SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Differentially expressed microRNAs and the antiproliferative role of miR-126 in small cell lung cancer

Edit Miko

Supervisor: Dr Beáta Scholtz

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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1. Introduction

1.1. Small cell lung cancer (SCLC).

Lung cancer is the leading cause of cancer-related mortality in the world. Neuroendocrine tumors of the lung can be divided into 4 subtypes: typical and atypical carcinoid tumors, large cell neuroendocrine cancers (LCNECs), and small cell lung cancer (SCLC). The 4 subtypes can be differentiated by morphology, immunohistochemistry, and specific patterns of chromosomal aberrations. Global gene expression profiling identifies only 2 prognostically different subtypes: carcinoid tumors and high-grade neuroendocrine tumors (HGNTs), the latter including SCLC and LCNEC.

SCLC accounts for close to 20 % of all lung cancer and is characterized by its rapid progression and frequent metastases. Its proliferation is fuelled in part by autocrine/paracrine growth stimulatory neuropeptides and by several aberrantly activated pro-proliferative signalling pathways. The predominant risk factor for SCLC is cigarette smoking, with smokers facing a 20–30-fold higher incidence of developing SCLC than non-smokers.

SCLC is sensitive to initial chemotherapy, but most patients relapse rapidly and become resistant to any therapy. Even with combination chemotherapy and radiotherapy treatments, the 5-year survival is only about 5% . Treatment is complicated by the fact that SCLC is almost always diagnosed as a late-stage disease.

1.2. Gene mutations and aberrant signal transduction in small cell lung cancer.

SCLC cells display many neuroendocrine characteristics and express neuroendocrine markers, like neuroendocrine cells like neural cell adhesion molecule (Ncam1), neuronspecific enzymes (e.g. L-DOPA decarboxylase, neuron-specific enolase) and neuropeptide hormones like bombesin/gastrin-releasing peptide (GRP). These neuroendocrine markers are highly expressed in SCLC tumors (80–100%) compared with NSCLC (0–20%). Therefore, the current hypothesis is that SCLC arises from neoplastic transformation of neuroendocrine cells in the pulmonary epithelium. There are several genetic abnormalities that are found characteristically in SCLC, or occur at significantly higher rates in SCLC than in NSCLC. Although loss of cell cycle control is a common characteristic in both lung cancer

types, the mechanism by which this genetic change affects the process of neoplastic transformation differs significantly.

The most frequently occuring genetic abnormalities in SCLC are inactivation of the retinoblastoma gene, p53 and FHIT tumor suppressor mutations. Many other genetic alterations occur in SCLC, including increased expression of the anti-apoptotic gene BCL-2, activation of autocrine pathways, increased telomerase function, reduced expression of matrix metalloproteinase inhibitors, and increased expression of vascular growth factors. C-MYC gene amplification and hypermethylation of the anti-apoptotic caspase-8 gene are seen exclusively in SCLC.

Several receptor tyrosine kinases, including c-Kit, c-Met and gastrin-releasing peptide (GRP) receptor are highly expressed in SCLC. Binding of these receptors to their ligands results in the activation of multiple proliferative signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)–Akt (protein kinase B) and mammalian target of rapamycin (mTOR) pathway. Activation of PI3K–Akt–mTOR pathway promotes tumorigenesis and therapeutic resistance, whereas its inhibition can lead to chemotherapy sensitization of SCLC cell lines.

1.3. RNA interference (RNAi).

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism which is triggered by double-stranded RNA (dsRNA), resulting mRNA degradation or inhibition of mRNA translation. The key players in RNAi are small regulatory RNAs, including small interfering RNAs (siRNAs), processed from longer dsRNAs and microRNAs (miRNAs), generated in a multistep process from endogenous primary transcripts (pri-miRNA). MicroRNAs play an important role in many biological processes such as development, differentiation, proliferation and apoptosis. Deregulated expression of miRNAs has been implicated in a wide range of pathologies, especially in cancer and several number of genes, including tumor suppressors and oncogenes are regulated by these small RNAs. MiRNAs interfere with gene expression by repressing protein translation, by accelerating mRNA degradation and by sequestrating mRNA to storage compartments.

The use of RNAi for therapies has been widely studied, expecially in viral infections, cancers, and inherited genetic disorders. RNAi technology is a potentially useful method to develop highly specific dsRNA-based gene-silencing therapeutics.

1.4. MicroRNA targets.

Determining the exact function of microRNAs requires identification of its target mRNAs. Target identification in plants is based on the perfect complementarity between miRNAs and target mRNAs.

In C. elegans, Drosophila and mammals most miRNAs are not perfectly complementary to their target mRNAs, which makes target identification quite complicated. MicroRNA may target over a hundred of different mRNAs, and a mRNA may be targeted by several different microRNAs. If negatively correlated expression levels of a miRNA:mRNA pair are detected across different tissue profiles, the mRNA is probably targeted by the miRNA. MiRNA targets appears to be regulated both at the mRNA and protein level, but some targets only show an effect at the protein level. Several computational programs exist to identify miRNA targets, such as TargetScan and PicTar. The number of targets predicted for a given miRNA ranges from 100 to 500, making validation studies difficult; therefore, verification of target genes through direct experimentation is critical for understanding miRNA function and improving target prediction algorithms.

1.5. The role of microRNAs in cancer.

A microRNA that is downregulated in cancer and targets an oncogene might normally function as a tumor suppressor, whereas an upregulated miRNA that targets a tumor suppressor might act as an oncogene.

Approximately 50% of miRNA genes located in cancer-associated genomic regions or in fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification or common breakpoint regions.

1.5.1. An example for tumor suppressor miRNAs: Let-7 and its role in lung cancer.

The gene for let-7 miRNA is located at a chromosome region that is usually deleted in human cancers. To identify the mechanism by which let-7 regulate proliferation pathways, let-7 family members were overexpressed in liver cancer cells, which resulted in the accumulation of cells in the G0 and G1 cell-cycle phase. Several genes involved in

promoting the G1-to-S and G2-to-M phase transition were identified as direct targets of let-7, including CDK6, CDC25 and CCND2.

Let-7 is expressed at low levels in lung cancers, and reduced let-7 expression was significantly associated with shortened postoperative survival, independent of disease stage. Overexpression of let-7 in lung adenocarcinoma cell lines inhibited lung cancer cell growth in vitro. In addition to the cell cycle regulatory proteins, let-7 negatively regulates RAS expression by pairing at the 3' UTR of RAS mRNA for translation repression. The correlation between reduced let-7 expression and increased RAS protein expression in the lung tumor samples relative to normal lung tissue suggests that let-7 functions as a tumor suppressor gene in lung oncogenesis.

1.5.2. An example for oncogenic miRNAs: the miR-17-92 cluster and its role in lung cancer.

The miR-17-92 cluster is a good example for oncogenic miRNAs. The cluster is located in the third intron of the C13orf 25 gene at the 13q31.3 chromosomal region, and produces seven miRNAs: miR-17-5p/3p, miR-18, miR-19a, miR-20a, miR-19b-1 and miR-92. The cluster is markedly overexpressed in lung cancers, especially in small-cell lung cancer (SCLC). Suppression of miR-17-5p and miR-20a with antisense oligonucleotides can induce apoptosis selectively in lung cancer cells overexpressing miR-17-92 and overexpression of the miR-17-92 cluster enhanced cell growth. All these suggest that marked overexpression of the miR-17-92 cluster may play a role in the development of lung cancers, especially in the most aggressive form, small-cell lung cancer. Studies about the role of miR-17-92 cluster in lung development showed that this cluster is normally required for the high proliferation and undifferentiated phenotype of lung cancer cells.

The miR-17-92 cluster regulates cell proliferation through a network including c-myc and E2F. C-Myc is a proto-oncogene, which regulates cell proliferation, growth and apoptosis; and the E2F family of transcription factors are essential in the regulation of the cell cycle and apoptosis. C-Myc can activate the expression of E2F and the miR-17-92 miRNA cluster, while miR-17-5p and miR-20a negatively regulate E2F translation. In the context of SCLC, the connection between the miR-17-92 cluster and the c-myc oncogene is interesting, because the members of myc gene family have been shown to be frequently amplified and/or overexpressed in small-cell lung cancers.

1.6. MicroRNAs as potential biomarkers and therapeutic targets in cancer.

Compared to mRNAs, microRNAs are relatively stable in solid tissue samples, or in the serum and other body fluids. Therefore, the profiles of circulating microRNAs have been explored in a variety of studies aiming at the identification of novel non-invasive biomarkers. The use of microRNAs as tumor markers for diagnosis and prognosis is under intensive research: if altered expression of a miRNA correlates with a malignant phenotype, it might be useful as diagnostic marker, and if altered expression of a miRNA predicts disease progress, such as future metastatic relapse, it may be utilized as a prognostic biomarker.

MiRNAs may also be used as therapeutic agents. If the expression of certain miRNAs is consistently reduced in a disease, miRNA-mimetics may be used. MiRNA mimetics are synthetic, double-stranded small RNAs containing the exact sequence of the endogenous miRNA. On the other hand, if the expression of certain miRNAs is increased in a disease, antagomiRs can be used to block miRNA expression specifically. Elmén and colleagues demostrated that uptake of the LNA-antimiR-122 in African green monkeys silences liver-specific miR-122 expression, and decreases total plasma cholesterol without hepatotoxicity. LNA-antimiR antisense oligonucleotides are quite stable in vivo, and due to their low toxicity, they may be well-suited for the development of novel therapeutic approaches targeting cancer-associated miRNAs.

MiRNAs regulate many genes, and the potential off-target effect of miRNA therapeutics is a major problems. These problems could be alleviated by engineering effective systems that deliver the synthetic miRNA oligonucleotides specifically to the diseased tissue and to cancer cells.

2. Aim of the studies

The aim of our first study was to characterize the miRNA expression profile of SCLC cell lines and primary SCLC tumors using miRNA microarray and qRT-PCR. Many microRNA genes are found in chromosomal regions with frequent genetic aberrations in tumors. Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC, but the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC. Therefore, we also analysed DNA copy number changes in SCLC cell lines and primary SCLC tumors for 5 genomic regions harboring prominently overexpressed miRNAs.

The second aim of our work was to study the function of downregulated microRNAs using gain-of-function experiments. miR-126 was uniformly downregulated miRNA in all SCLC samples, therefore, we studied the effect of miR-126 overexpression on SCLC cell proliferation and cell cycle progression. MicroRNAs can regulate gene expression by binding to the 3' untranslated region (3'UTR) of target mRNAs, inducing mRNA degradation or translation repression. Our analysis identified a novel target of miR-126 contributing to its effects on SCLC cell proliferation.

3. Materials and methods

3.1. Cell lines and human tissue samples

SCLC cell lines were purchased from the American Type Culture Collection (HTB-172, HTB-184, HTB-119=H69). The cell lines were maintened in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C with 5% CO2.
Normal human lung tissue samples were obtained from the 2nd Department of Surgery at the University of Debrecen. Peripheral blood samples were collected at the Pulmonology Clinic of University of Debrecen from tumor-free individuals. 23 archived formalin fixed, paraffin embedded (FFPE) SCLC tumor samples were obtained from the Department of Pathology at the University of Debrecen.

3.2. Microdissection of FFPE tumors

Blocks of tumors were cut to yield minimum 12 serial sections. The first and last sections (4 μm) were used for hematoxylin-eosin (HE) staining. 5 sections, cut at 20 μm to prevent loss of small RNA species during isolation, were used for microdissection. These sections were microdissected by an experienced pathologist to separate tumor and normal tissue, under direct observation with a microscope, guided by the HE-stained sections, using a fine needle. The remaining 4 μm sections were used for immunohistochemical staining.

3.3. Nucleic acid extraction

Total RNA was isolated from surgical lung samples and SCLC cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA was extracted from SCLC cell lines and peripherial blood mononuclear cells using the Qiagen Blood and Cell Culture kit, according to the instructions of the manufacturer. Total RNA and DNA was isolated from FFPE samples with RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer's protocol.

3.4. Microarray analysis

MiRNA profiling of normal lung and SCLC cell lines was performed using a service provider (LC Sciences, Houston, TX, http://www.lcsciences.com, miRNA dual-color microarray platform, Human V7.1).

The microarray interrogated 319 mature miRNAs, each represented by 4 individual probes on the chip. RNA from 3 SCLC cell lines (H69, HTB-172, HTB-184) was used for the microarray screen, compared to RNA from fresh surgical samples of lung tissue as normal control. Normal lung RNA was pooled from 6 samples isolated from surgical material, and the three SCLC cell lines were assayed individually on 3 chips, each chip comparing the expression profile of one cell line to the common normal lung reference.

MiRNAs with raw signals above the averaged microarray background +5 STDEV were selected for further analysis. Data was normalized to hsa-miR-342, and analyzed by the GeneSpring 7.3 software. Relative miRNA expression values (SCLC cell lines relative to normal lung) were derived after normalization from averaged values of 3 SCLC cell line microarrays, and averaged values of 3 normal lung microarrays.

3.5. MiRNA real-time quantitative RT-PCR analysis

Expression of selected miRNAs were tested by qRT-PCR analysis either by using the TaqMan MicroRNA Assays Early Access Kit or by individual miRNA qRT-PCR assays according to the manufacturer's protocol.

In the first screen we assayed 63 miRNAs, on pooled RNA samples isolated from 6 FFPE SCLC tumor samples. Pooling of RNA in this experiment was necessary due to the limiting amount of RNA obtained from the FFPE sections.

In subsequent experiments pooling of the RNA samples was no longer necessary, because we assayed fewer miRNAs per sample (7 downregulated and 8 overexpressed miRNAs in 17 SCLC tumors).

qPCR was performed on the ABI 7900 HT Sequence Detection System. Relative miRNA expression values (tumor vs normal) were calculated using the 2-ddCt method. MiRNA expression levels in FFPE SCLC primary tumor samples were compared to their own FFPE normal lung tissue counterparts, isolated from the vicinity from the tumor. MiRNA expression levels in SCLC cell lines were compared to fresh surgical normal lung tissue. The normalizing

gene was hsa-miR-342, which showed the smallest variation between normal lung, SCLC cell lines, and primary tumors.

3.6. Copy number analysis

Five primer sets were designed to measure the genomic copy number of five miRNAs (mir-183, mir-182, mir-95, mir-301 and the mir-17-92 cluster) in SCLC cell lines (H69, HTB-172, HTB-173, HTB-184), in four FFPE SCLC tumors and their normal tissue counterparts isolated by microdissection.

PBMC genomic DNA was mixed from the samples of 5 tumor-free individuals. Relative gene copy numbers were calculated using the 2-ddCt method. Since tumor cell lines and primary tumors harbor many amplified chromosomal regions, we tested several candidate normalizing genes. Out of these, PP1A, TBP and H19 genes proved to be suitable candidates to normalize miRNA copy number in our samples.

Copy numbers of the five miRNA genomic regions, and the TBP and H19 normalization genes were measured using SYBR Green I qPCR, and TaqMan assay was used to quantify PP1A.

Ct values for miRNAs and the normalizer genes were the average of 3 or 5 independent PCR measurements, each sample tested in duplicate in each PCR. Significance of differences for the means was assessed with paired t-test, using two tailed p value, CI 95%.

3.7. Reagents and transfection

miRNA Precursor Molecule hsa-miR-126 (PM12841) and Pre-miR Negative Control ≠1(AM17110) were obtained from Ambion. siRNAs targeting SLC7A5 (s15653), PLK2 (s64) and Silencer Select Negative Control≠1 siRNA (4390843) were obtained from Applied Biosystems. Cells were transfected with pre-miR-126, SLC7A5, PLK2 siRNAs and controls at a final concentration of 50 nM in all experiments, using Lipofectamine 2000 (Invitrogen). Cells were incubated with the transfection complexes for 6 h before replacing the medium. Cells were refed daily with fresh growth medium.

3.8. Establishing the growth curves

1.2 x 10^6 cell/well were plated in 6-well plates and transfected with miRNA precursors. 48 hours post-transfection 1/10^{th} of the cells were replated in 24-well plates, and grown further. Cell numbers were determined by trypan blue exclusion at different time points (48h, 72h, 96h) post-transfection. Results represent the mean of three independent experiments.

3.9. Cell cycle analysis

1.2 x 10^6 cell/well were plated in 6-well plates and transfected with pre-miRs and siRNAs. 48 hours post-transfection 1/5^{th} of the cells from each transfection were replated and grown further. Cells were harvested at 72 and 96 hours post-transfection, washed in PBS and fixed in ice-cold 95% methanol at -20 °C. Fixed cells were washed twice in PBS, then resuspended in 0.5 ml PBS containing propidium iodide (50 ug/ml) and RNase A (200 ug/ml) and were stained overnight at 4 °C.

Measurements were made on a FACSArray 96-well plate flow cytometric bioanalyzer (Becton Dickinson). The DNA dye was excited with the 532 nm laser line and emission was collected in the yellow channel in linear mode with a 585/42 nm bandpass filter. Cell clusters were gated out using FSC-A/SSC-A (Area) and FSC-W/SSC-W (Width) 2D-histograms. Fluorescence intensity data were fit with the automatic one cycle diploid model of the *Modfit LT 3.0* software (Verity Software House) with the AutoDebris Compensation, AutoAggregate Compensation and Apoptosis Model turned on. In the measurements the G1-G2 linearity ratio was around 1.92 and the R.C.S. of the fit (reduced chi-square, a measure of goodness of fit) was less than 5. All samples were prepared in triplicates and generally 50,000 cells were collected from each well.

3.10. QRT-PCR and semi-quantitative PCR

For quantification of mRNAs, mature miR-126 and RNU38B, reverse transcription was performed by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

RT primers for mature miR-126 and RNU38B were supplied by Applied Biosystems.

qPCR was performed with FastStart SYBR Green Master Mix (Roche) with 0.3 μM of forward and reverse primers on an ABI 7900 HT Sequence Detection System. The PCR program used for amplification was: 95°C 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec. HPRT1 was used for normalization.

Specific primers for semiquantitative PCR of mature miR-126 and RNU38B were supplied by Applied Biosystems. The PCR program used for semi-quantitative amplification was: 95°C for 10 min, followed by 15 cycles of 15 sec at 95°C and 60 sec at 60°C. PCR products were separated by agarose gel electrophoresis and visualized by EtBr staining.

3.11. Western blot

H69 cells were washed with PBS and lysed in 2X SDS loading buffer. Proteins were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membrane by electroblotting. Membranes were blocked with 2.5 % nonfat milk, and probed with primary antibody against human SLC7A5 (3157-1, 1:1000, Epitomics), PLK2 (Snk/H90, sc-25421, 1:100, Santa Cruz Biotechnology) and GAPDH (6C5, sc-32233, 1:1000, Santa Cruz Biotechnology). The membranes were further probed with horseradish peroxidase-conjugated secondary antibodies (1:10,000; anti-mouse or anti-rabbit; Amersham) and proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Pierce).

3.12. SLC7A5 3' UTR cloning and luciferase reporter assay

For luciferase reporter assays, 331 bp from 3'UTR of SLC7A5 gene, including the miR-126 target site, was amplified by PCR using F1 and R1 primers with XhoI and NotI sites. PCR was performed on H69 cDNA created by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The XhoI/NotI-digested PCR product was cloned into the XhoI/NotI digested psiCHECK2 dual luciferase vector (Promega). F1/R2 and F2/R1 primers were used to delete the miR-126 target site from the 3'UTR. After mixing the two PCR products, and digestion with XhoI and NotI, the 3'UTR fragment with a deleted miR-126 binding site was cloned into XhoI/NotI-digested psiCHECK2 vector.

H69 cells were cotransfected with 500 ng psiCHECK2 constructs (WT-UTR or DEL-UTR) and 50 nM pre-miR-126 or pre-miR-negative control in 6-well plates. 48 hours post-transfection, firefly and renilla luciferase activities were measured using Dual-Glo Luciferase

assay system (Promega) in a Perkin Elmer Victor3 V Multilabel Plate Reader. The ratio of the luminescent signals from renilla versus firefly was used to determine the target specificity of miR-126. All experiments were done in triplicate.

3.13. Statistical analyis

Statistical analyis was done using GraphPad Prism IV software. P values were calculated by paired t test. P values < 0.05 were considered significant.

3.14. Immunohistochemistry

Tissue microarrays of formalin-fixed paraffin-embedded surgical specimens representing primary SCLC tumors were constructed. These tumor specimens were characterised in our study as chromogranin-A/synaptophysin and thyroid-transcription factor-1 (TTF1) positive tumors (>70%).

Following hematoxylin-eosin staining, serial sections and antigen retrieving were made for immunohistochemistry (IHC) labeling using rabbit monoclonal antibody to SLC7A5 (1:300 dilution; Epitomics) for 1 hour at room temperature. Envision (biotin-free) peroxidase-based detection kit (Dako, Glostrup, Denmark) for mouse/rabbit antibodies was then used with the red AEC or brown DAB substrate-chromogen (Vector Labs) followed by hematoxylin nuclear counterstaining.

Alternatively, we performed double immunofluorescent (IF) staining where SLC7A5 antibody was visualized with the use of horse-radish peroxidase (HRP)-coupled anti-rabbit IgG$(Fab)_2$ and tyramide-FITC for green fluorescence followed by mAB to TTF1 and biotinylated secondary antibody treatments (all from Dako), developed with streptavidin-texas red for red fluorescence (Vector).

Finally, tissue-reactivities for the antibodies (percentage of positive cells) were evaluated for each case by using a 3D-Histotech-Zeiss slide-scanner and Mirax-viewer software program (3D-Histotech, Budapest, Hungary).

4. Results

4.1. MiRNA microarray expression profiling of SCLC cell lines and normal lung.

LC Sciences miRNA microarray platform, interrogating 319 mature miRNAs was performed to investigate the differential expression of miRNAs in small cell lung cancer. The miRNA expression profile of normal lung and three SCLC cell lines: HTB-184, HTB-172, and H69 was characterized. The reference normal lung RNA was pooled from 6 RNA samples isolated from surgically removed lung tissue.

We identified 19 significantly overexpressed miRNAs, with relative expression levels of at least 10-fold in SCLC cell lines compared to normal lung. MiR-9 and miR-7 showed the highest overexpression (> 50-fold) in SCLC cell lines, and miR-183, miR-182, miR-206, miR-95, miR-375 were expressed over 30-fold higher in SCLC cell lines than in normal lung tissue. We also identified 34 miRNAs that are downregulated in SCLC cell lines compared to normal lung, and a large region in 19q13.41 containing more than 30 downregulated miRNAs (miR-512 to miR-373).

Several differentially expressed miRNAs are organized in clusters, such as the miR-17-92 cluster, the miR-200b-429 cluster, or the miR-145/143 cluster and are transcribed as polycistronic transcripts. MiRNAs in one cluster are usually up- or downregulated in concert, indicating that they are members of the same regulatory unit. It is also evident that miRNAs present in multiple copies on different chromosomes (miR-29b, miR-199a), or closely related miRNAs (miR-199a/b, miR-200a/b/c, miR-181- c/d, miR-106a-363 and miR-17-92 clusters) are often coordinately regulated.

4.2. Validation of miRNA expression profile with qRT-PCR.

Based on the microarray experiment we selected 21 significantly up- or downregulated miRNAs for further validation; in addition, we also selected 41 miRNAs because of their potential role in cancer biology, based on literature data.

Expression levels of the 62 (+1 normalizer) miRNAs were validated by qRT-PCR in four sample types: three SCLC cell lines (pooled RNA from H69, HTB-184 and HTB-172), normal lung tissue samples (pooled RNA from 6 samples), formalin fixed, parraffin embedded (FFPE) primary SCLC tumor samples (pooled RNA, isolated from 6 samples),

FFPE normal lung tissue derived from the vicinity of the tumors (pooled RNA, isolated from 6 samples). The pooled RNA from 6 normal lung samples was the same that was used for the microarray experiment. Sections from FFPE tumor samples were microdissected under the microscope and RNA was isolated separately from the normal lung tissue and the tumor tissue.

In the qRT-PCR validation we identified 16 miRNAs that are overexpressed in primary SCLC tumors, as well as in SCLC cell lines. MiR-105, miR-301 and miR-17-5p were expressed over 40-fold higher in SCLC cell lines than in normal lung tissue, and miR-17-5p showed the highest overexpression (16.67-fold) in primary SCLC tumor samples.

qRT-PCR analysis also confirmed downregulation of 8 miRNAs in primary SCLC tumors and in SCLC cell lines. To detect microRNA downregulation in primary SCLC tumor samples was more difficult than in SCLC cell lines. This occured probably because of the presence of normal cells in the tumor samples, which probably decreased the difference between tumor and normal for several miRNA. The average relative expression level changes were 0.07-fold in SCLC cell lines, compared to 0.23-fold in pimary SCLC tumor samples for the same 8 miRNAs.

4.3. Expression patterns of selected miRNAs in SCLC cell lines and primary SCLC tumors.

7 downregulated and 8 overexpressed miRNAs were selected for further qRT-PCR analysis in 17 individual SCLC tumors.

QRT-PCR analysis verified that miR-126 is uniformly downregulated in SCLC cell lines, as well as in primary tumors. MiR-150, miR-223, miR-214 and miR-199a are also downregulated miRNAs in all SCLC cell lines, while miR-222 expression value is unchanged in two SCLC cell lines. MiR-150, miR-222 and miR-223 are downregulated in the majority of the primary tumor samples, whereas miR-29a, miR-214 and miR-199a showed a more variable expression pattern.

Four microRNAs (miR-301, miR-183, miR-106a, miR-105) were overexpressed in all SCLC cell lines, from these miRNAs miR-301 and miR-183 were uniformly overexpressed in SCLC tumors, and miR-106a, miR-25, and miR-95 were upregulated in the majority of the tumor samples. MiR-105, miR-374, miR185 showed a variable expression pattern in these

samples. We did not find any evidence of the tumor location (lung or metastatic site) influencing the expression of the miRNAs studied in these samples.

4.4. Gene amplification is not the general mechanism causing overexpression of several miRNAs in SCLC tumors.

Many microRNA genes are found in cancer-associated genomic regions, such as in minimal regions of loss of heterozygosity or minimal regions of amplification in tumors.
We used q-PCR to determine the copy numbers for 5 genomic regions harboring prominently overexpressed miRNAs: miR-17-92, miR-183/96, miR-182, miR-95 and miR-301 in SCLC cell lines and in primary SCLC tumors. Gene amplification was clearly detected for all 5 miRNA genomic regions in the SCLC cell lines including for the miR-17-92 cluster, which is in accordance with previous observations. However, in primary SCLC tumors only the miR-183/96/182 genomic region was characteristically amplified, in 3 out of 4 samples. Although miR-301 was uniformly and highly overexpressed in SCLC tumors, gene copy number gain was detected in only 1 sample, and another sample in fact lost one allele. Amplification was not detected for miR-95 or the miR-17-92 cluster in SCLC tumors.

4.5. Overexpression of miR-126 inhibits proliferation of SCLC cells by causing delay in the G1 phase.

Since our previous work has shown that miR-126 is uniformly under-expressed in primary SCLC tumors, in the next phase of our studies we investigated its role in regulating the proliferation of SCLC cells. H69 cells were transiently transfected with miR-126 precursor (pre-miR-126) or the negative control miRNA (NegmiR) using lipofectamine 2000 transfection reagent, and viable cell numbers were monitored for 96 hours using cell counting and trypan blue exclusion.

Transfection of pre-miR-126 into H69 cells resulted in increased miR-126 expression compared to non-transfected or NegMiR control-transfected cells. Overexpression of miR-126 resulted in a significantly decreased proliferation of H69 cells, evident from 72 hours post-transfection.

We verified the effect of miR-126 on SCLC cell proliferation in another SCLC cell line, HTB-172. We found that miR-126 overexpression inhibits the proliferation of HTB-172 cells similar to the H69 cell line.

Flow cytometric cell cycle analysis at two time points (72 and 96 hrs post-transfection) revealed an increasing percentage of miR-126-transfected H69 cells in the G1 phase over time, and a concomitant decrease in the percentage of cells in the G2/M phase, when compared to the negative control. This suggests that overexpression of miR-126 delays H69 cells in the G1 phase of the cell cycle.

4.6. Overexpression of miR-126 suppresses SLC7A5 expression at both the RNA and the protein level.

To identify potential targets for miR-126 that might play a role in regulating proliferation of SCLC cells, we first performed an in silico analysis using the miRNA target prediction databases TargetScan and PicTar. Six of the predicted targets were identified by both algorithms (CRK, PLK2, SLC7A5, PTPN9, FBXO33 and RGS3), and in fact, CRK and PLK2 are bona fide targets of miR-126 as proven by functional studies. However, with the exception of SLC7A5, none of the validated or doubly-predicted target genes are known to be overexpressed in SCLC cell lines or tumors. SLC7A5 protein overexpression in SCLC is in accordance with the previously described downregulation of miR-126 expression. We selected SLC7A5 for further studies to analyse the role of miR-126 in the cell cycle regulation of SCLC.

We next investigated the effect of miR-126 overexpression on SLC7A5 and PLK2 expression. miR-126 overexpression in H69 cells caused more than a 50% reduction in SLC7A5 mRNA levels, and also a slight suppression of PLK2 mRNA expression, as determined by qRT-PCR. Subsequent western blot analysis of SLC7A5 and PLK2 demonstrated that while miR-126 overexpression resulted in decreased SLC7A5 protein levels, PLK2 protein levels did not change significantly.

4.7. Suppression of SLC7A5 by RNAi delays SCLC cells in the G1 phase.

To better understand the effect of SLC7A5 in SCLC cell cycle control, we utilized RNA interference to specifically suppress SLC7A5 production in H69 cells, and performed cell cycle analysis by flow cytometry at 72 and 96 hours post-transfection.

Transfection of specific siRNA into H69 cells resulted in significantly lower SLC7A5 expression when compared to the negative control siRNA. Similarly to the effect of miR-126 overexpression, suppression of SLC7A5 resulted in an increasing percentage of transfected cells in the G1 phase over time, and a concomitant decrease in the percentage of cells in the G2/M phase, when compared to the negative control siRNA. In contrast, specific suppression of PLK2 expression by RNAi had no such effect on the cell cycle distribution of transfected H69 cells.

These results support the hypothesis that miR-126 inhibits proliferation and cause cell cycle delay of H69 SCLC cells by negatively regulating SLC7A5.

4.8. SLC7A5 is a direct target of miR-126.

To validate that SLC7A5 is a molecular target of miR-126, as suggested by the previous experiments, we constructed a luciferase reporter vector containing 331 bp of the SLC7A5 3' UTR, including the predicted miR-126 binding site (WT-UTR). We also constructed a control luciferase vector with the miR-126 binding site deleted from the SLC7A5 3' UTR (DEL-UTR). H69 cells were transiently transfected with the WT-UTR-luciferase or the DEL-UTR-luciferase vector and with pre-miR-126. Co-transfection of WT-UTR with pre-miR-126 resulted in a significant decrease of reporter activity as compared with the control; however, deletion of the miR-126 binding site from the SLC7A5 3' UTR abolished this effect of miR-126.

These data suggest that SLC7A5 is a direct functional target of miR-126 in H69-SCLC cell line.

4.9. SLC7A5 and miR-126 expression levels are inversely correlated in primary SCLC tumors.

The correlation of miR-126 and SLC7A5 expression was investigated in 12 primary SCLC tumor samples, using immunohistochemistry (IHC) with SLC7A5-specific antibody. SLC7A5 expression was not detectable in normal lung tissue, while SCLC tumors were positive for SLC7A5 protein expression - in fact, 8 tumors contained more than 70% SLC7A5-positive cells.

As demonstrated by the double IF stained specimens, the majority of tumor cells exhibited nuclear staining for TTF1 (typical feature for SCLC) with SLC7A5 co-expression. The tumor samples analysed with IHC were the same samples analysed before for aberrant miR-126 expression. Since all 12 SCLC tumors overexpressed SLC7A5 and under-expressed miR-126, the inverse correlation between the expression levels of miR-126 and its target could be corroborated in primary tumors.

5. Discussion

5.1. Differentialy expressed microRNAs in small cell lung cancer, and their biological functions in normal or neoplastic cells.

We combined microarray and qRT-PCR to identify microRNAs aberrantly expressed in small cell lung cancer. The microarray approach alone identified 19 overexpressed miRNAs, and more than 35 downregulated miRNAs in SCLC cell lines compared to normal lung. Out of these, 7 downregulated and 8 overexpressed miRNAs were selected for further qRT-PCR analysis in 17 individual SCLC tumors and 3 SCLC cell lines. The qRT-PCR analyses verified that miR-126 is a uniformly downregulated miRNA, while miR-301 and miR-183 are uniformly overexpressed microRNAs in all SCLC sample types. As described previously, miR-17-5p showed the highest expression level in primary SCLC tumors.

The microarray results showed for the first time that miR-374 and miR-506 can be deregulated in cancer, and, thus, may contribute to the tumorigenic phenotype. In addition, several miRNAs were not implicated before in lung cancer, such as overexpressed miR-429, miR-128a/b and miR-130b, and downregulated miR-135b, miR-511, miR-190, miR-296, miR-33, miR-502, and miR-325. The role of these miRNAs in neoplastic transformation is well documented, but quite variable depending on the actual tumor type.

A growing body of evidence supports that the overexpressed microRNAs are involved in normal development, differentiation and cancer, although little is known about their exact function in the development of lung cancer. Members of the miR-182/miR-183/miR-96 cluster were shown to be upregulated in several malignancies. MiR-183 was significantly up-regulated in hepatocellular carcinoma, inhibited apoptosis by repressing PDCD4 gene expression, and interestingly, could inhibit cancer metastasis. MiR-182 was shown to be strongly overexpressed in primary tumors of the lung, and regulated lung tumor growth. MiR-182 is frequently overexpressed in melanoma as well, and promotes melanoma metastasis. Less is known about miR-301; it is a crucial oncogene mediating proliferation and invasion in breast cancer, and, together with miR-95, it was shown to be overexpressed in pancreatic cancer.

Downregulated miRNA, miR-126 appears to have a complex role in regulating cellular proliferation. It has an anti-proliferative effect in several tumor types including SCLC, NSCLC, and colon cancer through targeting different members of the PI3K/Akt pathway, or in breast cancer cells, by targeting IRS1. In addition, miR-126 is a key regulator of vessel development, by targeting SPRED1 and PIK3R2 in endothelial cells and may be involved in the metastatic process, as evidenced by its effects on mammary or gastric carcinoma cell migration.

Both our results and previous observations showed that closely related miRNAs, or miRNAs with multiple copies on different chromosomes are often co-regulated, suggesting that aberrant transcriptional regulation plays an important role in the overexpression or downregulation of many miRNAs in SCLC.

Several studies investigating chromosomal aberrations in SCLC identified characteristically lost genomic regions. Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC. Interestingly, the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC, with the exception of the miR-200b/200a/429 cluster, which is located in 1p36.33 in the vicinity of the antiapoptotic gene TNFRSF4, frequently amplified in SCLC. Therefore, we analyzed DNA copy number changes in primary SCLC tumors for 5 genomic regions with overexpressed miRNAs: miR-17-92, miR-183/96, miR-182, miR-95 and miR-301. We identified one novel amplified region in SCLC: 7q32.2 contains the miR-183/96/182 cluster, and copy number gains were clearly detected in 3 of 4 SCLC tumors. In contrast, 3 other genomic regions studied showed no or infrequent amplification (miR-17-92, miR-95, and miR-301, respectively).

Some miRNAs have similar expression patterns in SCLC and NSCLC: miR-182, miR-375, miR-210, miR-200b and miR-301 are overexpressed, while miR-143, miR-145 and miR-126 are downregulated miRNAs in both lung cancer types. However, there are several miRNAs expressed differently between SCLC and NSCLC. For example, miR-98, miR-17-5p and miR-106a are overexpressed in SCLC cells compared to HBECs, but not in NSCLC cells. Similarly, miR-27a and miR-29a/b/c expression was downregulated in SCLC cells compared to HBECs, but not in NSCLC cells. These distinct miRNA expression pattern

suggests the possibility of developing miRNA profiling as a diagnostic tool for distinguishing SCLCs from NSCLC and normal lung. In this regard, miR-21 is an oncogenic miRNA upregulated in many solid tumors, and its detection in sputum samples of lung adenocarcinoma patients indicates that it may be utilized as a biomarker for the early detection of lung cancer . Interestingly, our study and others' have shown that miR-21 in not overexpressed in SCLC.

5.2. miR-126 inhibits proliferation of small cell lung cancer cells by targeting SLC7A5.

In our previous work we identified miR-126 as a downregulated microRNA in all examined SCLC samples. Several studies investigated the function of miR-126 in other types of cancer, but prior to our work, little was known about its role in SCLC. Previous studies have drawn a complex picture about the potential roles of miR-126 in cancer biology.

In several tumor types, miR-126 has a tumor-suppressor and pro-apoptotic effect, and is down-regulated accordingly in the tumor cells. Specifically, in NSCLC cells miR-126 can inhibit proliferation in vitro and in vivo by targeting VEGF or EGFL7, promotes apoptosis, and inhibits tumor cell adhesion, migration and invasion through regulating Crk function. Similarly, miR-126 also function as tumor suppressor in gastric cancer by affecting the proliferation and metastatic potential of gastric cancer cells, suppresses breast cancer metastasis and, importantly, the loss of miR-126 expression is associated with poor distal metastasis-free survival of breast cancer patients.

On the other hand, miR-126 is overexpressed in acute myeloid leukemias and has an anti-apoptotic effect in this tumor type. Thus, mir-126 is not an obligatory anti-proliferative miRNA; rather, it appears to have multiple functions depending on the cell type and the actual cellular environment. Based on the decreased expression of miR-126 in SCLC, we expected that it will have an anti-proliferative effect in this tumor type, which was confirmed by the proliferation assays and cell cycle analyses of miR-126-overexpressing H69 and HTB-172 cells.

The complexity of miR-126 functions (in tumor cells, and presumably in normal cells as well) is underscored by the observations, that not all validated miR-126 target mRNAs are affected by miR-126 in every cell type. For example, in SCLC cells miR-126 overexpression does not suppress PLK2 expression, even though PLK2 was shown to be a bona fide target for miR-126 in CBF AML cells, and plays a role in cell cycle and DNA damage checkpoint regulation.

A similar observation can be made for TOM1, which is targeted by miR-126 in CF airway epithelium cells, but not in MCF7 cells cells; or for SPRED1, which is targeted in HUVEC cells, but not in AML cell lines.
It is presently unclear how certain target mRNAs are presented to, and others are protected from miR-126 in these experimental setups, but the resulting target selectivity could contribute to the varied functions of miR-126.

Importantly, we identified SLC7A5 as a novel target of miR-126 in SCLC cells. This is of special interest, since with the exception of SLC7A5, none of the validated or doubly-predicted target genes for miR-126 are known to be overexpressed in SCLC cell lines or tumors. SLC7A5 (LAT1) is an L-type amino acid transporter, transporting large neutral amino acids through the plasma membrane. SLC7A5 is widely expressed in many cancer types, including SCLC, and was shown to play essential roles in cancer growth and survival. SLC7A5 expression levels are usually correlated to cancer progression and aggressiveness, and was shown that SLC7A5 expression may be utilized as a prognostic factor for patients with NSCLC.

We demonstrated that in SCLC cells, similarly to other tumor types, suppression of SLC7A5 expression has an anti-proliferative effect. SCL7A5 suppression or miR-126 overexpression both delay SCLC cells in the G1 phase, suggesting that the effect of miR-126 on the cell cycle is at least in part mediated through SLC7A5. Consequently, high level of SLC7A5 expression in SCLC cells may be maintained partly by decreased miR-126 expression, and, thus, may ensure efficient transport of essential amino acids in the rapidly proliferating tumor cells.

On the other hand, miR-126 may also be involved in a more direct regulation of the cell cycle in SCLC. For instance, several observations suggest that SLC7A5 may affect cell proliferation not just by providing nutrients, but also by activating nutrient-dependent growth regulatory pathways, such as the PI3K/Akt/mTOR pathway, which was shown before to be regulated by miR-126 in several experimental models. Class IA PI3Ks are the most studied class of PI3K family widely implicated in cancers. These kinases function as heterodimers composed of the regulatory subunit p85 (α,ß,p55γ) and the catalytic subunit p110(α,ß,δ). p85 ß is a bona fide target of miR-126 in breast, colon cancer and in endothelial cells. MiR-126

also regulates the PI3K/Akt pathway in NSCLC. A class III PI3K, vps34 is another member of PI3K family which plays a role in the regulation of mTOR activity in response to amino acid availability. Leucine, transported by SLC7A5, activates mTOR, which in turn phosphorylates p70S6 kinase 1 (ribosomal protein S6 kinase1) and 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1), leading to the production of growth promoting proteins.

SCLC represent ~20% of all cases of lung cancer. It is a very aggressive form of lung cancer, is highly metastatic and responsive to chemotherapy. Several studies suggest that different signaling pathways involved in cell proliferation and survival are upregulated in lung tumors, such as components of the PI3K/Akt/mTOR pathway. Aberrant activation of this pathway participate in tumor growth and progression.

Activated PI3K pathway promotes SCLC cell growth, metastasis and resistance of SCLC cells to various therapies. High level of phosphorylated Akt is detected in SCLC tumors and the level of mTOR effectors, S6K1 and S6K2 are also overexpressed in SCLC and correlate with chemoresistance and promote survival of SCLC tumors. SCLC cells exist in rich extracellular matrix environment which activates the PI3K/Akt pathway through ß1-integrin activation leading to chemoresistance and protection of apoptosis. Blocking PI3K activity or inhibition of mTOR with rapamycin promote apoptosis and decrease SCLC cell growth suggesting that blocking the mTOR pathway could be a very promising approach for SCLC treatment. AKT can also be activated by EGFR representing the connection between mTOR and EGFR pathways. Both pathways are active in SCLC and dual inhibition of these pathways could be a novel strategy to treat SCLC.

Our work has identified miR-126 as an important negative regulator of the growth and proliferation of SCLC cells, which probably fine-tunes the activity of the PI3K/Akt/mTOR network through multiple targets, including SLC7A5. Removal of miR-126 from the regulatory network may enhance the existing positive feedback between SLC7A5 and mTOR, and can contribute significantly to the proliferative potential of the tumor cells. In addition, miR-126 may regulate PI3K/Akt pathway by targeting PIK3R2/p85ß in SCLC cells as well. However, mir-126 may have additional functions in the tumor stroma: in normal endothelial cells it is a positive regulator of angiogenesis, and it is likely to have interesting functions in regulating the immune response. Therefore, more research is needed to understand the

complex role of miR-126 in the growth, survival and progression of SCLC tumors *in vivo*, and to determine how miR-126 may potentially be exploited as an anti-tumor agent.

6. Summary

MicroRNAs are small, non-coding RNAs that regulate gene expression by binding to the 3' untranslated region (3'UTR) of target mRNAs, inducing mRNA degradation or translation repression. Approximately 50% of miRNA genes are in cancer-associated genomic regions, suggesting that microRNAs play a significant role in tumor biology. Small cell lung cancer (SCLC) is a high-grade neuroendocrine tumor characterized by rapid progression and frequent metastasis.

We combined microarray and qRT-PCR analyses to identify microRNAs aberrantly expressed in small cell lung cancer.

The microarray approach alone identified 19 miRNAs that are significantly overexpressed, by at least 10-fold in SCLC cell lines compared to normal lung. Although a number of these miRNAs were implicated before in other tumor types, our results show the first time that miR-374 and miR-506 are deregulated in cancer, and, thus, may contribute to the tumorigenic phenotype. We also identified 35 miRNAs that are downregulated in SCLC cell lines compared to normal lung, and a large region in 19q13.41 containing more than 30 downregulated miRNAs (miR-512 to miR-373).

RNA samples from SCLC cell lines and a small number of microdissected primary SCLC tumors were analyzed with qRT-PCR as well. At first we identified 16 overexpressed and 8 downregulated miRNAs in primary SCLC tumor samples, as well as in SCLC cell line samples. MiR-17-5p showed the highest expression level (more than 15-fold) in primary SCLC tumors and the expression level of miR-17-5p was more than 30-fold in SCLC cell lines compared to normal lung, in line with the results of others.

7 downregulated and 8 overexpressed miRNAs were selected for further analysis in a larger panel of individual SCLC tumors and SCLC cell lines. qRT-PCR analysis verified that miR-126 is a uniformly downregulated miRNA, while miR-301 and miR-183 are uniformly overexpressed microRNAs in all SCLC sample types.

Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC. Interestingly, the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC. Therefore, we analyzed DNA copy number changes in

primary SCLC tumors for 5 genomic regions with overexpressed miRNAs. We identified one novel amplified region in SCLC: 7q32.2 contains the miR-183/96/182 cluster.

In our further work we demonstrated that miR-126 overexpression has a negative effect on SCLC cell proliferation, by delaying cells in the G1 phase of the cell cycle. Importantly, we identified SLC7A5 as a novel target of mir-126 in SCLC cells. miR-126 downregulates the expression of SLC7A5 at the translation level, and reduces mRNA stability simultaneously.

SLC7A5 (LAT1) is an L-type amino acid transporter widely expressed in many cancer types and was shown to play essential roles in cancer cell growth and survival. We demonstrated that in SCLC cells, similarly to other tumor types, suppression of SLC7A5 expression has an anti-proliferative effect. SCL7A5 suppression or miR-126 overexpression both delay SCLC cells in the G1 phase, suggesting that the effect of mir-126 on the cell cycle is mediated at least in part through SLC7A5. SLC7A5 provides the essential amino acids that act as signal to enhance growth of cancer cells through mammalian target-of-rapamycin (mTOR)-stimulated translation. Through different targets, miR-126 can negatively regulate PI3K/Akt pathway, which is aberrantly active in a large percentage of SCLC tumors. Therefore, miR-126 is an important negative regulator of the growth and proliferation of SCLC cells, which probably fine-tunes the activity of the PI3K/Akt/mTOR network through multiple targets, including SLC7A5.

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List of publications related to the dissertation

1. **Miko, E.**, Margitai, Z., Czimmerer, Z., Várkonyi, I., Dezső, B., Lányi, Á., Bacsó, Z., Scholtz, B.: miR-126 inhibits proliferation of small cell lung cancer cells by targeting SLC7A5. *FEBS Lett. 585* (8), 1191-1196, 2011.
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2. **Miko, E.**, Czimmerer, Z., Csánky, E., Boros, G., Buslig, J., Dezső, B., Scholtz, B.: Differentially expressed microRNAs in small cell lung cancer.
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List of other publications

3. Varga, I., Hutóczki, G., Petrás, M., Scholtz, B., **Miko, E.**, Kenyeres, A., Tóth, J., Zahuczky, G., Bognár, L., Hanzély, Z., Klekner, Á.: Expression of Invasion-Related Extracellular Matrix Molecules in Human Glioblastoma Versus Intracerebral Lung Adenocarcinoma Metastasis.
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4. Balogh, A., Paragh jr., G., Juhász, A., Köbling, T., Törőcsik, D., **Miko, E.**, Varga, V., Emri, G., Horkay, I., Scholtz, B., Remenyik, É.: Reference genes for quantitative real time PCR in UVB irradiated keratinocytes.
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