Title: Pretreatment microRNA expression impacting on epithelial to mesenchymal transition predicts intrinsic radiosensitivity in head and neck cancer cell lines and patients.

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STATEMENT OF TRANSLATIONAL RELEVANCE

In head and neck squamous cell carcinomas (HNSCC) radiation is a major treatment modality. Intrinsic radioresistance of tumor cells is one of the predominant causes of head and neck cancer recurrence. This phenomenon can only be examined by ex-vivo colony assays, but these take too much time to be clinically useful and do not reveal the biological mechanisms of intrinsic radioresistance. Using microRNA and messenger RNA expression profiles of HNSCC cell lines and tumors, we found that low expression of certain microRNAs that suppress epithelial to mesenchymal transition, measured prior to treatment, is causally related to intrinsic resistance to radiation. This finding provides an important step towards modification, and thereby improvement of the treatment of radioresistant tumors.
ABSTRACT

Purpose: Predominant causes of head and neck cancer recurrence after radiotherapy are rapid repopulation, hypoxia, fraction of cancer stem cells and intrinsic radioresistance. Currently, intrinsic radioresistance can only be assessed by ex-vivo colony assays. Besides being time-consuming, colony assays do not identify causes of intrinsic resistance. We aimed to identify a biomarker for intrinsic radioresistance to be used before start of treatment and to reveal biological processes that could be targeted to overcome intrinsic resistance.

Experimental design: We analyzed both micro- and messenger RNA expression in a large panel of HNSCC cell lines. Expression was measured on both irradiated and unirradiated samples. Results were validated using modified cell lines and a series of laryngeal cancer patients.

Results: MiRs, mRNAs and gene sets that correlated with resistance could be identified from expression data of unirradiated cells. The presence of epithelial to mesenchymal transition (EMT) and low expression of miRs involved in the inhibition of EMT were important radioresistance determinants. This finding was validated in two independent cell line pairs, in which the induction of EMT reduced radiosensitivity. Moreover, low expression of the most important miR (miR-203) was shown to correlate with local disease recurrence after radiotherapy in a series of laryngeal cancer patients.

Conclusions: These findings indicate that EMT and low expression of EMT-inhibiting miRs, especially miR-203, measured in pre-treatment material, causes intrinsic radioresistance of HNSCC, which could enable identification and treatment modification of radioresistant tumors.
BACKGROUND

Radioresistance of head and neck cancer

Radiotherapy is the most important treatment modality in head and neck cancer, with two thirds of patients treated with (chemo-)radiotherapy (1). With altered fractionated radiotherapy the locoregional control rates for earlier stages are encouraging, but for stage III-IV tumors locoregional control remains around 50% (2), leaving considerable need for improvement. Factors that contribute to control of the tumor are tumor site, stage, treatment schedule and dose, tumor volume and human papilloma virus (HPV) status (3–5). However, even after correcting for these factors, there are still differences in control rates. Such differences may result from differences in tumor micro-environment, tumor cell properties like hypoxia, rapid repopulation between fractions, the fraction of cancer stem cells or intrinsic radiosensitivity (6).

Intrinsic or cellular radiosensitivity is a term used to describe the process of one tumor cell being more resistant than another on the basis of different intracellular mechanisms, independent of microenvironmental factors.

An appropriate way to study intrinsic radiosensitivity is therefore in tissue culture in which potential confounding factors can be reduced or eliminated. It has indeed been shown that intrinsic cellular radiosensitivity significantly determines the outcome of radiotherapy in head and neck cancer (7). However, these data were attained using functional (cell survival) studies, giving limited or no information on genes or pathways involved, and thus providing little help to the treating physician on how to improve treatment for patients with radioresistant tumors. We therefore searched for genetic and thus potentially assessable and targetable factors that affect intrinsic radioresistance in head and neck cancer.
**Messenger RNA to study radioresistance**

Messenger RNA (mRNA) profiling has been used to study radioresistance in cell lines. To date, however, such experiments have been mostly performed on either one or two cell lines only, or on the NCI-60 cell line panel, which contains no HNSCC lines (8,9). Since it is known that radiosensitivity is partly dependent on the tissue of origin (e.g. lymphomas are more sensitive than solid tumors), use of such a cell line panel to predict HNSCC radiosensitivity is of questionable value. Therefore, Hall et al. attempted to identify a robust gene signature associated with intrinsic radiosensitivity on a series containing 16 cervical and 11 HNSCC cell lines. Unfortunately they failed to identify such a set (10). Possibly this could be attributed to the fact that messenger RNA levels alone give an incomplete picture of active processes in the cell, since other factors can influence translation to protein. Among these are microRNAs.

**MicroRNAs**

MicroRNAs (miRs) are genomically encoded small pieces of single stranded RNA of around 22 nucleotides each of which can silence hundreds of genes (11). More than 1,000 miRs have been identified so far, estimated to regulate expression of at least 60% of all genes (12). MiRs regulate gene expression by binding to their (partly) complementary sequence on mRNA molecules, resulting in reduced protein production (13,14). MiRs can reduce protein production by causing degradation of mRNAs or by inhibiting translation. Multiple modes of silencing thus seem to exist that can be active concurrently (15,16).

Ionizing radiation has been shown to induce significant changes in miR expression in 6 cancer cell lines (17). MiRs playing a role in radioresistance have been described, although experiments were done in cell line pairs and not in a larger panel of cell lines (18–20).

**Study goal**
The goal of this study was therefore to get a better insight into the genetic causes of intrinsic radioresistance in head and neck cancer cells focusing on miR expression. Using a large panel of HNSCC cell lines, we aimed to answer the following questions: 1. Do miR/mRNA expression changes induced by irradiation correlate with radioresistance? 2. Can we identify mRNAs that correlate with radioresistance? 3. Can we identify driving miRs that correlate with radioresistance? 4. If so, are these miRs and their targets related to certain pathways or processes? 5. Lastly, do these miRs correlate with radiotherapy response in patients with laryngeal cancer? The answers to these questions should lead to a better understanding of radioresistance in this disease and therefore provide guidance towards more individualized treatment.

**MATERIALS AND METHODS**

**Cell line selection and culture**

*Cell line selection.* All cell lines for hypothesis generation were obtained from Professor R. Grénman (University of Turku, Finland), who has a unique panel of more than 100 well characterized HNSCC cell lines with known radiosensitivity. We selected 32 HNSCC cell lines from different subsites (supplementary table 1). Cell lines previously treated with chemotherapy or derived from metastatic sites other than regional lymph nodes were excluded.

*Cell culture.* All cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 1% L-glutamine, 1% nonessential amino acids, 10% fetal bovine serum and antibiotics. Cells were incubated in humidified air with 5% CO2 at 37°C. Depending on the doubling time, cells were subcultured every 3-14 days to ensure exponential growth. Cells were used for experiments when they were around 60-70% confluent. Preferably low passages (10-20) were used.
**Validation cell lines.** The UT-SCC-43A and UT-SCC-43A-Snail cell lines were developed and provided by dr M. Takkunen (University of Helsinki, Finland) (21). The FaDu-cDNA3 and FaDu-HIF-ΔODD cell lines were developed and provided by prof. Kou-Juey Wu (National Yang-Ming University, Taiwan) (22). Both cell lines are human HNSCC, transfected with either the transcription factor snail or HIF-1α with a deleted oxygen degradation domain, thereby causing the cells to undergo epithelial to mesenchymal transition (EMT).

**Irradiation assay**

*Radiosensitivity assay.* Radiosensitivity of all cell lines was tested with a 96-well plate clonogenic assay, developed by Grénman et al (23,24). The radiosensitivity of a cell line was defined as the area under the survival curve, with measurements of the survival fraction at 6 different doses, each repeated at least 3 times. When a comparison was made between radioresistant and radiosensitive cell lines, the cut-off was set at a median area under the curve of 2.0.

*RNA collection after irradiation.* Cells were irradiated using a $^{137}$Cs irradiation unit with a dose rate of 0.662 Gy/min. Mock-irradiated cells were harvested for all cell lines, as well as cells at 2 and 6 hours after 4 Gy. At the given time points cells were rinsed with ice-cold PBS twice and then collected in RNA-Bee (Campro Scientific).

**RNA isolation from cell lines**

All steps from RNA isolation to microarray hybridization were performed at the institute’s central microarray facility. Cells in RNA-Bee were used to extract total RNA. The sample was then split into two for analysis of miR and mRNA separately. Messenger RNAs were further purified using the Rneasy Mini Kit and the RNase-Free DNase Set from Qiagen. The RNA was isolated and DNAse treated using the spin
columns according to the manufacturer's instructions. The Agilent 2100 bioanalyzer was used to confirm the presence of intact RNA.

**mRNA/miR microarrays in cell lines**

*Messenger RNA.* Biotin-labeled cRNA was generated using the Illumina® TotalPrep RNA Amplification kit (AMIL1791, Ambion Inc., Austin, TX). Briefly, to synthesize biotin-labeled cRNA, 350 ng of total RNA was reversed transcribed and subsequently amplified and labeled with biotin (in vitro transcription). Next, the cRNA (1,500 ng per array) was hybridized to v3 Illumina bead arrays according to the Manufacturer's instructions (Illumina, Inc., San Diego, CA). Array signals were developed by Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the BeadChip manual. Fluorescence intensities were measured with the scanner and averaged per probe. Background adjustment was done using the method from the affy package, after which data were log2 transformed and robust spline normalized. As a final step, annotations were updated using the lumiHumanAll package (25) in R and subsequently the data were aggregated per gene symbol: data from probes with the same gene symbol and a correlation greater than 0.7 were averaged.

*MicroRNAs.* Using the Exiqon miRCURY LNA microRNA Array kit (5th generation), 1ug total RNA was labeled with Hy3 and hybridized in a TECAN HS4800 Hybridization Station against the slides together with a reference pool of all samples (Hy5). The slides were scanned in a DNA Microarray Scanner (Model G250B, Serial number US22502518) from Agilent Technologies, which uses Scan Control software (Version A.6.11). After subtraction of the mean background signal, arrays were log2 transformed and normalized using the lowess method (using Imagene 6.0 software).

**Patient series**
**Patient selection.** Thirty-four patients treated at The Netherlands Cancer Institute between 2002 and 2010 were selected as a validation cohort. To avoid confounding by the addition of surgery or chemotherapy, a cohort consisting of patients with T2-3 laryngeal cancers was compiled. These patients were all treated with radiotherapy alone with a curative intent. The series was designed to be a matched cohort of 17 patients with local recurrences matched with 17 local cures. There were no significant differences between groups with and without local recurrence in age, gender, subsite, T-stage or treatment year (supplementary table 2).

**miR extraction.** Using the Roche High Pure miRNA Isolation Kit (REF: 05080576001), miRNAs were extracted from pre-treatment biopsies. Briefly, 5 slides of 5µm thick were deparaffinized and macrodissected, assuring that the sample consisted of at least 50% tumor cells. MiRs were further purified according to the manufacturer’s instructions.

**miRNA library preparation and sequencing**

The total RNA samples were quality controlled and quantified with the Agilent Technologies 2100 Bioanalyzer, using the RNA 6000 Nano kit. One µg of total RNA in a volume of 5 µl was used as input for the miR library preparation for Illumina sequencing (SR 50bp) using the TruSeq® Small RNA Sample Preparation kit (RS-200-0012 ) and Guide (Part # 15004197 Rev. E). Shortly, stepwise RNA ligation of 3’ and 5’ adapters to miRs introduce a specific index to every sample. The product was PCR amplified and pooled and purified using a 6% PAGE gel. Fragments of 145 – 160 bp were cut from the gel, washed and concentrated by ethanol precipitation and resuspended in nuclease free water. The small RNA library pools were quantified using a DNA 7500 chip with the Agilent Technologies 2100 Bioanalyzer. The pools were diluted to a concentration of 2 nM and passed on for sequencing onto an Illumina HiSeq2000.
machine and a stretch of 50 bp was sequenced according to manufacturer’s instructions. The FAST-Q data from the run were analyzed and quantified by comparing the data to the miR databases. Sequence reads (51bp) were mapped using the mirExpress pipeline. The reads were trimmed for adapter sequences upon alignment. During the alignment the identity was set to 0.9. Human mature and precursor sequences were downloaded from mirbase (version 20). The miR expression results that were generated for each sample were combined for further analysis. MiR counts were normalized to 100,000 reads per patient.

Analysis

Time course analyses were performed using the Biometric Research Branch (BRB) ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). This is a tool that performs a regression analysis of time course data, finding patterns that correlate with either time, class, or both. Pathways and networks were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Cell survival curves were generated and analyzed in GraphPad Prism 6.0. All other analyses were performed in R (26), using the Bioconductor packages (27) and our own scripts.

MiR target selection

Since most miR-mRNA interactions are predicted interactions based on the complementarity of their RNA sequences and not on experimentally validated interactions, a collection of the most likely mRNA targets was generated for each miR by analysis of validated interaction data from external databases. A maximum of 750 mRNA targets per miR were selected based on our own prediction model trained to predict experimentally validated targets from Tarbase 6.0 (28) on miR and target properties from TargetScanHuman 6.2 (14,29). A list of these interactions is available in supplementary text file 1.
RESULTS

Data overview

All tested cell lines responded to irradiation by profound changes in gene expression. To investigate whether this response correlates with radioresistance, we determined the abundance of 18,913 unique mRNAs at 0, 2 and 6 hours after 4 Gy and of 279 unique miRs at 0 and 6 hours after 4 Gy in 32 HNSCC cell lines (figure 1).

1. MiR/mRNA expression changes 2 and 6 hours after 4 Gy do not correlate with radioresistance

Thousands of mRNAs and miRs showed expression changes in one or more of the cell lines in response to 4 Gy. The time course plug-in in BRB-array tool identifies cell lines with similar gene up- or downregulation after irradiation. An expression response pattern common to all 32 cell lines involved 175 genes (supplementary figure 1), none of them encoding miRs. When analyzing these common response genes in Ingenuity Pathway Analysis (IPA), the most significant canonical pathways were associated with protein ubiquitination, cell cycle regulation and DNA double-strand break repair.

When genes with an altered expression 6 hours after 4 Gy (compared to baseline expression) were subjected to cluster analysis, two main response clusters became evident. Genes that were different between the two response clusters were analyzed in IPA, which showed that 11 cell lines in the first cluster had an activated TP53 and HNF4A response, while this response was inhibited in the other 21 cell lines. However, the two clusters showed no correlation with radioresistance (T-test p-value = 0.82).
The time course plug-in also searches for response patterns that are significantly different between two groups. Here we found that changes 2 and 6 hours after 4 Gy did not differ between the 14 radiosensitive and 18 resistant cell lines, neither in mRNA nor miR expression.

2. Messenger RNAs and radioresistance

The BRB time course plug-in further analyzes the difference between sensitive and resistant cell lines, independent of the time response. In this analysis 1226 genes with a stable expression over the 3 time points significantly correlated with radioresistance using a false discovery rate cut-off of < 0.05 (supplementary table 4). Additionally, separate T-tests were performed between the expression of the sensitive and resistant groups for each of the three time points. The three resulting p-values were then pooled per gene. The expression over time for the top-5 positively and negatively correlated genes (i.e. with the lowest pooled p-value) is shown in figure 2A and 2B. An IPA analysis showed that these 1226 genes corresponded mostly with the following molecular and cellular functions: cellular movement, cellular development, cellular growth and proliferation, cell-to-cell signaling and interaction and cell morphology. These functions are suggestive of a role for epithelial to mesenchymal transition (EMT), which describes a process in the cell that leads to loss of polarity, increased migratory and invasive capacity and reduced cell-cell contact (30).

3. Identification of miRs that correlate with radioresistance

To find driving miRs that influence radioresistance, we set three separate requirements: 1. to select miRs that were actively degrading their mRNA targets, there had to be a negative correlation between miR expression and expression of its targets, 2. a correlation between miR expression and radioresistance and 3. an inverse correlation of the target expression with radioresistance (compared to
the miR-radioresistance correlation). Using these criteria, the chance of finding false-positive results is brought down to a minimum and only relevant miRs are identified.

For this analysis miRs and mRNAs were filtered based on the interquartile range (IQR) of expression between the 32 cell lines to exclude uninformative values. This left 200 miRs and 13,041 mRNAs with an IQR higher than 0.5 for the analysis. Out of the 200 miRs, 39 were discarded because they had fewer than 5 predicted targets. After the filtering steps, the remaining 161 miRs had an average number of 506 predicted mRNA targets, as defined by our in silico generated miR-mRNA interaction database. Of these 161 miRs, 37 had a significantly negative miR-target Pearson correlation after multiple testing correction. P-values for the correlation between each miR and its targets were calculated using a 2-sided T-test of the Pearson correlations of the predicted mRNA targets for each miR versus the Pearson correlations of all other (random) mRNAs with the miR expression. P-values for the correlation between mRNA targets and radioresistance were calculated using the same approach, comparing the difference between all p-values for the Pearson correlations between the targets and radioresistance versus all p-values for the correlations between the non-target mRNAs and radioresistance. P-values for the difference in miR expression between sensitive and resistant cell lines over the 2 time points were obtained using the BRB time course plug-in. A significant correlation of the miR and its targets with radioresistance was observed for 12 of these 37 miRs, belonging to ten different miR families (table 1). Expression over time for the top five miR families can be seen in figure 2C. Of interest is that 292 of the earlier identified 1226 mRNAs that were significantly correlated with radioresistance are being regulated by one of these 12 miRs.

4. EMT correlates with radioresistance
From the data described in the second and third section, it appears that the loss of miRs downregulating EMT mRNAs were significantly correlated with the intrinsic radioresistance of these 32 HNSCC cell lines. To verify that EMT had a causal relation with radioresistance, we collected two HNSCC cell lines that had been forced to undergo EMT: UT-SCC-43A-Snail and FaDu-HIF-1α(ΔODD). Both Snail and HIF1α are known transcription factors for EMT. In cell culture, the Snail- or HIF1α-expressing cells were clearly mesenchymal, whereas the respective control cell lines UT-SCC-43A and FaDu-cDNA3 had an epithelial growth pattern. In these pairs we found that the cells that had undergone EMT were significantly more resistant to radiotherapy (figure 3), with areas under the survival curve increasing from 2.7 to 3.9 (p<0.0001) in the FaDu pair and from 2.6 to 4.6 (p<0.0001) in the UT-SCC-43A pair.

We further tested the correlation between radiosensitivity and processes known to influence radiotherapy response in the 32 cell lines, by using published gene sets for reactive oxygen species (31), hypoxia (32,33), proliferation (34), stem cells (single marker CD44 and the set from (35)), p53 [constructed ourselves, supplementary table 5], DNA repair [constructed ourselves, supplementary table 5] and intrinsic radiosensitivity (8,9). We also constructed our own HNSCC-EMT signature from the two pairs of HNSCC cell lines in which EMT was induced. This signature was constructed from genes with a fold change greater than 2 or under 0.5 between parental and EMT-induced strains. Additionally, only genes were selected that showed a fold change in the same direction (up- or downregulation) in both cell line pairs, which resulted in a set of 1189 genes (supplementary table 7).

For each cell line a score was generated for each gene set, by either calculating the mean expression of the genes in the set, or in the case of the HNSCC-EMT signature by calculating the Pearson correlation between the expression of the cell line and the average expression in FaDu-HIF-1α(ΔODD) and UT-SCC-43A-Snail cell lines for these 1189 genes. Next, scores for the gene sets were compared to the radiosensitivity values. Out of the different gene sets, the HNSCC-EMT gene set was the best predictor
of radiosensitivity (linear regression p-value: 0.001) in the panel of 32 HNSCC cell lines, with a Spearman correlation of 0.74 (p<0.0001). A plot of the HNSCC-EMT score against the radiosensitivity is shown in figure 4, the individual scores per cell line can be seen in supplementary table 6.

Of note is that the two EMT-inducible cell lines, although HNSCC cells, were not part of the 32 cell line panel, and thus were an independent test system, strengthening the interpretation of an EMT based mechanism for radioresistance.

5. MiRs predicting radiotherapy response in patients

The expression of the most significant miR in cell lines (miR-203) was tested in a pilot series of 34 patients with T2-3 larynx tumors, treated with radiotherapy. The 12 top miRs were analyzed. When two groups were created divided by the median expression, a trend was seen for higher recurrence percentages with low expression of miR-452 (HR = 0.5, p = 0.1), miR-200b (HR = 0.7, p = 0.4) and miR-141 (HR = 0.6, p = 0.4). However, only low miR-203 expression was significantly correlated with local recurrence in a multivariate cox regression (figure 5, HR = 0.364, log-rank p = 0.04). These findings are in line with the cell line data, i.e. loss of miR-203 expression leads to radioresistance.

DISCUSSION

It is not clear why some cells are radiosensitive and others are intrinsically radioresistant. By identifying the underlying mechanisms of radioresistance, it should become possible to personalize therapy where necessary, thereby achieving better treatment success rates. In this study, we correlated expression of micro- and messenger RNA to intrinsic radiosensitivity of head and neck cancer. In our HNSCC cell line panel, we found that a low expression of certain miRs was strongly correlated with radioresistance. Different analysis methods led to the conclusion that epithelial to mesenchymal
transition (EMT) was an important factor in radioresistance, namely, the top correlating mRNAs, miRs and gene sets were all involved in EMT and these findings were validated by testing two different cell lines engineered to undergo EMT, which caused an increase in resistance. Next, we have shown that low expression of the top miR (miR-203) predicting intrinsic radiosensitivity indeed corresponded to more local recurrences after radiotherapy in a patient series of laryngeal carcinomas. Since it has previously been reported that no major difference was detected in miR profiles among laryngeal, oropharyngeal, or hypopharyngeal cancers, we believe that this cohort could be representable for all of these subsites (36). It should be noted that results were obtained using multiple testing on a small series, needing further validation in a larger cohort of head and neck squamous cell carcinomas, preferably including head and neck tumors from different subsites.

Although separate EMT genes like fibronectin 1, Snail, Slug and E-cadherin have already been associated with radioresistance (37–40), it has not been clarified why EMT would cause radioresistance. We hypothesize that simultaneous with acquiring a mesenchymal phenotype, the mechanisms by which cells can become more resistant to irradiation are altered. EMT is mainly a description of a phenotype, but the fact that the acquisition of this phenotype is correlated with radioresistance may indicate it affects at least one of the three known mechanisms that lead to resistance: less damage upon irradiation, better repair of irradiation damage, or less cell death upon damage.

A first hypothesis could be that the evasion of DNA damage could lead to radioresistance (31). In a recent overview, Watson proposes that mesenchymal cancer cells possess heightened amounts of antioxidants that reduces damage caused by irradiation-induced reactive oxygen species (ROS) (41). Gammon et al. showed that within mesenchymal cancer cells under normoxic conditions, a subpopulation of cells with low oxygen and ROS levels can be found (42). Secondly, a more effective DNA damage repair system can lead to increased survival of cells after radiotherapy. This appears to be the case in breast cancer cell lines, in which it was shown that
HOXB9 induces both EMT and confers resistance to ionizing radiation by accelerating the DNA damage response (43). In another report it was shown that ATM-mediated Snail Serine 100 phosphorylation regulates cellular radiosensitivity (44).

Finally, damaged cells can evade cell death and thereby survive irradiation. Kurrey et al propose a model in ovarian cancer, in which EMT transcription factors Snail and Slug can antagonize p53-mediated apoptosis (40). TGF-β is also known to simultaneously invoke EMT and block apoptosis via PI3K signaling (45). In addition, another EMT-inducer, SIP1, has been ascribed anti-apoptotic properties (46). With the acquisition of an EMT phenotype, cells have been shown to increase autophagy: a lysosomal degradation pathway that can be used to increase survival of cells (47). Rouschop et al. demonstrated that inhibition of autophagy sensitized xenografts to irradiation (48).

In an attempt to confirm these hypotheses we tested different gene sets for reactive oxygen species, DNA repair, cell cycle phase and several means of cell death against the EMT gene set (supplementary table 7). From these analyses it appears that there is no single explanation for the radioresistance of the mesenchymal phenotype. The acquisition of a heightened EMT gene expression profile, corresponds to a higher expression of genes known to be expressed in G2, genes involved in DNA double-strand break repair and autophagy. This indicates that mesenchymal cells might become more resistant to radiotherapy by prolonging time spent in G2, more efficient double-strand break repair and the use of autophagy as a possible mechanism to evade cell death. ROS-scavenger or apoptosis gene sets showed no correlation with expression of EMT genes.

Our study is the first to identify miRs with their messenger RNA targets that are involved in radioresistance in HNSCC. By analyzing miRs together with their targets, a more realistic representation of what occurs in cells can be obtained. A pitfall remains the allocation of the correct targets to every
miR. Despite this possible confounding effect of wrongly allocated targets in the analysis, when studying the effect of all targets of one miR as a group, a reliable target effect can be observed. Future studies into correctly defining miR targets should improve this analysis method. The potential advantage of discovering miRs that are correlated with resistance is that, when used as therapeutic agents, they are able to target many genes at once, frequently within one pathway or network (49).

We observed that constitutive but not radiation-responsive genes correlated with radioresistance. These findings are consistent with findings of Birrell et al. on the yeast deletion mutant library (50) and the findings in the gene expression series of Amundson et al. who concluded that in the NCI-60 cell line panel ‘basal expression patterns discriminated well between radiosensitive and more resistant lines, possibly being more informative than radiation response signatures’ (8).

In conclusion, the pre-irradiation miR-203 status, determined by integrative miR and mRNA analyses, was the most powerful predictor of radioresistance in our HNSCC cell line panel. This EMT-inhibiting miR was decreased in patients with a local recurrence after radiotherapy. The fact that radioresistance could be best predicted from baseline expression suggests that future studies into intrinsic resistance should not focus on response to irradiation. If these findings can be translated to the clinical setting, it should be possible to predict radiotherapy outcome from a pre-treatment sample.

The next step would be to reverse EMT in vivo, possibly by restoring expression of miR-203. Since one miR can target many genes, EMT caused via different routes could potentially be inhibited by a single miR. Inhibition of EMT in vivo could not only make cells more radiosensitive but also more chemosensitive and less invasive, which together should lead to better patient survival.

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TABLE AND FIGURE LEGENDS

**Table 1. Relevant miRs correlated with radioresistance.**

Properties of the miRs and their associated mRNA targets that were significantly correlated with radioresistance. **Column 1:** miR name. **Column 2:** The number of predicted mRNAs that are being targeted by this miR. **Column 3:** A significant negative correlation between the miR and its predicted targets indicates that this miR is actively degrading its targets. **Column 4:** The direction of the miR expression in the group of resistant cell lines. **Column 5a:** p-values from the BRB array tools time course plug-in, representing the correlation between radioresistance (AUC) and the expression of the miR over the 2 measured time points. **Column 5b:** p-value of a 2-sided T-test comparing the difference between all p-values for the Pearson correlations between the predicted mRNA targets and radioresistance versus all p-values for the correlations between the non-target mRNAs and radioresistance. **Column 6:** all references for the described miR functions can be found in supplementary table 3. *Both member of miR family miR-200bc/429/548a. *Both member of miR family miR-141/200a.

**Figure 1. Overview of data.**

**Figure 2. MiR and mRNA expression differences between resistant and sensitive cell lines.**

mRNA and miR expression differences between sensitive and resistant cell lines over time. The differences between sensitive and resistant cell lines at the individual time points were all individually significant (T-test p-value < 0.05), except for miR-141/200a and miR-200bc/429. The differences between sensitive and resistant cells for these two miR families were only significant, when the measurements at both time points were considered. Error bars: mean + SEM.
Figure 3. Induction of EMT causes radioresistance.

Induction of EMT by HIF-1α (left panel) or Snail (right panel) leads to increased radioresistance.

Figure 4. HNSCC EMT score versus radiosensitivity.

Figure 5: Cells with a higher score for EMT (more mesenchymal) are more resistant to irradiation.

Figure 5. MiR-203 in patients

Seventeen patients with a local recurrence after radiotherapy were matched with 17 patients without a local recurrence. Patients with a high miR-203 expression had a significantly higher cure rate. The mean survival in this curve is 50% because of matching 1:1.
Table 1. Relevant miRs correlated with radioresistance.

<table>
<thead>
<tr>
<th>miR name</th>
<th>1. No of predicted mRNA targets</th>
<th>2. Significant negative miR-mRNA targets correlation? (p-value)</th>
<th>3. MiR expression in resistant cells up or down?</th>
<th>4. Correlation with radioresistance</th>
<th>5. Correlation with radioresistance</th>
<th>6. miR function</th>
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<td>miR-203a</td>
<td>541</td>
<td>Yes (1 \times 10^{-5})</td>
<td>Down</td>
<td>(&lt;1 \times 10^{-5})</td>
<td>3 \times 10^{-10})</td>
<td>Inhibit growth, self-renewal, migration, invasion and EMT</td>
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<td>miR-205-5p</td>
<td>545</td>
<td>Yes (3 \times 10^{-16})</td>
<td>Down</td>
<td>(&lt;1 \times 10^{-5})</td>
<td>8 \times 10^{-9})</td>
<td>Promote apoptosis, inhibit growth, migration, invasion and EMT</td>
</tr>
<tr>
<td>miR-452-5p</td>
<td>499</td>
<td>Yes (0.001)</td>
<td>Down</td>
<td>7 \times 10^{-4}\</td>
<td>2 \times 10^{-8}\</td>
<td>Reduce stem-like traits and tumorigenesis, EMT</td>
</tr>
<tr>
<td>miR-200b-3p$</td>
<td>562</td>
<td>Yes (1 \times 10^{-14})</td>
<td>Down</td>
<td>0.03</td>
<td>1 \times 10^{-15}\</td>
<td>Reduced proliferation, migration, invasion and EMT</td>
</tr>
<tr>
<td>miR-429$</td>
<td>562</td>
<td>Yes (5 \times 10^{-14})</td>
<td>Down</td>
<td>0.005</td>
<td>1 \times 10^{-15}\</td>
<td>Inhibit proliferation and EMT</td>
</tr>
<tr>
<td>miR-141-3p$</td>
<td>557</td>
<td>Yes (1 \times 10^{-9})</td>
<td>Down</td>
<td>0.02</td>
<td>6 \times 10^{-10}\</td>
<td>Inhibit EMT</td>
</tr>
<tr>
<td>miR-200a-3p$</td>
<td>554</td>
<td>Yes (8 \times 10^{-7})</td>
<td>Down</td>
<td>0.009</td>
<td>1 \times 10^{-9}\</td>
<td>Inhibit EMT</td>
</tr>
<tr>
<td>miR-7-5p</td>
<td>544</td>
<td>Yes (3 \times 10^{-14})</td>
<td>Down</td>
<td>0.04</td>
<td>4 \times 10^{-9}\</td>
<td>Inhibit invasion, self renewal and EMT, promote apoptosis</td>
</tr>
<tr>
<td>miR-138-5p</td>
<td>546</td>
<td>Yes (0.04)</td>
<td>Down</td>
<td>0.01</td>
<td>0.003</td>
<td>Inhibit proliferation, invasion, migration, modify DNA damage response</td>
</tr>
<tr>
<td>miR-34a-5p</td>
<td>539</td>
<td>Yes (0.0001)</td>
<td>Down</td>
<td>2 \times 10^{-4}\</td>
<td>1 \times 10^{-3}\</td>
<td>Inhibit proliferation, invasion, metastasis, stemness, EMT</td>
</tr>
<tr>
<td>miR-142-3p</td>
<td>522</td>
<td>Yes (0.03)</td>
<td>Down</td>
<td>0.04</td>
<td>5 \times 10^{-9}\</td>
<td>Maintenance of dendritic cells, inhibit growth and stemness</td>
</tr>
<tr>
<td>miR-33b-5p</td>
<td>483</td>
<td>Yes (0.0005)</td>
<td>Down</td>
<td>0.03</td>
<td>2 \times 10^{-5}\</td>
<td>Reduce proliferation, induce G1 arrest, cholesterol transport</td>
</tr>
</tbody>
</table>
Properties of the miRs and their associated mRNA targets that were significantly correlated with radioresistance.  

**Column 1:** miR name.  **Column 2:** The number of predicted mRNAs that are being targeted by this miR.  **Column 3:** A significant negative correlation between the miR and its predicted targets indicates that this miR is actively degrading its targets.  **Column 4:** The direction of the miR expression in the group of resistant cell lines.  **Column 5a:** p-values from the BRB array tools time course plug-in, representing the correlation between radioresistance (AUC) and the expression of the miR over the 2 measured time points.  **Column 5b:** p-value of a 2-sided T-test comparing the difference between all p-values for the Pearson correlations between the predicted mRNA targets and radioresistance versus all p-values for the correlations between the non-target mRNAs and radioresistance.  **Column 6:** all references for the described miR functions can be found in supplementary table 3. *Both member of miR family miR-200bc/429/548a. *Both member of miR family miR-141/200a.
Figure 1

**Data overview**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Radio-resistance</th>
<th>Experimental conditions</th>
<th>RNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 head and neck cancer cell lines</td>
<td>known</td>
<td>2 hours after 4 Gy</td>
<td>microRNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 hours after 4 Gy</td>
<td>mRNA</td>
</tr>
</tbody>
</table>

**Hypothesis generating**

- Mock-irradiated
- mRNA
- microRNA
- mRNA
- microRNA
- mRNA

**Hypothesis testing in cell lines**

- 2 cell line pairs +/- EMT
- unknown
- EMT induced by HIF1α-∆ODD
- mRNA
- EMT induced by snail
- mRNA

**Hypothesis testing in patients**

- 34 larynx cancer patients
- 17 cures
- Pre-treatment biopsy
- microRNA
- 17 recurrences
- Pre-treatment biopsy
- microRNA
**Figure 2**

**A** Top-5 mRNAs downregulated in resistant cells

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Expression (log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT6</td>
<td>8</td>
</tr>
<tr>
<td>ALDH3B2</td>
<td>6</td>
</tr>
<tr>
<td>TMPRSS13</td>
<td>4</td>
</tr>
<tr>
<td>ILDR1</td>
<td>2</td>
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<tr>
<td>GDPD3</td>
<td>0</td>
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</tbody>
</table>

Time after 4 Gy (hours)

**B** Top-5 mRNAs upregulated in resistant cells

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Expression (log2)</th>
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<tbody>
<tr>
<td>ARMCX1</td>
<td>10</td>
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<tr>
<td>SNRPN</td>
<td>8</td>
</tr>
<tr>
<td>SNURF</td>
<td>6</td>
</tr>
<tr>
<td>DNAJB5</td>
<td>4</td>
</tr>
<tr>
<td>TSPAN4</td>
<td>2</td>
</tr>
</tbody>
</table>

Time after 4 Gy (hours)

**C** Top-5 miRs downregulated in resistant cells

<table>
<thead>
<tr>
<th>miR</th>
<th>Expression (log2-ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-203</td>
<td>-1</td>
</tr>
<tr>
<td>miR-205/205ab</td>
<td>-2</td>
</tr>
<tr>
<td>miR-452/4676-3p</td>
<td>-3</td>
</tr>
<tr>
<td>miR-141/200a</td>
<td>-3</td>
</tr>
<tr>
<td>miR-200bc/429/548a</td>
<td>-3</td>
</tr>
</tbody>
</table>

Time after 4 Gy (hours)
Figure 3

Surviving fraction vs. Dose (Gy) for FaDu +/- HIF-1α(ΔODD) and UT-SCC-43A +/- Snail.

- FaDu-HIF-1α(ΔODD)
- FaDu-cDNA3
- UT-SCC-43A-Snail
- UT-SCC-43A

p-value <0.0001
Figure 4

$p$-value = 0.001
MiR-203 and local recurrence in 34 larynx cancer patients

Time (years)

Local recurrence free probability

No. at risk

<table>
<thead>
<tr>
<th></th>
<th>High</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17</td>
<td>14</td>
<td>11</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Low</td>
<td>17</td>
<td>11</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

\[p = 0.04\]
Pretreatment microRNA expression impacting on epithelial to mesenchymal transition predicts intrinsic radiosensitivity in head and neck cancer celllines and patients

Monique C. de Jong, Jelle ten Hoeve, Reidar Grenman, et al.

Clin Cancer Res  Published OnlineFirst August 11, 2015.

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