Identification of lethal microRNAs specific for head and neck cancer

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Abstract

Purpose: The prognosis of head and neck squamous cell carcinomas (HNSCC) remains disappointing and the development of novel anti-cancer agents is urgently awaited. We identified by a functional genetic screen microRNAs that are selectively lethal for head and neck cancer cells and not for normal cells, and investigated the genes targeted by them.

Experimental Design: A retroviral expression library of human microRNAs was introduced in HNSCC cell lines and normal oropharyngeal keratinocytes to identify tumor-selective lethal microRNAs. Potential downstream gene targets of these microRNAs were identified by gene expression profiling, and validated by functional assays.

Results: We identified six microRNAs that selectively inhibit proliferation of head and neck cancer cells. By gene expression profiling and 3'UTR assays we showed that the ataxia telangiectasia mutated (ATM) gene is a common target for at least two and likely three of these microRNAs. Specific inhibition of ATM resulted in a similar tumor-specific lethal effect, while the phenotype was reverted in rescue experiments.

Conclusions: These six microRNA might be developed as novel anti-cancer agents, and highlight ATM as an interesting novel therapeutic target for head and neck cancer.

Statement of translational relevance

Head and neck cancer is a disease with grim prognosis and novel treatments are urgently awaited. Here we identified microRNAs that show a tumor-selective lethal effect and may serve as a new therapeutic agents in head and neck cancer. Intratumoral injection in combination with electroporation of these microRNAs in head and neck tumors might be feasible, since access to the tumors is relatively easy. Importantly, we further show that the ATM gene is a target for some of these microRNAs and specific inhibitors of ATM showed a similar tumor-specific lethal effect. The application of ATM directed drugs might be a very interesting approach for trials specifically focusing on head and neck cancer.
Introduction

Head and neck squamous cell carcinoma (HNSCC) develops in the mucosal linings of the upper aerodigestive tract and contributes to approximately 5% of all cancers in the Western world (1, 2). Well-known risk factors are tobacco smoking, excessive consumption of alcohol containing beverages, and infection with the human papillomavirus (HPV) (3-5). About one third of the patients present with early stage tumors and receive single modality treatment, either surgery or radiotherapy. The five-year-survival rates for patients with early disease stages are over 90%. Unfortunately the majority of HNSCC patients present with advanced disease stages. These patients are treated with either a combination of surgery and radiotherapy or chemoradiation, the concurrent application of systemic cisplatin chemotherapy combined with locoregional radiotherapy. Patients with advanced disease stage frequently develop locoregional recurrences, distant metastasis and/or second primary tumors, which results in five-year-survival rates of less than 60% (1). Therefore, the development of novel anti-cancer agents to improve outcome is urgently awaited.

MicroRNAs (miRNAs) are ~22 nucleotide long, non-coding RNAs that are able to regulate the expression of multiple target genes (6). Classically, miRNAs regulate the expression of target genes through sequence-specific complementarity between the miRNA seed sequence and the 3’ untranslated region (UTR) of the target mRNAs (7). Perfect complementarity between the miRNA and the mRNA will generally target the mRNA for degradation, while imperfect complementarity of the miRNA to the 3’UTR will preferentially repress mRNA translation (8). However, insights on the interaction of miRNA with their target genes evolve continuously. Through this regulation at the posttranscriptional level, miRNAs are able to modulate the expression of numerous genes simultaneously, thereby regulating individual signaling pathways at multiple levels (9).

Several studies have shown the importance of miRNAs in cancer, including HNSCC. Altered miRNA expression profiles have been observed in both HNSCC cell lines and tumors when compared to the normal counterpart tissues (10, 11). The overexpression of
miR-21 is described in many tumor types, including HNSCC, and overexpression of miR-21 has also been associated with increased expression in preneoplastic lesions during malignant progression (10). A number of differentially expressed miRNAs were shown to be associated with poor prognosis, such as miR-21 and miR-211 (12, 13). Even more interestingly, recent studies have revealed that miRNAs may act as tumor suppressors by targeting oncogenes. For example, the miR-16 family inhibits cell cycle progression and induces apoptosis via the silencing of BCL2 (14), described in multiple tumor types including HNSCC. In a similar manner, ectopic expression of miR-181a resulted in decreased proliferation by targeting the oncogene KRAS (15).

In the present study we examined the potential of miRNAs for treatment of HNSCC. We hypothesized that miRNAs might cause tumor-specific cell death in HNSCC by targeting genes that might show a synthetic lethal interaction with one or more inactivated cancer genes. Several tumor-suppressing routes are inactivated in HNSCC amongst which the p53 and pRb pathways. Other genes or signaling routes may take over part of the lost functions and show a synthetic lethal interaction when inhibited (16). We used a human miRNA expression library in retroviral vectors to perform a functional genetic screen to specifically identify miRNAs that cause cell death of tumor cells and not of primary keratinocytes. We further investigated, by expression array analysis, the target genes of these miRNAs as functional inhibition of the target genes may elicit the same lethal phenotype, and could be performed by small molecules.
Material and Methods

Cell culture

Normal oral or oropharyngeal keratinocytes were isolated and cultured as previously described (17). Conditionally immortalized oropharyngeal keratinocytes (ciOKC) were generated by transformation of primary oropharyngeal keratinocytes with a temperature-sensitive SV40 large T-antigen. CiOKC were cultured in Keratinocyte Serum Free Medium (KSFM; Invitrogen, Breda, The Netherlands) supplemented with 0.1% bovine serum albumin, 25 mg bovine pituitary extract, 2.5 μg human recombinant EGF, 250 μg Amphotericin B (MP biomedicals, San Francisco, United States of America) and 250 μg gentamycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) at 32°C (18). VU-SCC-120, VU-SCC-OE, UM-SCC-6, SiHa, MCF7, HT29, U87 and Phoenix cells were cultured in DMEM, 5% FCS, 2 mM L-glutamine, 50 U/ml Penicillin and 50 μg/ml Streptomycin at 37°C and 5% CO2. The HNSCC cell lines used were all negative for the human papillomavirus and were sequenced for TP53 mutations. Cell line UM-SCC-6 was TP53 wild type, VU-SCC-120 contained two missense mutations (c.181_182CG>TT and c.527G>A) and VU-SCC-OE a truncating deletion (c.11_919del). Cell lines are authenticated regularly by their morphological characteristics and analysis of TP53 mutations and genetic markers.

Functional screen of human miRNA library

Amphotropic retroviral supernatants were produced for 370 annotated and putative miRNAs included in the human miRNA expression library (miR-Lib) with miR-Vec-Ctrl (scrambled sequence) as negative control (18, 19). The conditionally immortalized oropharyngeal keratinocytes were used for screening experiments as described previously (18). Both ciOKC and VU-SCC-120 cells (HNSCC cell line previously described as 93VU120 (20)) were transduced at two following days for four hours in the presence of 3 μg/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 48 hours the cells were subjected to blasticidin selection. For ciOKC this was two days 4 μg/ml and
subsequently 5 days of 8 µg/ml blasticidin (Sigma-Aldrich). For VU-SCC-120 the selection was performed with 10 µg/ml for seven days. For the initial screen, cell survival was assessed by visual inspection when the negative control (miR-Vec-Ctrl) had reached 100% confluency, and expressed as estimated percentage of the control (Supplementary Information). For subsequent validation experiments cell viability was quantified using the CellTiter-Blue® cell viability assay (Promega, Leiden, The Netherlands). The conversion of resazurin to resorufin was measured using the Infinite 200 plate reader (Tecan Group Ltd, Männedorf, Switzerland).

**RNA isolation from FFPE tissue**

Normal oropharyngeal mucosa was derived from three formalin-fixed paraffin-embedded (FFPE) specimens from patients who underwent uvulopalatopharyngoplasty. In addition, FFPE tumor biopsies were obtained from five HNSCC patients. The mucosal epithelium was microdissected from sections of the uvula specimen as previously described (21). Likewise, neoplastic areas were microdissected from tumor samples. Microdissected tissues were treated with 1 mg/ml of proteinase K for 17 hours at 56°C in buffer containing 100 mM TRIS-HCl (pH 9.0), 10 mM NaCl, 1% sodium dodecyl sulphate (SDS) and 5 mM EDTA (pH 8.2). Nucleic acids were isolated by phenol-chloroform extraction and precipitated by sodium acetate and ethanol according to standard protocols using glycogen as carrier. After the nucleic acids were washed with 70% ethanol, they were redissolved in RNAse-free water.

**Lentiviral shRNA ATM transduction**

Lentiviral vectors with short-hairpin RNA sequences targeting *ATM* transcripts were obtained from the MISSION® short-hairpin library of The RNAi Consortium (Sigma-Aldrich, Zwijndrecht, the Netherlands) that is available at VU University Medical Center. Viral supernatants were produced by co-transfection of HEK239T cells using FuGENE® 6
(Roche diagnostics, Woerden, The Netherlands) with the pLKO.1 short-hairpin vector together with the packaging and envelop vectors. Both ciOKC and VU-SCC-120 (HNSCC cell line) were transduced with lentiviruses at two following days for four hours in the presence of 3 µg/ml polybrene (Sigma-Aldrich). In total 48 hours after transduction cells were subjected to puromycin selection. For ciOKC this was two days 5 µg/ml and subsequently 5 days of 10 µg/ml puromycin (Sigma-Aldrich), and for VU-SCC-120 the selection was performed with 1 µg/ml for seven days.

**Quantitative Reverse Transcription-PCR**

Total RNA was isolated using the mirVana™ miRNA isolation kit (Ambion, Nieuwerkerk aan den IJssel, The Netherlands) according to the instructions of the manufacturer with the only modification that columns were eluted with 2x 25 µl elution buffer. Expression of hsa-miR-181a, hsa-miR-323, hsa-miR-326, hsa-miR-342, hsa-miR-345 and hsa-miR-371 was analyzed by Taqman® microRNA assays following the instructions of the manufacturer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). *ATM* expression was analyzed by Taqman® gene expression assay. Relative expression was calculated via the comparative CT method using the small nucleolar RNA transcript RNU44 (for microRNA analysis) or beta-glucuronidase, (*BGUS*: for *ATM* analysis) as internal references (22). Quantitative RT-PCR reactions without reverse transcriptase were carried out in parallel for each RNA sample to exclude signal by contaminating genomic DNA.

**Gene expression profiling**

The retroviral clones with miRNA genes miR-181a, miR-323, miR-326, miR-342, miR-345 and miR-371 and negative control miR-Vec-Ctrl were transiently transfected in VU-SCC-120 by FuGENE® 6 (Roche diagnostics). Total RNA was isolated 72 hours after transfection using the mirVana™ miRNA isolation kit (Ambion). Microarray hybridization
using the Agilent Low Input Quick Amplification labeling Kit and 4x44K Whole Human Genome Arrays was carried out according to the manufacturer (Agilent Technologies, Amstelveen, The Netherlands). Normalization of the gene expression data was performed within the R statistical software using the Limma-package, and comprised of RMA background correction, loess within-array normalization and A-quantile between-array normalization. Then, missing values were imputed using the impute-package (impute.knn with default settings). Finally, the slide and dye effect were removed by gene-wise linear regression using the log-intensity values. 

The log fold-changes between the reference group and each treated group were used to cluster the six treatments. This was carried out by means of hierarchical clustering with ward linkage, and the similarity defined both as the euclidean distance and as one minus the absolute value of the Spearman rank correlation measure. The grouping from hierarchical clustering was verified by means of principal component plots. Within each cluster the difference in gene expression between reference and treated samples were evaluated by means of a t-test. The multiplicity problem (many genes were tested) was addressed by application of the Benjamini-Hochberg procedure to the raw p-values to control the FDR (False Discovery Rate). Data are accessible under GEO number GSE34881.

**ATM inhibitor treatment**

Both ciOKC and VU-SCC-120 cells were subjected to a concentration range of 40 - 0.075 μM ATM inhibitor CP466722 (Axon Medchem, Groningen, The Netherlands). After 72 hours, cell viability was assessed with the CellTiter-Blue® cell viability assay.

**ATM 3’UTR reporter luciferase assay**

CiOKC cells were transiently co-transfected with a luciferase reporter construct containing the 3’UTR sequence of ATM (GeneCopoeia Inc, Rockville, MD, United States of
America) and one of the retroviral vectors containing the miR-181a, miR-323, miR-326 genes or negative control miR-Vec-Ctrl by FuGENE® 6. In total 72 hours after transfection, firefly and renilla luciferase activity was measured using the LucPair™ miR Dual Luciferase Assay Kit according to the instructions of the manufacturer (GeneCopoeia). Luciferase activity was measured using the Infinite 200 plate reader (Tecan Group Ltd, Männedorf, Switzerland).

**Lethal phenotype rescue**

VU-SCC-120 cells were transiently transfected with either pcDNA3.1(+)Flag-His-ATMwt (wild type ATM cDNA sequence, Addgene plasmid 31985) or pcDNA3.1(+) Flag-His-ATMkd (kinase-dead ATM cDNA sequence, Addgene plasmid 31986) (23). Amphotropic retroviral supernatants were produced for miR-181a, miR-326, miR-345 and miR-Vec-Ctrl (scrambled sequence) as negative control. VU-SCC-120-ATMwt, and VU-SCC-120-ATMkd cells were transduced with the microRNA expressing retroviruses at two following days for four hours in the presence of 3 µg/ml polybrene. After 72 hours cell viability was assessed by the CellTiter-Blue® cell viability assay.
Results

Identification of miRNAs lethal for HNSCC

To identify miRNAs that are lethal for head and neck cancer cells, we introduced a human miRNA expression library (miR-Lib) in HNSCC cell line VU-SCC-120 and close to normal conditionally immortalized primary oropharyngeal keratinocytes (ciOKC) (Figure 1A). The ciOKC cells have been immortalized by a temperature-sensitive SV40 large T antigen. When cultured at 32°C they are immortalized, while they become senescent when shifted to 39°C as a result of the inactivation of the large T-antigen. They behave, also at 32°C, as normal keratinocytes except for the immortalized phenotype (18).

The majority of miRNAs did not influence the survival of either tumor cells or ciOKC cells, or the lethal effect was similar in both models. However, a subset of 19 miRNAs (5.4%) specifically affected the head and neck cancer cell line, whereas the ciOKCs remained unaffected (Supplementary Table S1, Figure 1B). To verify the tumor-specific lethal effect of these miRNAs, we screened all nineteen miRNAs in two different ciOKC cell clones and three HNSCC cell lines (VU-SCC-OE, UM-SCC-6 and VU-SCC-120). We could not confirm the tumor-specific lethal effect of thirteen miRNAs. The remaining six miRNAs showed the expected phenotype (Supplementary Figure S1): a decreased proliferation in HNSCC cells but not in ciOKC cells and these miRNAs were further investigated. Visual inspection indicated that the tumor cells had disappeared from the wells indicating that they likely ceased proliferation and died. As yet, we did not elucidate the precise molecular mechanism behind the cell death.

For the initial large scale discovery screens we had to rely on the ciOKC cell model to study the effect of the miRNAs. In the subsequent small scale validation experiments we included primary keratinocytes. The miRNAs miR-181a, miR-323, miR-326, miR-342, miR-345 and miR-371 all showed a significant decrease in cell proliferation in three HNSCC cell lines, and not or only to a limited extent in primary keratinocytes (Figure 1C). The sequence identity of these six miRNAs was confirmed by Sanger sequencing (data not shown).
MiRNA expression in HNSCC

Since the ectopic expression of the six tumor-selective lethal miRNAs caused cell death in tumor cell lines but not or less in mucosal keratinocytes, we were interested in the expression of these miRNAs in HNSCC tumors and normal oral mucosa. Hence we determined expression levels for the six miRNAs in RNA extracted from both microdissected tumor and mucosal epithelium. MiR-371 was not expressed, neither in normal mucosa nor in the five tumors analyzed (data not shown). For the other five miRNAs expression was observed in all tumor and mucosal epithelium samples, but apparently at a low level (Figure 2). The expression levels of the miRNAs were in all cases lower than the expression of the RNU44 reference gene. In addition, only small differences in expression levels were observed between mucosa and tumor samples. The expression of miR-181a was slightly increased in tumors, but not significant (Figure 2A). For miR-326, miR-342 and miR-345 the expression level in tumor tissue was decreased when compared to normal mucosal epithelium, but not significantly. Only the expression level of miR-323 was significantly lower in tumor cells (Figure 2B).

Tumor-specific lethal phenotype in cell lines of different cancer types

We next questioned whether the effect observed in the HNSCC cell lines was specific for HNSCC. Therefore we tested the six miRNAs with HNSCC-specific lethal effects in other cancer cell lines. The introduction of the various miRNAs in cervical carcinoma cell line (SiHa) and breast carcinoma cell line (MCF7) had no effect on proliferation except for miRNA 181a (Supplementary Figure S2A and S2B). However, when the miRNAs were introduced in colon adenocarcinoma cell line (HT29) or glioblastoma cell line (U87) a decrease in cell proliferation was observed, although with a less severe phenotype as compared to the tested HNSCC cell lines (Supplementary Figure S2C and S2D). The effects observed vary with the specific microRNA. This strongly suggests that genes are targeted that show synthetic lethal interactions in relation to the mutational
status of specific cancer genes or deregulated signaling pathways in the various cell lines of different tissue origin, and it might be worthwhile to repeat these functional screens for other tumor types as it might reveal other candidate microRNAs. The lethal interaction is likely not related to a mutation in TP53 as all miRNAs showed the tumor-selective lethal effect in all three HNSCC cell lines while one cell line was TP53 wild type, one showed two missense mutations and one a large deletion (see Material and Methods for details).

**Target gene analysis**

MiRNAs regulate gene expression at the post-transcriptional level, so we were interested in the downstream gene targets of these six tumor-specific lethal miRNAs that might explain the cell growth inhibitory phenotype. First we performed an *in silico* analysis. There is a multitude of software tools available for target identification [reviewed in (24)], and we chose TargetScan and DIANA-microT. We also defined the overlap between genes detected by the two programs (Supplementary Table 4). There was a wide variety of potential genes identified with over 700 for miR-181a. This analysis did not give a direct clue.

Therefore we performed micro-array based gene expression analysis to identify candidate target genes. Since ectopic expression of these miRNAs caused a decrease in cell proliferation and cell death as phenotype in HNSCC cell lines, we were unable to analyze cells stably transduced with the miRNAs. We therefore decided to transiently transfect VU-SCC-120 cells with the retroviral vector plasmids instead of transduction with retroviral particles. Transient transfection is efficient, and overexpression can be observed almost immediately. RNA was isolated 72 hours post-transfection, the time point with high miRNA expression, but before cell death was observed (Supplementary Fig. S3). The expression level of the transfected miRNAs was also determined. Depending on the endogenous expression of the miRNAs in VU-SCC-120 cells, expression increased from 23 to 4,000 fold after transient transfection (Figure 3A and 3B).
Next, total RNA of the transfected cells was labeled and hybridized for gene expression profiling by microarray hybridization. We assumed that some of these six miRNAs might in fact target the same genes, and we therefore focused on a group-wise comparison. In the expression profiles we indeed observed significant correlations between miRNA associated mRNA expression profiles (Table 1). Highly significant correlations were observed for miR-181a and miR-326 (r=0.572), miR-326 and miR-345 (r=0.573), miR-323 and miR-371 (r=0.602) and miR-342 and miR-323 (r=0.577). Cluster analysis revealed two groups of each three miRNAs with downstream target effects that showed significant correlations (Figure 3C; Supplementary Tables S2 and S3). MiRNAs miR-181a, miR-326 and miR-345 clustered together in group A and group B was composed of miR-342, miR-371 and miR-323. The profiles of the miRNAs per group were combined and analyzed against the empty vector control (in tetraplicate hybridized on the arrays) to detect significant differentially expressed genes.

In total we observed 187 and 15 genes (FDR corrected p-value < 0.1) that were significantly differentially expressed genes in group A and B, respectively, as compared to the empty vector control. Subsequently, we applied several rankings on the differentially expressed genes to distinguish primary effects from secondary effects. First, we ranked the genes for a decreased level of expression, as miRNAs are considered to cause downregulation of expression of their target genes. Second, as for many genes multiple probes were present on the array, we subsequently ranked the genes for the number of probes per gene detected with an FDR corrected p-value <0.1 (Supplementary Tables S2 and S3). One of the most striking target genes that is apparently regulated by the miRNAs from group A is the ataxia telangiectasia mutated (ATM) gene. First the differences of many probes are highly significant given the limited sample size (four controls versus three miRNAs of group A analyzed in duplicate) and the conservative p-value adjustment. Second, in total 9 of 12 ATM probes were significantly regulated. Unfortunately, we did not find such an apparent lead target for the miRNAs in group B.
Knockdown of ATM causes tumor-specific lethality

ATM is a nuclear protein kinase that senses DNA damage and activated downstream pathways (24). To validate the observed decrease in expression we performed qRT-PCR for ATM in the same samples that were transiently transfected with the miRNAs and that were analyzed by micro-array hybridization. Indeed we confirmed that ATM expression is inhibited in the RNA samples from group A (Figure 4A).

Since ectopic expression of miR-181a, miR-326 and miR-345 results in tumor-specific lethality and down-regulation of ATM expression, we hypothesized that knockdown of ATM may also be accompanied by tumor-specific lethality in HNSCC cells. Therefore we introduced five lentiviral shRNA constructs designed to specifically knockdown ATM expression. Each shRNA sequence was complementary to a unique part of the ATM mRNA sequence. Introduction of the ATM shRNAs resulted in a minor inhibition of cell proliferation in ciOKC cells compared to cells transduced with a control construct (ctrl), but proliferation was significantly inhibited in HNSCC cell line VU-SCC-120, ranging from 21 to 90% (Figure 4B). The maximum window was observed with shRNA ATM1. It was at least 8 times more active in the tumor cell line as compared to the ciOKC cells. To check the knockdown, ATM expression was analyzed by qRT-PCR and expression levels were compared to the cells transduced with the control construct. Introduction of four out of five ATM shRNAs resulted in over 70% down-regulation of ATM expression levels. Only transduction with ATM shRNA number 5 did not result in significant down-regulation of ATM expression (Figure 4C).

ATM is a kinase and druggable by kinase inhibitors. To confirm the tumor-specific decrease in cell viability by ATM inhibition, we also subjected ciOKC and HNSCC cells to different concentrations of the commercially available specific ATM inhibitor CP466722. Analysis of cell viability shows that HNSCC cells are more sensitive to the inhibitor compared to the ciOKC cells (Figure 4D). The IC50 of 8.2 μM in ciOKC cells shifts to 2.6 μM in VU-SCC-120, a significant change of 3 fold (p-value <0.05 by T-testing).
Inhibition of ATM, either by microRNA overexpression, specific ATM shRNAs or kinase inhibitors results in a decrease in cell proliferation in HNSCC cells. When ATM is the direct effector, ectopic expression of ATM should rescue the HNSCC cells from cell death. To investigate this, VU-SCC-120 cells were transfected with either wild type ATM (ATMwt) or a kinase-dead mutant ATM (ATMkd) in an expression cassette that lacks the 3'UTR of ATM. Overexpression of ATMwt or ATMkd was confirmed by qRT-PCR (Supplementary Fig. S4). Next miRNAs, miR-181a, miR-326 and miR-345 were introduced in these two cell lines and compared to untransduced VU-SCC-120 (ctrl). The miRNAs all showed a decrease in cell proliferation in the untransduced VU-SCC-120 (Figure 4F). However, in the cells with the ATMwt expression construct, cell proliferation was rescued up to ~80% (miR-181a) and ~50% (miR-326 and miR-345). Rescue was not observed when the kinase-dead mutant ATM was ectopically expressed in VU-SCC-120 cells. These data indicate that the miRNAs inhibit ATM expression, which elicits the tumor-selective lethal phenotype and it depends on the kinase activity of ATM.

Targeting of ATM by miR-181a, -326- and -345

MiRNAs are known to regulate gene expression post-transcriptionally via binding to the 3'UTR or coding sequences of an mRNA sequence. To demonstrate an effect of miR-181a, miR-326 and miR-345 on the 3'UTR of the ATM gene miRNAs were cotransfected with a luciferase reporter construct cloned to the ATM 3'UTR sequence. Transfection of ciOKCs with miR-181a and miR-326 suppressed the activity of a luciferase reporter gene cloned to the 3'UTR of ATM. MiR-345 did show an effect, but not significant (Fig. 4E). The knockdown of ATM by the respective microRNAs seems to inversely correlate with the effect of ectopic ATM expression This suggests that some miRNAs might not target the 3'UTR, but the 5'UTR or coding sequences. These sequences are still present in the expression construct, only the 3'UTR of ATM has been substituted. In addition, ectopic ATM expression does not rescue the lethal phenotype to 100%, and we hypothesized that
another gene target might be involved as well. First, we re-inspected our expression data and found another gene significantly down-regulated in 9 of 10 available probes: CD40. We next checked the presence of microRNA target sites using RNA22 and TargetScan on both ATM and CD40. Multiple binding sites were found for these microRNAs in these genes, albeit mostly not in the 3'UTR region (Supplementary Fig. 5). The relevance of the various putative miRNA target sites, and the role of CD40 and its interaction with ATM requires further elucidation.
Discussion

In the present study we identified six miRNAs that, when ectopically expressed, lead to a tumor-specific inhibition of proliferation in HNSCC cells and not in primary keratinocytes. MiRNAs are generally classified into different families based on their sequence homology, most particularly of the seed sequence, and it is hypothesized that different miRNAs within the same family may have similar effects on gene expression. Although all miRNAs highlighted in this study have a tumor-specific lethal effect, and some even recognize at least one identical and apparently critical target gene, they do not belong to the same family.

Many miRNAs have already been implicated in cancer development and progression. For miR-345 a role in head and neck cancer development has been suggested previously by profiling studies. In these studies miRNA expression profiles in tumor samples were compared to normal mucosal epithelium, and it was shown that miR-345 showed an increased expression level in malignant cells (10).

MiR-181a has been shown by functional studies to have tumor-suppressive properties in HNSCC (15). Here we show a tumor-specific lethal effect for ectopic expression of these two miRs as well as miR-326, miR-342, miR-371 and miR-323 in head and neck cancer cells. We also show that a similar lethal effect was observed when those six miRNAs were introduced in colon cancer and glioblastoma cell lines, although there was a considerable variation in the lethal phenotype between cell lines of different origin. It might therefore be of interest to repeat similar screens in other tumor types. Unfortunately it is not always possible to have access to matched normal (or near normal) cells, which are required to allow a tumor-specific lethal screen and subsequent validation. Assuming that the identified hits are synthetic lethal with cancer-associated alterations in conserved cellular pathways such as cell cycle control or DNA repair, the precise source and histotype of the normal reference cells might be less critical, and normal fibroblasts may be sufficient.

MiRNAs regulate gene expression at the post-transcriptional level. One gene may
be regulated by multiple miRNAs, but also one miRNA may regulate multiple genes. Consequently, it is difficult to study downstream targets of a particular miRNA particularly as the target genes may also be involved in the regulation of expression of other genes. Therefore it is not surprising that many genes were shown to be significantly differentially expressed when the effect of the six tumor-specific lethal miRNAs was analyzed. Although the expression profiles of the six miRNAs were not similar, it was interesting to observe that two groups could be discriminated. Unfortunately, we could not identify an apparent candidate target gene in group B. Likely multiple repeats of the array analysis in multiple cell lines will be required to define more convincing candidate target genes. The limited sample size combined with the strict FDR correction of the p-values may have skewed the analyses. Moreover, miRNAs might also influence genes at the translational level, which would necessitate alternative approaches to identify the candidate target genes.

However, in group A several probes for ataxia telangiectasia mutated (ATM) showed significant differences. Using ATM 3'UTR luciferase reporter experiments we demonstrated that both miR-181a and miR-326 seem to directly target the 3'UTR sequence of ATM. We could not confirm this for miR-345. We also showed that specific down-regulation of ATM via shRNA knockdown elicits a similar tumor-specific lethal phenotype. Although there was a slight toxic effect in the normal squamous epithelial cells, the lethal phenotype observed in HNSCC cell lines was much more severe, and HNSCC cells are more sensitive to the specific ATM inhibitor CP466722. Finally, the tumor-selective phenotype of the miRNAs could be rescued by introduction of an ATM expression construct that lacks the 3'UTR of ATM itself, while the phenotype could not be rescued by expression of a kinase-dead mutant.

Intriguingly, there seemed an inverse correlation between reduction of luciferase activity with the ATM 3'UTR and the rescue level when expressing an ATM cDNA sequence without the 3'UTR, strongly suggesting that target sites in the coding cDNA sequence are highly relevant as well. When analyzing the potential target sites by RNA22 and TargetScan a binding site for miR-181a was found in the 3'UTR of ATM, but many
more in the coding cDNA sequence. Mutation of predicted target sites will be required to
proof the relevance of all of them, which will be a major future task. A worrying observation
is that the various prediction programs come up with different potential binding sites,
hampering a focused approach. Nonetheless such experiments will be required to establish
the ultimate proof of ATM targeting by these microRNAs of group A.

We also observed that the rescue level never reaches 100% cell survival. There
might be many explanations for this observation, but it suggests that also another target
gene might be involved, and we could pinpoint CD40 as a first hint. Additional experiments
will be required to proof this, and that combined targeted treatment might be of benefit.

Our data thusfar suggests that HNSCC cells are depending on ATM signaling for
their survival. When ATM expression is lost, either by shRNA knockdown or when targeted
by a miRNA, the cell is unable to survive, at least under culture conditions. ATM senses
DNA damage and phosphorylates CHEK2 which in turn activates p53 by phosphorylation.
This route plays a major role in maintaining genome integrity and cell cycle control (25).
Intriguingly, TP53 is mutated in two of the three HNSCC cell lines tested and ATM may
have become more critical to organize DNA repair or induce a cell cycle block during S/G2
phase after DNA damage independent of p53. The third cell line UM-SCC-6 is TP53 wild
type but it is unclear whether p53 is still functionally active. Detailed analyses of the
ATM/ATR-CHEK1/CHEK2 pathway should reveal which critical downstream signalling
route causes the lethal phenotype, and what the mechanism is. Obviously ATM has many
downstream targets, and the tumor-selective phenotype might be related to alterations in
other signalling routes. Nonetheless, our data suggest that ATM is an interesting drug
target for HNSCC.

Previously it has been shown that miR-181a targets KRAS, and this was put
forward as explanation that this particular microRNA inhibits proliferation of squamous
cancer cells (15). This seemed a little remarkable as KRAS mutations are hardly found in
HNSCC, suggesting that this is not the most critical driving gene or even pathway in
squamous oncogenesis. Based on our data, we assume that the targeting of ATM by miR-181a is the likely event that causes a proliferation stop and cell death in squamous cancer cells.

In summary, we show here that functional screens by microRNA expression libraries is an effective way to identify novel druggable targets. We showed that the ectopic expression of miRNAs such as miR-323, miR-345, miR-371, miR-181a, miR-342 and miR-326 may serve as a new treatment in HNSCC. Particularly in head and neck cancer where access to the tumor is relatively easy, introduction of miRNAs via for instance intratumoral injection combined with electroporation may be a therapeutic possibility in the future (26). Also the application of ATM inhibiting drugs might be a very interesting approach to study in clinical trials specifically focusing on HNSCC.

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Legends

Figure 1. Identification of 19 miRNAs with a potential tumor-specific lethal effect by a functional genetic screen. (A) Schematic representation of the primary tumor-specific lethality screen. Both ciOKC and VU-SCC-120 cells were retrovirally transduced with a human miRNA expression library. After blasticidin selection of transduced cells, cell density was assessed. The miRNAs that had little to no effect on cell density (70-100%) in ciOKC cells, but did result in low cell density (0-30%) in VU-SCC-120 cells were identified as potentially tumor-specific lethal. (B) The 19 miRNAs that were selected in the initial screen showed cell survival differences between ciOKC (blue bars) and VU-SCC-120 (red bars) cells. Cell density in duplicate wells was visually estimated by two independent observers, with standard deviation as error bars. (C) The effect of ectopic expression of the indicated six miRNAs on survival of primary oral keratinocytes (blue bars) and head and neck cancer cell lines VU-SCC-120 (red bars), VU-SCC-OE (yellow bars) and UM-SCC-06 (light green bars). Cell proliferation was quantified by the CellTiter-Blue assay. The average value of triplicate experiments is shown, with standard deviations as error bars. Significant differences are indicated by an asterisk* (p<0.05, Student’s t-test).

Figure 2. Box plots of the expression of the six lethal miRNAs in HNSCC tumors and normal mucosal epithelium. MiRNA levels were determined using qRT-PCR analysis on RNA of microdissected FFPE tumors and mucosal epithelium. DeltaCT values, Ct(miRNA)-Ct(RNU44), are depicted for (A) miR-181a (B) miR-323 (C) miR-326 (D) miR-342 and (E) miR-345. MiR-371 was not expressed in squamous tissues.

Figure 3. Ectopic expression of miRNAs and their effect on gene expression. VU-SCC-120 cells were transiently transfected with the six different miRNA plasmids and overexpression of the indicated miRNAs was compared to miR-Vec-Ctrl transfected cells for (A) miR-181a, miR-326, and miR-345 as well as (B) miR-323, miR-326 and miR-371.
Significant differences are indicated by an asterisk* (p<0.05, Student’s t-test). Two separate groups became apparent by hierarchical cluster analysis (C).

Figure 4. ATM expression is regulated by miR-181a, miR-326 and miR-345 from group A. (A) Endogenous ATM expression in ciOKC cells was analyzed by qRT-PCR after transient transfection with miR-Vec-Ctrl or the miRNA constructs indicated. In (B) the knockdown of endogenous ATM expression in ciOKC cells is depicted after lentiviral transduction with either a control construct (ctrl) or ATM shRNA constructs 1 to 5. In (C) cell proliferation of ciOKC (blue bars) and VU-SCC-120 (red bars) is depicted after ATM knockdown with five different shRNA constructs and control vector (ctrl). In (D) sensitivity of HNSCC cell lines VU-SCC-120 and ciOKC cells to the ATM drug CP466722 is determined. (E) Effect of the miRNAs of group A on ATM 3’UTR luciferase reporter construct. The 3’UTR of ATM was cloned behind the firefly luciferase. Firefly luciferase activity was normalized using renilla luciferase activity to correct for transfection efficiency. (F) Rescue experiments with ATM encoding expression constructs. Wild type ATM (ATM) or kinase-dead ATM (ATMkd) were transfected and the effect on the tumor-selective phenotype of miR-181a, miR-326, miR-345 or miR-Vec-Ctrl (miCtrl) compared to untransfected cells that have endogenous levels of ATM, but regulated by these miRNAs (ctrl). The average of triplicate experiments is shown with standard deviations as error bars. Significant differences are indicated with an asterisk* (p<0.05, Student’s t-test).
References


Table 1. Correlations of gene expression profiles between different microRNAs

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<th>miR-326</th>
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Figure 1

A

B

10 miRNAs with tumour-specific lethal phenotype

ctrl
miR-1
miR-15a
miR-181A
miR-183
miR-217
miR-218
miR-30C
miR-323
miR-326
miR-337
miR-342
miR-345
miR-370
miR-371
miR-372
miR-377
miR-409/412
miR-411
miR-X155

Blistatic growth
Cell density assessment
Retroviral transduction
Human miRNA expression library
370 putative and immortal miRNAs
1 miRNA: Vec (control, scrambled sequence, negative control)

VU-SCC-120
LOXC

Figure 2

**A**

DelCt (RNU44-miR181a)

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**B**

DelCt (RNU44-miR323)

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**C**

DelCt (RNU44-miR326)

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**D**

DelCt (RNU44-miR342)

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**E**

DelCt (RNU44-miR345)

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**Mucosa Tumour**

Research.
Figure 3

A

B

Fold expression

Fold expression

C

hsa-miR-181a

hsa-miR-326

hsa-miR-345

hsa-miR-342

hsa-miR-371

hsa-miR-323
Figure 4

A

B

C

D

Relative ATM expression

Cell Proliferation (%)

Relative ATM expression

Cell viability (%)

ATM inhibitor (uM)

ctrl miR-181a miR-326 miR-345

ciOKC VU-SCC-120 ctrl ATM1 ATM2 ATM3 ATM4 ATM5

ciOKC10 VU-SCC-120

ctrl ATM1 ATM2 ATM3 ATM4 ATM5
Figure 4

E

F

E

F

Relative Luciferase Activity

Relative Cell Proliferation

miCtrl  miR-181a  miR-326  miR-345

miCtrl  miR-181a  miR-326  miR-345

Ctrl  ATM  ATM kd

*
Clinical Cancer Research

Identification of lethal microRNAs specific for head and neck cancer

Marlon Lindenbergh-van der Plas, Sanne R. Martens-de Kemp, Michiel de Maaker, et al.

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