miR-7 AND miR-34a SEQUENCE CLONING AND EXPRESSION IN A1235 GLIOBLASTOMA CELL LINE

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Abstract: miRNAs are small non-coding RNAs which have an important role in signalling circuits regulating different cell processes. miR-7 and miR-34a are known as tumour suppressors, and both of them can interfere with cell proliferation, differentiation, apoptosis and migration. We constructed plasmids containing pri-miRNA sequences for these two miRNAs and introduced them into the A1235 glioblastoma cell line. Clones containing increased expression of processed miR-7 and miR-34a were obtained. The proliferation and sensitivity to alkylation agent of transfected cells were similar to those of control cells. Our results indicate that an increase in miR-7 and miR34 expression alone in A1235 glioblastoma cells is not sufficient to change their proliferation or sensitivity to the influence of alkylating agents.

INTRODUCTION

MicroRNA (miRNA) discovery revealed a completely new world of cellular control mechanisms involved in transcription and translation. These mechanisms have developed as far back as in unicellular organisms and have been found to have a role in multicellular organism differentiation and regulation in both plants and animals.¹ miRNAs belong to the non-coding RNAs (ncRNA) which are classified into two groups according to their length, those longer than 300 nucleotides (nt) and the second group, shorter than 300 nt. The small ncRNAs are further divided into small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs) and miRNAs. The microRNAs are short RNA sequences of 21-22 nt which take part in RNA silencing by binding to target sequences in mRNAs, typically resulting in repressed gene or protein expression.² DNA sequences coding for miRNA are dispersed across the genome. Most of the canonical miRNAs in the human genome are co-located within introns but can also be present within coding sequences. The biogenesis of miRNAs is under tight temporal and spatial control, and the miRNA expression programme depends on the cell and tissue type. They are regulated by their promoters, still not well-characterized, which are usually transcribed by RNA pol II. miRNA genes are transcribed into primary miRNA (pri-miRNA) transcripts about 1 kb pairs long, which are further processed by Drosha-containing complexes. The resulting hairpin precursor miRNAs (pre-miRNAs) are transported to the cytoplasm, where the Dicer complex removes the loop region from pre-miRNAs, releasing a small double stranded RNA molecule. One strand of the duplex is bound by Argonaute to form a miRNA-induced silencing complex (miRISC), which targets mRNAs for regulation.³,⁴ Transcriptional regulation outcomes by miRNAs can be mRNA destabilization or degradation, translational repression and, in some cases, even activation of gene expression. It is supposed that miRNA sequences perfectly matching mRNA lead to target molecule degradation. On the other hand, most of the genomic

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miRNAs are not perfectly complementary to the target mRNA, and they lead to the decrease in protein expression. Most of the miRNAs target 3'UTR mRNA, but they can also pair coding RNA sequences.\(^3,4\) It is supposed that human cells are able to express more than 2500 miRNAs. Although their role in the regulation of many genes and their involvement in signalling circuits regulating processes such as differentiation and carcinogenesis is known, the question of how, in general, they choose their target mRNAs still remains. Hypothetically, each mRNA has numerous potential miRNA binding sites, so each miRNA has many possible targets. Yet, their activity is often cell-type specific, and their targets should be proven experimentally.\(^3\)

In this article, we cloned a DNA sequence for miR-7 and miR-34a, under the CMV promoter, in order to express them in a glioblastoma cell line. Both of these miRNAs are known to act as tumour suppressors, influencing cell proliferation and apoptosis.\(^5,6,7\) miR-7 is one of the most investigated miRNAs, conserved from annelids to humans and found to have a role in nerve cell differentiation and tumorigenesis.\(^7\) miR-34a is ubiquitously expressed, but with the highest expression level in the brain. It is involved in the signalling circuits of p53 and epithelial-mesenchymal master transcription factors and thus in the regulation of cell cycle arrest, apoptosis, senescence and migration.\(^8\) Changes in miR expression can also influence the cell sensitivity on chemotherapeutics. Expression of miR-34a can be upregulated by p53, and A1235 cells have wild type p53.\(^3,8\) miR-7 was found to influence the expression of poly(ADP ribose) polymerase and possibly interfere with alkylation damage repair.\(^9,10\) Our results indicate that an increase in miR-7 and miR34 expression alone in A1235 glioblastoma cells is not sufficient to change their proliferation or sensitivity to the influence of alkylating agents.

**MATERIAL AND METHODS**

**Amplification of DNA sequences for miRNA and cloning into plasmids**

pri-miR DNA sequences were amplified from genomic DNA isolated from the glioblastoma cell line A1235. DNA was isolated using a standard procedure.\(^11\) Sequences were amplified using specific primers and Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) according to the manufacturer's protocol. Primers for pri-miR-7-2 were: F: 5'-GGAGAGAGAGAATGAGCCTTCCTTG-3' and R: 5'-GGAGACTCCACGTTGACTCTCTAAAG-3'. For miR-34a, the conditions were: 98°C for 45 s, followed by 38 cycles comprising 98°C for 15 s, 58°C for 30 s and 72°C for 1 min and finishing with elongation on 72°C for 2 min. Amplified sequences were cut from the agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA). A-overhangs were added to the sequence using Taq polymerase reaction,\(^12\) and the sequence was cloned into pGEM-T Easy vector (Promega, USA), according to the manufacturer's protocol. Plasmids were introduced into competent *Escherichia coli* XL10-Gold strain (Stratagene, USA) by chemical transformation, following the instructions from the manufacturer. White colonies grown on X-gal and IPTG agar plates were selected and analysed for the insertion. Plasmids containing the insertion were isolated using Wizard Plus SV Minipreps DNA Purification System (Promega), according to the protocol.

DNA sequences cloned into pGEM vectors were cut out by *EcoRI* restriction endonuclease (New England Biolabs, USA) and cloned into pcDNA3 plasmid (Invitrogen, USA). pcDNA3 plasmid was first linearized by *EcoRI* and dephosphorylated on its 5' ends with calf intestinal alkaline phosphatase (CIAP, TaKaRa, Japan) and the insert was ligated in pcDNA3 plasmid using T4 ligase (Promega).

pcDNA3 plasmids with inserted sequences were introduced into *E. coli* by chemical transformation. Colonies positive for inserted sequences were detected by PCR reaction and the miR-34a and miR-7 DNA sequence orientation was determined by restriction digestion with *PstI* and *Xhol*, respectively, according to standard procedure (New England Biolabs). Chosen clones were sequenced (ABI Prism 310, Applied Biosystems). The control plasmid used was pcDNA3-mRFP (pcDNA3-mRFP was a gift from Doug Golenbock (Addgene plasmid # 13032; http://n2t.net/addgene:13032; RRID:Addgene_1303213)).

**Cell culture, transfection and growth assessment**

Human glioblastoma cell line A1235 was grown in DMEM (Sigma-Aldrich, USA) with the addition of 10 % foetal calf serum (Sigma-Aldrich), under the standard conditions.\(^13\) For transfection of control pcDNA-mRFP and cloned plasmids, Lipofectamine 3000 Reagent (Thermo Fisher Scientific, USA) was used according to the manufacturer's protocol. Cells were selected for two weeks in 400 μg/mL genetecin (Merck, Germany) to obtain stable colonies which were further isolated. The presence of the plasmid sequence in the cells was detected in the DNA isolated from the cells by PCR reaction using primers for CMV promoter (F: 5'-CGTCAATGGTGGTGAGTATT-3' and R: 5'-CGTGAGTCAAAACGGTATC-3'). The cell transfection with constructed plasmids was done several times. Both pooled colonies (in general from several

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tens of colonies) and single colonies (5-10 colonies for each transfection) were isolated. The analysis was done on pooled colonies. Cell microphotographs were taken by the inverted microscope Zeiss Axiovert 40 CFL. For growth curve, cells were seeded in a 48-well plate and every day one set of cells was counted on the haemocytometer grid under the light microscope (Axiovert 40 CFL, Zeiss). Trypan blue exclusion test was used for viable cell detection. For growth assessment in the presence of chemotherapeutics, cells were treated with 5 and 10 μM N-methyl-N'-nitro N-nitrosoguanidine (MNNG) (a kind gift from dr. B. Brdar), one day after seeding, and counted under the microscope.

qRT-PCR analysis

For miRNA detection in the cells, total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. For miRNA detection, TaqMan Advanced miRNA Assays (Applied Biosystems, USA) were used with predesigned primers and probes (hsa-miR 34a-5p, Assay ID 47048, Assay A25576; hsa-miR-7-5p, Assay ID 478341, Assay A25576). As a control miR, hsa-miR-432-5p was used (Assay ID 478090). qRT-PCR was done on 7500 Fast Real-Time PCR System (Applied Biosystems).

Statistical analysis

Data were statistically analyzed using the software package Microsoft Office. The significance of independent two-tailed Student’s t-test was set at p-value <0.05.

RESULTS

pcDNA3-miR-34a and pcDNA3-miR-7 plasmid construction

To determine the effect of miR-34a and miR-7 on the A1235 glioblastoma cell line, we constructed two expression plasmids containing the sequences for each miRNA. DNA sequences coding for pri-miRNAs were cloned into the pcDNA3 plasmid to allow the cell machinery to do the pri-miRNA processing. The pri-miRNA sequences for miR-34a and miR-7-2 were cloned as DNA sequences of 1293 nt and 975 nt respectively, using specific primers and genomic DNA. Both sequences were first cloned by TA-cloning into pGEM plasmids and then recloned into pcDNA3. The sequences were checked by restriction analysis and sequencing (data not shown). Maps of cloned plasmids pcDNA3-miR-7 and pcDNA3-miR-34a are presented on Figure 1.

miR expression in the glioblastoma cell line

To see the effect of the plasmids pcDNA3-miR-7 and pcDNA3-miR-34a expression in the A1235 cells, plasmids were introduced into the cells by transfection, and stable clones were obtained after antibiotic selection. The morphology of the cells transfected by each of the plasmids was the same as the parental (Figure 2). The presence of the transfected plasmids was checked on DNA isolated from the clones, by PCR amplification of the CMV promoter sequence (data not shown).

Figure 1. Plasmid maps. A: pcDNA3-miR-7; B: pcDNA3-miR-34a.
Figure 2. Microphotography of A1235 cells in cultures showing the morphology of parental A1235 cells and cells transfected with plasmids coding for red fluorescent protein RFP, miR-7 and miR-34a. RFP: A1235 cells transfected with plasmid coding for RFP; miR-7: cells transfected with plasmid coding for primiR-7; miR-34a: cells transfected with plasmid coding for primiR-34a. Pictures were taken at magnification of 100x, scale bar = 50 µm.

To analyze the expression of the processed miRNA, TaqMan Advanced miRNA Assay was used. Total RNA was isolated from cells transfected with pcDNA3-miR-7, pcDNA3-miR-34a and control cells transfected with pcDNA3-mRFP. Specific TaqMan assays were used for the detection of miR-7-5p and miR-34a. According to assay protocol, the expression was compared to miR-423 chosen as miR with a stable level of expression. The assay protocol is based on the ligation of the specific linkers to miRNAs and amplification of the ligated sequences. In the second step, sequences are amplified with miRNA-specific primers. The results obtained by miRNA amplification in the transfected cells are presented on Figure 3. Expression of both miRNAs was significantly increased in the corresponding clones. A1235 cells transfected with pcDNA3-miR-7 had around 100 times higher expression of miR-7 in comparison with cells transfected with pcDNA3-RFP and pcDNA3-miR-34a. Cells transfected with pcDNA3-

miR-34a had around 8 times higher expression of miR-34a in comparison with control plasmid and pcDNA-miR-7.

Growth of cells with increased expression of miRs

As miR-34a and miR-7 are known as tumor suppressors, we analyzed the growth of A1235 cells transfected with miR plasmids. Cell growth was assessed by cell counting under the microscope. Growth curves obtained are presented in Figure 4. A. There was no significant growth inhibition in comparison with nontransfected cell and in comparison to cells transfected with control plasmid.

We also compared the growth of the transfected cells in the presence of alkylating agent MNNG, to see if transfected miRs influence the cell DNA damage response. It is known that both miRNAs can act as tumor
suppressors and influence cell cycle arrest and apoptosis. Therefore, we treated the cells with low concentrations of MNNG (5 and 10 µM) which cause the growth arrest in A1235 cells. A1235 cells are considered sensitive to alkylation damage.14 Figures 4. B and C show that the alkylation caused growth arrest in all cell strains similarly, and there were no significant differences in growth inhibition between control RFP cells and cells transfected with miR-7 and miR-34a, respectively.

DISCUSSION

miRNAs take part in the complex signaling circuits and are involved in the processes of differentiation, migration, proliferation, as well as in carcinogenesis.15 Many miRNAs act as tumor suppressors and oncogenes, and the same miRNA can even have both of these roles depending on the tumor type. The two miRNAs, miR-7 and miR-34a, which we expressed in a glioblastoma cell line act as tumor suppressors and belong to the most investigated miRNAs.

Between 700 and 1000 genes were found in different cell types to be miR-34a targets.5,6,16,17 Many of them are linked to cell proliferation and growth, as well as tumorigenesis. miR-34a was found to be a target of p53, and thus involved in the cell cycle arrest, apoptosis and senescence as responses to DNA damage.5,18 Beside regulation by p53, miR-34a can also be regulated p53-independently.5 The main signaling pathways influenced are TGF-beta, Wnt, MAPK, Notch and Akt, as miR-34a directly targets some of their members, such as Notch-1, Notch-2, CDK6, c-Met and Rictor.19 Also, its proapoptotic activities are exerted through the regulation of Bcl2, BIRC3 and other genes.5 It was found that this miR can take part in cellular reprogramming and expression of proteins linked with stem cell features, such as CD44, Sox and Nanog, as well as in epithelial-mesenchymal transition, influencing signaling circuits of its master transcription factors.6,20 Pre-miR-34a expression was found to be
Figure 4. Proliferation and growth inhibition curves of normal and transfected glioblastoma A1235 cells after treatment with N-methyl N’-nitro-N’-nitroso guanidine (MNNG). A: untransfected (A1235) or cells transfected with pcDNA3-mRFP (RFP), pcDNA3-miR-34a (miR-34a) and pcDNA3-miR-7 (miR-7) were grown for 5 days and their proliferation followed by cell counting and Trypan blue exclusion test. B and C: cell line growth was followed after treatment with 5 µM (B) or 10 µM (C) MNNG.

decreased in glioblastomas and its expression is often compromised during neuroblastoma development. Also, transient expression of mir-34a was found to decrease glioblastoma and neuroblastoma cell line' proliferation. Because of its wide area of activities involved in tumor suppression, there were even attempts to use miR as a therapy for some solid and hematologic tumors.

In our experiments, A1235 glioblastoma cells were transfected with a plasmid coding DNA for the miR-34a sequence. Using qPCR, it was shown that cells express an 8-fold increase in the amount of mature miR-34a. However, the cells did not show any morphology changes nor significant changes in the growth rate in comparison with the control cells. The explanation for these results can be the fact that colonies with a higher proliferation rate were isolated and the possibility that only cells which in some way overcame the arrest during the selection process of stable transfectants survived.

Also, it is possible that the glioblastoma cell line used harbor mutations in molecules involved in Akt signaling, leading to the constitutive activation of this pathway, and thus developed resistance to miR modulation. Constitutive activation of the Akt pathway is often present in glioblastomas.

Treatment with DNA damaging alkylation agent caused cell arrest in A1235 cells, similarly in wild type and cells with increased expression of miR-34a. It is supposed that miR-34a is a downstream effector of p53, but having wild type p53, it is possible that its activation was sufficient for the arrest. We did not observe additional cell apoptosis.

Another miR expressed in a glioblastoma cell line was miR-7, also considered a tumor suppressor and involved in the cell growth regulation. It was found to downregulate the cell proliferation influencing EGFR expression and downstream Akt kinase and MAPK pathways through downregulation of several genes.
molecules. It can also regulate the expression of Ack1 (associated cdc42 kinase 1) and Pak1 (p21-activated kinase 1) and interfere with the cell growth and migration, angiogenesis, as well as with apoptosis. Although it was found that miR-7 can also act as oncomir and increase the cell proliferation in several types of carcinoma, its expression is linked to the process of differentiation and inhibition of the proliferation in glioblastoma and nerve cells. Its expression is often decreased in glioblastoma and other types of brain tumors in comparison with normal tissue. It is detected as one of the miRNAs forming "miR signature" in neural stem and neural cancer stem cell.

In our experiments, A1235 cells with high expression of mature miR-7 were obtained. The cells were transfected with a miR DNA sequence, and the miR processing was dependent on the cellular machinery. The protocol used supposed the detection of processed miRNAs. It is interesting that Kefas et al. found specific regulation of miR and inhibition of the production of mature molecules despite the high expression of pri-miRNA. It was found that specific molecules expressed in some cell types bind to the conserved terminal loop of pri-miR-7 and inhibit processing. The expression of these inhibitors seems to be regulated during the process of neural cell differentiation, during which miR-7-1 processing increases and expression of inhibitor proteins decreases. Yet, some experiments were done on a glioblastoma cell line constitutively expressing high levels of processed miR-7, obviously not causing growth arrest. Jia et al. showed that glioblastoma cell line resistant to alkylating agents had low expression of miR-7, and its overexpression sensitized the cells to chemotherapeutics and suppressed the stemness of the cells. This miR-7 activity was found to be mediated by YY1 downregulation. A1235 cells with increased expression of miR-7 seemed to be resistant to proliferation inhibition. The reasons could be the same as in the case of miR-34a cells: selection of cells resistant to inhibition, either by its parental signaling background or by changes obtained during stable clone establishment. Another explanation, although hypothetical, can be the expression of specific miR-7 inhibitors, such as possibly circular ciRS-7 (or CDRIas).

To conclude, we detected an increase in the expression of the processed miRNAs, miR-7 and miR-34a, in the glioblastoma cell line A1235 transfected with expression plasmids containing DNA sequences for pri-miRNAs. Although acting as tumor suppressors, miR-7 and miR-34a did not have a significant influence on the cell growth and response to the chemotherapeutics in these settings. Further investigation is needed to analyses the activities and targets of miR-7 and miR-34a in this and other cell lines.

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