs miR-1 and miR-21 in the growth of RCC cells compared to that of non-malignant renal cells. Materials and Methods: Four malignant cell lines (Caki-1, 786-O, RCC4, A498) were examined regarding their cell growth, microRNA and telomerase expression, and were compared to non-malignant RC-124 renal cells. Results: Inconsistencies appeared in the panel of RCC cells regarding antiproliferative and proliferative properties of miR-1 and miR-21, respectively. Notably, and most likely due to immortalization, non-malignant RC-124 cells exhibited telomerase expression and activity, and were compared to non-malignant RC-124 renal cells. Results: Inconsistencies appeared in the panel of RCC cells regarding antiproliferative and proliferative properties of miR-1 and miR-21, respectively. Notably, and most likely due to immortalization, non-malignant RC-124 cells exhibited telomerase expression and activity. Conclusion: miR-1 and miR-21 functionality in cancer progression, particularly in tumor growth, may be more dependent on the individual cellular context and may reflect RCC heterogeneity. Thus, both microRNAs, in combination with other stratifying biomarkers, may be useful in terms of RCC diagnosis, prognosis, or treatment response.

Tumorigenesis gives cells an advantage in survival by an increase of proliferation, angiogenesis, immunomodulation and immortality (1-4). Due to its delayed diagnosis until advanced stages, renal cell cancer (RCC) is known as the deadliest neoplasm of the urinary tract (1, 5). Thus, it is more necessary than ever to understand its tumor biology and explore new targets for further therapeutic approaches and prognostic options (6). Former studies suggested that among others, a misguided microRNA expression pattern may be involved in tumor initiation and progression in RCC (7, 8). Among such mechanisms, small regulatory RNAs, so-called microRNAs (miRs), can operate as controllers of different processes in tumorigenesis, such as cell migration and differentiation, as well as metastasis and apoptosis (9-11). miR-1 and miR-21 are two of numerous representatives of microRNAs. miR-1, initially found in cardiac muscle cells (12), but also found in various tissues (13-15), was strongly suspected of acting as a tumor suppressor due to its anti-oncogenic properties (16) in prostate, breast and colorectal cancer (13-15); interestingly, the type of tissue in fact determines the biological behavior of miR-1 (9). Sadly, the current data situation for the expression of miR-1 in RCC leaves much to be desired. For this reason, our study aimed to expand the first findings in order to gain a better understanding of the tumor biology of RCC. Similarly to miR-1, miR-21 was also detected in various malignant tissues, such as prostate and lung cancer and their metastases, pancreatic cancer and colorectal cancer (17-21). However, in contrast to miR-1, miR-21 appears to be an oncomir. There are preliminary investigations, showing miR-21 to appear as an oncomir in the case of RCC (22, 23).

The fact that cancer cells have a further survival advantage by extending their chromosomal ends by means of endogenic telomerase, not only raises a problem, but also provides a therapeutic approach in cancer therapy (4). In order to gain deeper insight into these issues, as far as we are aware, we are the first to investigate one non-malignant

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Key Words: Renal cell carcinoma, microRNA-1, microRNA-21, proliferation.
and four established RCC cell lines regarding their miR-1 and miR-21 expression and effect on cell proliferation, additionally determining both telomerase expression and activity.

Materials and Methods

Cell culture. Human RCC cell lines Caki-1, 786-O, A498 (all Cell Lines Service, Eppelheim, Germany), and RCC4 (Sigma-Aldrich, München, Germany) and the non-malignant renal cell line RC-124 (Cell Lines Service) were applied in this study. Caki-1 and A498 cells were propagated in minimum essential medium (MEM) supplemented with 79.6 mg/l non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin (P/S), and 10% fetal bovine serum (FBS; all PAN Biotech, Aidenbach, Germany). 786-O cells were propagated in RPMI-1640 medium containing 2 mM L-glutamine, 1% P/S, and 10% FBS (all PAN Biotech), RCC4 cells were propagated in Dulbecco’s modified Eagle’s medium with 1 mM sodium pyruvate, 1% P/S, and 10% FBS (all PAN Biotech), and RC-124 cells were propagated in McCoy’s 5a medium supplemented with 2 mM L-glutamine, 1% P/S, and 10% FBS (all PAN Biotech). All cells were passaged twice a week in a humidified atmosphere at 37°C with 5% CO₂.

Proliferation assay. Cellular proliferation of cells was assessed by cell counting (CASY Cell Counter and Analyzer Model TT; Roche Applied Science, Mannheim, Germany). Adherent cells were detached by trypsin/ethylenediaminetetra-acetic acid treatment, suspended in CASYton (1:100 dilution; Roche Applied Science) and 400 μl of the cell suspension was analyzed in triplicates. Measurement was performed using a capillary of 150 μm in diameter and cell line-specific gate settings to discriminate between living cells, dead cells, and cellular debris: 6.9 μm/14.7 μm for 786-O, 6.5 μm/12.75 μm for RC-124, 8.5 μm/15.75 μm for RCC4, 7.2 μm/15.75 μm for A-498, and 8.57 μm/15.4 μm for Caki-1 cells.

RNA preparation and cDNA synthesis. Total RNA was prepared using peqGOLDTrifast Reagent (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer’s instructions. Subsequently, total RNA concentration was determined utilizing a Nanodrop 2000c UV/vis spectrophotometer (Peqlab Biotechnology) and RNA was stored at −80°C. To perform the reverse transcription, 100 ng of total RNA were used with Superscript III Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions. For quantitative analysis of human telomerase reverse transcriptase (hTERT) mRNA expression, 60-90 ng cDNA was analyzed using a sequence detector (7500 Fast Real-Time PCR System; Life Technologies) and TaqMan target mixes (Assay-on-Demand Gene expression reagents; Thermo Scientific: ID Hs00972656m1).

For the determination of telomerase activity, TRAPeze RT Telomerase Detection Kit (Merck Millipore, Darmstadt, Germany) was used according to the manufacturer’s instructions. Cells were lysed by repeated freeze and thaw cycles in 3-[(Scholamidopropl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer followed by 30 min of incubation on ice. After centrifugation at 16,000 x g for 20 min at 4°C, aliquots of the supernatant were stored at −80°C. Protein concentration of extract was determined with DC Protein Assay (Bio-Rad). Using an ABI Prism 7500 Fast real-time cycler (Life Technologies), samples were incubated for 30 min at 30°C and amplified by PCR (45 cycles: 15 s at 94°C, 1 min at 59°C and 10 s at 45°C). The threshold cycle values (Ct) were determined from semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with standard curves generated from standard telomeric repeats provided with the kit.

Statistics. For data evaluation, the graphics and statistical software Graph Pad Prism v. 5.01 (GraphPad Software, La Jolla, CA, USA) was used. Statistical analyses were performed by using the unpaired Student’s t-test, with acceptance of significance at p<0.05.

Results

The non-malignant cell line RC-124 was established from primary cell preparations of non-tumor kidney tissue of a male carcinoma patient in 1998. Although frequently used as a non-malignant control, nothing is known about the experimental modality of cell immortalization which resulted in the RC-124 cell line. Since telomerase-transduced immortalization is often used for the generation of cell lines, we examined telomerase expression and activity in RC-124 cells compared to malignant RCC cell lines.

Notably, non-malignant RC-124 cells clearly transcribed the telomerase catalytic subunit hTERT mRNA (Figure 1A) accompanied by pronounced enzymatic activity (Figure 1B). Regarding the four malignant cell lines Caki-1, 786-O, RCC4 and A498, telomerase expression and activity was explicitly detectable, as expected for cancer cells. What is striking here is the fact that telomerase expression and activity in highly proliferative Caki-1 and 786-O cells was significantly higher than in the poorly proliferative RCC4.
and A498 cells. Compared to the four malignant cell lines, non-malignant RC-124 cells displayed relatively high expression of hTERT and very high telomerase activity.

Within the scope of our investigations, we examined the cellular growth of the non-malignant renal cell line RC-124 compared to the four RCC cell lines. As shown in Figure 2, the malignant cell lines Caki-1, 786-O, RCC4 and A498 have a different growth pattern compared to the non-malignant RC-124 cell line. Furthermore, even within the malignant cell lines, inconsistencies were found. The two malignant cell lines Caki-1 and 786-O exhibited higher proliferation compared to RC-124, whereas RCC4 and A498 cells exhibited a markedly lower increase of proliferation within 120 h, even lower than that of non-malignant RC-124 cells, in spite of being malignant. The strikingly different growth characteristics, especially the subdued growth of malignant cell lines A498 and RCC4, prompted us to investigate whether two different microRNAs influence the described findings.

Figure 3 shows the expression of the microRNAs miR-1 and miR-21 in the non-malignant and the four malignant cell lines. For Caki-1, as a highly proliferative cell line, the expression of miR-1, was not only weaker compared to the non-malignant RC-124 cells, but in fact was also the weakest in comparison to all other cell lines. In order of strength, miR-1 expression in RCC-4 cells followed that of Caki-1 cells. It is striking that Caki-1, as a highly proliferative cell line in our studies, exhibited a distinctly opposite expression of miR-1 compared to A498, a cell line with reduced proliferation compared to non-malignant RC-124 cells, whereas RCC4 and A498 cells expressed lower miR-1 compared to non-malignant RC-124 cells. The expression of miR-21 in Caki-1 cells was low, whereas in A498 cells it was high. However, regarding the expression of miR-21 in Caki-1 and RCC-4 cells, a contrasting expression pattern was observed. As already recognized in proliferative behavior, Caki-1 cells lead in the expression of miR-21. In general, the differences in miR-21 expression between cell lines are not as great as in the expression of miR-1.

Discussion

As RCC is the most common type of kidney cancer, with an increasing incidence (7, 24), it is important to explore and understand the tumor biology of this malignancy in order to find new early diagnostic markers and therapeutic approaches. Cancer cells acquire various mechanisms by which they escape apoptosis, become immortal, or provide to them at least a survival advantage compared to non-malignant
cells (25). Thus, tumor formation is mediated by ensuring adequate blood supply through the tumor cells themselves, by initiating angiogenesis (2), immunomodulation (3) and the attainment of immortality, not only by escaping apoptosis, but also by the presence of telomerase (4).

One strategy tumor cells use to hijack and reprogram regulatory pathways is the modulation of microRNAs, acting as tumor suppressors or oncogenes depending on tumor entity, and consequently playing a role in carcinogenesis. After their first discovery in the 1990s, focus was particularly on the ability of microRNAs to regulate gene expression, especially with regard to cancer (7, 26). MicroRNAs are a class of small non-coding RNAs which can interfere with mRNA and are able to regulate gene expression at the post-transcriptional level (7). Several studies have dealt with the effects of different microRNAs in RCC, such as miR-21, but not miR-1. Cell proliferative properties of miR-1 have been investigated in various tumor tissues, such as ovarian, lung, colorectal, prostate, bladder and endometrial cancer, as well as RCC (14, 27-32).

However, the thesis that miR-1 functions as a tumor suppressor, supported by the above-mentioned investigations, cannot be regarded as being set in stone. In context of a study on gastric cancer, miR-1 was shown to be associated with a higher incidence of liver metastases (33). This finding was confirmed by two studies on ovarian carcinoma cells, which also provided initial indications that miR-1 can lose its role as a classic tumor suppressor (27, 34). Although the former studies dealt with other malignant entities, a similar situation is also apparent for RCC within the framework of the present investigations. Unfortunately, to the best of our knowledge, there are only two investigations regarding miR-1 expression in RCC (32, 35), describing miR-1 as a tumor suppressor, by targeting transgelin-2 mRNA (32). Our results indicate a more complex regulation and may point to some individual aspects of miR-1 functionality in RCC development. In the case of RCC cell lines Caki-1 and A498, miR-1 appears to function as a tumor suppressor. In Caki-1 cells, a low level of miR-1 was accompanied by a high rate of proliferation, whereas the explicitly elevated miR-1 level in A498 cells led to attenuated cellular growth. In contrast to this, miR-1 in 786-O and RCC4 cells appeared to be dysregulated regarding the proliferative capacity. Even though miR-1 is strongly expressed in 786-O cells, the cell line was found to exhibit rather highly proliferative behavior comparable to that of Caki-1 cells. In contrast, while RCC4 cells demonstrated a very low expression of miR-1 out of the panel of RCC cell lines, RCC4 proliferation was very low.

Our findings confirm previous work showing miR-2 is pro-oncogenic in different malignancies. Thus, in all tested RCC cell lines, as well as in the non-malignant RC-124 cell line, miR-21 expression was relatively similar. The suspected role of miR-21 as an oncomir is also confirmed by the proliferative behavior of Caki-1 cells, with their having the strongest proliferation and miR-21 expression in our analyses. Liu et al. reported that the expression of miR-21 in RCC was not only associated with a poor prognosis, but also had an impact on recurrence. They found that expression of miR-21 was greater in malignant A498 cells compared to the kidney cell line HRPTEpiC and miR-21 suppresses the p53/p21-cyclin E2-B-cell-associated X protein/caspase-3 signaling pathway (36).

Malignant A498 cells did not show higher miR-21 expression in our study compared to that of the non-malignant cells, however, the differences in expression were not very pronounced. This might be because of our selection of another cell line for control cells, RC-124 rather than HRPTEpiC. In a review by Gu et al., miR-21 was shown to
be a promising marker and therapeutic target in RCC (7). It is also known that miR-21 drives RCC cell proliferation and invasion by mediating post-transcriptional regulation of phosphatase and tensin homolog that in turn increased an oncogenic pathway (7, 37). In addition, the post-transcriptional down-regulation of the programmed cell death protein 4 tumor-suppressor gene by miR-21 is another example of miR-21 acting as an oncomir (38).

The possibility that cancer cells can proliferate unhindered is not only due to the presence of microRNA, but also due to the fact that cancer cells have the ability to prevent the degradation of their chromosomal ends. Telomeres are the tails of the chromosomes, and are variable in length. Human telomeres, with a length of about 10 kb, are shortened at each cell division. If telomeres reach a length of 4 kb after approximately 50-100 cell divisions, this is associated with a transition of the cell into the resting phase of the cell cycle (4) and a shrinking of chromosomal ends (39). This in turn means that the cell cannot divide anymore.

Telomerase, a reverse transcriptase that counteracts telomere degradation by restoration, gives the cell a longer or infinite lifespan (40). In humans, telomerase activity is low in or absent from the majority of cells (4). In contrast to non-malignant cells, however, cancer cells have a high telomerase activity, which generates a further survival advantage – they are able to divide infinitely (40). In accordance with the detailed knowledge about the role of telomerases in cancer cells, it is not surprising that our analyses have shown that in highly proliferative RCC cell lines, Caki-1 and 786-O, both the expression of telomerase and its activity were particularly pronounced, in contrast to the more weakly proliferative RCC4 and A498 cells. The strong telomerase expression in the non-malignant RC-124 cells, however, was certainly provoked by genetic engineering with the aim of generating an immortalized cell line.

All in all, it can be seen that our finding, namely miR-21 functioning as an oncomir, have been reported in past studies (7, 36-38). Additionally, we were able to show that not only in ovarian cancer (27, 34), but also in RCC, the facade of miR-1 as a classic tumor suppressor continues to crumble. Due to the fact that the exact underlying mechanisms in RCC are not yet cataloged, comprehensive research is necessary to establish miR-1 as well as miR-21 in tumor cells as a new prognostic marker in RCC.

Conclusion

As far as we know, this is the first study to compare miR-1 and miR-21 in terms of their expression and their proliferative capacities in four established RCC cell lines compared to a non-malignant renal cell line. Notably, miR-1 and miR-21 functionality in cancer progression may be more dependent on the individual cellular context, e.g. cancer stage, as expected. These results may question the general classification system of microRNAs as bearing anti-oncogenic or oncogenic cellular functions in all stages of all malignancy. Additionally, and most likely due to immortalization, non-malignant RC-124 cells exhibited telomerase expression and activity.

miR-1 and miR-21 may be also useful as biomarkers for tumor heterogeneity and, in combination with other stratifying biomarkers, for RCC diagnosis, prognosis, or treatment response.

Competing Interests

The Authors declare that they have no competing interests.

Acknowledgements

The Authors thank Katja Wittig for excellent technical assistance and are grateful to Rüdiger Bode for fruitful discussion.

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Accepted April 27, 2017

in vivo 31: 625-630 (2017)