Aus dem Institut für Neuropathologie der Heinrich-Heine-Universität Düsseldorf

Direktor: Prof. Dr. med. Guido Reifenberger

Identification and functional analyses of microRNAs involved in the malignant progression of astrocytic gliomas

Dissertation

zur Erlangung des Grades eines Doktors der Medizin

Der Medizinischen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Bastian Malzkorn

2010

Als Inauguraldissertation gedruckt mit Genehmigung
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez.:
Dekan: Prof. Dr. med. Joachim Windolf
Referent: Prof. Dr. med. Guido Reifenberger
Korreferent: Prof. Dr. med Rainer Haas

Wesentliche Teile dieser Arbeit wurden veröffentlicht in:

Malzkorn B, Wolter M, Liesenberg F, Grzendowski M, Stühler K, Meyer HE, Reifenberger G. Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathology. 2009 Aug (published online ahead of print). Available from: http://dx.doi.org/10.1111/j.1750-3639.2009.00328.x

Malzkorn B, Wolter M, Reifenberger G. MicroRNA: Biogenesis, regulation, and role in primary brain tumors. In: Erdmann, VA, Reifenberger G, Barciszewski J, editors. Therapeutic Ribonucleic Acids in Brain Tumors. Springer; 2009. pp. 327-354

Für meine Eltern und meinen Bruder

Contents

1 Introduction

1.1 Astrocytic gliomas

Astrocytic gliomas are primary central nervous system (CNS) tumors that can be subdivided into two major groups according to their growth pattern. The first more common group comprises diffusely infiltrating astrocytic tumors. Within this group the World Health Organization (WHO) classification of tumors of the central nervous system differentiates diffuse astrocytoma, anaplastic astrocytoma, glioblastoma and gliomatosis cerebri [1]. The second group consists of tumors demonstrating a more circumscribed growth, i.e. pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma.

This study focused on the investigation of diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma. Diffuse astrocytoma of WHO grade II inherently tends to locally recur and spontaneously progress to anaplastic astrocytoma WHO grade III and eventually secondary glioblastoma WHO grade IV [1].

1.1.1 Epidemiology

Astrocytic gliomas represent the majority of primary CNS tumors. Nonetheless, the annual incidence rates are rather small and range from 1.3/1,000,000 population (diffuse astrocytoma) to 3/100,000 population (glioblastoma). The mean age of diagnosis is 46 years for diffuse astrocytomas and 50 years for anaplastic astrocytomas. Primary glioblastomas that develop *de novo* are diagnosed at a mean age of 64 years,

whereas secondary glioblastomas that evolve by spontaneous malignant progression of low-grade gliomas usually develop in patients younger than 45 years.
The median time of progression from lower-grade gliomas to secondary glioblastomas is about five years. Despite aggressive treatment the prognosis of astrocytic tumors is very poor. Median 5-year survival rates range from 45 % (diffuse astrocytomas WHO grade II) to only 2,9 % (glioblastoma WHO grade IV). [All epidemiological data cited were calculated by the Central Brain Tumor Registry of the United States [2]]. In addition to tumor grading, the prognosis of diffusely infiltrating astrocytomas depends on the age at diagnosis, initial clinical performance score, extent of resection, and the *MGMT* promoter methylation status [3].

1.1.2 Clinical presentation, diagnosis, therapy

Diffusely infiltrating astrocytomas most frequently arise in the cerebral hemispheres affecting the frontal and temporal lobes, albeit they may be located in all parts of the brain. Tumor infiltration can even reach the contralateral hemisphere [4]. Depending on tumor location, patients with astrocytic gliomas may present variable focal neurological deficits. Initially epileptic seizures are common symptoms. Fast growing tumors with huge perifocal edema evoke more severe symptoms mainly due to mass shift and an increase of intracranial pressure [4].
On magnetic resonance imaging (MRI), diffuse astrocytomas present as intraaxial lesions that are typically hypointense on T1-weighted images and hyperintense on T2-weighted images. They demonstrate well-defined or ill-defined margins and usually lack contrast enhancement. Anaplastic astrocytomas are heterogeneously hyperintense on T2-weighted images with gadolinium contrast enhancement being common.

Glioblastoma typically presents with ring enhancements due to necrotic areas in the center of the tumor. Neither contrast enhancement nor other markers of malignancy allow to definitely asses glioma grade by imaging techniques. Therefore the diagnosis has to be assured by neuropathological analysis of bioptic material that can determine the histological type and grade (see section "Tumor grading"; for review on glioma imaging see Reference [5]).

The spectrum of glioma management comprises observation, surgery, radiotherapy, chemotherapy and palliative approaches. Recent clinical guidelines recommend individual therapeutic regimes that take into account histological type, WHO grade and possible extent of tumor resection, as well as age and constitution of the patient [6]. In the future, targeted molecular therapy will hopefully improve prognosis. Several tyrosine kinase inhibitors and other new drugs targeting key pathways of glioma pathogenesis are already in clinical phase I and II trials [7].

1.1.3 Tumor grading

Histopathological analysis of bioptic material is the gold-standard of glioma classification. The different WHO grades of malignancy are defined by characteristic histological findings: Diffuse astrocytoma is a well-differentiated tumor without any signs of anaplasia. Anaplastic astrocytoma shows a higher degree of nuclear pleomorphism, increased cellularity, and an elevated mitotic activity. Glioblastoma can be differentiated from anaplastic astrocytoma by prominent microvascular proliferation and necroses. Several immunohistochemical markers help to identify glial lineage and tumor grading. [4].

The adjusted prognosis (taking into account age, performance score, and extent of

resection) of individual patients varies within each histological group. Molecular diagnostics aim at the identification of molecular prognostic factors and markers of response to therapy. For example *MGMT* promoter methylation - which is already part of routine molecular diagnosis in neuropathology centers - is associated with the response of glioblastomas to alkylating chemotherapy [8, 9, 10, 4].

1.1.4 Molecular pathology of astrocytoma progression

The molecular basis of astrocytoma progression has been investigated in previous studies by analyzing chromosomal and genetic aberrations as well as changes in mRNA expression levels (for review see [4]). Collectively, these studies revealed that about 60 % of diffuse astrocytomas carry *TP53* mutations, usually accompanied by loss of heterozygosity (LOH) on 17p, thus resulting in complete abrogation of wild-type p53 function in the tumor cells [4]. In addition, *IDH1* or *IDH2* gene mutations, overexpression of *PDGFA* and *PDGFRA* as well as gains on chromosome 7 are commonly found in these tumors [4, 11, 12]. Anaplastic astrocytomas often carry additional chromosomal alterations, such as deletions on chromosomes 6, 9p, 11p, 19q, and 22q. Relevant target genes on 9p are the *CDKN2A*, $p14^{ARF}$, and *CDKN2B* tumor suppressor genes. Moreover, *CDK4* or *CDK6* amplification or inactivating alterations of *RB1* are detectable in a subset of anaplastic astrocytomas [13]. Glioblastomas show various chromosomal and genetic alterations that lead to the inactivation of different tumor suppressor genes (e.g. *DCC*, *PTEN*, *EMP3*, *MGMT*, *CTMP*) as well as the aberrant activation of proto-oncogenes (e.g. *EGFR*, *CDK4*, *MDM2*, *CDK6*, *CCND1/3*, *MDM4*, *PDGFRA*) [4]. Interestingly, the pattern of genetic aberrations in primary glioblastomas, which present *de novo* with a short clinical history, is distinct

from that of secondary glioblastomas, which develop by progression from pre-existing lower grade gliomas (Figure 1) [14]. For example, primary glioblastomas show frequent *EGFR* amplification and *PTEN* mutation while secondary glioblastomas, similar to diffuse and anaplastic astrocytomas, are characterized by frequent mutation in the *TP53* and *IDH1* genes [11, 12, 14]. Notably, most of the molecular alterations at the gene and transcript levels that have been detected in glioblastomas can be assigned to certain functional pathways, in particular the phosphoinositol 3 kinase/Akt, mitogen-activated kinase, p53 and pRb signalling cascades [4, 15].

1.2 MicroRNAs (miRNAs)

MicroRNAs are approximately 22 nucleotides long, endogenous, single-stranded, non-protein-coding RNA molecules that post-transcriptionally regulate gene expression.

1.2.1 History of miRNAs

The discovery of the miRNA pathway and its role in the regulation of gene expression is closely linked to the history of RNA interference (RNAi). In 1990, van der Kroll and co-workers [16] as well as Napoli and colleagues [17] attempted to produce deep purple petunia flowers and therefore over-expressed a pigment synthesis enzyme. Surprisingly, they generated predominantly white flowers, which was caused by a "co-suppression" of the transgenic and endogenous pigment synthesis enzyme gene. A similar phenomenon was reported in 1992 by Romano and Macino [18], whose introduction of homologous RNA sequences resulted in "quelling" of the endogenous gene

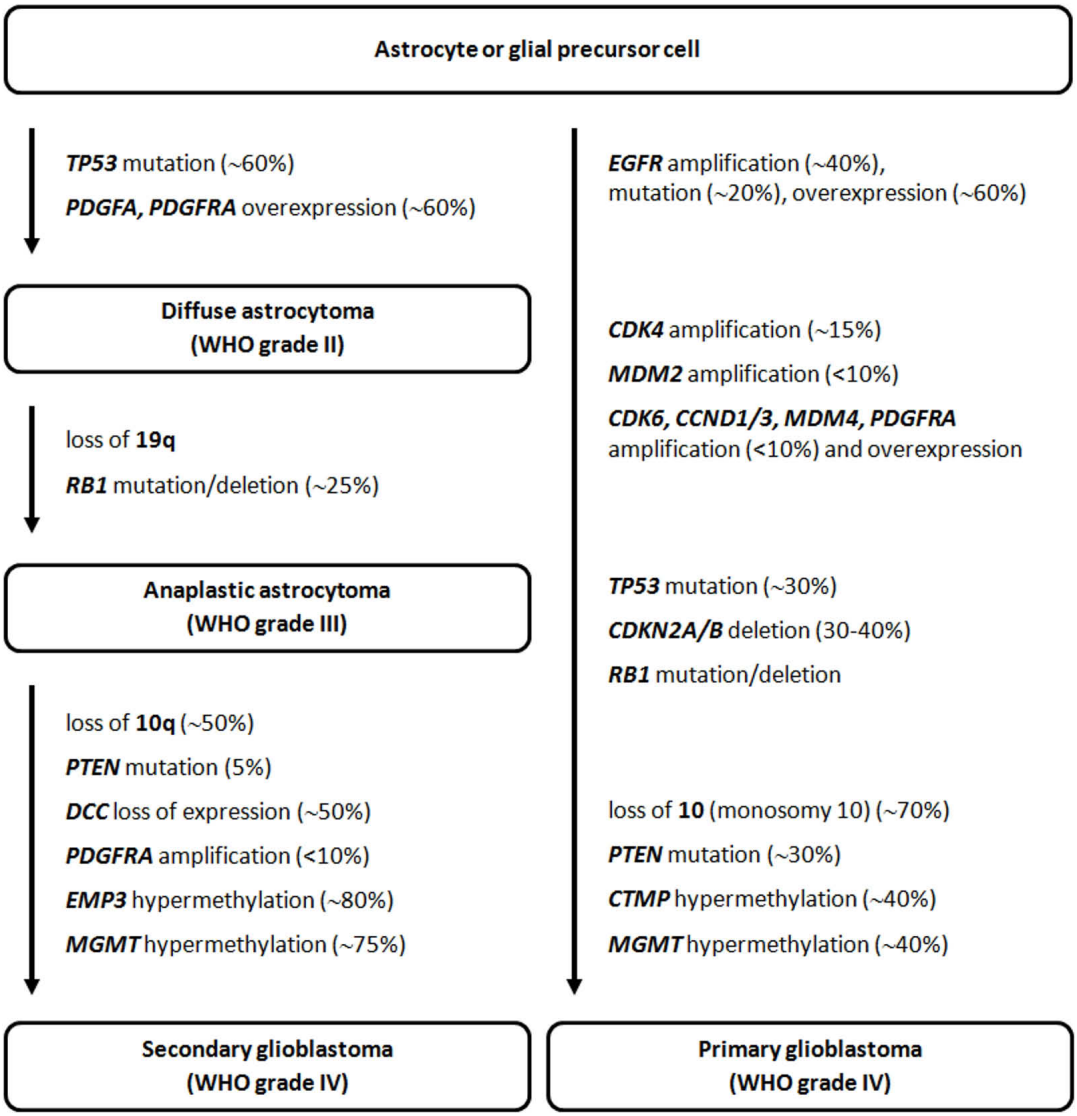


Figure 1: Molecular alterations in diffuse astrocytomas, anaplastic astrocytomas, and glioblastomas. Characteristic genetic and epigenetic aberrations that distinguish secondary glioblastoma from primary (*de novo*) glioblastoma are depicted. See section "Molecular pathology of astrocytoma progression" for more details; (according to Reference [14] with modifications by Riemenschneider and Reifenberger [4]).

in *Neurospora crassa*. In 1995, Guo and Kemphues observed that the introduction of sense or antisense RNA to *par-1* mRNA led to degradation of the *par-1* message in *Caenorhabditis elegans* [19]. Thus, the dogma was questioned that only antisense RNA leads to RNA silencing via hybridization with mRNA and subsequent inhibition of translation or degradation. Three years later Fire and coworkers [20] elaborately demonstrated that gene silencing in *Caenorhabditis elegans* was actually triggered by dsRNA, which contaminated ss-RNA preparations in previous studies, and thereby provided an explanation for co-suppression, quelling and silencing by sense mRNA. Furthermore, they concluded from their experiments, that dsRNA acts specifically in a posttranscriptional, non-stoichometrical mechanism. Mello and Fire termed this phenomenon RNAi and speculated that "dsRNA could be used by the organism for physiological gene silencing" [20]. In 2006, they were awarded the Nobel Prize in Physiology/Medicine for this breakthrough in RNAi research. Further investigations uncovered siRNAs - 21- to 25-nucleotide fragments of the original dsRNA - as the effector molecules of RNAi [21]. The isolation of Dicer as the enzyme that processed dsRNA into siRNAs [22] and of RNA-induced Silencing Complex (RISC) as effector complex [23], gave further insight into the mechanisms of RNAi.

Even before mechanistic details of RNAi were elucidated, Lee and coworkers discovered the first miRNA in 1993. They identified a 22-nucleotide RNA transcript derived from the *lin-4* locus of *Caenorhabditis elegans* that did not encode a protein and was proposed to be derived from a 65 nt hairpin precursor [24]. Previous experiments showed that loss-of-function mutations in *lin-4* as well as gain of function of the protein-coding gene *lin-14* disrupted the developmental timing of nematodes [25]. Lee and collaborators [24] then demonstrated that the 22-nucleotide *lin-4* RNA repressed the protein levels of *lin-14*. These authors supposed that *lin-4* regulates *lin-14*

mRNA by interactions between the miRNA and the 3'-untranslated region (UTR) of its mRNA target. The identification of the second miRNA *let-7* in *Caenorhabditis elegans* [26] showed that miRNAs are no oddity of nematode biology since the *let-7* sequence - in contrast to the sequence of *lin-4* - is evolutionary conserved. The discoveries in the field of RNAi further accelerated the revelation of a new mechanism of gene regulation. Hutvagner and colleagues demonstrated that the hairpin *let-7* precursor is processed by Dicer [27], similar to the processing of exogenous dsRNAs in RNAi. MicroRNAs were shown to associate with Ago proteins [28] that had been identified as key proteins in RISCs [29]. Later, a mechanism of translational inhibition by miRNAs was described [30]. Within a few years, details of nuclear miRNA processing could be explored. The primary transcription by DNA-Polymerase II was evidenced [31], the role of Drosha in nuclear processing of primary miRNA transcripts was elucidated [32] and Exportin5 was identified as nuclear export protein of pre-miRNAs [33]. Besides the investigations of details of the miRNA pathway, several cloning efforts expanded the number of known miRNAs [34, 35]. Up to the present 718 human miRNA genes are registered in the miRBase sequence database (August, 2009), Landgraf and colleagues [35] provided evidence for expression of 340 distinct human mature miRNAs transcribed from 416 miRNA genes.

1.2.2 Genomic organization

MicroRNA genes are dispersed in the entire genome except for the Y chromosome. Most miRNAs are evolutionary conserved in closely related species and many have homologs in distant species. For unequivocal annotation the first letters of each miRNA name represent the species (e.g. "hsa" = *Homo sapiens*). It is common

practice to use the short forms of miRNA names without this prefix, if the context clearly implies the respective species.

A mature miRNA can be encoded by several miRNA genes (annotated: hsa-miR-...-1, and hsa-miR-...-2, etc.) that are located in different regions of the genome. In addition, there are miRNA paralogs with nearly identical sequences (annotated: hsa-miR-...a, hsa-miR-...b, etc.) or highly related miRNAs which share a common seed sequence. Paralogous and highly related miRNAs constitute a miRNA family [35] because they probably target a similar set of regulated genes.

The majority of mammalian miRNA gene loci are part of defined transcription units - protein coding or non-coding. Many miRNA genes are found in the introns of transcription units with the sense sequence on the same strand [36]. Nevertheless, miRNA genes may be located at various positions (Figure 2 A): (I) intergenic miRNA genes, without any relation to a transcription unit and with the sense sequence on the plus- or minus-strand of DNA, (II) exonic, and (III) intronic miRNA genes with the sense sequence on the same strand or the opposite strand in relation to the orientation of the transcription unit [37]. Some miRNA genes are present in either an exon or an intron depending on the pattern of alternative splicing [36], others overlap the intron-exon boundary [37]. The host transcript and miRNAs usually have similar expression profiles suggesting that they are transcribed from the same promoter [38].

A distinctive feature of the genomic organization of miRNAs is that many miRNAs are found in clusters [35], which are transcribed as polycistronic primary transcripts [39] (Figure 2 B). MicroRNAs in a cluster are often related to each other suggesting that the cluster may have developed by gene duplication.

1.2.3 Biogenesis

MicroRNA biogenesis is a multistep process - the first two steps are localized in the nucleus followed by maturation in the cytoplasm (basic principles of miRNA biogenesis are summarized in Figure 2 C). Most miRNA genes are transcribed by RNA-polymerase II. The primary transcripts are polyadenylated and capped [31, 25]. Nonetheless, a miRNA cluster on chromosome 19 that is located between Alu repeats was shown to be transcribed by RNA-polymerase III [40]. The long primary transcript of a miRNA gene (pri-miRNA) is polycistronic for clustered miRNA genes [39]. A monocistronic pri-miRNA is composed of a doublestranded stem of about 33 base pairs, that is divided into a lower and upper stem, with the latter containing the mature miRNA, a terminal loop and two flanking single-stranded segments [41]. After transcription the pri-miRNA is "cropped" by a protein complex called the "microprocessor" [32, 42]. The microprocessor complex comprises the RNase III endonuclease Drosha that cleaves both strands of the ds-RNA between upper and lower stem of the pri-miRNA [32] and the ds-RNA binding protein Dgcr8 that recognizes the ss-RNA-ds-RNA junction to bring the catalytical center of Drosha into the right position [41]. Other protein components that support microprocessing *in vivo* are p72/Ddx17, p62/Ddx5 and hnRNPU1-like [42]. Cropping releases an approximately 70-nucleotides long hairpin precursor of the miRNA (pre-miRNA) with a characteristic 5'-phosphate and a 3'-2-nucleotide overhang [32]. Recently an alternative way of nuclear processing, which bypasses the microprocessor has been discovered. Pre-miRNA can also assemble after "mirtrons" - short introns with sequences that allow formation of an intramolecular hairpin structure - are spliced out of the mRNA of protein coding genes. In contrast to the Drosha cleavage products, the pre-miRNAs

that fold into hairpin structures spontaneously after debranching of the spliced lariat have single nucleotide overhangs at both arms of the pre-miRNA stem [43, 44]. For further processing, pre-miRNAs are exported to the cytoplasm by Exportin5-RanGTP, which also seems to protect nuclear pre-miRNAs from degradation [33]. In the cytoplasm, another RNAse III enzyme termed Dicer cleaves the pre-miRNA hairpin between its stem and its terminal loop and thereby releases a short miRNA duplex with 2-nucleotide 3'-overhangs that contain the mature miRNA and a complementary miRNA (annotated: hsa-miR-... and hsa-miR-...*) [27]. Dicer works in an ATP-independent manner in a protein complex with Trbp and Ago. This complex is termed miRNA RISC loading complex (mRLC). The mRLC processes pre-miRNAs to miRNA duplexes and unwinds the miRNA duplex in order to load the mature miRNA on miRISCs [45, 46, 47], also annotated miRNA containing ribonucleoproteins (miRNPs). Inherent thermodynamic asymmetry decides which of the two strands of a miRNA duplex will be loaded on miRNPs. The RNA strand whose 5'-end is less stably bound to the opposite strand forms the effective miRNA which is incorporated into miRNPs [48, 49, 50]. Recently, first functions of Ago2 in miRLCs have been elucidated. At least for some miRNAs with perfect complementarity in the stem Ago2 cleaves the 3'-arm of the pre-miRNA generating an Ago2-cleaved precursor miRNA (ac-pre-miRNA) that consists of a shortened hairpin bound to a fragment of about eleven to twelve nucleotides. This intermediate may further facilitate strand selection and removal of the passenger strand [51]. After strand selection the miRLC disassembles and the core miRNP containing Ago proteins and miRNA is released to mediate miRNA effects [46]. Until now, precise mechanisms of miRNP assembly are a matter of research and other proteins may be involved in this process.

1.2.4 MicroRNA effector mechanisms

MicroRNAs down-regulate gene expression by different mechanisms after base-pairing to their target mRNAs and perhaps other nucleic acids. More than one-third of human genes appear to be regulated via highly conserved miRNA target sites [52]. Three post-transcriptional mechanisms of target gene down-regulation have been experimentally validated in many different studies: (I) endonucleolytical cleavage of mRNAs, (II) translational inhibition, and (III) destabilization of mRNAs (Figure 2 D).

MicroRNAs do not function as naked RNAs but as components of ribonucleoprotein complexes (miRNPs), that contain a member of the Ago protein family [53], which at least partly determines the mechanism of gene regulation, and other proteins [54]. Another determinant of miRNA function is the complementarity of the miRNA to its target sites, which are predominantly located in the 3'-UTR of protein coding genes but sometimes are also found in the open reading frame [52, 55]. Perfect complementarity between miRNPs and their target mRNA sites leads to RNAi-like endonucleolytical cleavage of the target mRNA between the nucleotides bound to residues 10 and 11 of the miRNA [21, 56]. Only miRNPs that contain the Ago2 isoform can cleave target mRNA [29, 57]. In animals, base pairing between miRNA and target mRNA is imperfect in most cases [58] and induces translational inhibition or mRNA destabilization. These effector mechanisms seem to be executed by all Ago family members [59] and require several conditions of miRNA target sites: perfect complementarity to the seed sequence (nucleotides 2-7 of the miRNA), no complementarity to the central region of the miRNA to prevent endonucleolytical cleavage, reasonable complementarity to the 3'-region of the miRNA (especially residues 13-16), location at the ends of 3'-UTR but not too close to the stop codon. In addition,

multiple miRNA target sites are necessary for effective, co-operative down-regulation of gene expression [60]. The molecular details of translational repression by miRNPs are still unclear. MiRNPs inhibit protein synthesis at the initiation step by competing with eIF4E for cap-binding and thereby precluding 40S initiation complex assembly [30, 61] or by recruiting eIF6A that prevents joining of the 60S ribosomal subunit to the initiation complex [62]. Other data indicate that miRNP-mediated inhibition of translation targets post-initiation steps, as small RNAs also inhibit IRES-dependent translation, repressed mRNAs are associated with active polyribosomes, and silencing by short RNAs decreases translational readthrough [63]. MiRNPs that bind to their targets with imperfect complementarity can also induce mRNA destabilization via recruitment of decapping and deadenylation complexes [64]. Investigations of the compartmentalization of miRNA effector machinery revealed a connection of miRNP, targeted mRNAs, and P-bodies that comprise high concentrations of enzymes involved in mRNA degradation. Either as a cause or as a consequence of inhibiting protein synthesis, translation repression by miRNPs delivers mRNAs to P-bodies [65].

During recent years, new mechanisms of miRNA-mediated gene regulation have been discovered. In *Arabidopsis* miRNAs directly or indirectly recruit chromatin modifying complexes after binding to the mRNA target sites to epigenetically down-regulate target gene expression [66]. Epigenetic silencing also seems to be an effector mechanism in mammalian cells [67]. These findings indicate that miRNAs possibly induce a more specific epigenetic regulation than the already validated post-transcriptional regulation of proteins of the methylation machinery [55]. The fact that *miR-29b* is enriched in the nucleus due to a 3'- terminal motif directed nuclear import may encourage the search for unknown functions of miRNA in the mammalian nucleus [68]. Another field yet to be investigated is the role of miRNAs in cell-cell communication

as miRNAs have been detected in exosomes. Some miRNAs have a higher concentration in exosomes than in the cytoplasm suggesting that these species may be uniquely secreted via exosomes [69]. Recently, *miR-184* was shown to interfere with Ship2 suppression mediated by *miR-205* [70] possibly demonstrating an effector mechanism that cross-links different miRNA networks. Moreover, miRNA-mediated gene regulation was shown not to be restricted to silencing: *miR-369* up-regulates expression of Tnfα upon cell cycle arrest after binding to a 3'-UTR AU-rich element(ARE) and recruiting a protein complex that contains Ago2 and Fxr1 [71].
Whatever the details of effector mechanisms are, it is noteworthy that a specific miRNA may regulate hundreds of target genes simultaneously, while a single miRNA target can be regulated by multiple miRNAs. To investigate these intricate networks several target prediction algorithms have been developed that can be browsed via an online resource, which also highlights experimentally validated targets (free online access is available via www.diana.pcbi.upenn.edu/miRGen [37]).

1.2.5 Regulation of miRNA expression and function

The miRNA pathway is regulated - or may be misregulated in disease - at different stages. MicroRNA transcription can be de-regulated as a consequence of genetic alteration ([72, 73]) and is modulated by transcriptional activators (e.g. c-Myc [74], p53 [75], Hif [76]) or repressors (eg c-Myc[77], p53 [75]). In addition, the transcription of some miRNAs is epigenetically controlled by DNA methylation (e.g. *miR-127* [78]). Some studies depicted post-transcriptional regulation of miRNA expression [79, 80, 81]. For example Smad proteins were shown to enhance cropping of *pri-miR-21* [82] and hnRNP A1 is required for processing of *pri-miR-18a* [83]. Interestingly all Ago

proteins post-transcriptionally increase production or stability of mature miRNAs [51], thus demonstrating how closely miRNA regulation, biogenesis (Ago2 produces ac-pre-miRNAs), and function (Ago proteins mediate miRNA effects) are cross-linked. Besides regulation of transcription and processing, other regulatory mechanisms may directly interfere with miRNA function. For example the RNA-binding protein Dnd1 inhibits miRNA access to its target mRNA and thereby prevents miRNA mediated inhibition [84]. Apobec3g de-represses miRNA mediated protein translation inhibition by enhancing the association of miRNA targeted mRNAs with polysomes and by facilitating the dissociation of miRNA-targeted mRNA from P-bodies [85]. In addition, the sequestration of targeted mRNA in P-bodies is reversible due to stress stimuli [86]. Several interesting findings demonstrated that genetic alterations (translocation [87] or mutations [88, 89, 90, 91]) of miRNA targets can also interfere with miRNA functions. These alterations can disrupt miRNA mediated regulation on the one hand [87, 90, 91], but establish new miRNA:mRNA target interactions on the other hand [89].

Upstream regulation of miRNA function can involve multiple regulators, which are sometimes regulated by the miRNA itself, thus demonstrating feed-back regulation (e.g. E2F/*miR-20a* [92]). All in all, miRNAs take part in complex regulatory networks - upstream and downstream of the mature miRNA - that may influence almost every investigated cellular process [93].

1.2.6 MicroRNA in cancer

Functional analyses of the first identified miRNAs *lin-4* and *let-7* demonstrated that both regulate cell growth and differentiation [94, 26], i.e. processes whose misregula-

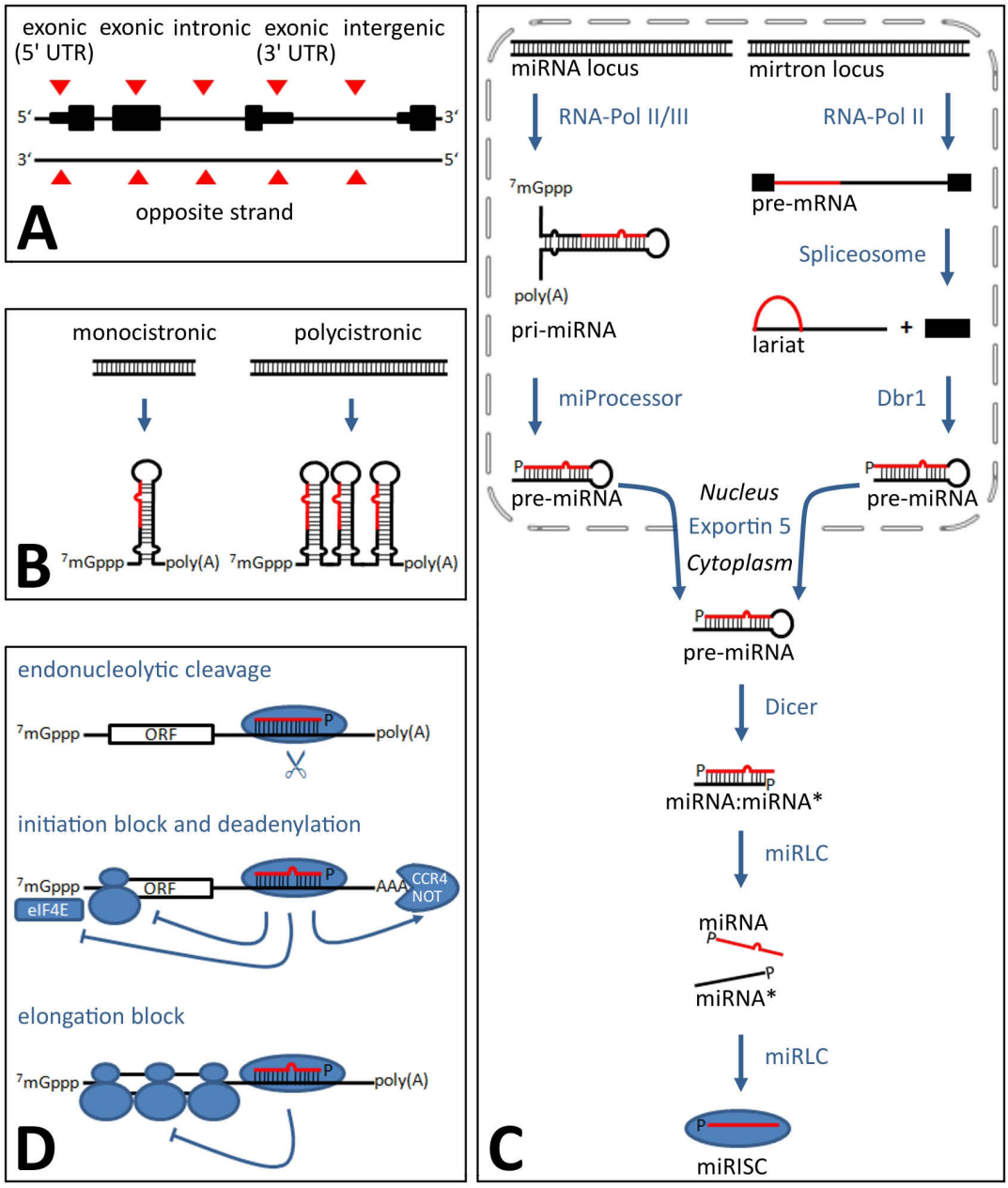


Figure 2: Synopsis of miRNA genomic organization (A/B), biogenesis (C), and effector mechanisms (D). A) MicroRNAs have their own transcription units (intergenic) or are transcribed with other genes. Intronic and exonic miRNA loci are found in protein coding genes and non-coding transcription units (not depicted). MicroRNAs are encoded on the plus- or minus-strand of DNA.

Figure 2: Synopsis of miRNA genomic organization (A/B), biogenesis (C), and effector mechanisms (D) (continued). B) Many miRNA loci are clustered, which results in transcription as a polycistron. Other miRNA loci are transcribed monocistronically. **C)** MicroRNA genes are transcribed by RNA-polymerase II (or III) to generate the primary transcripts (pri-miRNAs). The first processing step is mediated by the microprocessor complex (containing the nuclear RNase III Drosha) and results in a 70-nucleotide stem-loop (pre-miRNA). This classical nuclear processing is bypassed when certain short introns (mirtrons) spontaneously constitute pre-miRNAs after splicing of the pre-mRNA and Dbr1-mediated debranching of the excised lariat. Pre-miRNAs are transported into the cytoplasm by Exportin-5 and its cofactor Ran-GTP. After export, the cytoplasmic RNase III Dicer promotes the second processing step to produce miRNA duplexes. The duplex is separated and one strand is selected as the mature miRNA, which is integrated in miRNA-induced-silencing-complex (miRISC), whereas the other strand (miRNA*) is degraded. **D)** MiRISCs that contain Ago2 and bind to their target mRNA with perfect complementarity perform endonucleolytical cleavage of their targets. In humans most miRNAs bind to their target with imperfect complementarity, which may induce several processes, including blocking of initiation and promotion of deadenylation after binding of miRISCs to the target 3'-UTR. Some data also indicate an inhibition of elongation.

tion is closely related to tumorigenesis. Several global analyses corroborated that deregulation of miRNA function may contribute to tumorigenesis. Interestingly, about half of the known human miRNAs map within fragile regions of the genome that are associated with various cancers [73]. In addition, the inhibition of key proteins of miRNA biogenesis has been linked to the aggressiveness of cancer [95, 96, 81], which is in line with the finding of generally reduced miRNA levels in cancer [97]. Lu and co-workers suggested that expression profiling of miRNAs is a more accurate method of classifying cancer subtypes than using the mRNA expression profiles of protein-coding genes. In their study, miRNA profiles reflected the developmental lineage and differentiation state of the tumors, supporting a role for miRNA profiles in molecular diagnostics [97].

The first miRNAs that proved to have tumor-suppressive effects were *miR-15a* and *miR-16*. The expression of these miRNAs was found to be down-regulated and the *miR-15a-16-1* cluster on 13q14 showed frequent deletions in patients with B-CLL [98].

Moreover, putatively tumor-predisposing germ-line mutations have been reported [99]. Further investigations demonstrated that *miR-15a* and *miR-16* negatively regulate the anti-apoptotic protein Bcl2 and thereby induce apoptosis [100]. Deletion of the *miR-15a-16-1* cluster is a favorable prognostic factor as compared to other genetic aberrations in B-CLL [99] possibly because the deleted miRNA genes have paralogs that maintain the miRNA family's inhibition of oncogene expression [101]. The first miRNAs that were shown to be oncogenic are those of the *miR-17-92* cluster [102].

In general the role of miRNAs in tumorigenesis is explained by the following model inferred from the biology of miRNA-mediated gene regulation. A miRNA that reduces proto-oncogene expression may act as a tumor suppressor, whose expression is often reduced in cancer due to various mechanisms (described above). Vice versa, miRNAs that inhibit tumor suppressor gene expression may be oncogenic, and these miRNAs are frequently over-expressed in malignancies. In some cases, viral miRNAs act as oncogenes [103]. MicroRNAs that have been reported as being silenced or over-expressed in human cancers target regulators of proliferation [104], apoptosis [100], angiogenesis [105], invasion, metastasis[106], reaction to hypoxia [107], and "stemness" of cancer stem cells [108].

Until now, many miRNAs have been shown to be involved in tumorigenesis. The recent review article by Lee and Dulta [109] provides lists of miRNAs up- or down-regulated in several cancers, as well as tables that summarize relevant miRNA regulators and targets. Notably, a tumor-suppressive role of a miRNA in a defined tumor entity is not mutually exclusive with oncogenic functions in other cancers due to the complexity of miRNA regulatory networks.

What are the clinical implications of the revelation of the miRNA cancer connection? As mentioned above miRNAs might serve as diagnostic molecular markers of cancer.

In some studies a small set of miRNAs is sufficient as diagnostic tool. For example the ratio of the expression levels of two miRNAs clearly discriminated pancreatic cancers, chronic pancreatitis, and normal pancreas [110]. Moreover, de-regulated miRNA expression correlates with prognostic features in several cancers. The first report on a prognostic significance of aberrant miRNA expression described shortened post-operative survival in patients with reduced *let-7* levels in lung cancer [111]. However, the expectations of scientists and clinicians are beyond grouping patients on the basis of new prognostic markers. Hopefully, miRNAs are new therapeutic targets to improve survival. A few studies have already demonstrated first therapeutic approaches: Changing the cellular levels of miRNAs *in vitro* affected the potencies of several anti-cancer chemotherapeutics [112]. Re-expression of the epigenetically silenced *miR-203* in Philadelphia chromosome positive CML cell lines reduced bcr-abl expression, and thereby inhibited proliferation [113]. Restoration of silenced *miR-203* expression - either directly or through the application of demethylating drugs - might represent a novel therapeutic approach that sensitizes cancer cells for imatinib and could still be effective in imatinib-resistant cases if the miRNA target sites are unchanged [113].

1.2.7 MicroRNA in glioma

Early studies of miRNA expression in the brain indicated that specific patterns of miRNA expression can be recognized during brain development [114, 115]. Furthermore, a role of miRNAs in neuronal differentiation was outlined [116, 117]. Therefore, it is likely that misregulation of miRNA expression contributes to brain tumorigenesis. Interestingly, one of the miRNAs identified as temporally regulated during brain

development (*miR-9*) together with *miR-92b* is specifically over-expressed in brain primary tumors [118]. The expression levels of these two miRNAs distinguished between primary brain tumors and tumors from other tissues as well as their metastases to the brain [118].

To date, the role of miRNAs in pituitary adenomas, medulloblastomas, and gliomas has been analyzed in more detail. Differential miRNA expression in gliomas was first evidenced by Ciafre and colleagues [119]. In an approach to investigate the role of miRNAs in gliomas they used microarrays to determine the expression of 245 miRNAs in nine primary glioblastomas and surrounding glial tissue from the same patient, as well as in glioblastoma cell lines. Microarray data were confirmed by Northern Blot analyses. Two miRNAs (*miR-221* and *miR-21*) were shown to be up-regulated and the brain enriched miRNAs *miR-128*, *miR-181a*, *miR-181b* and *miR-181c* were shown to be down-regulated in glioblastoma [119].

In addition, miRNA expression was found to correlate with the glioma malignancy grade. In a stem-loop reverse transcription (RT) polymerase chain reaction (PCR) analysis of nine glioma samples and three glioma cell lines *miR-181a* and *miR-181b* expression levels were significantly lower in high-grade gliomas than in low-grade gliomas [120]. Furthermore, *miR-181a* and *miR-181b* functioned as tumor suppressors in glioma cells by inducing apoptosis and inhibiting anchorage-independent growth and invasion [120].

MicroRNAs can act as regulators of known molecular pathways that mediate glioma development or progression. For example, reduced expression of *miR-7* in five glioblastoma samples as compared to adjacent tissue was determined by real-time RT-PCR. This miRNA decreased cell viability and invasiveness, targeted Egfr expression directly, and suppressed Akt pathway activation independent of its Egfr inhibition [121].

First experimental data also suggest that glioma cells can alter miRNA levels in endothelial cells and thereby induce neoangiogenesis. The *miR-296* level was elevated in primary tumor endothelial cells isolated from human brain tumors as compared to normal brain endothelial cells. In primary human brain microvascular endothelial cells, the level of *miR-296* was increased by glioma cells or angiogenic growth factors. The miRNA *miR-296* contributed to angiogenesis by suppressing Hgs expression, which led to reduced Hgs-mediated degradation of the growth factor receptors Vegfr2 and Pdgfrβ. Moreover, inhibition of *miR-296* decreased angiogenesis in tumor xenografts *in vivo* [122].

There are also studies suggesting that specific miRNA signatures display "stemness" of glioma cells and that alterations of miRNA levels may induce differentiation. For example, expression levels of *miR-124* and *miR-137* were shown to be decreased in high-grade astrocytomas relative to non-neoplastic brain tissue. During differentiation of mouse neural stem cells following growth factor withdrawal their levels were increased. Furthermore, transfection of *miR-124* or *miR-137* induced morphological changes and marker expression consistent with neuronal differentiation in mouse oligodendroglioma-derived stem cells and $CD133^+$ human glioblastoma-derived stem cells. Over-expression of *miR-124* or *miR-137* also induced cell cycle arrest in glioblastoma cells and decreased expression of Cdk6 and phophorylated Rb [108]. A microarray analyses of $CD133^+$ and $CD133^-$ cells identified several differentially regulated miRNAs. For example *miR-451* was under-expressed in $CD133^+$ cells as compared to $CD133^-$ cells. Further functional characterization showed that *miR-451* may be activated by Smad proteins and inhibits cell growth as well as neurosphere formation of glioma cells [123].

Re-expression of tumor-suppressive miRNAs or inhibition of oncogenic miRNAs can

reduce tumor mouse xenograft growth *in vivo*. Microarray profiling of glioblastoma samples and adjacent human brain [124] confirmed decreased *miR-128* levels in tumor samples as reported by other studies [119, 125]. Over-expression of *miR-128* reduced glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo*. Moreover, the Bmi-1 oncogene was identified as a target of *miR-128* that is regulated through a single *miR-128* binding site in its 3'-UTR. The miRNA *miR-128* causes changes in glioma cells consistent with the down-regulation of Bmi-1, including a reduction in self-renewal capacity [124]. Another miRNA microarray analysis of 5 glioblastoma patients [126], early passage cultures from 4 patients, and 6 glioblastoma cell lines versus non-neoplastic brain tissues and astrocyte cultures validated the elevated *miR-21* levels in glioblastoma reported by Ciafre and collaborators [119]. Moreover, functional characterization revealed that *miR-21* increases apoptotic cell death [126]. The putatively oncogenic *miR-21* was further investigated as a candidate for molecularly targeted therapy. The combination of *miR-21* knockdown and neural precursor cells expressing a secretable variant of the cytotoxic agent Trail (Npc-s-Trail) promoted apoptosis in glioma cells and decreased tumor growth in mouse xenografts *in vivo*. These findings indicate a possible role for LNA-anti-miR-21 in sensitizing glioma cells for cytotoxic agents by overcoming resistance to apoptosis [127].

1.3 Goals and experimental approach of this study

The aim of this study was to identify miRNAs whose expression is specifically associated with the spontaneous malignant progression of astrocytic gliomas. A second goal was to functionally characterize such miRNA candidates. The project was started by comparing the expression profiles of 157 miRNAs in primary diffuse astrocytomas

and recurrent secondary glioblastomas from four individual patients. For further validation six miRNAs were selected and their expression changes during astrocytoma progression were determined: By means of targeted real-time stem-loop RT-PCR the four patients that were part of the screening experiments and three additional patients with low-grade primary astrocytomas and recurrent anaplastic astrocytomas were investigated. In addition, a series of 14 diffuse astrocytomas and 13 independent secondary glioblastomas was analyzed. In a second step, two promising miRNA candidates (*miR-184* and *miR-17*) with validated progression-associated expression were selected for further characterization. Duplex-PCR analyses were performed to determine wether genetic aberrations cause the differential miRNA expression upon progression or not. Furthermore, these two miRNAs were functionally characterized by inhibition of miRNA function or miRNA re-expresion. The influence on cell viability, proliferation, apoptosis, and invasion was observed. In addition, Affymetrix-chip based expression profiling and two-dimensional difference gel electrophoresis (2D-DIGE) based proteomic analyses were applied to identify sets of transcripts and proteins that are directly or indirectly regulated by *miR-184* and *miR-17*.

2 Materials and Methods

2.1 Patient samples and extraction of nucleic acids

All tumors were selected from the collection of frozen brain tumor tissue specimens at the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf, and investigated in accordance with protocols approved by the institutional review board (study number 2767). The tumors were classified according to the World Health Organization (WHO) classification of tumors of the central nervous systems [1].
Parts of each tumor were snap-frozen immediately after operation and then stored at -80 °C. Only specimens with a histologically estimated tumor cell content of 80 % or more were used for the molecular analyses. MicroRNA expression profiles were determined in primary low-grade and recurrent high-grade tumor pairs derived from four independent patients. These patients had been originally operated on WHO grade II gliomas (three diffuse astrocytomas and one astrocytoma-predominant oligoastrocytoma) and then developed spontaneous recurrences that were histologically classified as glioblastomas (WHO grade IV). None of the patients had been treated by radio- or chemotherapy in between both operations. For validation purposes, tumors were investigated from three additional patients with primary WHO grade II gliomas (two diffuse astrocytomas and one astrocytoma predominant oligoastrocytoma) and recurrent high-grade gliomas (two anaplastic astrocytomas and one astrocytoma-predominant anaplastic oligoastrocytoma, all corresponding to WHO grade III). All seven patients were included in previous studies addressing genetic alterations and differential mRNA expression associated with astrocytoma progression [128, 129]. An additional valida-

tion step performed in co-operation with M. Wolter (Department of Neuropathology, Heinrich-Heine-University, Düsseldorf) involved the analysis of 14 diffuse astrocytomas and 13 secondary glioblastomas from 27 independent patients.
DNA and RNA were extracted from unfixed frozen tumor samples by ultracentrifugation over caesium chloride as described elsewhere [128]. As non-neoplastic references, two commercially available RNA samples were obtained from adult human brain tissue (Clontech, Mountain View, CA; Stratagene, Cedar Creek, TX). For the second validation step these control samples were supplemented by an additional set of nine non-neoplastic brain RNAs obtained from different commercial sources (Ambion, Austin, TX; Biochain, Hayward, CA). Commercially available universal human RNA (Stratagene) was used to calibrate the real-time RT-PCR experiments.

2.2 MicroRNA sequences and miRNA target prediction

MicroRNA sequences and potential target genes were retrieved from the Sanger Institute mirBase registry and mirBase target database release 10.1 (free online access is availible via http://microrna.sanger.ac.uk/index.shtml).

2.3 Real-time RT-PCR analyses

Reverse transcription of mature miRNAs was carried out with stem-loop primers specific for each investigated miRNA (Figure 3 A). Real-time PCR was performed on an ABI PRISM® 5700 system using the Applied Biosystems Early Access miRNA kit with dye-labeled TaqMan® probes to monitor amplification according to the

manufacturer's protocol (Applied Biosystems, Foster City, CA; Figure 3 B). Fluorescent data were converted into cycle threshold measurements by the SDS software and exported to Microsoft Excel. Fold expression changes relative to universal human RNA were calculated with the $2^{-\Delta\Delta C_T}$ method [130]. As recommened by the manufacturer, the expression level of *let-7a* was used as a reference, which showed robust expression in the investigated samples, as well as in glioma samples of a previous study [108]. Sequences of all primers used for miRNA amplification are listed at http://www.appliedbiosystems.com. MicroRNAs were regarded as showing progression-associated increased expression, if a more than twofold higher expression level was detected in the secondary glioblastoma as compared to the respective primary low-grade glioma with a raised expression level in the secondary glioblastoma sample as compared to a non-neoplastic brain tissue. Progression-associated decreased expression was assumed, if the recurrent tumor demonstrated a more than twofold lower expression level as compared to the respective primary tumor with a lowered expressionn level in the secondary glioblastoma sample as compared to a non-neoplastic brain tissue. In the validation experiments performed for selected miRNAs (*miR-16*, *miR-17*, *miR-19a*, *miR-20a*, *miR-140*, *miR-184*) amplification of *let-7a* cDNA was measured by incorporation of SYBR® green fluorescent dye (Invitrogen, Carlsbad, CA) into the double-stranded DNA, while the individual target miRNAs were detected by TaqMan® technology (Applied Biosystems). The independent validation series of 14 diffuse astrocytomas, 13 secondary glioblastomas, and nine non-neoplastic brain tissue samples was investigated for expression of the six selected candidate miRNAs using TaqMan® based asssays.

For expression analyses of potential miRNA target transcripts, 1 μg of total RNA of each tumor was reverse-transcribed into cDNA using SuperScript® reverse tran-

scriptase (Invitrogen) and random primers. The mRNA expression levels were detected by real-time PCR on the StepOnePlus™ system (Applied Biosystems) using incorporation of SYBR® green fluorescent dye into the double-stranded PCR products. The expression level of each transcript was normalized to the expression level of *ARF1* transcripts (NCBI GenBank accession no. M36340, for primer sequences see Table 1).

2.4 Cluster analyses

Complete linkage hierarchical clustering was performed using the software Cluster. Results were visualized using the TreeView software (both Eisen Software, Berkeley, CA).

2.5 Duplex PCR analyses

The *miR-184* locus was analyzed for homozygous deletion and the *miR-17* locus for gene amplification by duplex-PCR assays (for primer sequences see Table 1). The PCR products were separated by electrophoresis in 3 % agarose gels and the ethidium bromide-stained bands were recorded with the Gel-Doc™ 1000 system (Bio-Rad, Hercules, CA). Quantitative analysis of the signals obtained for each miRNA locus and a reference gene (*APRT*, *WI3306*) was performed with the Molecular-Analyst software (Bio-Rad). Increases in the target gene/reference gene ratio of more than threefold of the ratio obtained for constitutional DNA were considered as evidence for gene amplification. Reduction in the target gene/reference gene ratio below 0.3-fold was considered as evidence for homozygous deletion.

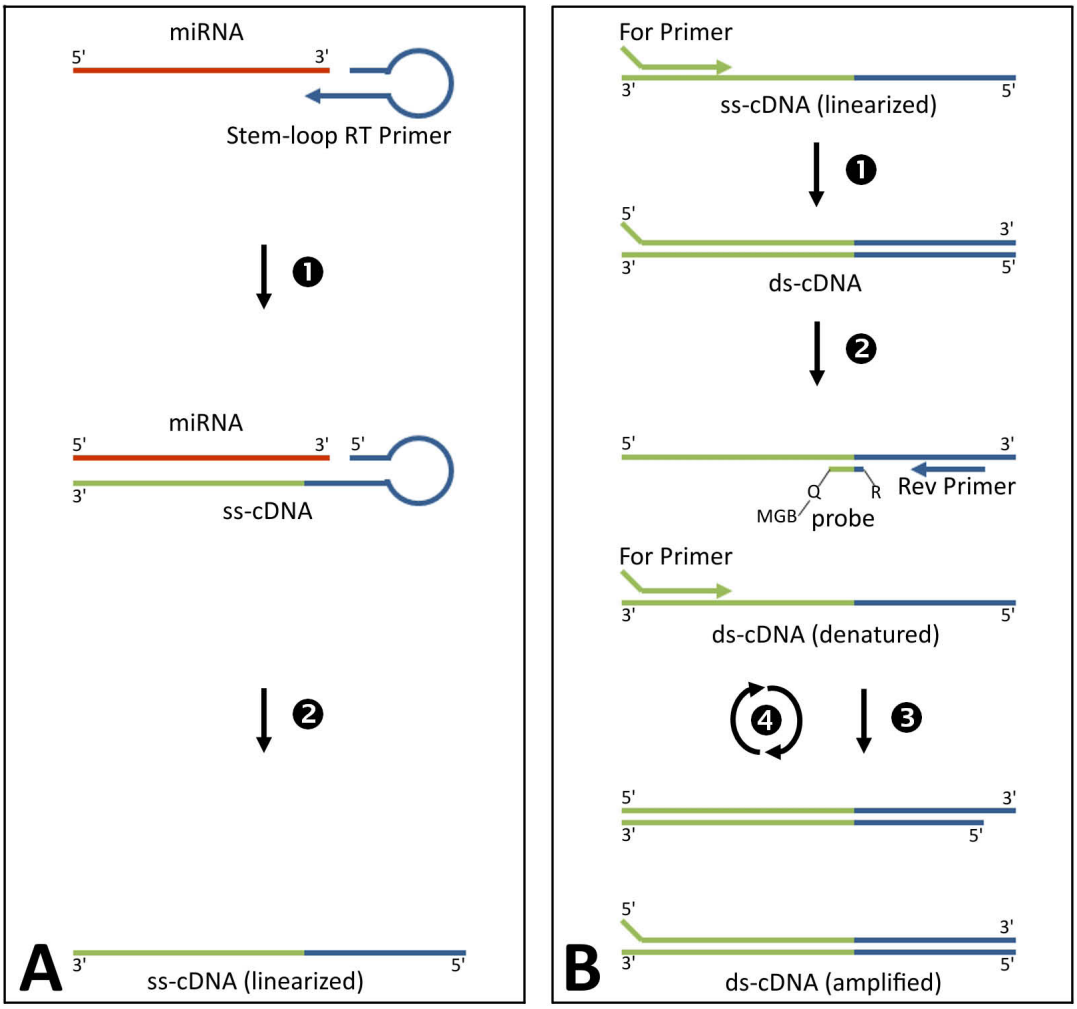


Figure 3: Principle of stem-loop RT-PCR. A) To prime the reverse transcription of mature miRNAs, stem-loop RT primers specific for each investigated miRNA were annealed to the 3' end of the mature miRNA (1). Then the first strand of cDNA was synthesized by reverse transcriptase. **B)** Annealing of the forward primer to the single-stranded cDNA initiated the polymerisation of the second strand of cDNA (1). Thereafter, the double-stranded cDNA was denatured, which was followed by annealing of forward and reverse primers. A TaqMan probe containing reporter (R), quencher (Q), and minor groove binder (MGB) bound specifically to a complementary sequence between the forward and reverse primer sites. During amplification the probe was fragmented by the 5'-exonuclease activity of DNA-polymerase. Thereby the fluorescent reporter dye was separated from the quencher molecule that suppressed reporter fluorescence by Förster-type energy transfer as long as located in close proximity to the reporter molecule. Therefore the fluorescence signal increased in a stoichiometrical way during each reaction cycle allowing to monitor amplification quantitatively (3). After duplication of the amount of cDNA the next amplification cycle started with denaturing of cDNA (4).

Table 1: Primer sequences used for duplex-PCR analysis of miRNA loci and expression analyses of putative miRNA targets.

	Primer sequences		amplicon
ID	forward	reverse	size (bp)
miR-17	5'-tcaaagtgcttacagtgcaggt-3'	5'-aaaagcactcaacatcagcag-3'	134
miR-184	5'-tcctgcaaagcttcatcaaa-3'	5'-cggaggctgtgagtgtcaat-3'	132
APRT	5'-cctggtgaagatcacggaat-3'	5'-gcagtgttgtggctccag-3'	185
WI-3306	5'-gtaatgcaggtttggccatt-3'	5'-tgctctgttctcaggcagac-3'	213
AKR1C3	5'-gatcctcaacaagccaggac-3'	5'-tctcgttgagatcccagagc-3'	147
AKT2	5'-aacacaaggaaagggaacca-3'	5'-acctagctcgggacagctc-3'	146
CDC25A	5'-ctctggacagctcctctcgt-3'	5'-gctggagctacacagggaag-3'	87
CENTG1	5'-gcagctgagttcccttcg-3'	5'-tagctgcagcgtttcatgtc-3'	110
CTBP1	5'-catcatcgtccggattgg-3'	5'-ccggtacaggttcaggatgt-3'	145
MAZ	5'-actgtggcaagagcttctcc-3'	5'-cctcgtgtcgtactgtgtgc-3'	147
NRN1	5'-acgaacatcaagaccgtgtg-3'	5'-tcgaataagctgccttggat-3'	149
S100A16	5'-ctggagaggaggcagactga-3'	5'-ccttctccagctccgtgtag-3'	85
SH3GL1	5'-ggaccaagctggatgatgac-3'	5'-tggacaccgtgttgagcat-3'	150

2.6 Transfection of cultured glioma cells

The glioma cell lines A172 and T98G were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown as monolayer cultures in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (HIFBS) and penicillin/streptomycin (Invitrogen/GIBCO, Carlsbad, CA) in 5 % CO_2 humidified incubator at 37 °C.

The miRNA *miR-184* was over-expressed in glioma cells by transient transfection of 25 nM *miR-184* Pre-miR™ miRNA precursor (pre-184; Ambion) with siPORT™ NeoFx™ transfection reagent (Ambion) according to the manufacturer's protocol (2 µl siPORT™ NeoFX™ per 1 ml final volume). Function of *miR-17* was silenced by transfection of 50 nM Anti-miR™ miRNA inhibitors (anti-17; Ambion). To normalize for side-effects not caused by specific miRNA overexpression or inhibition, cells were transfected with commercially available negative control oligonucleotides (Pre-miR™ negative control, Anti-miR™ negative control; Ambion). 24 h before transfection, cells were detached by trypsin, diluted to a concentration of 10,000 cells/ml in DMEM supplemented with 10 % HIFBS, and then dispensed into cell culture plates. Oligonucleotide solution and transfection reagent were diluted in 50 µl OptiMEM® each (Invitrogen/GIBCO, Carlsbad, CA). Then both dilutions were mixed and incubated for 20 minutes at room temperature. Cell culture medium was replaced by 900 µl DMEM supplemented with 10 % HIFBS and the mixture of oligonucleotide and transfection reagent was added.

2.7 *In vitro* assays for functional analyses

To determine the influence of miRNA modulation on cell viability, a standard colorimetric assay was used (Sigma, Seelze, Germany). 72 h after transfection 10 μl 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well of a 96-well cell culture plate. Metabolically active cells are able to reduce the tetrazolium salt MTT to colored formazan. After incubation at 37 °C for 4 h, the reaction was stopped by solubilization with 150 μl dimethylsulfoxide. Absorbance at a wave length of 570 nm was measured using an enzyme linked immunosorbent assay (ELISA) plate reader (Tecan, Crailsheim, Germany) to determine the amount of formazan that was produced. Absorbance at a wavelength of 630 nm was subtracted to normalize for different amounts of cell debris.

The proliferation rate of cells transfected with pre-184 or anti-17 was compared to cells transfected with respective controls using a commercially available BrdU incorporation assay (Roche, Mannheim, Germany) according to the manufacturer's protocol. BrdU labeling solution was added 48 h after transfection and cells were fixed after 24 h of incubation at 37 °C.

Apoptotic activity in transfected and control cells was determined with a fluorometric caspase-3/7 assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Caspase substrate was added 72 h after transfection and fluorometric measurements were performed after an incubation time of 2 h at room temperature.

Invasive growth properties of transfected and control cells were quantified with a transwell assay (Figure 4; BD Biosciences, San Jose, CA). BD BioCoat^{TM} Matrigel^{TM} invasion chambers 8.0 μm and BD BioCoat^{TM} 8.0 μm control inserts were rehydrated in a 24-well companion plate with serum-free DMEM at 37 °C for 2 h. Cells were

mechanically detached from 10 cm-dishes 48 h after transfection and suspended in serum-free DMEM at a concentration of 70,000 cells/ml. 750 μl of medium containing 10 % fetal bovine serum as chemo-attractant were pipetted into each well of an additional 24-well companion plate. Inserts were transfered to this plate and 500 μl of cell suspension were dispensed in each well followed by incubation at 37 °C for 24 h. Afterwards, cells that had not migrated through the membrane (and the Matrigel^{TM} coating) were removed with cotton swabs. The inserts were put into methanol for 1 min to fix the migrated cells at the other side of the membrane. Membranes were stained with hematoxylin and eosin, sliced out of the inserts and fixed on a glass slide. A representative sector was counted under the microscope at 100-fold magnification. To normalize for different proliferation and unspecific migration through the pores of the membrane the number of cells that migrated through each Matrigel^{TM}-coated membrane was divided by the average number of cells that migrated through control membranes without Matrigel^{TM} coating.

Each experimental group in the individual functional tests consisted of six replicates. Two-sided student's t-tests were applied to compare results between transfected and control cells. p-values of less than 0.05 were considered to indicate significant differences. The results of each *in vitro* experiment were converted into arbitrary units (a.u.) for better comparability of different experiments setting the average measurement of the control cells at 100 a.u..

2.8 Microarray expression profiling

A172 and T98G glioma cells were washed twice with phosphate-buffered saline (PBS) 72 h after transfection and then lysed with Trizol® reagent (Invitrogen) to extract

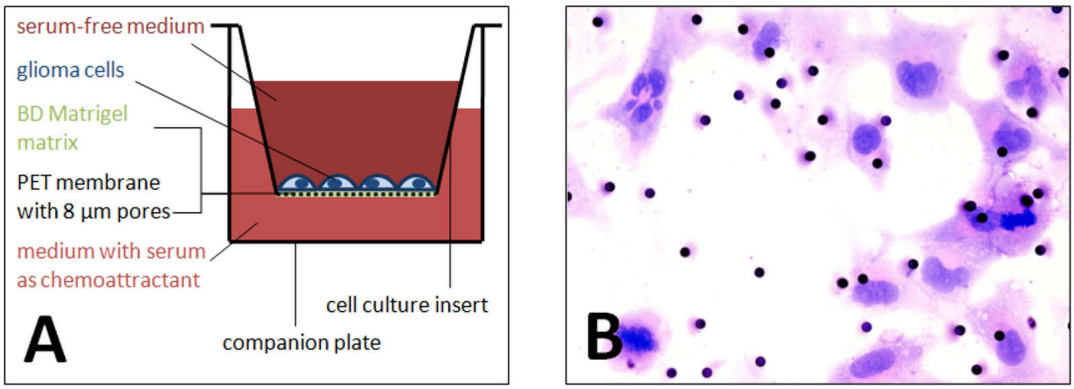


Figure 4: In vitro invasion assay. A) Glioma cells were suspended in serum free medium and pipetted into BD BioCoat^{TM} Matrigel^{TM} invasion chambers. On the other side of the coated porous membrane medium containing fetal bovine serum served as chemo-attractant for glioma cells and initiated their migration **B)** After an incubation time of 24 h all cells at the inside of the invasion chambers, that had not invaded the artificial extracellular matrix and migrated through the pores, were removed. The migrated cells were hematoxylin and eosin stained. The microscopic photography shows the 8.0 μm pores (dark circles) and stained glioma cells.

the RNA according to the manufacturer's protocol. Expression profiles of specifically transfected versus control-transfected cells were determined by hybridization to Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) in co-operation with G. Röder (Department of Oncological Chemistry, Heinrich-Heine-University, Düsseldorf). A global scaling strategy was used that sets the average signal intensity of the array to a default target signal of 500 and a comparative analysis of the specifically transfected samples versus the control samples was performed. Changes with p-values < 0.05 were considered as statistically significant. Genes with a signal log ratio (transfected/negative control) > 1 or $<$ -1 were regarded as being differentially regulated.

2.9 Proteomic analyses using 2D-DIGE and mass spectrometry (MS)

A172 and T98G glioma cells were put on ice 72 h after transfection, washed with cold PBS twice and then suspended mechanically in 1 ml PBS, followed by centrifugation at 1,000 x g for 5 min. After removal of the supernatant, the cell pellet was snap-frozen in liquid nitrogen and stored at -80 °C. For cell lysis a 100 μg pellet was mixed with 148 μl of lysis buffer (TrisHCl 30 mmol/L, thiourea 2 mol/L, urea 7 mol/l, CHAPS 4 %, pH 8.5). Cell lysis was completed by subsequent sonification (6 x 10 s pulses on ice). Centrifugation at 12,000 x g for 15 min was performed to remove cell debris. The protein samples with miRNA overexpression or inhibition of miRNA function were labeled with Cy3 minimal CyDye^{TM} (GE Health Care, Munich, Germany) and the control-transfected samples were labeled with Cy5 minimal CyDye^{TM} (GE Health Care) according to the manufacturer's instructions. An internal standard composed of equal amounts of all samples of one cell line was then labeled with Cy2 minimal CyDye^{TM} (GE Health Care). Two-dimensional gel electrophoresis was performed using carrier ampholyte-based isoelectric focusing (IEF) as described elsewhere [131]. After gel scanning the protein patterns were differentially analysed using Decyder (GE Healthcare) as described elsewhere [132]. For protein identification by matrix-assisted laser desorption/ionization (MALDI)-MS silver post-staining was performed using a MS-compatible protocol [133]. In-gel digestion of proteins was performed with trypsin and obtained peptides were subjected to MALDI-MS analysis and identified using the Mascot algorithm for searching the International Protein Index (IPI) protein database (http://www.ebi.ac.uk). 2D-DIGE experiments were carried out in co-operation with M. Grzendowski (Medical Proteome Center (MPC), Ruhr-University, Bochum).

2.10 SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

For protein extraction from cell cultures, cells were put on ice and washed with cold PBS twice. Then 400 μl lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5 % TritonX-100, 0.5 % deoxycholate) was added to each 10 cm-dish. Cell lysates were centrifuged to remove cell debris. Supernatants were separated on 10 % tris-glycin SDS polyacrylamide gels. For Western blot analysis of tumor tissues, the protein fraction obtained by ultracentrifugation over caesium chloride was used. Excess of guanidine hydrochloride salt load was removed by replacement of solvent with sample buffer using ultrafiltration applying Microcons® (Millipore, Schwalbach, Germany, 3 kDa Cut-off). The proteins were then denatured and separated by gel electrophoresis on precasted NuPAGE® Novex® 4-12 % Bis-Tris Midi gels (Invitrogen, Karlsruhe, Germany). Separated proteins were transferred to Hybond-P PVDF membranes (Amersham Biosciences, Piscataway, NJ) using a Trans Blot Cell Blot module (Bio-Rad). The membranes were blocked with 5 % bovine serum albumine (BSA) in PBS-T (0,05 % Tween 20 in phosphate-buffered saline) and probed with antibodies against Akt2 (1:100, Cell Signaling, Danvers, MA: #2962) and α-tubulin antibody (Sigma, Taufkirchen, Germany: T9026) or β-actin (1:10000, Sigma-Aldrich, St. Louis, MO: A5541). Primary antibody binding was detected by anti-mouse or anti-rabbit antibodies linked to horseradish peroxidase followed by incubation with Immobilon Western HRP Substrate luminol reagent and peroxidase solution (Millipore, Billerica, MA). Chemiluminescence was recorded using the LAS-300 mini system (Fujifilm Life Science, Stanford, CT). Akt2 protein levels in glioma tissues were determined in co-operation with M. Grzendowski (MPC, Ruhr-University, Bochum).

3 Results

3.1 Identification of miRNAs that are differentially expressed between diffuse astrocytoma and secondary glioblastoma

The expression levels of 157 miRNAs were determined in four primary gliomas of WHO grade II and their corresponding recurrent secondary glioblastomas of WHO grade IV by stem-loop real-time RT-PCR. Two miRNAs (*miR-184* and *miR-328*) exhibited a progression-associated down-regulation, i.e., a more than twofold lower expression in the secondary glioblastoma as compared to the corresponding primary diffuse astrocytoma, in the majority of patients, i.e., two of three or three of four investigated patients respectively (Table 2). Twelve miRNAs (*miR-9*, *miR-15a*, *miR-16*, *miR-17*, *miR-19a*, *miR-20a*, *miR-21*, *miR-25*, *miR-28*, *miR-130b*, *miR-140*, *miR-210*) showed a progression-associated up-regulation in the majority of investigated patients (Table 2). Unsupervised hierarchical clustering was performed on the basis of the expression levels of these fourteen miRNAs. Clustering analysis clearly differentiated between primary and recurrent gliomas (Figure 5).

3.2 Validation of differentially expressed miRNAs

3.2.1 Additional individual patients with astrocytoma progression

From the set of 14 miRNAs demonstrating progression-associated differential expression *miR-16*, *miR-17*, *miR-19a*, *miR-20a*, *miR-140*, and *miR-184* were selected for validation experiments. MicroRNAs that had been investigated in gliomas before were

Table 2: MicroRNAs with progression-associated differential expression in four patients with primary glioma that recurred as secondary glioblastoma. Expression levels of 157 miRNAs were determined. MicroRNAs were regarded as showing progression-associated up-regulation, if they showed a fold change (recurrent/primary tumor) > 2 with a raised expression level in the secondary glioblastoma sample as compared to a non-neoplastic brain tissue. Progression-associated down-regulation was defined in an analogous manner as a fold-change (recurrent/primary tumor) < 0.5 with a lowered expression level in the secondary glioblastoma sample. Only miRNAs with progression-associated expression in the majority of patients are listed. Patient numbers 1-4 encode the individual patient; case-numbers encode primary (AII) and recurrent glioma (sGBIV).

		Fold change(AII/sGBIV)			
	Patient No.	1	2	3	4
	Case No. (AII)	A72D	A201D	A128D	OA23D
	Case No. (sGBIV)	GB239D	GB240D	GB119D	GB175D
Progression-associated up-regulation	*miR-9*	3.16	5.03	3.75	3.19
	miR-15a	2.50	4.50	1.36	7.81
	miR-16	3.72	4.82	2.69	3.24
	miR-17	3.89	12.64	7.52	*n.d.*
	miR-19a	1.46	2.85	3.88	16.28
	miR-20a	1.27	7.06	5.21	5.43
	miR-21	0.75	5.98	3.64	*n.d.*
	miR-25	0.72	6.13	4.44	6.66
	miR-28	0.75	4.47	7.46	4.58
	miR-130b	2.94	5.39	7.04	11.88
	miR-140	3.56	1.54	5.72	4.21
	miR-210	*n.d.*	1.49	17.45	6.89
Progression-associated down-regulation	*miR-184*	0.28	0.17	0.56	0.02
	miR-328	0.13	8.06	0.41	0.30

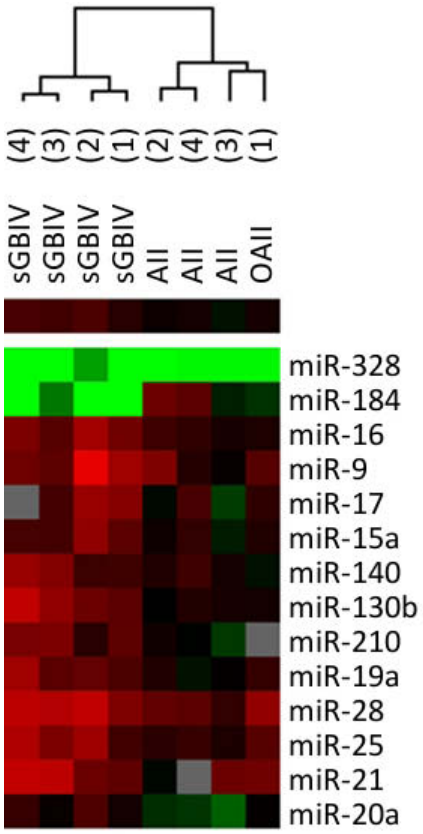


Figure 5: MicroRNAs with progression-associated expression in patients with low-grade primary glioma that recurred as secondary glioblastoma. The 14 miRNAs with progression-associated expression in the majority of patients were selected for the classification of gliomas by cluster analysis. Hierarchical clustering was performed and results were visualized using Cluster and TreeView (both Eisen Software). Clustering analysis clearly differentiates between primary and recurrent gliomas. [AII: diffuse astrocytoma, OAII: low-grade oligoastrocytoma sGBIV: secondary glioblastoma; numbers encode the individual patient; green: low expression level and red: high expression level related to a non-neoplastic human brain tissue, grey: not determined]

not further analyzed. In addition, the number of candidate miRNAs for validation experiments was restricted taking into account the fraction of investigated patients demonstrating progression-associated expression, reported differential expression in other tumor entities, genomic organziation and predicted targets of each miRNA.

By targeted real-time stem-loop RT-PCR expression levels were determined in the in the four original patients' tumor samples and in tumor samples from three additional patients whose gliomas progressed from WHO grade II to WHO grade III on recurrence. These analyses confirmed progression-associated expression of all selected miRNAs in the majority of the original patients. Furthermore, *miR-17*, *miR-20a*, and *miR-184* exhibited progression-associated differential expression in at least two of the three additional patients (Table 3).

Table 3: Results of validation experiments performed for six selected miRNAs in the original four patients and three additional patients with primary low-grade and recurrent anaplastic astrocytoma. Expression of each miRNA was determined by real-time RT-PCR using a modified experimental setup as compared to the screening experiments (see chapter "Materials and Methods").

	Original patients (n=4) recurrent/primary tumor		Additional patients (n=3) recurrent/primary tumor
miRNA	screening	validation	
miR-16	4/4 up	3/4 up	1/3 up
miR-17	3/3 up	3/4 up	3/3 up
miR-19a	3/4 up	2/3 up	0/2 up
miR-20a	3/4 up	3/3 up	2/3 up
miR-140	3/4 up	3/4 up	1/3 up
miR-184	3/4 down	3/4 down	3/3 down

3.2.2 Independent tumor samples of different WHO grades

A second validation step based on the analysis of an independent series of fourteen diffuse astrocytomas and thirteen secondary glioblastomas (Figure 6) demonstrated a significantly higher expression of *miR-17* in secondary glioblastomas as compared to diffuse astrocytomas. Median expression levels of *miR-16*, *miR-19a*, *miR-20a* and *miR-140* also were increased in secondary glioblastomas as compared to low-grade gliomas, however, for *miR-16* and *miR-19a* the differences were not statistically significant (Figure 6). Expression levels of *miR-16*, *miR-19a*, *miR-20a* and *miR-140* were significantly lower in the tumors as compared to the non-neoplastic brain tissues. The median expression level of *miR-184* was significantly decreased in secondary glioblastomas relative to diffuse astrocytomas and non-neoplastic brain tissue, respectively (Figure 6). Based on these results *miR-184* and *miR-17* were selected as candidates for further analyses.

3.3 Copy number analyses of the *miR-184* and *miR-17* loci

Duplex-PCR assays were performed to investigate aberrations in the gene copy number at the *miR-184* and the *miR-17* loci. However, neither homozygous deletion of the *miR-184* locus (Figure 7) nor amplification of the *miR-17* locus (Figure 8) was detected in any of the seven primary or recurrent glioma samples from the individual patients with glioma progression.

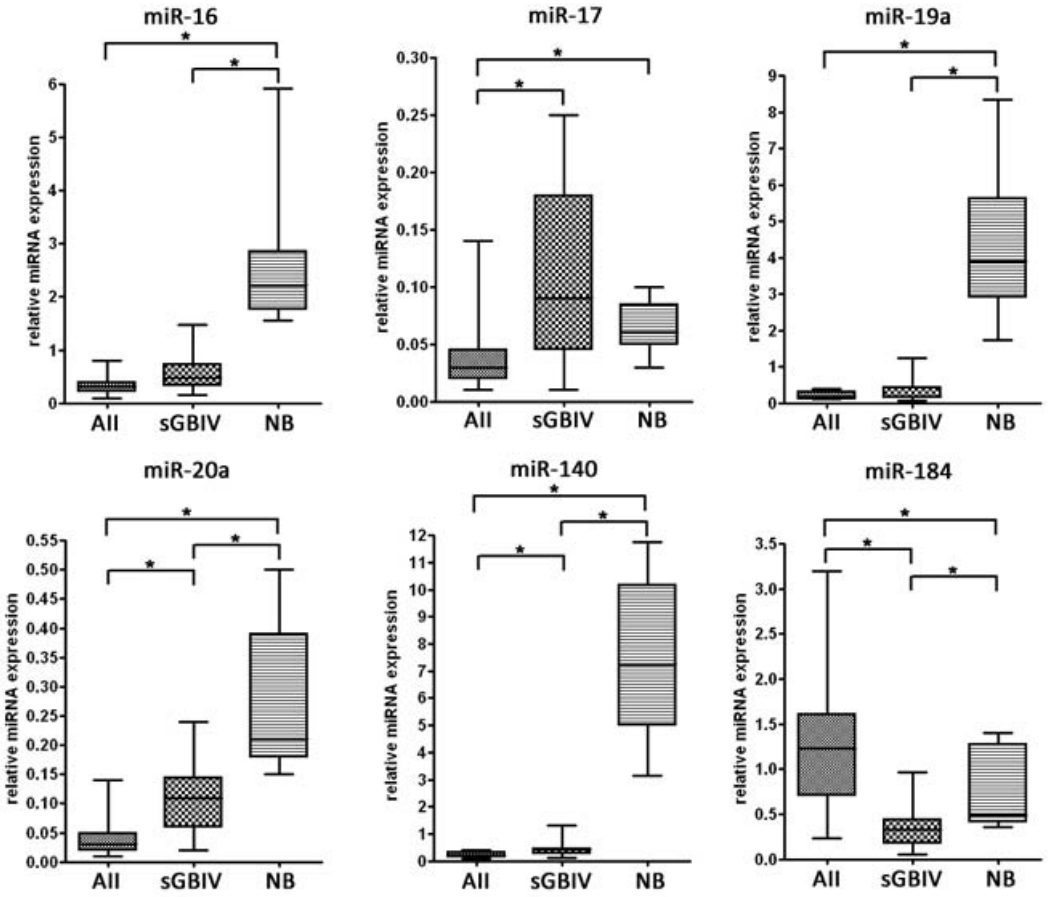


Figure 6: Results of validation experiments performed for the six selected miRNA candidates in an independent series of 14 diffuse astrocytomas (AII), 13 secondary glioblastomas (sGBIV), and nine non-neoplastic brain tissue samples (NB). Expression levels of the individual miRNAs were determined by using TaqMan®-based PCR assays. Box plots are depicted indicating median, lower and upper quartile, as well as sample maximum and sample minimum of normalized expression values. Asterisks indicate significant expression differences ($p < 0.05$)

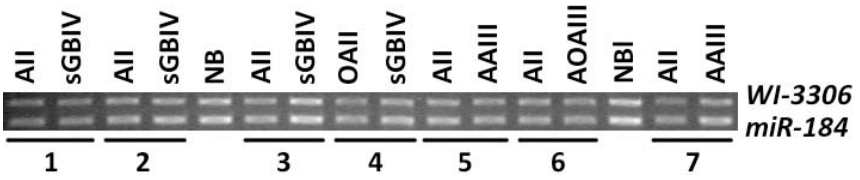


Figure 7: Duplex-PCR analysis of *miR-184* locus. Regions of *miR-184* locus (15q25.1) and a reference gene locus (*WI3306*; 2q21) were amplified by PCR. The PCR products were separated by electrophoresis in 3 % agarose gels and the ethidium bromide-stained bands were recorded. Note that the DNA-bands of *miR-184* have nearly the same intensity as the corresponding *WI3306* bands indicating similar gene dosage. [AII: diffuse astrocytoma, OAII: low-grade oligoastrocytoma, AAIII: anaplastic astrocytoma, AOAIII: anaplastic oligoastrocytoma, sGBIV: secondary glioblastoma, NB: non-neoplastic brain tissue, NBl: normal blood]

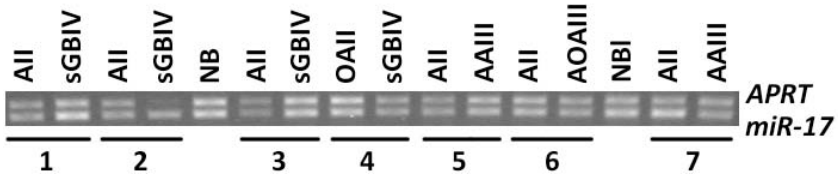


Figure 8: Duplex-PCR analysis of *miR-17* locus. Regions of *miR-17* locus (13q31.3) and a reference gene locus (*APRT*; 16q24) were amplified by PCR. The PCR products were separated by electrophoresis in 3 % agarose gels and the ethidium bromide-stained bands were recorded. Note, that - except for one sample - the DNA-bands of *miR-17* have nearly the same intensity as the corresponding *APRT* bands indicating similar gene dosage. The secondary glioblastoma sample of patient No. 2 showed a more intense *miR-17* band as compared to the reference band. Validation experiments using a *WI3306* as reference did not substantiate amplification of *miR-17* locus in this sample (data not shown). [AII: diffuse astrocytoma, OAII: low-grade oligoastrocytoma, AAIII: anaplastic astrocytoma, AOAIII: anaplastic oligoastrocytoma, sGBIV: secondary glioblastoma, NB: non-neoplastic brain tissue, NBl: normal blood]

3.4 Functional effects of *miR-184* overexpression and *miR-17* inhibition in human glioma cells.

To investigate the biological effects of progression-associated miRNA expression, *miR-184* was over-expressed in A172 and T98G glioma cells by transfection of *miR-184* precursors. In addition, *miR-17* was inhibited in these glioma cell lines by transfection of complementary miRNA inhibitors. The glioma cell lines were efficiently transfectable with nearly 100 % of cells showing an uptake of fluorescent-labeled oligonucleotides (Figure 9 A, 10 A). The effect of transfection on the respective miRNA expression levels was determined by real-time stem-loop RT-PCR. In cells transfected with miRNA precursors *miR-184* expression level was markedly increased as compared to control-transfected cells (Figure 9 B). The expression level of *miR-17* was effectively decreased in glioma cells transfected with the respective miRNA inhibitors as compared to glioma cells transfected with control oligonucleotides (Figure 10 B).
Overexpression of *miR-184* in A172 and T98G glioma cells significantly ($p < 0{,}05$) decreased cell viability and proliferation (Figure 9 C, 9 E). In T98G cells, *miR-184* additionally reduced invasiveness in Matrigel^{TM} assays (Figure 9 D). Overexpression of *miR-184* increased apoptotic activity in A172 cells, but reduced apoptotic activity in T98G cells (Figure 9 F).
Inhibition of *miR-17* significantly reduced cell viability of A172 and T98G cells and increased apoptotic activity in T98G cells (Figure 10 C, 10 F). Invasiveness or proliferation was not significantly changed in cells with inhibited *miR-17* function (Figure 10 D, 10 E).

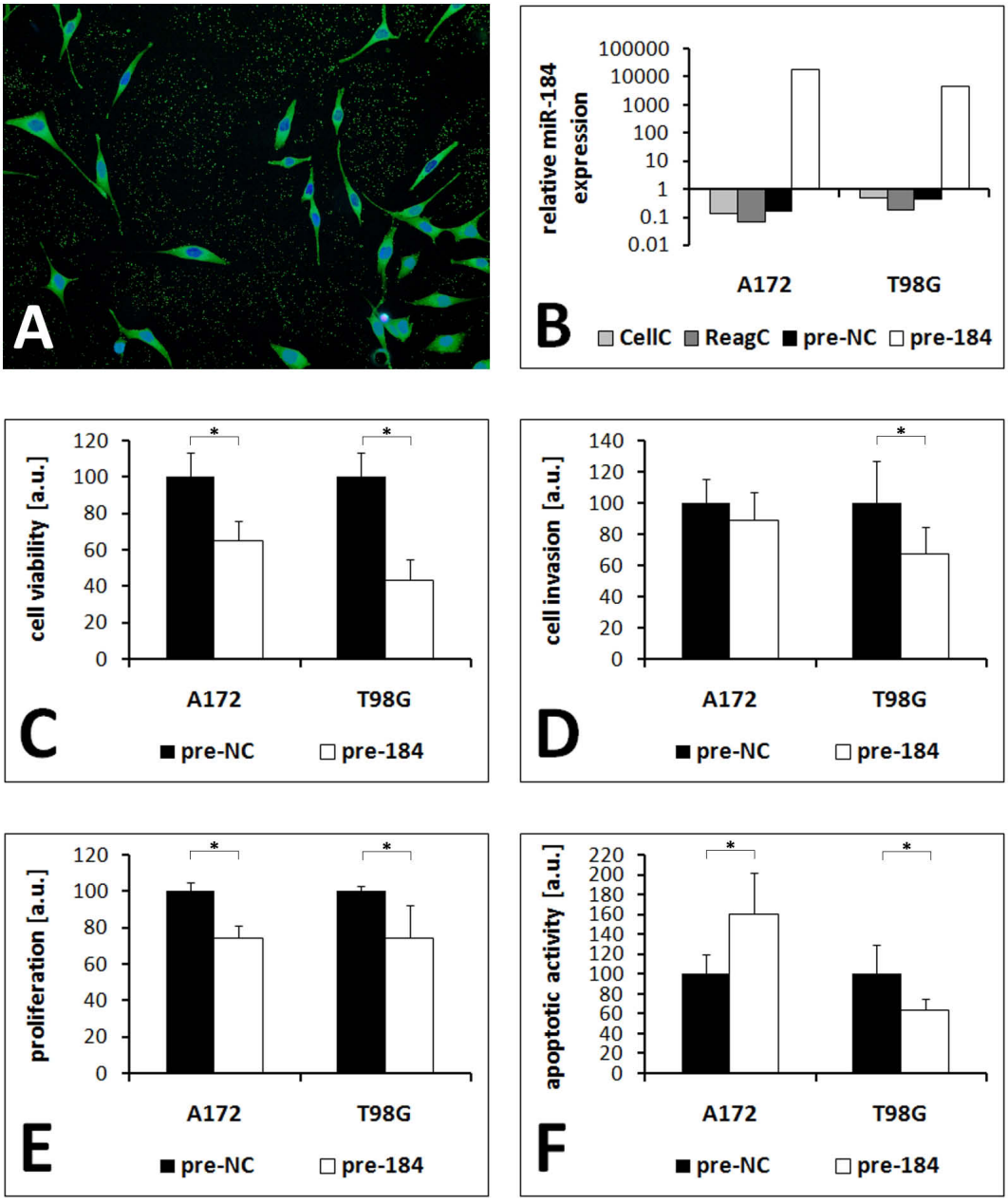


Figure 9: *In vitro* effects of *miR-184* overexpression in glioma cells. A) As a control for transfection efficacy, A172 cells were transfected with a fluorescent-labeled oligonucleotide and counterstained with DAPI. Note that nearly all cells emit green fluorescence indicating efficient transfection.

Figure 9: *In vitro* effects of *miR-184* overexpression in glioma cells (continued). **B)** Overexpression of *miR-184* in A172 and T98G cells after transfection of *miR-184* precursors. Expression levels relative to non-neoplastic human brain were determined by real-time stem-loop RT-PCR and normalized to *let-7a*. **C)** Cell viability was determined using an MTT-assay. **D)** Cell invasion was quantified using a transwell-assay with Matrigel^{TM} coated membranes. **E)** Proliferation was measured using a BrdU-assay. **F)** For the comparison of apoptotic activity a fluorometric caspase-3/-7 assay was used. [The results of *in-vitro* assays were converted into arbitrary units (a.u.) setting the average result in the corresponding control cells as 100 a.u.. Error bars show standard deviations, asterisks indicate significant differences (p-value < 0.05). CellC: cell control, ReagC: cells treated with transfection reagent, pre-NC: cells transfected with negative control, pre-184: cells transfected with *miR-184* precursors.]

3.5 Protein and mRNA expression profiling of glioma cells after *miR-184* overexpression or *miR-17* inhibition

Protein and mRNA expression profiles of glioma cells were determined after *miR-184* overexpression and *miR-17* inhibition in order to identify transcripts and proteins that are regulated - either directly or indirectly - following specific miRNA inhibition or overexpression of these miRNAs.

Proteomic analysis revealed 42 distinct protein spots that were regulated in A172 cells, and 74 distinct protein spots that were regulated in T98G cells following overexpression of *miR-184* ($p < 0.05$). MALDI-time-of-flight(TOF)/TOF MS identified 11 and 14 non-redundant proteins down-regulated in A172 and T98G cells, respectively (Supplementary Table 1 and Supplementary Figures 1 and 2). In T98G cells the predicted *miR-184* target nucleophosmin 1 (Npm1, OMIM 16040) was down-regulated (average fold-change = -1.6) after overexpression of *miR-184* (Figure 13 A). The proteins Hnrpk and Hnrpm were up-regulated in both cell lines.

Affymetrix chip profiling revealed 1380 differentially regulated mRNAs in A172 cells, and 784 differentially regulated mRNAs in T98G cells transfected with *miR-184* pre-

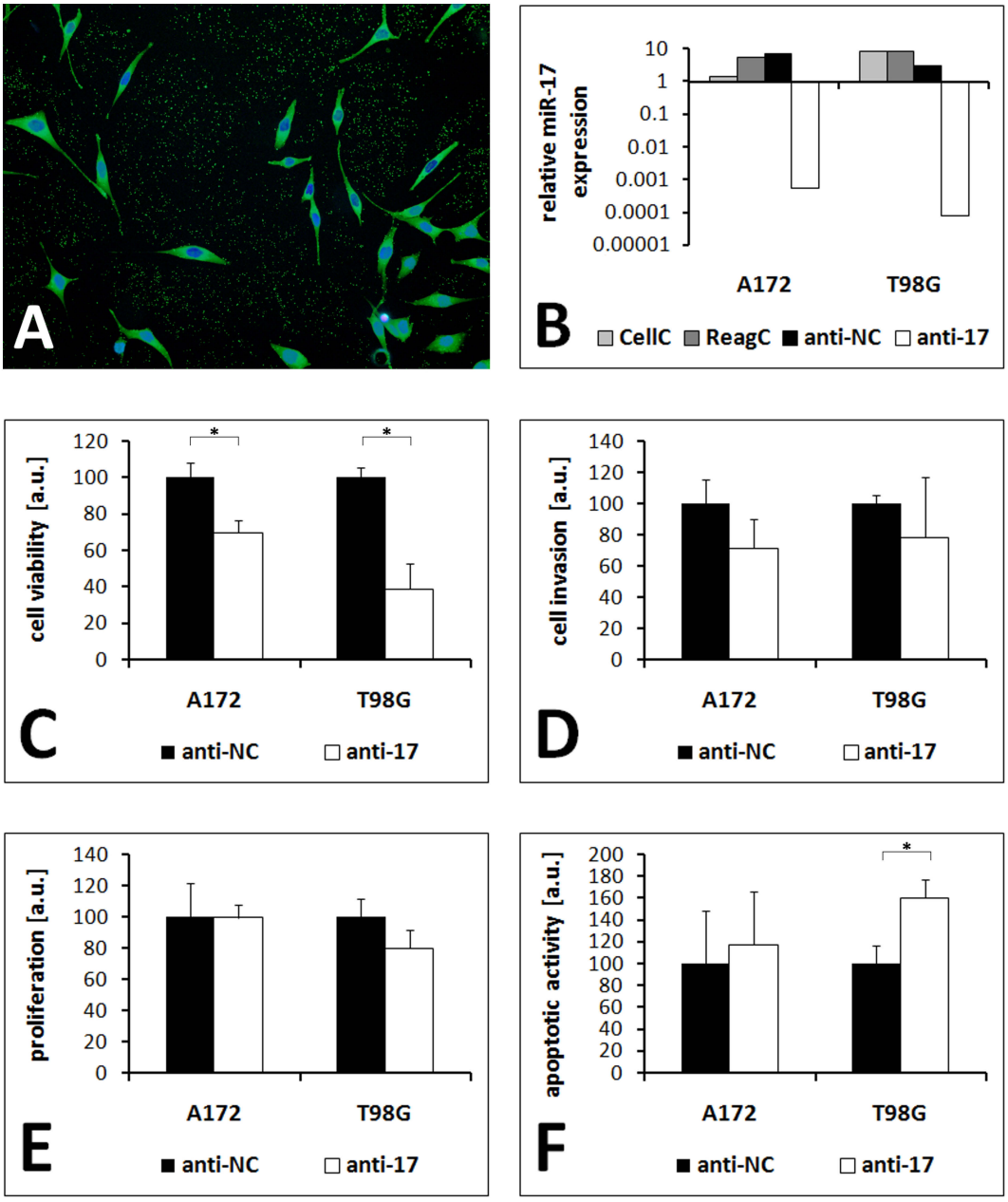


Figure 10: *In vitro* effects of *miR-17* inhibition in glioma cells. A) A) As a control for transfection efficacy, A172 cells were transfected with a fluorescent-labeled oligonucleotide and counterstained with DAPI. Note that nearly all cells emit green fluorescence indicating efficient transfection.

Figure 10: *In vitro* effects of *miR-17* inhibition in glioma cells (continued). B) Inhibition of *miR-17* in A172 and T98G cells after transfection of *miR-17* inhibitors. Expression levels relative to non-neoplastic human brain were determined by real-time stem-loop RT-PCR and normalized to *let-7a*. **C)** Cell viability was determined using an MTT-assay. **D)** Cell invasion was quantified using a transwell-assay with Matrigel^{TM} coated membranes. **E)** Proliferation was measured using a BrdU-assay. **F)** For the comparison of apoptotic activity a fluorometric caspase-3/-7 assay was used. [The results of *in-vitro* assays were converted into arbitrary units (a.u.) setting the average result in the corresponding control cells as 100 a.u.. Error bars show standard deviations, asterisks indicate significant differences (p-value < 0.05). CellC: cell control, ReagC: cells treated with transfection reagent, anti-NC: cells transfected with negative control, anti-17: cells transfected with *miR-17* inhibitors.]

cursors as compared to control cells (signal-log-ratio > 1 or $<$ -1, $p < 0.05$). A total of 17 non-redundant transcripts corresponding to predicted targets of *miR-184* showed decreased expression in both analyzed cell lines (Table 4).

Real-time RT-PCR analysis confirmed the differential mRNA expression of *AKR1C3*, *AKT2*, *CDC25A*, *CTBP1*, *S100A16*, *MAZ*, *NRN1* and *SH3GL1* when comparing *miR-184* precursor-transfected versus control-transfected cells.

Among these candidates, Akt2 was selected for additional Western Blot analysis, which confirmed decreased expression of Akt2 protein in A172 and T98G cells after transfection of *miR-184* precursors (Figure 11). In addition, Western blot analysis of an independent series of five diffuse astrocytomas and five secondary glioblastomas revealed increased Akt2 protein expression in the secondary glioblastomas (Figure 12).

Proteomic analysis revealed that inhibition of *miR-17* resulted in differential expression of 39 protein spots in A172 cells, and 83 protein spots in T98G cells ($p < 0.05$). MALDI-TOF/TOF MS identified 16 non-redundant proteins up-regulated in A172 cells and 33 non-redundant proteins in T98G cells (Supplementary Table 2 and Supplementary Figures 3 and 4). In T98G cells the predicted *miR-17* target Pold2 showed elevated expression (average fold-change = 1.3) after transfection of *miR-17* inhibitors

(Figure 13 B). Three non-redundant proteins (Hspa1a/b, Eno1, Cct5) without predicted *miR-17* binding sites were up-regulated in both cell lines.

Expression profiling at the transcript level identified 475 regulated mRNAs in A172 cells, and 125 regulated mRNAs in T98G cells transfected with the anti-17 miRNA inhibitors as compared to control-transfected cells (signal-log-ratio > 1 or < -1, change-p-value < 0.05). However, none of these transcripts corresponded to a predicted target mRNA of *miR-17* and there was no overlap of regulated transcripts between the two cell lines.

Table 4: Potential target genes of *miR-184* identified by expression profiling using Affymetrix GeneChip® Human Genome U133 plus 2.0 arrays. The mRNA expression profiles of glioma cells with *miR-184* overexpression (pre-184) and glioma cells transfected with negative controls (pre-NC) were compared. The list contains targets of *miR-184* as predicted by mirBase target database with signal log ratios (pre-184/pre-NC) < -1 and change p-values < 0.05 in both investigated glioma cell lines (A172 and T98G).

		signal log ratio (pre-184/pre-NC)	
Gene Symbol	Affy-No.	A172	T98G
CDC25A	1555772_a_at	−1.5	−1.2
CDC25A	204695_at	−1.6	−1.1
CENTG1	1555907_at	−1.0	−1.3
LITAF	200704_at	−1.9	−1.3
ARHGDIA	201167_x_at	−2.1	−1.6
ARHGDIA	211716_x_at	−2.7	−1.3
ARHGDIA	213606_s_at	−2.8	−1.8
SH3GL1	201851_at	−2.5	−1.3
CTBP1	203392_s_at	−2.1	−1.5
CTBP1	212863_x_at	−1.9	−1.3
CTBP1	213980_s_at	−2.2	−1.5
DAG1	205417_s_at	−1.8	−1.0
AKR1C3	209160_at	−2.6	−2.1
PPAP2B	209355_s_at	−1.4	−2.2
PPAP2B	212226_s_at	−1.5	−1.5
MAZ	212064_x_at	−1.1	−1.0
NRN1	218625_at	−1.7	−2.1
KLC2	218906_x_at	−2.2	−1.3
LRRC8A	224624_at	−1.7	−1.6
LRRC8A	233487_s_at	−2.2	−1.1
NSMCE1	224666_at	−2.3	−1.7
AKT2	225471_s_at	−1.7	−1.1
TMEM142A	226531_at	−2.3	−1.8
S100A16	227998_at	−2.6	−1.4

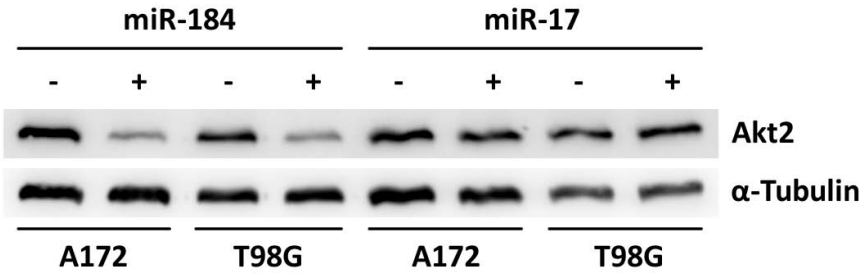


Figure 11: Down-regulation of Akt2 in glioma cells overexpressing *miR-184*. A172 and T98G cells were transfected with *miR-184* precursors (+) or corresponding controls (-). Western blots were probed with antibodies aganinst Akt2 and α-Tubulin as loading control. As a further control, glioma cells transfected with inhibitors of miR-17 (+) or respective controls (-) were probed. Note markedly reduced Akt2 protein levels in A172 and T98G cells transfected with *miR-184* precursors but not with *miR-17* inhibitors or controls.

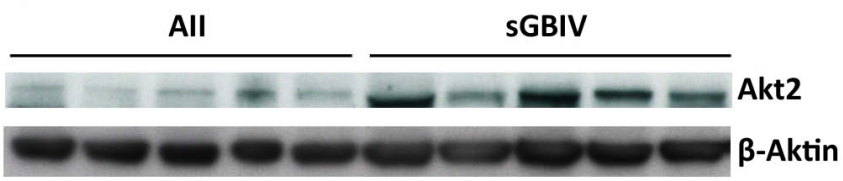


Figure 12: Expression of Akt2 protein in diffuse astrocytomas as compared to secondary glioblastomas. Western blots containing protein extracts from five diffuse astrocytomas (AII) and five secondary glioblastomas (sGBIV) were probed with antibodies against Akt2 and β-Actin as a loading control. Note increased Akt2 protein levels in secondary glioblastomas as compared to the low-grade diffuse astrocytomas.

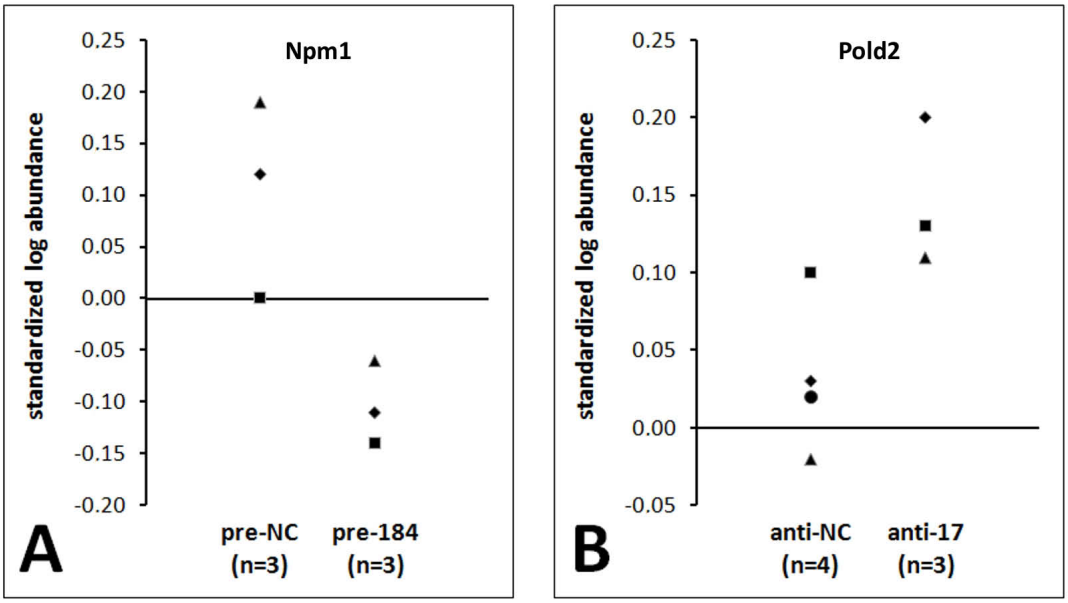


Figure 13: Proteome changes in T98G glioma cells following *miR-184* overexpression (A) or *miR-17* inhibition (B). A) Expression of Npm1 was found to be significantly ($p < 0.05$) down-regulated in T98G cells transfected with *miR-184* precursors (pre-184) as compared to control-transfected cells (pre-NC). **B)** Protein expression of Pold2 was significantly ($p < 0.05$) up-regulated in T98G cells transfected with *miR-17* inhibitors (anti-17) as compared to control-transfected cells (anti-NC). Npm1 is a predicted target of *miR-184* and Pold2 is a predicted target of *miR-17* according to the Sanger Institute mirBase target database (http://microrna.sanger.ac.uk/index.shtml).

4 Discussion

4.1 A set of miRNAs exhibits progression-associated differential expression in astrocytic gliomas

Screening the expression levels of 157 miRNAs in four patients with primary low-grade astrocytoma that recurred as secondary glioblastoma, 14 miRNAs with progression-associated expression in at least three of four patients were identified. The role of these miRNAs in gliomas and in other human cancers will be discussed here, knowing that an oncogenic effect in certain cancers is not mutually exclusive with tumor suppressive effects in other cancers and vice versa (see section "miRNA in cancer"). Moreover, the own data only show a correlation between astrocytoma progression and miRNA expression levels for the miRNAs discussed in this section, which, however, does not necessarily implicate a causal link.

In line with the own data, *miR-9* (1q23.1(-) intronic, 5q14.3(-) intergenic, 15q26.1(+) intergenic) was reported as being up-regulated in oligodendroglioma [134], glioblastoma [119] and B-CLL [135], thus suggesting a tumor-promoting function in gliomas and CLL. Overexpression of *miR-9* in primary brain tumors relative to metastases in the brain could be of diagnostic value [136]. Inhibition of *miR-9* reduced SK-Hep-1 cell invasion and led to E-Cadherin up-regulation [137]. However, studies on epithelial cancers revealed epigenetic silencing and/or down-regulation of *miR-9-1* in advanced breast and ovarian carcinomas [138, 139], which would fit to a tumor suppressive function.

The clustered miRNAs *miR-15a* and *miR-16-1* (13q14.3(-) intronic; *miR-16* also 3q25.33(+) intronic) were reported to be deleted and/or down-regulated in a subset of B-CLL [98, 140]. In addition, germ-line mutations in the miRNA genes were observed in CLL-patients [99]. In pituitary adenomas *miR-15a* and *miR-16-1* are expressed to a lesser degree than in normal pituitary. The expression level inversely correlates with adenoma diameter [141]. Loss of p53 function [75] and gain of PKCα [142] are putative mechanisms of down-regulation of *miR-15* and *miR-16*. The functional characterization of *miR-15-16-1* cluster revealed, that both miRNAs induce apoptosis by targeting Bcl2 in hematopoietic cell lines [100] and gastric cancer cell lines [143]. In acute promyelocytic leukemia cell lines retinoic acid-induced granulopoietic differentiation is correlated with an up-regulation of *miR-15* and *miR-16-1* [144]. Both miRNAs restrict cellular growth and cell cycle progression in an Rb dependent way targeting Cyclin D1 and Cyclin E and other regulators of cell cycle *in vitro* [145, 146, 142]. On the other hand, serous ovarian carcinomas showed increased *miR-16* expression relative to normal ovarian tissue [147], which is in accordance with the own finding of *miR-15a* and *miR-16* up-regulation in high-grade gliomas. Further detailed investigation of the *miR-15* family (including *miR-15a*, *miR-15b*, *miR-16*, *miR-195*) in glioma is required to address the question of functional consequences of *miR-15a* and *miR-16* overexpression in gliomas.

The present study also detected higher expression of *miR-140* (16q22.1(+) intronic) in secondary glioblastomas. These results are in line with findings of increased *miR-140* levels that are linearly associated with the degree of Kaposi sarcoma transformation [148], while studies on ovarian cancer revealed reduced *miR-140* levels as compared to normal ovarian tissue [149]. Taken together, these findings indicate tissue-specific differences in the function of certain miRNAs, thus emphasizing the complexity of

miRNA-associated regulatory networks and the importance of validating miRNA function in the appropriate cell systems.

The demonstration of a progression-associated up-regulation of *miR-21* is concordant with many other investigations that identified oncogenic functions of this miRNA: *miR-21* is overexpressed in glioblastomas [126, 119], squamous cell carcinoma of tongue [150], ovarian cancer [149, 147], cervical cancer [151], hepatocellular carcinomas [152], pancreas tumors [153, 154, 155], CLL [140], solid cancers of prostate, colon, stomach, lung, and breast [153]. However, a recent study did not observe statistically significant differences of *miR-21* expression between independent low-grade astrocytomas and secondary glioblastomas [156], which is probably due to high interindividual differences in the expression of this miRNA. In pancreatic endocrine tumors *miR-21* overexpression is correlated with Ki67 proliferation index and liver metastasis [154]. High expression levels of *miR-21* correlate with poor survival in colon cancer [157]. Inhibition of *miR-21* function decreases cell growth in breast cancer cells *in vitro* and *in vivo* [158]. Furthermore inhibition of *miR-21* increases apoptosis in breast cancer cells [158] and in glioblastoma cells *in vitro* [126] and *in vivo* [127]. Also, effects of *miR-21* inhibition in colorectal cells on invasion, intravasation and lung metastasis have been observed [159]. Some data about regulation of *miR-21* expression are published: In multiple myeloma cells *miR-21* is induced by Stat3 and is thereby involved in the IL6-dependent survival pathway of these cells [160]. Phorbol-12-myristate-13-acetate treatment raises *miR-21* expression in a promyelocytic cell line by an Ap1 dependent activation of transcription. Interestingly, this regulation does not affect the overlapping coding gene *TMEM49*. Demonstrating a double-negative feedback regu-

lation *miR-21* down-regulates Nfb1, an inhibitor of *miR-21* expression [161]. Several studies reported target genes contributing to *miR-21* function: The anti-apoptotic Bcl2 is a putative target gene of *miR-21* in breast cancer cells. In hepatocellular cancer cells Pten, which is a important tumorsuppressor in glioblastomas [162], is an experimentally validated target of *miR-21* [163]. Further validated target genes of *miR-21* are Tpm1 and Maspin in breast cancer [164, 165], Pdcd4 in colon carcinoma [159], Sprouty2 in cardiocytes [166], and Marcks in prostata cancer cells [167]. Functional analyses of *miR-21* in glioblastoma cells revealed oncogenic properties due to inhibition of the p53, transforming growth factor beta and mitochondrial apoptosis pathways [168]. Moreover, therapeutic implications of *miR-21* cancer treatment has been reported: Changing the cellular levels of *miR-21* significantly influences the growth inhibitory effect of several anticancer drugs *in vitro* [112]. In addition, targeted inhibition of *miR-21* sensitized glioma cells for cytotoxic agents, pointing to a potential novel approach for targeted glioma therapy [127].

The miRNA *miR-25* is located on 7q (7q22.1(-) intronic, clustered with *miR-106b* and *miR-93*), a region frequently gained in diffuse astrocytomas [4]. The observed up-regulation in secondary glioblastomas is in line with a report on *miR-25* overexpression in glioblastomas [119]. Also, *miR-25* is up-regulated in gastric cancers and targets p57 [169]. In esophageal neoplasias the *miR-106b-25* polycistron is activated by genomic amplification and is potentially involved in progression and proliferation via suppression of p21 and Bim [170]. To analyze the functional significance of *miR-25* up-regulation in glioma progression, however, a detailed analysis of the corresponding miRNA-family, i.e. *miR-25*, *miR-92a* and *miR-92b*, would be necessary.

Similarly, *miR-28* (3q28 (+) intronic) and *miR-130b* (22q11.21(+) intergenic, clustered with *miR-301b*), both demonstrating progression-associated up-regulation in the investigated patients, each belong to distinct families of closely related miRNAs, i.e., *miR-28* and *miR-151* as well as *miR-130a*, *miR-130b*, *miR-301a* and *miR-301b*, respectively. Hence, it remains to be explored whether the up-regulation of a single family member has significant biological effects or may be compensated for by loss of function of other family members. Nevertheless, in line with the own findings *miR-28* showed increased expression in renal cell carcinomas as compared to normal kidney tissue [171].

The up-regulation of *miR-210* (11p15.5(-) intergenic) in gliomas is interesting because *miR-210* was reported to be induced by hypoxia in breast carcinoma cells by an HIF-1α/VHL dependent pathway [76]. Hypoxia induced *miR-210* suppresses Rad52 which is part of the homology-dependent DNA-repair system [172]. The expression level of *miR-210* in breast cancer [76] and pancreatic tumors [173] is inversely correlated with the patients' survival. In endothelial cells, inhibition of *miR-210* reduced cell growth, induced apoptosis and inhibited the formation of capillary-like structures stimulated by hypoxia [174]. Thus, elevated *miR-210* expression in glioblastomas may be triggered by the regional hypoxia in these tumors which is of prognostic significance for patients with glioblastoma [175]. In addition, an investigation of serum from diffuse large B-cell lymphoma indicated a diagnostic potential of serum *miR-210* levels [176].

Analyzing matched primary low-grade and recurrent high-grade gliomas *miR-328* displayed a progression-associated down-regulation. It maps to 16q (16q22.1(-) intronic),

a region frequently lost in pediatric high-grade astrocytomas [177]. However, data on expression of this particular miRNA in cancer have not been reported to date. A role in multidrug resistance of cancer cells was implied by a recent study demonstrating that *miR-328* negatively regulates Abcg2 protein expression and that repression of Abcg2 expression via a *miR-328*-mediated pathway is translated into significantly increased drug sensitivity in cancer cells [178].

4.2 The miRNA *miR-184* is a putative suppressor of astrocytoma progression

Concordant with decreased *miR-184* levels in prostate cancer as compared to adjacent tissue [179], the expression of *miR-184* (15q25.1) was down-regulated during progression from low-grade to anaplastic glioma and secondary glioblastoma, respectively. Furthermore, overexpression of *miR-184* significantly decreased cell viability and proliferation of glioma cells, suggesting a tumor suppressive role of this miRNA in gliomas. Interestingly, *miR-184* increased apoptotic activity in A172 cells but reduced apoptotic activity and invasive growth in T98G cells. This divergent influence on apoptotic rates in T98G and A172 cells is probably related to the different genetic background of these cell lines, with the *TP53* gene being mutant in T98G but wild-type in A172 cells [180]. Cell type specific effects are also indicated by the fact that overexpression of *miR-184* in neuroblastoma cell lines decreased proliferation and induced apoptosis [181] while proliferation was decreased in squamous cell carcinomas by inhibition of *miR-184* [150, 182].

Interestingly, studies in an ischemia-induced retinal neovascularization model revealed

lower *miR-184* expression in ischemic retina whereas high expression levels were determined in the avascular cornea and lens. Moreover reduced ischemia-induced retinal neovascularisation was observed after intraocular injection of *miR-184* precursors [183]. Thus, similar to the up-regulation of *miR-17* and *miR-210*, the down-regulation of *miR-184* in high-grade gliomas may be linked to hypoxia and probably contributing to tumor neoangiogenesis. On the other hand, inhibitory effects of N-myc on *miR-184* transcription have been reported in neuroblastoma [181].

Amplification of *MYCN* is a possible mechanism leading to *miR-184* down-regulation during astrocytoma progression, but systematic analyses of gene amplifications in glioblastomas reported only a few percent of glioblastomas with *MYCN* amplification [184]. More recently, epigenetic regulation of *miR-184* in the mouse brain has been analyzed and suggested a paternal-specific expression of this particular miRNA [185]. Thus, the observed progression-associated down-regulation of *miR-184* may be caused by diverse molecular alterations, with the own findings excluding homozygous gene deletion as a mechanism in gliomas.

With respect to *miR-184* targets, possible regulation of c-Myc [150], Frizzeled 4 [183] and *miR-205* [70] has been reported. Npm1 was identified as being significantly down-regulated in T98G cells after transfection with *miR-184* precursors. Npm1 is an ubiquitously expressed nucleolar phosphoprotein that has been implicated in the pathogenesis of epithelial and hematopoietic malignancies. Depending on gene dosage and expression level *NPM1* seems to function as either an oncogene or a tumour suppressor gene (for review see [186]). Furthermore, increased *NPM1* mRNA levels were found in glioblastomas [187], and siRNA-mediated inhibition of Npm1 reduced the proliferation and increased apoptosis of neural stem cells [188]. Thus, *miR-184*-mediated down-regulation of Npm1 may contribute to its anti-proliferative and anti-apoptotic effects

in glioma cells. Affymetrix chip-based expression profiling identified 17 predicted targets of *miR-184* whose expression was at least twofold reduced in A172 and T98G cells after transfection of *miR-184* precursors. Nine of these candidate targets with putative oncogenic functions ((*AKR1C3* [189, 190, 191, 192, 193, 194, 195, 196, 197], *CTBP1* [198, 199, 200, 201, 202, 203], *S100A16* [204, 205, 206], *CENTG1* [207, 208, 209], *MAZ* [210, 211], *NRN1* [212, 213], *SH3GL1* [214, 106], *CDC25A* [215, 216], *AKT2* [217, 218, 219, 220]) were selected for targeted analysis, which validated their down-regulation at the mRNA level.

In addition, Western blot analysis showed that Akt2 protein expression is decreased in *miR-184* overexpressing glioma cells and increased in secondary glioblastomas relative to diffuse astrocytomas. To definitely prove that down-regulation of the predicted *miR-184* target Akt2 after *miR-184* re-expression is caused by a direct interaction of the miRNA and the target 3'-UTR, further investigations by 3'UTR-luciferase assays are nescessary. Nevertheless, an inhibition of Akt2 by *miR-184* likely contributes to the proliferation-inhibitory and pro-apoptotic effects observed in glioma cells that re-express *miR-184*: Akt2 gene amplification has been found in several human malignancies and is linked to cell growth, proliferation, survival and various aspects of intermediary metabolism (reviewed in Reference [221, 217, 222]). However, neither amplification nor mRNA-overexpression of *AKT2* were detected in a large series of glioblastomas [162]. Thus, down-regulation of *miR-184* in high-grade gliomas may lead to increased Akt2 protein levels and thereby contribute to the aberrant activation of the phosphoinositol 3-kinase /Akt pathway commonly seen in these tumors [162].

4.3 Increased expression of miRNAs encoded by the *miR-17-92* cluster may promote astrocytoma progression

The *miR-17-92* cluster (13q31.3(+) intronic) comprising *miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1* and *miR-92a-1*, is trancribed as a polycistron. Due to ancient duplications [223] there are two paralogs of the *miR-17-92* cluster (*miR-106b-25* cluster and *miR-106a-363* cluster) in the human genome. Focussing on functional similarities the miRNAs of the *miR-17-92* cluster can be regarded as members of four genefamilies according to their seed sequences (*miR-17* family: *miR-17*, *miR-20a*, *miR-20b*, *miR-106a*, *miR-106b*, *miR-93*; *miR-18* family: *miR-18a*, *miR-18b*; *miR-19* family: *miR-19a*, *miR-19b*; *miR-25* family: *miR-25*, *miR-92a*, *miR-363*) [224].

Screening for the expression of *miR-17*, *miR-19a*, *miR-20a* and *miR-92a* in the matched pairs of primary low-grade and recurrent high-grade astrocytomas revealed progression-associated up-regulation of *miR-17*, *miR-19a* and *miR-20a*. in the recurrent glioblastomas of two patients *miR-92a* was up-regulated but down-regulated in one patient. Such differences in the expression levels of individual members of the *miR-17-92* cluster may be explained by independent post-trancriptional regulation or alterations of the paralogous *miR-106a-92* cluster. In line with the own data, miRNAs of the *miR-17-92* cluster were found to be overexpressed in advanced neuroblastomas with *MYCN* amplification [225], in hematopoietic malignancies [99, 88, 226], lung cancers [102, 153], hepatocellular carcinomas [152, 227], bladder cancers [171], thyreoid cancers [228], and cancers of breast, colon, pancreas, and prostate [153].

In B-cell lymphomas, overexpression of the *miR-17-92* cluster was related to increased gene dosage [88]. In contrast, none of the investigated glioma patients showed amplification of the respective gene locus. However, other mechanisms leading to over-

expression of the *miR-17-92* cluster have been reported: Strikingly, expression of the *miR-17-92* cluster does not correlate with expression of the host gene C13orf25 [229]. C-Myc transactivates expression of the *miR-17-92* cluster [74]. In a mouse model of hepatocellular tumorigenesis activation of Pparα inhibits *let-7c*, which leads to a disinhibition of c-Myc and a subsequent increase in the expression of the *miR-17-92* cluster that is critical for Pparα induced hepatocellular proliferation [230]. N-Myc was also shown as inducer of miRNAs encoded by the *miR-17-92* cluster [231, 225, 232]. Further known transactivators of *miR-17-92* cluster expression are the transcription factors E2f1-3 [92, 233, 234], Stat3 [235], and NK-like homeodomain proteins [236]. Myc proteins and E2f1-3 may be overexpressed in malignant gliomas .

The *in vitro* analyses of glioma cells after inhibition of *miR-17* demonstrated influences on cell viability and apoptotic activity. These results are in accordance with studies that have demonstrated oncogenic effects of the *miR-17-92* cluster in different cell types. For example, overexpression of the *miR-17-92* cluster in lung cancer cells significantly increased proliferation [102]. In anaplastic thyreoid cancer cell lines, inhibition of *miR-17*, *miR-17** and *miR-19a* reduced cell growth [228]. Furthermore, inhibition of *miR-17* and *miR-20a* induced apoptosis in lung cancer cells [237], while *miR-20a* showed anti-apoptotic effects in prostata cancer cells [92]. Suggesting a role in tumor angiogenesis *miR-17-92* cluster transduction into Kras transformed colonozytes lacking p53 leads to formation of larger, better perfused tumors [105]. *In vitro* or *in vivo* treatment with antagomiR-17 inhibited the growth of *MYCN*-amplified neuroblastoma [231]. On the other hand, the *miR-17-92* locus is deleted in a fraction of breast cancers [72] and *miR-17* overexpression caused reduced proliferation in breast cancer cells [238]. Taken together, these findings point to cell type-specific roles of this particular miRNA cluster.

Several targets of miRNAs belonging to the *miR-17-92* cluster have been experimentally validated, including members of the E2F family [74, 92], Rbl2 [239], Aib1 [238], p21 and Bim [231], as well as an isoform of p63 [240]. In addition, the Pten tumor suppressor protein, which is frequently inactivated in glioblastoma [241], has been identified as a target of *miR-17* and *miR-19a* in mouse embryonic fibroblasts [242]. Using proteomic analyses, Pold2 was identified as a predicted target that was up-regulated by inhibition of *miR-17* in T98G cells. Pold2 is the 50 kdA regulatory subunit of the DNA polymerase delta complex and involved in DNA repair mechanisms [243, 244]. Inactivating point mutations of the proof-reading Pold1 domain of this complex increase susceptibility to cancer development in the mouse model [245]. If a decrease in Pold2 abundance has similar effects on cancer susceptibility, up-regulation of *miR-17* may lead to alterations of DNA repair in gliomas. However, this hypothesis requires experimental validation. In addition, a detailed analysis of members of *miR-17* gene family could show how closely their effects on target genes are connected and if an overexpression of *miR-17* can be compensated for by down-regulation of other family members.

4.4 Summary

In summary, 14 miRNAs were shown to be differentially expressed between primary WHO grade II gliomas and secondary glioblastomas. Among these, *miR-17*, *miR-19a*, *miR-20a*, *miR-21*, *miR-25*, *miR-28*, and *miR-210* have been previously implicated as potentially oncogenic miRNAs in different malignancies and accordingly were up-regulated in glioma progression. The miRNAs *miR-9*, *miR-15a*, *miR-16-1* and *miR-*

140 were reported as putative tumor suppressors in non-glial tumors but demonstrated progression-associated up-regulation in gliomas, indicating tissue-specific functional effects of these miRNAs. Two miRNAs with progression-associated differential expression, i.e., *miR-130b* and *miR-328*, have not been implicated in cancer before.

In vitro analyses of glioma cell lines revealed evidence for *miR-184* being a suppressor of glioma progression while *miR-17* likely promotes glioma progression. Using mRNA and protein expression profiling, putative targets were identified that were differentially expressed in glioma cells following overexpression of *miR-184* or inhibition of *miR-17*, respectively, including Npm1 and Akt2 (*miR-184*) as well as Pold2 (*miR-17*). Additional studies are needed to characterize the molecular mechanisms underlying the observed progression-associated miRNA expression changes and to prove that the identified candidate targets are directly regulated by miRNA-specific binding to the respective transcripts. Furthermore, the potential roles of miRNAs in the molecular diagnostics of gliomas and as novel therapeutic targets warrant further exploration.

5 References

[1] Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, editors. WHO Classification of Tumours of the Central Nervous System. 3rd ed. Lyon: IARC Press; 2007.

[2] CBTRUS. Statistical Report: Primary Brain Tumors in the United States. Central Brain Tumor Registry of the United States, 1998-2002; 2005.

[3] Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, et al. Long-term survival with glioblastoma multiforme. Brain. 2007 Oct;130(Pt 10):2596–2606. Available from: http://dx.doi.org/10.1093/brain/awm204.

[4] Riemenschneider MJ, Reifenberger G. Astrocytic tumors. In: von Deimling A, editor. Gliomas (Series: Recent Results in Cancer Research Vol. 171). Springer; 2009. pp. 3–24.

[5] Ricci PE, Dungan DH. Imaging of low- and intermediate-grade gliomas. Semin Radiat Oncol. 2001 Apr;11(2):103–112. Available from: http://dx.doi.org/doi:10.1053/srao.2001.21420.

[6] Bogdahn U, Grisold W, Groß M, Kortmann RD, Merlo A, Reifenberger G, et al. Gliome. In: Diener HC, Putzki N, editors. Leitlinien für die Diagnostik und Therapie in der Neurologie, 4. überarb. Auflage. Georg Thieme Verlag; 2008. pp. 774–788.

[7] Rich JN, Bigner DD. Development of novel targeted therapies in the treatment of malignant glioma. Nat Rev Drug Discov. 2004 May;3(5):430–446. Available from: http://dx.doi.org/10.1038/nrd1380.

[8] Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med. 2000 Nov;343(19):1350–1354. Available from: http://dx.doi.org/10.1056/NEJM200011093431901.

[9] Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005 Mar;352(10):997–1003. Available from: http://dx.doi.org/10.1056/NEJMoa043331.

[10] Herrlinger U, Rieger J, Koch D, Loeser S, Blaschke B, Kortmann RD, et al. Phase II trial of lomustine plus temozolomide chemotherapy in addition to radiotherapy in newly diagnosed glioblastoma: UKT-03. J Clin Oncol. 2006

Sep;24(27):4412–4417. Available from: http://dx.doi.org/10.1200/JCO.2006.06.9104.

[11] Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol. 2008 Dec;116(6):597–602. Available from: http://dx.doi.org/10.1007/s00401-008-0455-2.

[12] Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med. 2009 Feb;360(8):765–773. Available from: http://dx.doi.org/10.1056/NEJMoa0808710.

[13] Ichimura K, Ohgaki H, Kleihues P, Collins VP. Molecular pathogenesis of astrocytic tumours. J Neurooncol. 2004 Nov;70(2):137–160. Available from: http://dx.doi.org/10.1007/s11060-004-2747-2.

[14] Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol. 2007 May;170(5):1445–1453. Available from: http://dx.doi.org/10.2353/ajpath.2007.070011.

[15] Network CGAR. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008 Oct;455(7216):1061–1068. Available from: http://dx.doi.org/10.1038/nature07385.

[16] van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell. 1990 Apr;2(4):291–299. Available from: http://www.plantcell.org/cgi/reprintframed/2/4/291.

[17] Napoli C, Lemieux C, Jorgensen R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell. 1990 Apr;2(4):279–289. Available from: http://dx.doi.org/10.1105/tpc.2.4.279.

[18] Romano N, Macino G. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol Microbiol. 1992 Nov;6(22):3343–3353. Available from: http://dx.doi.org/10.1111/j.1365-2958.1992.tb02202.x.

[19] Guo S, Kemphues KJ. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell. 1995 May;81(4):611–620. Available from: http://dx.doi.org/10.1016/0092-8674(95)90082-9.

[20] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998 Feb;391(6669):806–811. Available from: http://dx.doi.org/10.1038/35888.

[21] Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001 Jan;15(2):188–200. Available from: http://dx.doi.org/10.1101/gad.862301.

[22] Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001 Jan;409(6818):363–366. Available from: http://dx.doi.org/10.1038/35053110.

[23] Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature. 2000 Mar;404(6775):293–296. Available from: http://dx.doi.org/10.1038/35005107.

[24] Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993 Dec;75(5):843–854. Available from: http://dx.doi.org/10.1016/0092-8674(93)90529-Y.

[25] Lee R, Feinbaum R, Ambros V. A short history of a short RNA. Cell. 2004 Jan;116(2 Suppl):S89–92, 1 p following S96. Available from: http://dx.doi.org/10.1016/S0092-8674(04)00035-2.

[26] Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000 Feb;403(6772):901–906. Available from: http://dx.doi.org/10.1038/35002607.

[27] Hutvágner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. 2001 Aug;293(5531):834–838. Available from: http://dx.doi.org/10.1126/science.1062961.

[28] Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, et al. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. 2002 Mar;16(6):720–728. Available from: http://dx.doi.org/10.1101/gad.974702.

[29] Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science. 2001 Aug;293(5532):1146–1150. Available from: http://dx.doi.org/10.1126/science.1064023.

[30] Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005 Sep;309(5740):1573–1576. Available from: http://dx.doi.org/10.1126/science.1115079.

[31] Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004 Dec;10(12):1957–1966. Available from: http://dx.doi.org/10.1261/rna.7135204.

[32] Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003 Sep;425(6956):415–419. Available from: http://dx.doi.org/10.1038/nature01957.

[33] Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003 Dec;17(24):3011–3016. Available from: http://dx.doi.org/10.1101/gad.1158803.

[34] Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. 2001 Oct;294(5543):853–858. Available from: http://dx.doi.org/10.1126/science.1064921.

[35] Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007 Jun;129(7):1401–1414. Available from: http://dx.doi.org/10.1016/j.cell.2007.04.040.

[36] Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004 Oct;14(10A):1902–1910. Available from: http://dx.doi.org/10.1101/gr.2722704.

[37] Megraw M, Sethupathy P, Corda B, Hatzigeorgiou AG. miRGen: a database for the study of animal microRNA genomic organization and function. Nucleic Acids Res. 2007 Jan;35(Database issue):D149–D155. Available from: http://dx.doi.org/10.1093/nar/gkl904.

[38] Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005 Mar;11(3):241–247. Available from: http://dx.doi.org/10.1261/rna.7240905.

[39] Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 2002 Sep;21(17):4663–4670. Available from: http://dx.doi.org/10.1093/emboj/cdf476.

[40] Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol. 2006 Dec;13(12):1097–1101. Available from: http://dx.doi.org/10.1038/nsmb1167.

[41] Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell. 2006 Jun;125(5):887–901. Available from: http://dx.doi.org/10.1016/j.cell.2006.03.043.

[42] Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The Microprocessor complex mediates the genesis of microRNAs. Nature. 2004 Nov;432(7014):235–240. Available from: http://dx.doi.org/10.1038/nature03120.

[43] Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC. Mammalian mirtron genes. Mol Cell. 2007 Oct;28(2):328–336. Available from: http://dx.doi.org/10.1016/j.molcel.2007.09.028.

[44] Chan SP, Slack FJ. And now introducing mammalian mirtrons. Dev Cell. 2007 Nov;13(5):605–607. Available from: http://dx.doi.org/10.1016/j.devcel.2007.10.010.

[45] Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 2005 Aug;436(7051):740–744. Available from: http://dx.doi.org/10.1038/nature03868.

[46] Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. 2005 Dec;19(24):2979–2990. Available from: http://dx.doi.org/10.1101/gad.1384005.

[47] Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005 Nov;123(4):631–640. Available from: http://dx.doi.org/10.1016/j.cell.2005.10.022.

[48] Tomari Y, Zamore PD. Perspective: machines for RNAi. Genes Dev. 2005 Mar;19(5):517–529. Available from: http://dx.doi.org/10.1101/gad.1284105.

[49] Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 2003 Oct;115(2):209–216. Available from: http://dx.doi.org/10.1016/S0092-8674(03)00893-6.

[50] Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003 Oct;115(2):199–208. Available from: http://dx.doi.org/10.1016/S0092-8674(03)00759-1.

[51] Diederichs S, Haber DA. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell. 2007 Dec;131(6):1097–1108. Available from: http://dx.doi.org/10.1016/j.cell.2007.10.032.

[52] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005 Jan;120(1):15–20. Available from: http://dx.doi.org/10.1016/j.cell.2004.12.035.

[53] Tolia NH, Joshua-Tor L. Slicer and the argonautes. Nat Chem Biol. 2007 Jan;3(1):36–43. Available from: http://dx.doi.org/10.1038/nchembio848.

[54] Peters L, Meister G. Argonaute proteins: mediators of RNA silencing. Mol Cell. 2007 Jun;26(5):611–623. Available from: http://dx.doi.org/10.1016/j.molcel.2007.05.001.

[55] Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. RNA. 2008 May;14(5):872–877. Available from: http://dx.doi.org/10.1261/rna.972008.

[56] Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science. 2004 Apr;304(5670):594–596. Available from: http://dx.doi.org/10.1126/science.1097434.

[57] Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004 Jul;15(2):185–197. Available from: http://dx.doi.org/10.1016/j.molcel.2004.07.007.

[58] Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. Cell. 2002 Aug;110(4):513–520. Available from: http://dx.doi.org/10.1016/S0092-8674(02)00863-2.

[59] Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA. 2004 Oct;10(10):1518–1525. Available from: http://dx.doi.org/10.1261/rna.7131604.

[60] Grimson A, Farh KKH, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell. 2007 Jul;27(1):91–105. Available from: http://dx.doi.org/10.1016/j.molcel.2007.06.017.

[61] Kiriakidou M, Tan GS, Lamprinaki S, Planell-Saguer MD, Nelson PT, Mourelatos Z. An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell. 2007 Jun;129(6):1141–1151. Available from: http://dx.doi.org/10.1016/j.cell.2007.05.016.

[62] Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, et al. MicroRNA silencing through RISC recruitment of eIF6. Nature. 2007 Jun;447(7146):823–828. Available from: http://dx.doi.org/10.1038/nature05841.

[63] Petersen CP, Bordeleau ME, Pelletier J, Sharp PA. Short RNAs repress translation after initiation in mammalian cells. Mol Cell. 2006 Feb;21(4):533–542. Available from: http://dx.doi.org/10.1016/j.molcel.2006.01.031.

[64] Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. 2006 Jul;20(14):1885–1898. Available from: http://dx.doi.org/10.1101/gad.1424106.

[65] Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. 2005 Jul;7(7):719–723. Available from: http://dx.doi.org/10.1038/ncb1274.

[66] Bao N, Lye KW, Barton MK. MicroRNA binding sites in Arabidopsis class III HD-ZIP mRNAs are required for methylation of the template chromosome. Dev Cell. 2004 Nov;7(5):653–662. Available from: http://dx.doi.org/10.1016/j.devcel.2004.10.003.

[67] Gonzalez S, Pisano DG, Serrano M. Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. Cell Cycle. 2008 Aug;7(16):2601–2608. Available from: http://www.landesbioscience.com/journals/cc/article/6541/.

[68] Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. Science. 2007 Jan;315(5808):97–100. Available from: http://dx.doi.org/10.1126/science.1136235.

[69] Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007 Jun;9(6):654–659. Available from: http://dx.doi.org/10.1038/ncb1596.

[70] Yu J, Ryan DG, Getsios S, Oliveira-Fernandes M, Fatima A, Lavker RM. MicroRNA-184 antagonizes microRNA-205 to maintain SHIP2 levels in epithelia. Proc Natl Acad Sci U S A. 2008 Dec;105(49):19300–19305. Available from: http://dx.doi.org/10.1073/pnas.0803992105.

[71] Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007 Dec;318(5858):1931–1934. Available from: http://dx.doi.org/10.1126/science.1149460.

[72] Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, et al. microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci U S A. 2006 Jun;103(24):9136–9141. Available from: http://dx.doi.org/10.1073/pnas.0508889103.

[73] Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A. 2004 Mar;101(9):2999–3004. Available from: http://dx.doi.org/10.1073/pnas.0307323101.

[74] O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature. 2005 Jun;435(7043):839–843. Available from: http://dx.doi.org/10.1038/nature03677.

[75] Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A, et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle. 2007 Jul;6(13):1586–1593. Available from: http://www.landesbioscience.com/journals/cc/article/4436/.

[76] Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, et al. hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res. 2008 Mar;14(5):1340–1348. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-07-1755.

[77] Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet. 2008 Jan;40(1):43–50. Available from: http://dx.doi.org/10.1038/ng.2007.30.

[78] Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006 Jun;9(6):435–443. Available from: http://dx.doi.org/10.1016/j.ccr.2006.04.020.

[79] Michael MZ, Connor SMO, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003 Oct;1(12):882–891. Available from: http://mcr.aacrjournals.org/content/1/12/882.long.

[80] Obernosterer G, Leuschner PJF, Alenius M, Martinez J. Post-transcriptional regulation of microRNA expression. RNA. 2006 Jul;12(7):1161–1167. Available from: http://dx.doi.org/10.1261/rna.2322506.

[81] Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev. 2006 Aug;20(16):2202–2207. Available from: http://dx.doi.org/10.1101/gad.1444406.

[82] Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008 Jul;454(7200):56–61. Available from: http://dx.doi.org/10.1038/nature07086.

[83] Guil S, Cáceres JF. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. Nat Struct Mol Biol. 2007 Jul;14(7):591–596. Available from: http://dx.doi.org/10.1038/nsmb1250.

[84] Kedde M, Strasser MJ, Boldajipour B, Vrielink JAFO, Slanchev K, le Sage C, et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell. 2007 Dec;131(7):1273–1286. Available from: http://dx.doi.org/10.1016/j.cell.2007.11.034.

[85] Huang J, Liang Z, Yang B, Tian H, Ma J, Zhang H. Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members. J Biol Chem. 2007 Nov;282(46):33632–33640. Available from: http://dx.doi.org/10.1074/jbc.M705116200.

[86] Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell. 2006 Jun;125(6):1111–1124. Available from: http://dx.doi.org/10.1016/j.cell.2006.04.031.

[87] Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science. 2007 Mar;315(5818):1576–1579. Available from: http://dx.doi.org/10.1126/science.1137999.

[88] He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. Nature. 2005 Jun;435(7043):828–833. Available from: http://dx.doi.org/10.1038/nature03552.

[89] Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibé B, et al. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet. 2006 Jul;38(7):813–818. Available from: http://dx.doi.org/10.1038/ng1810.

[90] Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GSA, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. Proc Natl Acad Sci U S A. 2007 Aug;104(33):13513–13518. Available from: http://dx.doi.org/10.1073/pnas.0706217104.

[91] Kapeller J, Houghton LA, Mönnikes H, Walstab J, Möller D, Bönisch H, et al. First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome. Hum Mol Genet. 2008 Oct;17(19):2967–2977. Available from: http://dx.doi.org/10.1093/hmg/ddn195.

[92] Sylvestre Y, Guire VD, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, et al. An E2F/miR-20a autoregulatory feedback loop. J Biol Chem. 2007 Jan;282(4):2135–2143. Available from: http://dx.doi.org/10.1074/jbc.M608939200.

[93] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008 Feb;9(2):102–114. Available from: http://dx.doi.org/10.1038/nrg2290.

[94] Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993 Dec;75(5):855–862. Available from: http://dx.doi.org/10.1016/0092-8674(93)90530-4.

[95] Karube Y, Tanaka H, Osada H, Tomida S, Tatematsu Y, Yanagisawa K, et al. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. Cancer Sci. 2005 Feb;96(2):111–115. Available from: http://dx.doi.org/10.1111/j.1349-7006.2005.00015.x.

[96] Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007 May;39(5):673–677. Available from: http://dx.doi.org/10.1038/ng2003.

[97] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005 Jun;435(7043):834–838. Available from: http://dx.doi.org/10.1038/nature03702.

[98] Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002 Nov;99(24):15524–15529. Available from: http://dx.doi.org/10.1073/pnas.242606799.

[99] Calin GA, Ferracin M, Cimmino A, Leva GD, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005 Oct;353(17):1793–1801. Available from: http://dx.doi.org/10.1056/NEJMoa050995.

[100] Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005 Sep;102(39):13944–13949. Available from: http://dx.doi.org/10.1073/pnas.0506654102.

[101] Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006 Apr;6(4):259–269. Available from: http://dx.doi.org/10.1038/nrc1840.

[102] Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. 2005 Nov;65(21):9628–9632. Download der pdf hakt. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-05-2352.

[103] Samols MA, Skalsky RL, Maldonado AM, Riva A, Lopez MC, Baker HV, et al. Identification of cellular genes targeted by KSHV-encoded microRNAs. PLoS Pathog. 2007 May;3(5):e65. Available from: http://dx.doi.org/10.1371/journal.ppat.0030065.

[104] le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. 2007 Aug;26(15):3699–3708. Available from: http://dx.doi.org/10.1038/sj.emboj.7601790.

[105] Dews M, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, et al. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet. 2006 Sep;38(9):1060–1065. Available from: http://dx.doi.org/10.1038/ng1855.

[106] Ma LH, Liu H, Xiong H, Chen B, Zhang XW, Wang YY, et al. Aberrant transcriptional regulation of the MLL fusion partner EEN by AML1-ETO and its implication in leukemogenesis. Blood. 2007 Jan;109(2):769–777. Available from: http://dx.doi.org/10.1182/blood-2006-02-003517.

[107] Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, et al. A microRNA signature of hypoxia. Mol Cell Biol. 2007 Mar;27(5):1859–1867. Available from: http://dx.doi.org/10.1128/MCB.01395-06.

[108] Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, Yu M, et al. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med. 2008;6:14. Available from: http://dx.doi.org/10.1186/1741-7015-6-14.

[109] Lee YS, Dutta A. MicroRNAs in Cancer. Annu Rev Pathol. 2008 Sep;Available from: http://dx.doi.org/10.1146/annurev.pathol.4.110807.092222.

[110] Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene. 2007 Jun;26(30):4442–4452. Available from: http://dx.doi.org/10.1038/sj.onc.1210228.

[111] Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004 Jun;64(11):3753–3756. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-04-0637.

[112] Blower PE, Chung JH, Verducci JS, Lin S, Park JK, Dai Z, et al. MicroRNAs modulate the chemosensitivity of tumor cells. Mol Cancer Ther. 2008 Jan;7(1):1–9. Available from: http://dx.doi.org/10.1158/1535-7163.MCT-07-0573.

[113] Bueno MJ, de Castro IP, de Cedrón MG, Santos J, Calin GA, Cigudosa JC, et al. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. Cancer Cell. 2008 Jun;13(6):496–506. Available from: http://dx.doi.org/10.1016/j.ccr.2008.04.018.

[114] Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA. 2003 Oct;9(10):1274–1281. Available from: http://dx.doi.org/10.1261/rna.5980303.

[115] Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, et al. Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 2004;5(9):R68. Available from: http://dx.doi.org/10.1186/gb-2004-5-9-r68.

[116] Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 2004;5(3):R13. Available from: http://dx.doi.org/10.1186/gb-2004-5-3-r13.

[117] Makeyev EV, Zhang J, Carrasco MA, Maniatis T. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol Cell. 2007 Aug;27(3):435–448. Available from: http://dx.doi.org/10.1016/j.molcel.2007.07.015.

[118] Nass D, Rosenwald S, Meiri E, Gilad S, Tabibian-Keissar H, Schlosberg A, et al. MiR-92b and miR-9/9* Are Specifically Expressed in Brain Primary Tumors and Can Be Used to Differentiate Primary from Metastatic Brain Tumors. Brain Pathol. 2008 Jul;Available from: http://dx.doi.org/10.1111/j.1750-3639.2008.00184.x.

[119] Ciafrè SA, Galardi S, Mangiola A, Ferracin M, Liu CG, Sabatino G, et al. Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun. 2005 Sep;334(4):1351–1358. Available from: http://dx.doi.org/10.1016/j.bbrc.2005.07.030.

[120] Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, et al. hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res. 2008 Oct;1236C:185–193. Available from: http://dx.doi.org/10.1016/j.brainres.2008.07.085.

[121] Kefas B, Godlewski J, Comeau L, Li Y, Abounader R, Hawkinson M, et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res. 2008 May;68(10):3566–3572. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-07-6639.

[122] Würdinger T, Tannous BA, Saydam O, Skog J, Grau S, Soutschek J, et al. miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells. Cancer Cell. 2008 Nov;14(5):382–393. Available from: http://dx.doi.org/10.1016/j.ccr.2008.10.005.

[123] Gal H, Pandi G, Kanner AA, Ram Z, Lithwick-Yanai G, Amariglio N, et al. MIR-451 and Imatinib mesylate inhibit tumor growth of Glioblastoma stem cells. Biochem Biophys Res Commun. 2008 Nov;376(1):86–90. Available from: http://dx.doi.org/10.1016/j.bbrc.2008.08.107.

[124] Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, Nuovo G, et al. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer Res. 2008 Nov;68(22):9125–9130. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-2629.

[125] Zhang WH, Poh A, Fanous A, Eastman A. DNA damage-induced S phase arrest in human breast cancer depends on Chk1, but G(2) arrest can occur independently of Chk1, Chk2 or MAPKAPK2. Cell Cycle. 2008 Mar;7(11).

[126] Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res. 2005 Jul;65(14):6029–6033. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-05-0137.

[127] Corsten MF, Miranda R, Kasmieh R, Krichevsky AM, Weissleder R, Shah K. MicroRNA-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. Cancer Res. 2007 Oct;67(19):8994–9000. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-07-1045.

[128] van den Boom J, Wolter M, Kuick R, Misek DE, Youkilis AS, Wechsler DS, et al. Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. Am J Pathol. 2003 Sep;163(3):1033–1043. Available from: http://ajp.amjpathol.org/cgi/content/abstract/163/3/1033.

[129] van den Boom J, Wolter M, Blaschke B, Knobbe CB, Reifenberger G. Identification of novel genes associated with astrocytoma progression using suppression subtractive hybridization and real-time reverse transcription-polymerase chain reaction. Int J Cancer. 2006 Nov;119(10):2330–2338. Available from: http://dx.doi.org/10.1002/ijc.22108.

[130] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402–408. Available from: http://dx.doi.org/10.1006/meth.2001.1262.

[131] Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis. 1995 Jun;16(6):1034–1059. Available from: http://dx.doi.org/10.1002/elps.11501601175.

[132] Sitek B, Sipos B, Klöppel G, Schmiegel W, Hahn SA, Meyer HE, et al. Application of fluorescence dye saturation labeling for differential proteome analysis of 1,000 microdissected cells from pancreatic ductal adenocarcinoma precursor lesions. Methods Mol Biol. 2008;425:1–14. Available from: http://dx.doi.org/10.1007/978-1-60327-210-0_1.

[133] Nesterenko MV, Tilley M, Upton SJ. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J Biochem Biophys Methods. 1994 Apr;28(3):239–242. Available from: http://dx.doi.org/10.1016/0165-022X(94)90020-5.

[134] Nelson PT, Baldwin DA, Kloosterman WP, Kauppinen S, Plasterk RHA, Mourelatos Z. RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. RNA. 2006 Feb;12(2):187–191. Available from: http://dx.doi.org/10.1261/rna.2258506.

[135] Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci U S A. 2004 Aug;101(32):11755–11760. Available from: http://dx.doi.org/10.1073/pnas.0404432101.

[136] Nass D, Rosenwald S, Meiri E, Gilad S, Tabibian-Keissar H, Schlosberg A, et al. MiR-92b and miR-9/9* are specifically expressed in brain primary tumors and can be used to differentiate primary from metastatic brain tumors. Brain Pathol. 2009 Jul;19(3):375–383. Available from: http://dx.doi.org/10.1111/j.1750-3639.2008.00184.x.

[137] Hao-Xiang T, Qian W, Lian-Zhou C, Xiao-Hui H, Jin-Song C, Xin-Hui F, et al. MicroRNA-9 reduces cell invasion and E-cadherin secretion in SK-Hep-1 cell. Med Oncol. 2009 Jul;Available from: http://dx.doi.org/10.1007/s12032-009-9264-2.

[138] Lehmann U, Hasemeier B, Christgen M, Müller M, Römermann D, Länger F, et al. Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol. 2008 Jan;214(1):17–24. Available from: http://dx.doi.org/10.1002/path.2251.

[139] Laios A, O'Toole S, Flavin R, Martin C, Kelly L, Ring M, et al. Potential role of miR-9 and miR-223 in recurrent ovarian cancer. Mol Cancer. 2008;7:35. Available from: http://dx.doi.org/10.1186/1476-4598-7-35.

[140] Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood. 2007 Jun;109(11):4944–4951. Available from: http://dx.doi.org/10.1182/blood-2006-12-062398.

[141] Bottoni A, Piccin D, Tagliati F, Luchin A, Zatelli MC, degli Uberti EC. miR-15a and miR-16-1 down-regulation in pituitary adenomas. J Cell Physiol. 2005 Jul;204(1):280–285. Available from: http://dx.doi.org/10.1002/jcp.20282.

[142] Cohen EEW, Zhu H, Lingen MW, Martin LE, Kuo WL, Choi EA, et al. A feed-forward loop involving protein kinase Calpha and microRNAs regulates tumor cell cycle. Cancer Res. 2009 Jan;69(1):65–74. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-0377.

[143] Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, et al. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer. 2008 Apr;123(2):372–379. Available from: http://dx.doi.org/10.1002/ijc.23501.

[144] Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A, et al. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene. 2007 Jun;26(28):4148–4157. Available from: http://dx.doi.org/10.1038/sj.onc.1210186.

[145] Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, et al. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol. 2007 Mar;27(6):2240–2252. Available from: http://dx.doi.org/10.1128/MCB.02005-06.

[146] Bandi N, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, et al. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. Cancer Res. 2009 Jul;69(13):5553–5559. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-4277.

[147] Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, et al. MicroRNA Expression Profiles in Serous Ovarian Carcinoma. Clin Cancer Res. 2008 May;14(9):2690–2695. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-07-1731.

[148] O'Hara AJ, Chugh P, Wang L, Netto EM, Luz E, Harrington WJ, et al. Pre-micro RNA signatures delineate stages of endothelial cell transformation in Kaposi sarcoma. PLoS Pathog. 2009 Apr;5(4):e1000389. Available from: http://dx.doi.org/10.1371/journal.ppat.1000389.

[149] Iorio MV, Visone R, Leva GD, Donati V, Petrocca F, Casalini P, et al. MicroRNA signatures in human ovarian cancer. Cancer Res. 2007 Sep;67(18):8699–8707. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-07-1936.

[150] Wong TS, Liu XB, Wong BYH, Ng RWM, Yuen APW, Wei WI. Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue. Clin Cancer Res. 2008 May;14(9):2588–2592. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-07-0666.

[151] Lui WO, Pourmand N, Patterson BK, Fire A. Patterns of known and novel small RNAs in human cervical cancer. Cancer Res. 2007 Jul;67(13):6031–6043. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-06-0561.

[152] Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, et al. Down-regulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem. 2006 Oct;99(3):671–678. Available from: http://dx.doi.org/10.1002/jcb.20982.

[153] Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006 Feb;103(7):2257–2261. Available from: http://dx.doi.org/10.1073/pnas.0510565103.

[154] Roldo C, Missiaglia E, Hagan JP, Falconi M, Capelli P, Bersani S, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol. 2006 Oct;24(29):4677–4684. Available from: http://dx.doi.org/10.1200/JCO.2005.05.5194.

[155] Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer. 2007 Mar;120(5):1046–1054. Available from: http://dx.doi.org/10.1002/ijc.22394.

[156] Conti A, Aguennouz M, Torre DL, Tomasello C, Cardali S, Angileri FF, et al. miR-21 and 221 upregulation and miR-181b downregulation in human grade II-IV astrocytic tumors. J Neurooncol. 2009 Jul;93(3):325–332. Available from: http://dx.doi.org/10.1007/s11060-009-9797-4.

[157] Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA. 2008 Jan;299(4):425–436. Available from: http://dx.doi.org/10.1001/jama.299.4.425.

[158] Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. Oncogene. 2007 Apr;26(19):2799–2803. Available from: http://dx.doi.org/10.1038/sj.onc.1210083.

[159] Asangani IA, Rasheed SAK, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene. 2008 Apr;27(15):2128–2136. Available from: http://dx.doi.org/10.1038/sj.onc.1210856.

[160] Löffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermüller J, Kretzschmar AK, et al. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood. 2007 Aug;110(4):1330–1333. Available from: http://dx.doi.org/10.1182/blood-2007-03-081133.

[161] Fujita S, Ito T, Mizutani T, Minoguchi S, Yamamichi N, Sakurai K, et al. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. J Mol Biol. 2008 May;378(3):492–504. Available from: http://dx.doi.org/10.1016/j.jmb.2008.03.015.

[162] Knobbe CB, Reifenberger G. Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol. 2003 Oct;13(4):507–518. Available from: http://dx.doi.org/10.1111/j.1750-3639.2003.tb00481.x.

[163] Meng F, Henson R, Wehbe-Janek H, Smith H, Ueno Y, Patel T. The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. J Biol Chem. 2007 Mar;282(11):8256–8264. Available from: http://dx.doi.org/10.1074/jbc.M607712200.

[164] Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem. 2007 May;282(19):14328–14336. Available from: http://dx.doi.org/10.1074/jbc.M611393200.

[165] Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 2008 Mar;18(3):350–359. Available from: http://dx.doi.org/10.1038/cr.2008.24.

[166] Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, et al. MicroRNA-21 Targets Sprouty2 and Promotes Cellular Outgrowths. Mol Biol Cell. 2008 May;Available from: http://dx.doi.org/10.1091/mbc.E08-02-0159.

[167] Li T, Li D, Sha J, Sun P, Huang Y. MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. Biochem Biophys Res Commun. 2009 Jun;383(3):280–285. Available from: http://dx.doi.org/10.1016/j.bbrc.2009.03.077.

[168] Papagiannakopoulos T, Shapiro A, Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res. 2008 Oct;68(19):8164–8172. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-1305.

[169] Kim YK, Yu J, Han TS, Park SY, Namkoong B, Kim DH, et al. Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. Nucleic Acids Res. 2009 Apr;37(5):1672–1681. Available from: http://dx.doi.org/10.1093/nar/gkp002.

[170] Kan T, Sato F, Ito T, Matsumura N, David S, Cheng Y, et al. The miR-106b-25 polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim. Gastroenterology. 2009 May;136(5):1689–1700. Available from: http://dx.doi.org/10.1053/j.gastro.2009.02.002.

[171] Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, et al. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol. 2007;25(5):387–392. Available from: http://dx.doi.org/10.1016/j.urolonc.2007.01.019.

[172] Crosby ME, Kulshreshtha R, Ivan M, Glazer PM. MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res. 2009 Feb;69(3):1221–1229. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-2516.

[173] Greither T, Grochola L, Udelnow A, Lautenschläger C, Würl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumours associates with poorer survival. Int J Cancer. 2009 Jun;Available from: http://dx.doi.org/10.1002/ijc.24687.

[174] Fasanaro P, D'Alessandra Y, Stefano VD, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine-kinase ligand Ephrin-A3. J Biol Chem. 2008 Apr;Available from: http://dx.doi.org/10.1074/jbc.M800731200.

[175] Spence AM, Muzi M, Swanson KR, O'Sullivan F, Rockhill JK, Rajendran JG, et al. Regional Hypoxia in Glioblastoma Multiforme Quantified with [18F]Fluoromisonidazole Positron Emission Tomography before Radiotherapy: Correlation with Time to Progression and Survival. Clin Cancer Res. 2008 May;14(9):2623–2630. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-07-4995.

[176] Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol. 2008 May;141(5):672–675. Available from: http://dx.doi.org/10.1111/j.1365-2141.2008.07077.x.

[177] Rickert CH, Sträter R, Kaatsch P, Wassmann H, Jürgens H, Dockhorn-Dworniczak B, et al. Pediatric high-grade astrocytomas show chromosomal imbalances distinct from adult cases. Am J Pathol. 2001 Apr;158(4):1525–1532. Available from: http://ajp.amjpathol.org/cgi/content/abstract/158/4/1525.

[178] Pan YZ, Morris ME, Yu AM. MicroRNA-328 negatively regulates the expression of breast cancer resistance protein (BCRP/ABCG2) in human cancer cells. Mol Pharmacol. 2009 Jun;75(6):1374–1379. Available from: http://dx.doi.org/10.1124/mol.108.054163.

[179] Schäfer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, et al. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer. 2009 Aug;Available from: http://dx.doi.org/10.1002/ijc.24827.

[180] Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol. 1999 Jul;9(3):469–479. Available from: http://dx.doi.org/10.1111/j.1750-3639.1999.tb00536.x.

[181] Chen Y, Stallings RL. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. Cancer Res. 2007 Feb;67(3):976–983. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-06-3667.

[182] Wong TS, Ho WK, Chan JYW, Ng RWM, Wei WI. Mature miR-184 and squamous cell carcinoma of the tongue. ScientificWorldJournal. 2009;9:130–132. Available from: http://dx.doi.org/10.1100/tsw.2009.12.

[183] Shen J, Yang X, Xie B, Chen Y, Swaim M, Hackett SF, et al. MicroRNAs Regulate Ocular Neovascularization. Mol Ther. 2008 May;Available from: http://dx.doi.org/10.1038/mt.2008.104.

[184] Collins VP. Amplified genes in human gliomas. Semin Cancer Biol. 1993 Feb;4(1):27–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8448375.

[185] Nomura T, Kimura M, Horii T, Morita S, Soejima H, Kudo S, et al. MeCP2-dependent repression of an imprinted miR-184 released by depolarization. Hum Mol Genet. 2008 Apr;17(8):1192–1199. Available from: http://dx.doi.org/10.1093/hmg/ddn011.

[186] Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. Nat Rev Cancer. 2006 Jul;6(7):493–505. Available from: http://dx.doi.org/10.1038/nrc1885.

[187] Yokota T, Kouno J, Adachi K, Takahashi H, Teramoto A, Matsumoto K, et al. Identification of histological markers for malignant glioma by genome-wide expression analysis: dynein, alpha-PIX and sorcin. Acta Neuropathol. 2006 Jan;111(1):29–38. Available from: http://dx.doi.org/10.1007/s00401-005-1085-6.

[188] Qing Y, Yingmao G, Lujun B, Shaoling L. Role of Npm1 in proliferation, apoptosis and differentiation of neural stem cells. J Neurol Sci. 2008 Mar;266(1-2):131–137. Available from: http://dx.doi.org/10.1016/j.jns.2007.09.029.

[189] Palackal NT, Lee SH, Harvey RG, Blair IA, Penning TM. Activation of polycyclic aromatic hydrocarbon trans-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. J Biol Chem. 2002 Jul;277(27):24799–24808. Available from: http://dx.doi.org/10.1074/jbc.M112424200.

[190] Nagaraj NS, Beckers S, Mensah JK, Waigel S, Vigneswaran N, Zacharias W. Cigarette smoke condensate induces cytochromes P450 and aldo-keto reductases in oral cancer cells. Toxicol Lett. 2006 Aug;165(2):182–194. Available from: http://dx.doi.org/10.1016/j.toxlet.2006.03.008.

[191] Rizner TL, Smuc T, Rupreht R, Sinkovec J, Penning TM. AKR1C1 and AKR1C3 may determine progesterone and estrogen ratios in endometrial cancer. Mol Cell Endocrinol. 2006 Mar;248(1-2):126–135. Available from: http://dx.doi.org/10.1016/j.mce.2005.10.009.

[192] Amin SA, Huang CC, Reierstad S, Lin Z, Arbieva Z, Wiley E, et al. Paracrine-stimulated gene expression profile favors estradiol production in breast tumors. Mol Cell Endocrinol. 2006 Jul;253(1-2):44–55. Available from: http://dx.doi.org/10.1016/j.mce.2006.04.029.

[193] Fung KM, Samara ENS, Wong C, Metwalli A, Krlin R, Bane B, et al. Increased expression of type 2 3alpha-hydroxysteroid dehydrogenase/type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma. Endocr Relat Cancer. 2006 Mar;13(1):169–180. Available from: http://dx.doi.org/10.1677/erc.1.01048.

[194] Ragel BT, Couldwell WT, Gillespie DL, Jensen RL. Identification of hypoxia-induced genes in a malignant glioma cell line (U-251) by cDNA microarray analysis. Neurosurg Rev. 2007 Jul;30(3):181–7; discussion 187. Available from: http://dx.doi.org/10.1007/s10143-007-0070-z.

[195] Byrns MC, Steckelbroeck S, Penning TM. An indomethacin analogue, N-(4-chlorobenzoyl)-melatonin, is a selective inhibitor of aldo-keto reductase 1C3 (type 2 3alpha-HSD, type 5 17beta-HSD, and prostaglandin F synthase), a potential target for the treatment of hormone dependent and hormone independent malignancies. Biochem Pharmacol. 2008 Jan;75(2):484–493. Available from: http://dx.doi.org/10.1016/j.bcp.2007.09.008.

[196] Ducray F, Idbaih A, de Reynies A, Bieche I, Thillet J, Mokhtari K, et al. Anaplastic oligodendrogliomas with 1p19q codeletion have a proneural gene expression profile. Mol Cancer. 2008 May;7(1):41. Available from: http://dx.doi.org/10.1186/1476-4598-7-41.

[197] Wang S, Yang Q, Fung KM, Lin HK. AKR1C2 and AKR1C3 mediated prostaglandin D(2) metabolism augments the PI3K/Akt proliferative signaling pathway in human prostate cancer cells. Mol Cell Endocrinol. 2008 Apr;Available from: http://dx.doi.org/10.1016/j.mce.2008.04.004.

[198] Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. Blood. 2001 May;97(9):2815–2822. Available from: http://bloodjournal.hematologylibrary.org/cgi/content/short/97/9/2815.

[199] Poser I, Golob M, Weidner M, Buettner R, Bosserhoff AK. Down-regulation of COOH-terminal binding protein expression in malignant melanomas leads to induction of MIA expression. Cancer Res. 2002 Oct;62(20):5962–5966. Available from: http://cancerres.aacrjournals.org/cgi/content/full/62/20/5962.

[200] Nadauld LD, Phelps R, Moore BC, Eisinger A, Sandoval IT, Chidester S, et al. Adenomatous polyposis coli control of C-terminal binding protein-1 stability regulates expression of intestinal retinol dehydrogenases. J Biol Chem. 2006 Dec;281(49):37828–37835. Available from: http://dx.doi.org/10.1074/jbc.M602119200.

[201] Paliwal S, Pande S, Kovi RC, Sharpless NE, Bardeesy N, Grossman SR. Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. Mol Cell Biol. 2006 Mar;26(6):2360–2372. Available from: http://dx.doi.org/10.1128/MCB.26.6.2360-2372.2006.

[202] Jin W, Scotto KW, Hait WN, Yang JM. Involvement of CtBP1 in the transcriptional activation of the MDR1 gene in human multidrug resistant cancer cells. Biochem Pharmacol. 2007 Sep;74(6):851–859. Available from: http://dx.doi.org/10.1016/j.bcp.2007.06.017.

[203] Chen YW, Paliwal S, Draheim K, Grossman SR, Lewis BC. p19Arf inhibits the invasion of hepatocellular carcinoma cells by binding to C-terminal binding protein. Cancer Res. 2008 Jan;68(2):476–482. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-07-1960.

[204] Yao R, Lopez-Beltran A, Maclennan GT, Montironi R, Eble JN, Cheng L. Expression of S100 protein family members in the pathogenesis of bladder tumors. Anticancer Res. 2007;27(5A):3051–3058. Available from: http://ar.iiarjournals.org/content/27/5A/3051.abstract.

[205] Bianchi L, Canton C, Bini L, Orlandi R, Ménard S, Armini A, et al. Protein profile changes in the human breast cancer cell line MCF-7 in response to SEL1L gene induction. Proteomics. 2005 Jun;5(9):2433–2442. Available from: http://dx.doi.org/10.1002/pmic.200401283.

[206] Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, et al. Global gene expression profiling of circulating tumor cells. Cancer Res. 2005 Jun;65(12):4993–4997. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-04-4330.

[207] Knobbe CB, Trampe-Kieslich A, Reifenberger G. Genetic alteration and expression of the phosphoinositol-3-kinase/Akt pathway genes PIK3CA and PIKE in human glioblastomas. Neuropathol Appl Neurobiol. 2005 Oct;31(5):486–490. Available from: http://dx.doi.org/10.1111/j.1365-2990.2005.00660.x.

[208] Chan CB, Ye K. PIKE GTPase are phosphoinositide-3-kinase enhancers, suppressing programmed cell death. J Cell Mol Med. 2007;11(1):39–53. Available from: http://dx.doi.org/10.1111/j.1582-4934.2007.00014.x.

[209] Liu X, Hu Y, Hao C, Rempel SA, Ye K. PIKE-A is a proto-oncogene promoting cell growth, transformation and invasion. Oncogene. 2007 Jul;26(34):4918–4927. Available from: http://dx.doi.org/10.1038/sj.onc.1210290.

[210] Bataller L, Wade DF, Graus F, Rosenfeld MR, Dalmau J. The MAZ protein is an autoantigen of Hodgkin's disease and paraneoplastic cerebellar dysfunction. Ann Neurol. 2003 Jan;53(1):123–127. Available from: http://dx.doi.org/10.1002/ana.10434.

[211] Wang X, Southard R, Allred C, Talbert D, Wilson M, Kilgore M. MAZ drives tumor-specific expression of PPAR gamma 1 in breast cancer cells. Breast Cancer Res Treat. 2007 Sep;Available from: http://dx.doi.org/10.1007/s10549-007-9765-7.

[212] Raggo C, Ruhl R, McAllister S, Koon H, Dezube BJ, Früh K, et al. Novel cellular genes essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus. Cancer Res. 2005 Jun;65(12):5084–5095. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-04-2822.

[213] Jan SL, Meur NL, Cazes A, Philippe J, Cunff ML, Léger J, et al. Characterization of the expression of the hypoxia-induced genes neuritin, TXNIP and IGFBP3 in cancer. FEBS Lett. 2006 Jun;580(14):3395–3400. Available from: http://dx.doi.org/10.1016/j.febslet.2006.05.011.

[214] So CW, Caldas C, Liu MM, Chen SJ, Huang QH, Gu LJ, et al. EEN encodes for a member of a new family of proteins containing an Src homology 3 domain and is the third gene located on chromosome 19p13 that fuses to MLL in human leukemia. Proc Natl Acad Sci U S A. 1997 Mar;94(6):2563–2568. Available from: http://www.pnas.org/content/94/6/2563.abstract.

[215] Louie MC, Revenko AS, Zou JX, Yao J, Chen HW. Direct control of cell cycle gene expression by proto-oncogene product ACTR, and its autoregulation underlies its transforming activity. Mol Cell Biol. 2006 May;26(10):3810–3823. Available from: http://dx.doi.org/10.1128/MCB.26.10.3810-3823.2006.

[216] Stolfi C, Fina D, Caruso R, Caprioli F, Fantini MC, Rizzo A, et al. Mesalazine negatively regulates Cdc25A protein expression and promotes accumulation of colon cancer cells in S-Phase. Carcinogenesis. 2008 May;Available from: http://dx.doi.org/10.1093/carcin/bgn122.

[217] Bellacosa A, Testa JR, Moore R, Larue L. A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities. Cancer Biol Ther. 2004 Mar;3(3):268–275. Available from: http://www.landesbioscience.com/journals/cbt/article/703/.

[218] Koul D, Shen R, Bergh S, Sheng X, Shishodia S, Lafortune TA, et al. Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma. Mol Cancer Ther. 2006 Mar;5(3):637–644. Available from: http://dx.doi.org/10.1158/1535-7163.MCT-05-0453.

[219] Mizoguchi M, Betensky RA, Batchelor TT, Bernay DC, Louis DN, Nutt CL. Activation of STAT3, MAPK, and AKT in malignant astrocytic gliomas: correlation with EGFR status, tumor grade, and survival. J Neuropathol Exp Neurol. 2006 Dec;65(12):1181–1188. Available from: http://dx.doi.org/10.1097/01.jnen.0000248549.14962.b2.

[220] Riemenschneider MJ, Betensky RA, Pasedag SM, Louis DN. AKT activation in human glioblastomas enhances proliferation via TSC2 and S6 kinase signaling. Cancer Res. 2006 Jun;66(11):5618–5623. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-06-0364.

[221] Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal. 2002 May;14(5):381–395. Available from: http://dx.doi.org/10.1016/S0898-6568(01)00271-6.

[222] Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med. 2005;9(1):59–71. Available from: http://dx.doi.org/10.1111/j.1582-4934.2005.tb00337.x.

[223] Tanzer A, Stadler PF. Molecular evolution of a microRNA cluster. J Mol Biol. 2004 May;339(2):327–335. Available from: http://dx.doi.org/10.1016/j.jmb.2004.03.065.

[224] Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell. 2008 Apr;133(2):217–222. Available from: http://dx.doi.org/10.1016/j.cell.2008.04.001.

[225] Schulte JH, Horn S, Otto T, Samans B, Heukamp LC, Eilers UC, et al. MYCN regulates oncogenic MicroRNAs in neuroblastoma. Int J Cancer. 2008 Feb;122(3):699–704. Available from: http://dx.doi.org/10.1002/ijc.23153.

[226] Venturini L, Battmer K, Castoldi M, Schultheis B, Hochhaus A, Muckenthaler MU, et al. Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood. 2007 May;109(10):4399–4405. Available from: http://dx.doi.org/10.1182/blood-2006-09-045104.

[227] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene. 2006 Apr;25(17):2537–2545. Available from: http://dx.doi.org/10.1038/sj.onc.1209283.

[228] Takakura S, Mitsutake N, Nakashima M, Namba H, Saenko VA, Rogounovitch TI, et al. Oncogenic role of miR-17-92 cluster in anaplastic thyroid cancer cells. Cancer Sci. 2008 Jun;99(6):1147–1154. Available from: http://dx.doi.org/10.1111/j.1349-7006.2008.00800.x.

[229] Sikand K, Slane S, Shukla G. Intrinsic expression of host genes and intronic miRNAs in prostate carcinoma cells. Cancer Cell Int. 2009 Aug;9(1):21. Available from: http://dx.doi.org/10.1186/1475-2867-9-21.

[230] Shah YM, Morimura K, Yang Q, Tanabe T, Takagi M, Gonzalez FJ. Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. Mol Cell Biol. 2007 Jun;27(12):4238–4247. Available from: http://dx.doi.org/10.1128/MCB.00317-07.

[231] Fontana L, Fiori ME, Albini S, Cifaldi L, Giovinazzi S, Forloni M, et al. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. PLoS ONE. 2008;3(5):e2236. Available from: http://dx.doi.org/10.1371/journal.pone.0002236.

[232] Northcott PA, Fernandez-L A, Hagan JP, Ellison DW, Grajkowska W, Gillespie Y, et al. The miR-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by N-myc in sonic hedgehog-treated cerebellar neural precursors. Cancer Res. 2009 Apr;69(8):3249–3255. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-08-4710.

[233] Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem. 2007 Jan;282(4):2130–2134. Available from: http://dx.doi.org/10.1074/jbc.C600252200.

[234] Aguda BD, Kim Y, Piper-Hunter MG, Friedman A, Marsh CB. MicroRNA regulation of a cancer network: consequences of the feedback loops involving miR-17-92, E2F, and Myc. Proc Natl Acad Sci U S A. 2008 Dec;105(50):19678–19683. Available from: http://dx.doi.org/10.1073/pnas.0811166106.

[235] Brock M, Trenkmann M, Gay RE, Michel BA, Gay S, Fischler M, et al. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ Res. 2009 May;104(10):1184–1191. Available from: http://dx.doi.org/10.1161/CIRCRESAHA.109.197491.

[236] Nagel S, Venturini L, Przybylski GK, Grabarczyk P, Schmidt CA, Meyer C, et al. Activation of miR-17-92 by NK-like homeodomain proteins suppresses apoptosis via reduction of E2F1 in T-cell acute lymphoblastic leukemia. Leuk Lymphoma. 2009 Jan;50(1):101–108. Available from: http://dx.doi.org/10.1080/10428190802626632.

[237] Matsubara H, Takeuchi T, Nishikawa E, Yanagisawa K, Hayashita Y, Ebi H, et al. Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. Oncogene. 2007 Sep;26(41):6099–6105. Available from: http://dx.doi.org/10.1038/sj.onc.1210425.

[238] Hossain A, Kuo MT, Saunders GF. Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol. 2006 Nov;26(21):8191–8201. Available from: http://dx.doi.org/10.1128/MCB.00242-06.

[239] Lu Y, Thomson JM, Wong HYF, Hammond SM, Hogan BLM. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. Dev Biol. 2007 Oct;310(2):442–453. Available from: http://dx.doi.org/10.1016/j.ydbio.2007.08.007.

[240] Manni I, Artuso S, Careccia S, Rizzo MG, Baserga R, Piaggio G, et al. The microRNA miR-92 increases proliferation of myeloid cells and by targeting p63 modulates the abundance of its isoforms. FASEB J. 2009 Jul;Available from: http://dx.doi.org/10.1096/fj.09-131847.

[241] Knobbe CB, Merlo A, Reifenberger G. Pten signaling in gliomas. Neuro Oncol. 2002 Jul;4(3):196–211. Available from: http://neuro-oncology.dukejournals.org/cgi/reprint/4/3/196?view=long&pmid=12084351.

[242] Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nat Immunol. 2008 Apr;9(4):405–414. Available from: http://dx.doi.org/10.1038/ni1575.

[243] Hindges R, Hübscher U. DNA polymerase delta, an essential enzyme for DNA transactions. Biol Chem. 1997 May;378(5):345–362. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9191022.

[244] Pavlov YI, Frahm C, McElhinny SAN, Niimi A, Suzuki M, Kunkel TA. Evidence that errors made by DNA polymerase alpha are corrected by DNA polymerase delta. Curr Biol. 2006 Jan;16(2):202–207. Available from: http://dx.doi.org/10.1016/j.cub.2005.12.002.

[245] Goldsby RE, Hays LE, Chen X, Olmsted EA, Slayton WB, Spangrude GJ, et al. High incidence of epithelial cancers in mice deficient for DNA polymerase delta proofreading. Proc Natl Acad Sci U S A. 2002 Nov;99(24):15560–15565. Available from: http://dx.doi.org/10.1073/pnas.232340999.

6 Supplementary Figures and Tables

Supplementary Table 1: Differential protein expression caused by *miR-184* overexpression. A172 and T98G cells were transfected with *miR-184* precursors (pre-184) or negative controls (pre-NC). 2D-DIGE analyses were performed to identify differentially expressed proteins. Note that Npm1 is a predicted target of *miR-184*. [Spot No.: identification number of the protein spot on the gel images (Supplementary Figures 1 and 2); Accession (International Protein Index (IPI) accession number), Protein Name, and Gene Symbol: specification of the identified protein and the referring gene; Av. ratio: averaged fold change observed between the cells transfected with pre-184 related to pre-NC-transfected cells or the cells transfected with pre-NC related to pre-184-transfected cells, positive values refer to up-regulation and vice versa negative values refer to down-regulation in pre-184-transfected cells as compared to cells transfected with pre-NC; p-Value: value gained by performing an equal variance two tailed Student's T-test; Seq. p*I* and Seq. MW: isoelectric point and molecular weight calculated from the amino acid sequence based on database information; Seq. Coverage: ratio of the number of amino acids explained by peptides from experimental mass spectrometry data, to the total number of amino acids (protein sequence) in %; Mascot Score: probability score of the chosen search engine Mascot, a score greater than 67 is significant ($P < 0.05$).]

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
up-regulated in A172 cells transfected with pre-184 as compared to control-transfected (pre-NC) cells									
1	IPI00514561.1	Heterogeneous nuclear ribonucleoprotein K	HNRPK	1.4	0.028	5.3	47.5	60.3	253.0
2	IPI00216746.1	Heterogeneous nuclear ribonucleoprotein K	HNRPK	1.4	0.033	5.0	51.0	44.2	177.0
3	IPI00024175.3	Proteasome subunit alpha type-7	PSMA7	1.3	0.049	9.3	27.9	70.6	202.0
4	IPI00472102.3	61 kDa protein	HSPD1	1.3	0.043	5.6	61.2	40.9	87.0
5	IPI00383296.5	Heterogeneous nuclear ribonucleoprotein M	HNRPM	1.3	0.034	9.6	73.6	51.1	86.2
6	IPI00514561.1	Heterogeneous nuclear ribonucleoprotein K	HNRPK	1.3	0.011	5.3	47.5	44.4	148.0
7	IPI00219018.7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2	0.048	9.3	36.0	60.0	189.0
8	IPI00383296.5	Heterogeneous nuclear ribonucleoprotein M	HNRPM	1.2	0.023	9.6	73.6	57.5	284.0
9	IPI00013881.6	Heterogeneous nuclear ribonucleoprotein H	HNRPH1	1.2	0.049	5.9	49.2	63.9	230.0
10	IPI00220271.3	Alcohol dehydrogenase	AKR1A1	1.2	0.012	6.4	36.5	26.8	71.6
11	IPI00847536.1	heat shock 70kDa protein 1B	HSPA1B; HSPA1A	1.2	0.050	5.4	70.0	56.2	268.0

continued on the next page

Supplementary Table 1: Differential protein expression caused by *miR-184* re-expression. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
12	IPI00013894.1	Stress-induced-phosphoprotein 1	STIP1	1.2	0.015	6.4	62.6	47.1	216.0
down-regulated in A172 cells transfected with pre-184 as compared to control-transfected (pre-NC) cells									
13	IPI00418169.3	annexin A2	ANXA2	−6.6	0.044	9.2	40.4	45.1	114.0
14	IPI00295081.1	Tubulin gamma-1 chain	TUBG1	−2.3	0.002	5.7	51.1	22.8	121.0
15	IPI00018206.3	Aspartate amino-transferase, mitochondrial precursor	GOT2	−1.8	0.005	9.8	47.4	49.5	127.0
16	IPI00021828.1	Cystatin-B	CSTB	−1.6	0.049	7.9	11.1	61.2	91.3
17	IPI00293167.4	Stromal cell-derived factor 2 precursor	SDF2	−1.6	0.033	7.0	23.0	50.7	103.0
18	IPI00024540.3	Endophilin-B2	SH3GLB2	−1.4	0.043	5.6	43.9	26.1	72.4
19	IPI00025796.3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial precursor	NDUFS3	−1.4	0.045	7.8	30.2	56.8	196.0
20	IPI00027341.1	Macrophage-capping protein	CAPG	−1.4	0.032	5.9	38.5	30.7	85.2
21	IPI00793199.1	annexin IV	ANXA4	−1.4	0.044	5.8	36.1	63.2	152.0
22	IPI00008530.1	60S acidic ribosomal protein P0	RPLP0	−1.3	0.025	5.6	34.3	46.4	103.0
23	IPI00012011.6	Cofilin-1	CFL1	−1.3	0.039	9.1	18.5	77.7	149.0
24	IPI00012011.6	Cofilin-1	CFL1	−1.2	0.047	9.1	18.5	45.8	65.9
up-regulated in T98G cells transfected with pre-184 as compared to control-transfected (pre-NC) cells									
1	IPI00216308.5	Voltage-dependent anion-selective channel protein 1	VDAC1	2.8	0.012	9.2	30.8	61.1	190.0
2	IPI00021439.1	Actin, cytoplasmic 1	ACTB	2.2	0.045	5.2	41.7	33.1	94.7
3	IPI00021439.1	Actin, cytoplasmic 1	ACTB	2.2	0.044	5.2	41.7	34.1	95.4
4	IPI00025491.1	Eukaryotic initiation factor 4A-I	EIF4A1	2.1	0.040	5.2	46.1	35.2	80.6
5	IPI00026260.1	Nucleoside diphosphate kinase B	NME1; NME2	2.0	0.045	9.4	17.3	59.9	90.5
6	IPI00607693.2	carboxylesterase 1 isoform c precursor	CES1	1.9	0.009	6.2	62.4	18.0	69.2
7	IPI00465439.5	Fructose-bisphosphate aldolase A	ALDOA	1.8	0.016	9.2	39.4	32.7	82.2
8	IPI00021439.1	Actin, cytoplasmic 1	ACTB Actin.	1.8	0.010	5.2	41.7	78.4	209.0
9	IPI00843748.1	89 kDa protein	VCP	1.8	0.036	5.0	89.3	30.5	142.0
10	IPI00026260.1	Nucleoside diphosphate kinase B	NME1; NME2	1.8	0.010	9.4	17.3	59.9	90.5
11	IPI00022082.7	Septin-8	SEPT8	1.7	0.020	5.7	49.8	46.2	150.0
12	IPI00021439.1	Actin, cytoplasmic 1	ACTB	1.7	0.019	5.2	41.7	32.3	74.4
13	IPI00021439.1	Actin, cytoplasmic 1	ACTB	1.7	0.016	5.2	41.7	40.5	150.0

continued on the next page

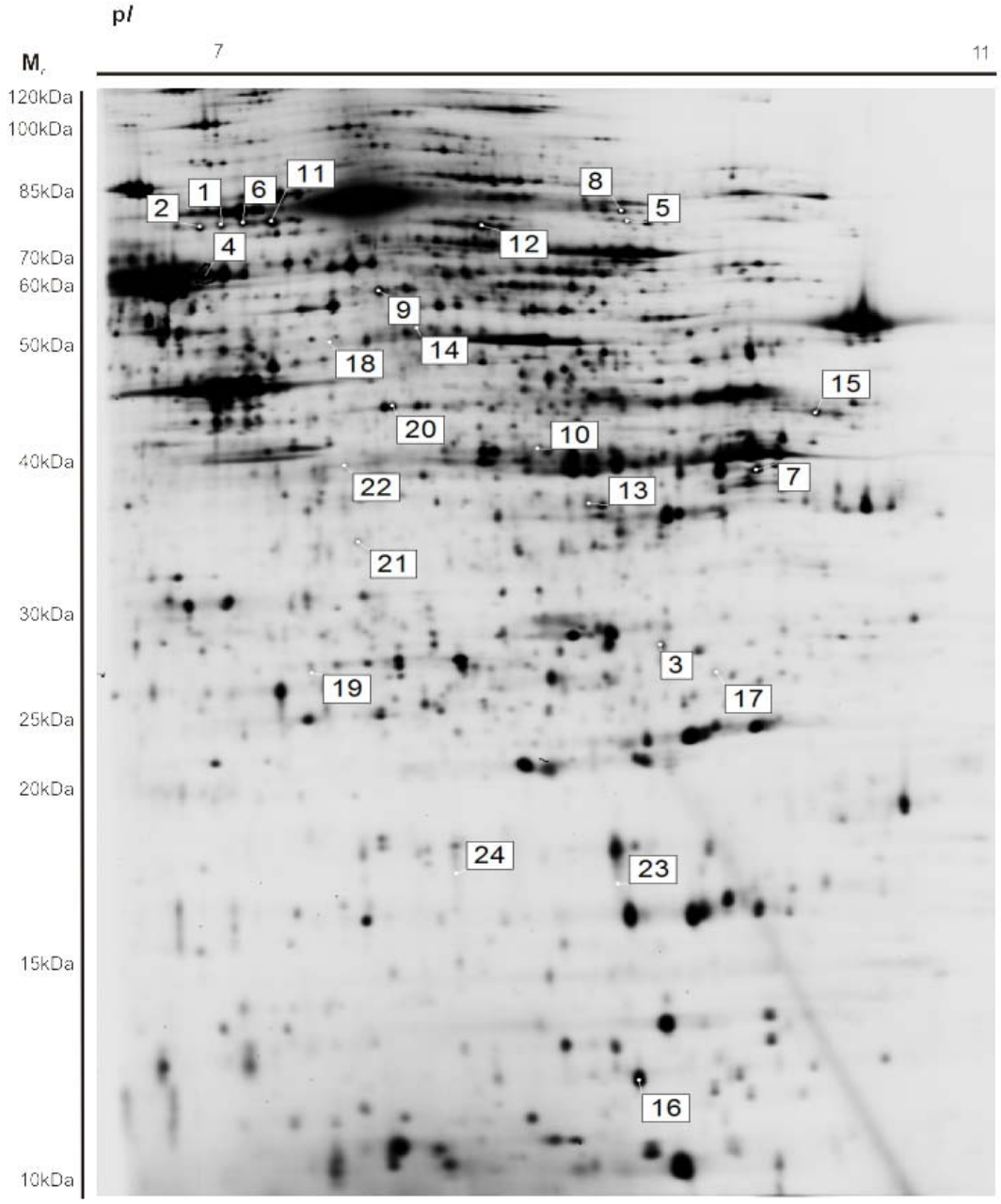
Supplementary Table 1: Differential protein expression caused by *miR-184* re-expression. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
14	IPI00021440.1	Actin, cytoplasmic 2	PSPHL; ACTG1	1.7	0.035	5.2	41.8	25.9	77.8
15	IPI00021440.1	Actin, cytoplasmic 2	PSPHL; ACTG1	1.7	0.031	5.2	41.8	62.7	164.0
16	IPI00003881.5	Heterogeneous nuclear ribonucleoprotein F	HNRPF	1.7	0.012	5.3	45.6	48.9	144.0
17	IPI00003362.2	HSPA5 protein	HSPA5	1.6	0.048	4.9	72.4	52.5	351.0
18	IPI00017855.1	Aconitate hydratase, mitochondrial precursor	ACO2	1.6	0.019	7.9	85.4	51.3	292.0
19	IPI00171903.2	Heterogeneous nuclear ribonucleoprotein M	HNRPM	1.6	0.039	9.6	77.5	19.6	68.8
20	IPI00011229.1	Cathepsin D precursor	CTSD	1.5	0.024	6.1	44.5	31.3	125.0
21	IPI00387144.4	Tubulin alpha-1B chain	TUBA1B	1.5	0.015	4.8	50.1	49.9	127.0
22	IPI00744692.1	Transaldolase	TALDO1	1.5	0.008	6.4	37.5	44.8	165.0
23	IPI00291419.5	Acetyl-CoA acetyltransferase, cytosolic	ACAT2	1.5	0.020	6.5	41.3	42.6	140.0
24	IPI00465439.5	Fructose-bisphosphate aldolase A	ALDOA	1.5	0.048	9.2	39.4	72.5	234.0
25	IPI00396378.3	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNP A2B1	1.4	0.026	9.3	37.4	34.6	88.4
26	IPI00002966.1	Heat shock 70 kDa protein 4	HSPA4	1.4	0.008	5.0	94.2	23.6	101.0
27	IPI00021439.1	Actin, cytoplasmic 1	ACTB	1.4	0.019	5.2	41.7	44.5	125.0
28	IPI00216746.1	Heterogeneous nuclear ribonucleoprotein K	HNRPK	1.4	0.018	5.0	51.0	40.5	150.0
29	IPI00021439.1	Actin, cytoplasmic 1	ACTB	1.4	0.030	5.2	41.7	58.4	170.0
30	IPI00418471.6	Vimentin	VIM	1.3	0.049	4.9	53.6	53.0	153.0
31	IPI00455315.4	Annexin A2	ANXA2	1.3	0.010	8.5	38.6	63.7	171.0
32	IPI00334190.4	Stomatin-like protein 2	STOML2	1.3	0.007	7.7	38.5	45.2	94.2
33	IPI00783586.1	43 kDa protein	NDRG1	1.3	0.018	5.4	42.8	29.2	66.0
34	IPI00297477.3	U2 small nuclear ribonucleoprotein A'	SNRPA1	1.3	0.030	9.4	28.4	29.4	120.0
35	IPI00305383.1	Cytochrome b-c1 complex subunit 2, mitochondrial precursor	UQCRC2	1.3	0.039	9.3	48.4	39.7	132.0
36	IPI00179964.5	Polypyrimidine tract-binding protein 1	PTBP1	1.2	0.001	9.8	57.2	25.2	73.3
37	IPI00002966.1	Heat shock 70 kDa protein 4	HSPA4	1.1	0.005	5.0	94.2	57.0	303.0
down-regulated in T98G cells transfected with pre-184 as compared to control-transfected (pre-NC) cells									
38	IPI00549248.4	Nucleophosmin	NPM1	−1.6	0.026	4.5	32.6	34.4	95.1
39	IPI00017855.1	Aconitate hydratase, mitochondrial precursor	ACO2	−1.6	0.050	7.9	85.4	46.0	225.0

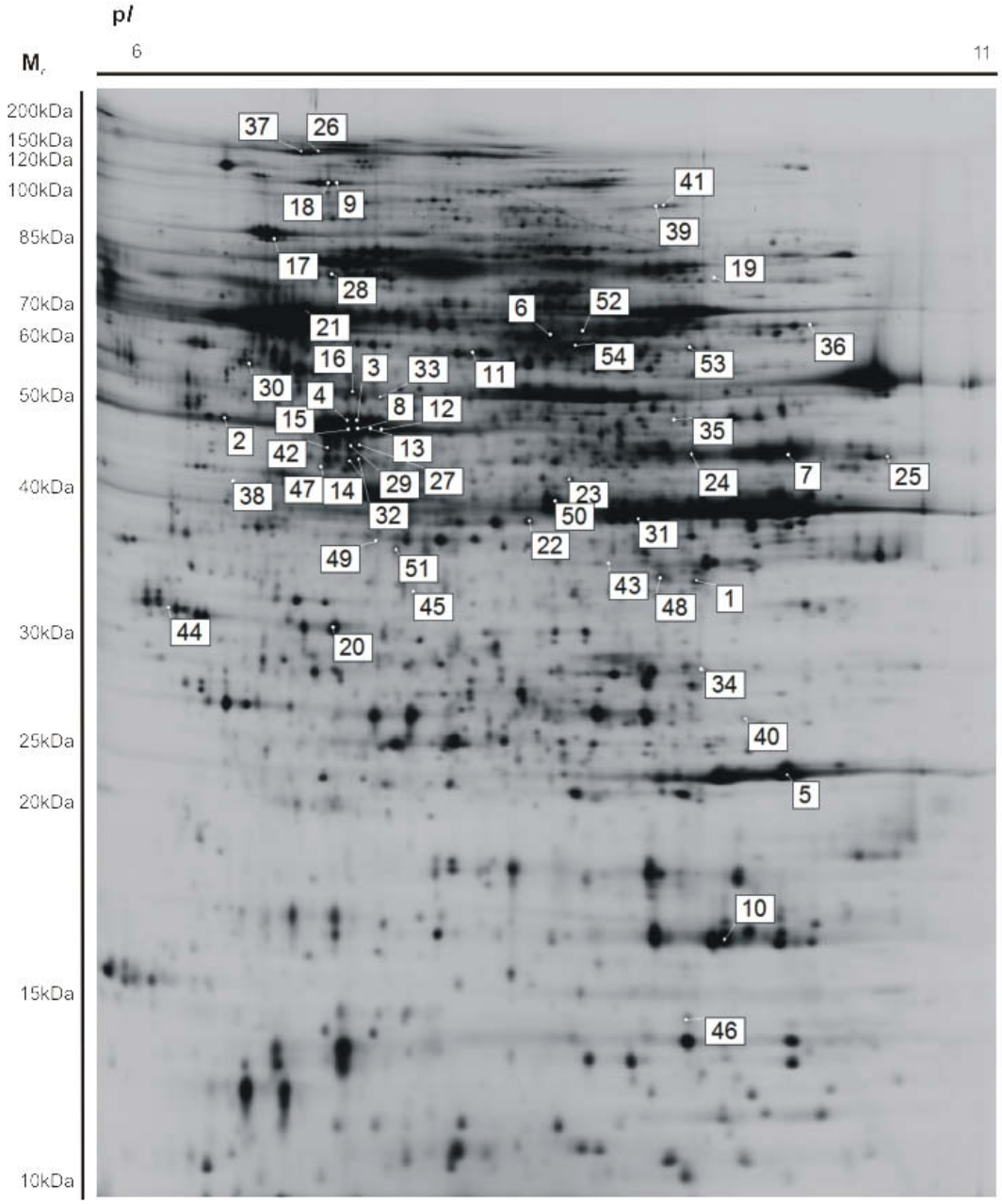
continued on the next page

Supplementary Table 1: Differential protein expression caused by *miR-184* re-expression. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
40	IPI00015833.1	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3	CHCHD3	−1.5	0.043	9.3	26.1	52.4	197.0
41	IPI00017855.1	Aconitate hydratase, mitochondrial precursor	ACO2	−1.5	0.023	7.9	85.4	48.2	214.0
42	IPI00005087.1	Tropomodulin-3	TMOD3	−1.4	0.041	4.9	39.6	53.1	225.0
43	IPI00411706.1	S-formylglutathione hydrolase	ESD	−1.4	0.018	6.6	31.4	60.3	114.0
44	IPI00010779.4	Tropomyosin alpha-4 chain	TPM4	−1.4	0.005	4.5	28.5	44.8	178.0
45	IPI00021440.1	Actin. cytoplasmic 2	PSPHL; ACTG1	−1.4	0.001	5.2	41.8	35.5	115.0
46	IPI00029744.1	Single-stranded DNA-binding protein, mitochondrial precursor	SSBP1	−1.4	0.046	9.9	17.2	41.2	88.6
47	IPI00021439.1	Actin, cytoplasmatic 1	ACTB	−1.3	0.033	5.2	41.7	42.7	98.2
48	IPI00641950.3	Lung cancer oncogene 7	GNB2L1	−1.3	0.012	9.6	37.9	70.6	198.0
49	IPI00549885.4	Pyruvate dehydrogenase E1 component subunit beta. mitochondrial precursor	PDHB	−1.3	0.033	5.6	37.2	26.7	72.2
50	IPI00218918.5	Annexin A1	ANXA1	−1.3	0.029	6.7	38.7	35.3	95.7
51	IPI00015018.1	Inorganic pyrophosphatase	PPA1	−1.3	0.029	5.5	32.6	74.0	212.0
52	IPI00289800.7	Glucose-6-phosphate 1-dehydrogenase	G6PD	−1.2	0.037	6.4	59.2	48.0	92.3
53	IPI00759575.1	Glutathione reductase, mitochondrial precursor	GSR I	−1.2	0.005	8.6	51.7	58.9	193.0
54	IPI00289800.7	Glucose-6-phosphate 1-dehydrogenase	G6PD	−1.2	0.007	6.4	59.2	77.5	375.0



Supplementary Figure 1: Representative proteome pattern of A172 cells transfected with *miR-184* precursors revealed by 2D-DIGE. Image analysis and subsequent MS-analyses led to the identification of 24 significantly regulated proteins (data obtained in collaboration with M. Grzendowski, MPC, Ruhr-University, Bochum).



Supplementary Figure 2: Representative proteome pattern of T98G cells transfected with *miR-184* precursors revealed by 2D-DIGE. Image analysis and subsequent MS-analyses led to the identification of 54 significantly regulated proteins (data obtained in collaboration with M. Grzendowski, MPC, Ruhr-University, Bochum).

Supplementary Table 2: Differential protein expression caused by inhibition of *miR-17*. A172 and T98G cells were transfected with *miR-17* inhibitors (anti-17) or negative controls (anti-NC). 2D-DIGE analyses were performed to identify differentially expressed proteins. Note that Pold2 is a predicted target of *miR-17*. [Spot No.: identification number of the protein spot on the gel images (Supplementary Figures 3 and 4); Accession (International Protein Index (IPI) accession number), Protein Name, and Gene Symbol: specification of the identified protein and the referring gene; Av. ratio: averaged fold change observed between the cells transfected with anti-17 related to anti-NC-transfected cells or the cells transfected with anti-NC related to anti-17-transfected cells, positive values refer to up-regulation and vice versa negative values refer to down-regulation in anti-17-transfected cells as compared to cells transfected with anti-NC; p-Value: value gained by performing an equal variance two tailed Student's T-test; Seq. p*I* and Seq. MW: isoelectric point and molecular weight calculated from the amino acid sequence based on database information; Seq. Coverage: ratio of the number of amino acids explained by peptides from experimental mass spectrometry data, to the total number of amino acids (protein sequence) in %; Mascot Score: probability score of the chosen search engine Mascot, a score greater than 67 is significant ($P < 0.05$).]

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
up-regulated in A172 cells transfected with anti-17 as compared to control-transfected (anti-NC) cells									
1	IPI00003362.2	HSPA5 protein	HSPA5	1.4	0.023	4.9	72.4	42.4	214.0
2	IPI00216746.1	Heterogeneous nuclear ribonucleoprotein K	HNRPK	1.3	0.026	5.0	51.0	41.8	197.0
3	IPI00845339.1	heat shock 70kDa protein 1A	HSPA1B; HSPA1A	1.3	0.025	5.4	70.0	51.0	193.0
4	IPI00003865.1	Heat shock cognate 71 kDa protein	HSPA8	1.2	0.022	5.2	70.9	51.7	220.0
5	IPI00219757.13	Glutathione S-transferase P	GSTP1	1.2	0.013	5.3	23.3	57.6	109.0
6	IPI00021440.1	Actin, cytoplasmic 2	PSPHL; ACTG1	1.2	0.026	5.2	41.8	25.3	76.5
7	IPI00396485.3	Elongation factor 1-alpha 1	EEF1A1	1.2	0.047	9.7	50.1	37.0	107.0
8	IPI00007765.5	Stress-70 protein, mitochondrial precursor	HSPA9	1.2	0.050	5.8	73.6	56.4	331.0
9	IPI00843975.1	Ezrin	VIL2	1.2	0.022	5.9	69.4	57.8	224.0
10	IPI00304925.5	Heat shock 70 kDa protein 1	HSPA1B; HSPA1A	1.2	0.001	5.4	70.0	43.2	147.0
11	IPI00479209.1	KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDRBS1	1.2	0.033	7.7	45.9	34.4	121.0
12	IPI00219219.3	Galectin-1	LGALS1	1.2	0.007	5.2	14.7	60.0	126.0
13	IPI00465248.5	Alpha-enolase	ENO1	1.2	0.012	7.7	47.1	52.1	177.0
14	IPI00010720.1	T-complex protein 1 subunit epsilon	CCT5	1.1	0.010	5.3	59.6	67.5	240.0
15	IPI00000105.4	Major vault protein	MVP	1.1	0.010	5.2	99.3	20.2	86.9

continued on the next page

Supplementary Table 2: Differential protein expression caused by inhibition of *miR-17*. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
16	IPI00007765.5	Stress-70 protein, mitochondrial precursor	HSPA9	1.1	0.003	5.8	73.6	49.9	216.0
17	IPI00465248.5	Alpha-enolase	ENO1	1.1	0.049	7.7	47.1	55.8	189.0
18	IPI00009771.6	Lamin-B2	LMNB2	1.1	0.016	5.4	69.9	31.8	163.0
19	IPI00414696.1	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNP A2B1	1.1	0.019	9.1	36.0	57.8	187.0
20	IPI00000105.4	Major vault protein	MVP	1.1	0.033	5.2	99.3	35.1	183.0
down-regulated in A172 cells transfected with anti-17 as compared to control-transfected (anti-NC) cells									
21	IPI00016832.1	Proteasome subunit alpha type-1	PSMA1	−1.6	0.042	6.2	29.5	54.4	101.0
22	IPI00396485.3	Elongation factor 1-alpha 1	EEF1A1	−1.3	0.049	9.7	50.1	45.2	127.0
23	IPI00479186.5	Pyruvate kinase isozymes M1/M2	PKM2	−1.3	0.043	9.0	57.9	75.3	350.0
up-regulated in T98G cells transfected with anti-17 as compared to anti-NC									
1	IPI00002922.5	F-box-like/WD repeat-containing protein TBL1XR1	TBL1XR1	1.5	0.032	5.2	55.6	33.5	88.9
2	IPI00168554.1	Sulfiredoxin-1	SRXN1; SCRT2	1.4	0.016	9.1	14.3	65.0	84.0
3	IPI00465248.5	Alpha-enolase	ENO1	1.4	0.015	7.7	47.1	56.5	195.0
4	IPI00017855.1	Aconitate hydratase, mitochondrial precursor	ACO2	1.3	0.039	7.9	85.4	36.4	172.0
5	IPI00657680.1	Uncharacterized protein PDIA3	PDIA3	1.3	0.001	6.4	54.9	50.5	228.0
6	IPI00297779.7	T-complex protein 1 subunit beta	CCT2	1.3	0.029	6.0	57.5	43.0	97.1
7	IPI00025616.1	DNA polymerase subunit delta-2	POLD2	1.3	0.033	5.2	51.3	29.2	68.9
8	IPI00025512.2	Heat shock protein beta-1	HSPB1	1.3	0.037	6.0	22.8	33.7	72.0
9	IPI00182728.2	Vacuolar protein sorting-associating protein 4B	VPS4B	1.3	0.039	6.9	49.3	30.4	76.7
10	IPI00218342.10	C-1-tetrahydrofolate synthase. cytoplasmic	MTHFD1	1.3	0.036	7.0	101.5	57.6	405.0
11	IPI00032826.1	Hsc70-interacting protein	ST13	1.3	0.013	5.0	41.3	32.8	69.6
12	IPI00011126.6	26S protease regulatory subunit 4	PSMC1	1.2	0.010	5.8	49.2	39.8	140.0
13	IPI00025252.1	Protein disulfide-isomerase A3 precursor	PDIA3	1.2	0.009	5.9	56.7	46.9	204.0
14	IPI00784104.1	Sequestosome-1	SQSTM1	1.2	0.004	5.2	38.6	54.8	134.0
15	IPI00444704.2	G-rich sequence factor 1	GRSF1	1.2	0.038	5.4	48.0	32.5	88.7
16	IPI00643920.2	Transketolase	TKT	1.2	0.016	8.5	67.8	56.7	177.0

continued on the next page

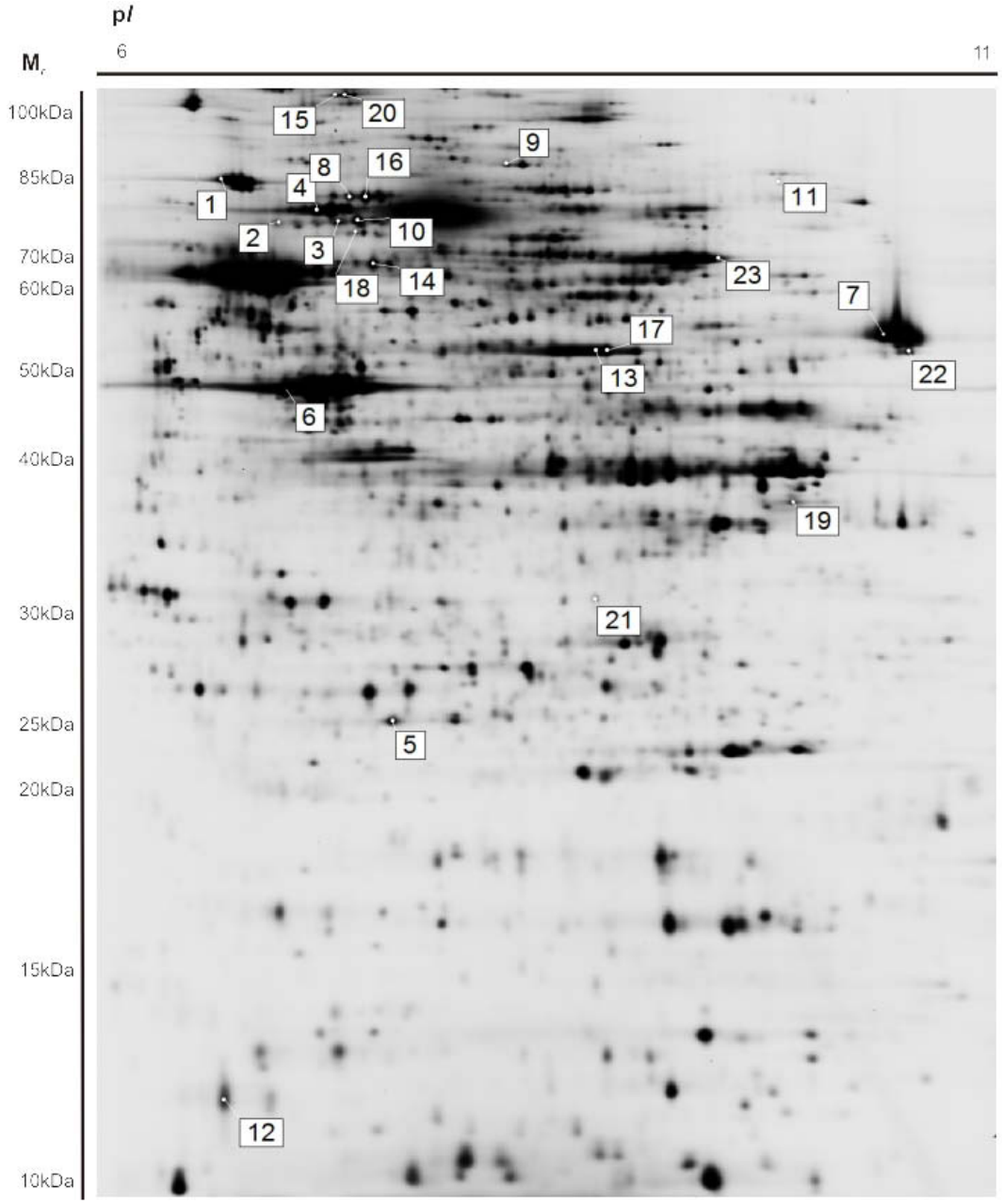
Supplementary Table 2: Differential protein expression caused by inhibition of *miR-17*. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
17	IPI00010720.1	T-complex protein 1 subunit epsilon	CCT5	1.2	0.002	5.3	59.6	46.6	90.8
18	IPI00783357.1	Sequestosome-1	SQSTM1	1.2	0.012	5.0	47.7	48.6	148.0
19	IPI00289800.7	Glucose-6-phosphate 1-dehydrogenase	G6PD	1.2	0.010	6.4	59.2	63.7	295.0
20	IPI00304925.5	Heat shock 70 kDa protein 1	HSPA1B; HSPA1A	1.2	0.046	5.4	70.0	54.4	224.0
21	IPI00219018.7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2	0.016	9.3	36.0	57.9	123.0
22	IPI00303476.1	ATP synthase subunit beta, mitochondrial precursor	ATP5B	1.2	0.028	5.1	56.5	63.9	224.0
23	IPI00018452.1	Copine-1	CPNE1	1.2	0.044	5.4	59.0	25.1	90.6
24	IPI00550689.3	UPF0027 protein C22orf28	C22orf28	1.2	0.028	6.9	55.2	60.4	224.0
25	IPI00008475.1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	HMGCS1	1.1	0.014	5.1	57.3	44.4	180.0
26	IPI00012795.3	Eukaryotic translation initiation factor 3 subunit 2	EIF3I	1.1	0.006	5.3	36.5	42.2	126.0
27	IPI00031420.3	UDP-glucose 6-dehydrogenase	UGDH	1.1	0.011	6.9	55.0	79.6	166.0
28	IPI00150961.2	membrane component chromosome 11 surface marker 1 isoform 1	CAPRIN1	1.1	0.032	5.0	78.3	22.0	81.6
29	IPI00021405.3	Lamin-A/C	LMNA	1.1	0.012	6.6	74.1	47.7	235.0
30	IPI00746165.2	WD repeat-containing protein 1	WDR1	1.1	0.038	6.2	66.2	59.6	271.0
31	IPI00395646.1	Thioredoxin domain containing 5	TXNDC5; MUTED	1.1	0.045	5.7	43.6	42.7	118.0
32	IPI00465439.5	Fructose-bisphosphate aldolase A	ALDOA	1.1	0.045	9.2	39.4	34.6	101.0
33	IPI00029079.5	GMP synthase	GMPS	1.1	0.004	6.4	76.7	37.5	172.0
34	IPI00440493.2	ATP synthase subunit alpha, mitochondrial precursor	ATP5A1	1.1	0.028	9.6	59.7	37.4	126.0
35	IPI00306369.3	tRNA	NSUN2	1.1	0.031	6.3	86.4	46.9	186.0
36	IPI00788802.1	Transketolase variant (Fragment)	TKT	1.1	0.003	8.8	67.9	46.1	206.0
down-regulated in T98G cells transfected with anti-17 as compared to control-transfected (anti-NC) cells									
37	IPI00012011.6	Cofilin-1	CFL1	−1.7	0.007	9.1	18.5	59.0	125.0
38	IPI00171199.5	Proteasome subunit alpha type-3	PSMA3	−1.5	0.005	5.1	27.6	33.9	99.4
39	IPI00216691.5	Profilin-1	PFN1	−1.3	0.027	9.4	15.0	60.7	81.2
40	IPI00015842.1	Reticulocalbin-1 precursor	RCN1	−1.2	0.011	4.7	38.9	44.4	149.0

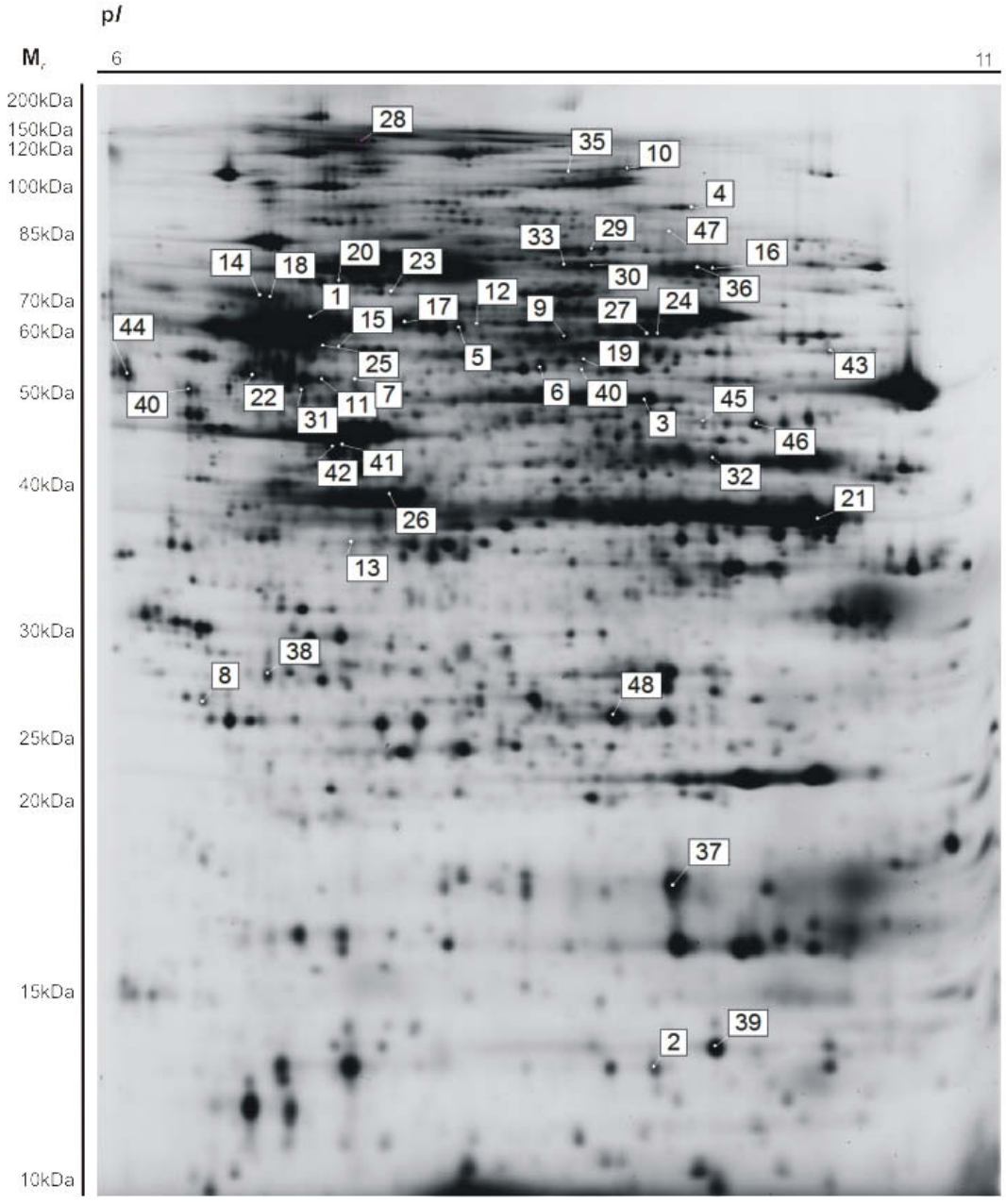
continued on the next page

Supplementary Table 2: Differential protein expression caused by inhibition of *miR-17*. (continued)

Spot No.	Accession	Protein	Gene Symbol	Av. Ratio	p-Value	Seq. p*I*	Seq. MW	Seq. Coverage	Mascot Score
41	IPI00096066.2	Succinyl-CoA ligase [GDP-forming] beta-chain, mitochondrial precursor	SUCLG2	−1.2	0.006	6.1	46.5	23.6	70.8
42	IPI00021440.1	Actin, cytoplasmic 2	PSPHL; ACTG1	−1.2	0.004	5.2	41.8	70.7	213.0
43	IPI00334175.3	Polypyrimidine tract-binding protein 1	PTBP1	−1.1	0.040	9.7	59.0	29.1	89.5
44	IPI00014537.3	Calumenin precursor	CALU	−1.1	0.010	4.3	37.1	52.4	142.0
45	IPI00011107.2	Isocitrate dehydrogenase [NADP], mitochondrial precursor	IDH2	−1.1	0.015	9.6	50.9	19.5	72.4
46	IPI00290416.3	Obg-like ATPase 1	OLA1	−1.1	0.000	8.6	44.7	73.2	258.0
47	IPI00386114.1	Splicing factor 1	SF1	−1.1	0.042	9.7	68.3	18.6	74.0
48	IPI00465028.7	Triosephosphate isomerase	TPI1	−1.1	0.045	5.6	30.8	50.3	133.0



Supplementary Figure 3: Representative proteome pattern of A172 cells transfected with *miR-17* inhibitors revealed by 2D-DIGE. Image analysis and subsequent MS-analyses led to the identification of 23 significantly regulated proteins (data obtained in collaboration with M. Grzendowski, MPC, Ruhr-University, Bochum).



Supplementary Figure 4: Representative proteome pattern of T98G cells transfected with *miR-17* inhibitors revealed by 2D-DIGE. Image analysis and subsequent MS-analyses led to the identification of 48 significantly regulated proteins (data obtained in collaboration with M. Grzendowski, MPC, Ruhr-University, Bochum).

7 Abbreviations

2D-DIGE	two-dimensional difference gel electrophoresis
AAIII	anaplastic astrocytoma, WHO grade III
AII	diffuse astrocytoma, WHO grade II
ac-pre-miRNA	Ago2-cleaved precursor miRNA
anti-17	*miR-17* inhibiting oligonucleotide
anti-NC	negative control for miRNA inhibition experiments
AOAIII	anaplastic oligoastrocytoma, WHO grade III
ARE	adenine/uracil(AU)-rich element
ATP	adenosine tri-phosphate
B-CLL	B-cell chronic lymphocytic leukemia
Bis-Tris	Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane
BrdU	Bromodeoxyuridine
BSA	bovine serum albumine
CD133	cluster of differentiation 133
cDNA	copy deoxyribonucleic acid
CHAPS	3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonic acid
CML	chronic myeloid leukemia
CNS	central nervous system
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's modification Eagle's medium
DNA	deoxyribonucleic acid
ds-	double-stranded *(prefix)*
ELISA	enzyme linked immunosorbent assay
HIFBS	heat-inactivated fetal bovine serum

hsa-	homo sapiens *(prefix in miRNA names)*
IEF	isoelectric focusing
IPI	International Protein Index
IRES	internal ribosomal entry site
kDa	Kilodaltons
LOH	loss of heterozygosity
MALDI	matrix-assisted laser desorption/ionization
miRNA	micro ribonucleic acid
miRNP	miRNA containing ribonucleoprotein
MPC	Medical Proteome Center
MRI	magnetic resonance imaging
mRLC	miRNA RISC loading complex
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NB	non-neoplastic brain tissue
NBl	normal blood
OAII	diffuse oligoastrocytoma, WHO grade II
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
P-bodies	processing bodies
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pre-184	synthetic *miR-184* precursor molecule
pre-miRNA	miRNA precursor
pre-NC	negative control for miRNA overexpression experiments

pri-miRNA	miRNA primary transcript
PVDF	polyvinylidene difluoride
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
sGBIV	secondary glioblastoma, WHO grade IV
siRNA	small interfering RNA
ss-	single-stranded *(prefix)*
TOF	time-of-flight
TrisHCl	Tris(hydroxymethyl)aminomethane hydrochloride
UTR	untranslated region
WHO	World Health Organization

Genes, transcripts, and proteins are abbreviated according to the National Center for Biotechnology Information (NCBI) GenBank short forms of the gene names (free online access is available via http://www.ncbi.nlm.nih.gov/).
Gene symbols in capital letters refer to the gene locus or the mRNA transcript. If only the first letter of the gene symbol is an upper-case character, the abbreviation refers to the respective protein.
The annotation of miRNAs is explained in the section “Genomic organization”.

8 Abstract

Diffuse Astrozytome des WHO (World Health Organization) Grades II tendieren zur spontanen Progression zu anaplastischen Astrozytomen (WHO Grad III) oder sekundären Glioblastomen (WHO Grad IV). MicroRNAs (miRNAs) sind in der Regel nur 22 Nukleotide kurze, endogene, einzelsträngige, nicht für Proteine kodierende RNA Moleküle, die regulatorisch in die Genexpression eingreifen. Maligne Tumoren zeigen häufig Veränderungen in der Expression und Funktion verschiedener miRNAs. In der vorliegenden Arbeit wurde eine mögliche Rolle von miRNAs in der Progression von primär niedriggradigen zu sekundär hochgradigen Gliomen untersucht. Hierfür wurden von vier Patienten mit primären WHO Grad II Gliomen, die im Rezidiv einen spontanen Progress zu sekundären WHO Grad IV Glioblastomen aufwiesen, Expressionsprofile von 157 miRNAs mittels quantitativer PCR-Analysen erstellt. Diese Experimente ergaben für 12 miRNAs (*miR-9*, *miR-15a*, *miR-16*, *miR-17*, *miR-19a*, *miR-20a*, *miR-21*, *miR-25*, *miR-28*, *miR-130b*, *miR-140* und *miR-210*) eine erhöhte Expression und für zwei miRNAs (*miR-184* und *miR-328*) eine verminderte Expression in den Rezidiv- im Vergleich zu den zugehörigen Primärtumoren. Die Progressions-assoziierte differentielle Expression von sechs der miRNAs (*miR-16*, *miR-17*, *miR-19a*, *miR-20a*, *miR-140* und *miR-184*) wurde in niedriggradigen Primär- und hochgradigen Rezidivtumoren von drei zusätzlichen Patienten sowie in einer Serie von 27 unabhängigen Patienten, darunter 13 Patienten mit einem diffusen Astrozytom und 14 Patienten mit einem sekundären Glioblastom, untersucht. Hierbei wurden die Progressions-assoziierte Herunterregulation von *miR-184* und die Hochregulation von *miR-17* in beiden Validierungsgruppen bestätigt. Die differentielle Expression dieser beiden miRNA Kandidaten wurde nicht durch Amplifikation beziehungsweise Deletion der zugehörigen Genloci verursacht. Funktionelle Analysen der beiden miRNAs *in vitro* erbrachten miRNA-spezifische Einflüsse auf Vitalität, Proliferation, Apoptose und invasives Wachstumspotential von A172 und T98G Gliomzelllen. Die Bestimmung von mRNA- und Protein-Expressionsprofilen nach Überexpression von *miR-184* oder Inhibition von *miR-17* in den Gliomzellen ergab eine Reihe von Transkripten und Proteinen, die differentiell durch Modulation der Expression der miRNAs exprimiert wurden. So wurde nach Überexpression von *miR-184* für das *AKT2* Gen sowohl auf mRNA- als auch auf Proteinebene eine deutlich verminderte Expression in Gliomzellen nachgewiesen. Dieses Ergebnis steht im Einklang mit einer geringeren Akt2 Proteinexpression in diffusen Astrozytomen im Vergleich zu sekundären Glioblastomen. Akt2 ist ein vorhergesagtes Target von *miR-184*, so dass indirekte oder direkte Regulationsmechanismen die differentielle Akt2 Expression bedingen könnten. Darüber hinaus wurde Npm1 mit Hilfe der 2D-Fluoreszenz-Differenz-Gelelektrophorese als weiteres vorhergesagtes Target von *miR-184* identifiziert, das in Gliomzellen, die *miR-184* überexprimieren, herunterreguliert war. Mit Pold2 wurde ein mögliches Target von *miR-17* gefunden, das nach Inhibition von *miR-17* in Gliomzellen hochreguliert wurde. Zusammenfassend sprechen die eigenen Ergebnisse für eine wichtige Rolle einer veränderten Expression bestimmter miRNAs in Gliomen und weisen mit *miR-184* und *miR-17* auf zwei neue hochinteressante Kandidaten hin, die für die spontane maligne Progression von primär niedriggradigen zu sekundär hochgradigen Gliomen von Bedeutung sind.

A Eidesstattliche Erklärung

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Insbesondere habe ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen.

Die Arbeit wurde bisher noch von keiner medizinischen Fakultät abgelehnt.

Ich bin mir darüber klar, dass der Bruch der obigen Eidesstattlichen Versicherung in jedem Fall zur Folge hat, dass die Fakultät die Promotion widerruft.

B Danksagung

Mein erster Dank gilt Herrn Prof. Dr. Guido Reifenberger, der mir dieses aktuelle und spannende Thema anvertraut hat, mein Dissertationsprojekt fortwährend mit großem Interesse begleitete und mir Gelegenheiten eröffnete, selbstständig erste Schritte in allen Bereichen des wissenschaftlichen Arbeitens – von der Pipette bis zur Publikation – zu gehen.

Frau Dr. Marietta Wolter gebührt mein herzlicher Dank. Sie stand mir während der gesamten Doktorarbeit mit exzellentem fachlichen Rat zur Seite und unterstützte den Fortschritt der Arbeit tatkräftig. Darüberhinaus fieberte sie bei Experimenten und Vorträgen mit und half mit viel Humor auch über kleinere Durststrecken hinweg.

Ich danke Herrn Dr. Michael Grzendowski für die Zusammenarbeit bei den Proteomanalysen und die interessanten Labortage in Bochum. Herrn Dr. Gernot Röder sei für die Hybridisierung der Affymetrixchips gedankt.

Allen Mitarbeiter des Instituts für Neuropathologie danke ich für Ihre Unterstützung und das freundschaftliche Miteinander im Labor, sowie die ausgelassene Stimmung bei Aktivitäten nach getaner Arbeit.

Zuletzt möchte ich mich bei meiner Freundin, meiner Familie und meinen Freunden bedanken, die diese Arbeit auf ihre Weise unterstützt haben und auch für nächtliche und wochenendliche Laborsitzungen Verständnis aufbrachten.

C Curriculum vitae

Bastian Malzkorn

geboren am 26. Mai 1983 in Hilden

Schullaufbahn

1989-1993	Gemeinschaftsgrundschule Zur Verlach, Hilden
1993-2002	Helmholtz Gymnasium, Hilden
06/2002	Allgemeine Hochschulreife

Zivildienst

2002-2003	Zivildienst im St. Vinzenz Krankenhaus, Düsseldorf

Studium

2003-2009	Studium der Medizin an der Heinrich-Heine-Universität Düsseldorf
09/2005	1. Abschnitt der Ärztlichen Prüfung
2008-2009	Praktisches Jahr am Universitätsklinikum Düsseldorf, Wahlfach Neurologie
11/2009	2. Abschnitt der Ärztlichen Prüfung

Stipendium

2003	Aufnahme in die Förderung der Studienstiftung des deutschen Volkes
08/2004	Sommerakademie der Studienstiftung in Salem: Arbeitsgruppe Neuroprotektion
08/2008	Sommerakademie der Studienstiftung in Neubeuren: Arbeitsgruppe Stammzellbiologie und Tissue Engineering

Promotion

2006-2008	Experimenteller Teil der Doktorarbeit am Institut für Neuropathologie der Universität Düsseldorf

Präsentation von Ergebnissen der Doktorarbeit

11/2006	Vortrag beim miRNA Symposium des BMFZ, Düsseldorf
06/2007	Posterpräsentation am TdwN, Düsseldorf
08/2007	Vortrag auf der 2. Klausurtagung des BMFZ, Wermelskirchen
11/2007	Posterpräsentation beim NGFN-Meeting, Heidelberg
01/2008	Vortrag im Rahmen der European RNA Symposia Tour, Düsseldorf
06/2008	Posterpräsentation beim Treffen der NOA, Bochum
10/2008	Vortrag auf der 53. Jahrestagung der DGNN, Würzburg

Publikationen

08/2009	Malzkorn B, Wolter M, Liesenberg F, Grzendowski M, Stühler K, Meyer HE, Reifenberger G. Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathology. 2009 Aug (published online ahead of print).
08/2009	Malzkorn B, Wolter M, Reifenberger G. MicroRNA: Biogenesis, regulation, and role in primary brain tumors. In: Erdmann, VA, Reifenberger G, Barciszewski J, editors. Therapeutic Ribonucleic Acids in Brain Tumors. Springer; 2009. pp. 327-354