Abstract  

Purpose: MicroRNA (miRNA) expression alterations have been described in cancer. Many cancers are characterized by areas of hypoxia, enhanced hypoxia-inducible factor (HIF) levels, and increased expression of hypoxically regulated genes, all of which correlate with patient outcome. We examined hypoxia-induced miRNA expression changes to identify markers of survival in breast cancer.

Experimental Design: We used microarrays to analyze miRNA expression changes induced by hypoxia in MCF7 breast cancer cell lines and validated results by quantitative-PCR (Q-PCR). Small interfering RNA against HIF-1α and HIF-2α, and RCC4 cells transfected with the von Hippel-Lindau (VHL) protein were used to investigate HIF dependency of miRNA expression. miRNA Q-PCR assays were done on 219 early breast cancer samples with long-term follow-up. Correlation of expression with clinical variables was done using Pearson and Spearman’s rank tests, univariate, and Cox multivariate analysis.

Results: hsa-miR-210 induction was the most significant change under hypoxia by microarray analysis (3.4-fold, \( P < 0.001 \)). hsa-miR-210 expression changes were validated by Q-PCR and detected in other cancer cell lines. Using small interfering RNAs and RCC4 cells transfected with VHL, we showed that the regulation by hypoxia of hsa-miR-210 was mediated by the HIF-1α/VHL transcriptional system but not HIF-2α. hsa-miR-210 expression levels in breast cancer samples correlated directly with a hypoxia score based on the expression of 99 genes. hsa-miR-210 expression levels showed an inverse correlation with disease-free and overall survival, significant in both univariate and multivariate analyses.

Conclusions: We show that hsa-miR-210 overexpression is induced by hypoxia in a HIF-1α– and VHL-dependent fashion and its expression levels in breast cancer samples are an independent prognostic factor.

Hypoxia in cancer appears as a consequence of the growth of a malignant tumor but can also act to promote tumor development. Hypoxic conditions in solid malignancies may confer resistance to conventional therapies and are associated with a poorer prognosis (1–3). The exposure of cells to hypoxia leads to the coordinated regulation of many genes. The protein products of these genes have a wide variety of critical roles in processes such as energy metabolism, angiogenesis, growth, and apoptosis. Studies of the mechanisms underlying the regulation of such genes have implicated a central role for the transcription factor hypoxia-inducible factor (HIF), which exists as a heterodimer of an α and β subunit (4). In the presence of oxygen, HIF-α molecules undergo prolyl hydroxylation which is catalyzed by three homologous 2-oxoglutarate–dependent dioxygenases, PHD1, PHD2, and PHD3. The von Hippel-Lindau (VHL) protein recognizes and binds to two specific hydroxyprolyl residues in HIF-1α and HIF-2α, and facilitates ubiquitination leading to rapid proteasomal degradation. Further oxygen-regulated control of HIF-α is achieved by another dioxygenase (FIH-1), which catalyzes the formation of a specific hydroxyasparaginyl residue in HIF-α, reducing its binding to the transcriptional coactivator p300 (for reviews, see refs. 5, 6). The HIF system has been directly implicated in the responses of tumor cells to hypoxia (7, 8). Many cancers are
characterized by enhanced HIF levels and increased expression of hypoxically regulated genes which correlate with both tumor aggression and patient outcome (8, 9). Furthermore, the majority of renal carcinomas exhibit VHL mutations which lead to enhanced HIF action. Recently, comprehensive gene array studies (9–12) have emphasized the dominant role of the HIF transcriptional system and the HIF peptidyl hydroxylases in the regulation of gene expression by hypoxia. However, other mechanisms of gene regulation by hypoxia are likely and include control of mRNA stability (13), translation (14), and regulation mediated by microRNAs (miRNA).

miRNAs are noncoding RNA oligonucleotides that have emerged as important regulators of gene expression (15, 16). More than 500 genes encoding miRNAs have been identified in mammals. In their mature form, miRNAs specifically bind to the 3′-untranslated region of target mRNAs, leading to either mRNA degradation or inhibition of translation (15) and they may also be able to influence transcription of target genes by promoter activation (17) or transcriptional silencing (18). Differential expression levels of particular miRNAs have been observed in several tumor types when compared with normal tissue (19). miRNA copy number variation seems to be common in cancer (20) and overexpression of specific miRNAs can be sufficient for oncogenesis (21). miRNA levels seem to be regulated by external stimuli and their transcription is mediated at least in part by RNA polymerase II (22), although posttranscriptional regulation has also been reported (23). However, the precise mechanisms underlying inducible production, the role of transcription factors and the effects of hypoxia on miRNAs require further definition.

In order to determine the possible role of miRNAs in hypoxic gene regulation and to identify hypoxically regulated miRNAs that may have relevance in tumor pathogenesis, we have surveyed changes in miRNA expression levels in response to hypoxia. We have characterized in detail the hypoxic regulation of one specific miRNA (hsa-miR-210) and examined the mechanism of its regulation. hsa-miR-210 exhibits HIF-1α, VHL, and HIF prolyl hydroxylase–mediated regulation and therefore extends the mechanisms by which HIF is able to exert its effects on gene expression. During the course of this work, Kulshreshtha and colleagues also showed hsa-miR-210 induction by hypoxia in several cell lines and its regulation by HIF-1α using other methods (24). Our work is consistent with these results, but in addition, reveals the relationship of hsa-miR-210 levels with cancer pathogenesis and prognosis. We hypothesized that levels of hsa-miR-210 expression in cancer would correlate with degree of hypoxia and tumor behavior. To test this, we have examined the expression of this miRNA in human breast cancers according to the REMARK guidelines (25).

Materials and Methods

Patients with breast cancer. Two hundred and nineteen patients with early first primary breast cancer, treated in Oxford between 1989 and 1992, were studied. Ethical approval for analysis of samples and notes was obtained from the local research ethics committee. Patients received surgery followed by adjuvant chemotherapy, adjuvant hormone therapy, both of these therapies, or no adjuvant treatment. Tamoxifen was used as endocrine therapy for 5 years for premenopausal and postmenopausal patients if estrogen receptor (ER)–positive. In patients who were <50 years of age, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil were administered if the tumors were lymph node–positive, or ER-negative and/or >3 cm in diameter. Patients >50 years of age with ER-negative, lymph node–positive tumors also received cyclophosphamide, methotrexate, and 5-fluorouracil for six cycles, at a thrice weekly intravenous regimen. The data set was complete for age, nodal status, definitive surgery, relapse, and survival. The patient demographics are provided in Supplementary Table S1 and include the patient’s age, tumor size, grade, histology, nodal status, and ER status; Data were collected from clinical and pathological records. Of these, 146 patients were <50 years of age, and 73 have follow-up survival data of 6.3, 9.1, and 9.3 years; all other patients alive have >10 years follow-up.

Methods for selection of samples based on hypoxia score. A signature of 99 genes up-regulated under hypoxic conditions has been derived in head and neck tumors in vitro and it has been assessed for its prognostic value in a head and neck breast cancer data set (26). Here, this signature was used to calculate a hypoxic score for 73 of the 219 breast cancer samples in which gene expression data were available. The hypoxic score was calculated by considering the median expression value of the 99 probes set present in the hypoxic signature as previously described (26). In brief, Affymetrix HG-U133A and B gene expression microarray experiments had been previously done on 73 of the 219 samples, and the details of the processing and normalization have been described elsewhere (27, 28). Expression values were logged (log2) and HG-U133A and B arrays were mapped to the Affymetrix HG-U133 Plus 2.0 arrays where the hypoxic signature was originally derived.

The score is a measure of the level of expression of genes in the hypoxia signature: a high positive score reflects a higher than average expression of the genes in the hypoxia signature and indicates that the sample is hypoxic, a low negative score reflects a lower than average expression of the genes in the hypoxia signature and indicates that the sample is normoxic (26).

Cell lines and culture conditions. The human cell lines studied were breast adenocarcinoma cell line MCF7, hepatoblastoma cell line Hep3B, uterine cervix adenocarcinoma cell line HeLa, and renal cancer cell line RCC4 stably transfected with either an empty vector or VHL (29). All cell types were grown in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 2 mmol/L of l-glutamine. Expression of cell cultures to hypoxia was undertaken in an In vivo Hypoxia Work Station (Ruskin Technologies) in parallel with cells maintained in normoxic conditions (21% oxygen). All experiments were done in triplicate from independent cell cultures.

RNA extraction. RNA was extracted from cells using the mirVana miRNA Isolation Kit (Ambion). RNA was extracted and purified from liquid nitrogen–frozen breast tumor samples or normal breast tissue for the controls using Tri-reagent (Sigma-Aldrich) and ethanol precipitation. In both cases, RNA quality and abundance were determined after extraction using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, respectively).

miRNA microarrays. Microarrays were generated using the mirVana Probe Set version 1 (Ambion). Probes were spotted onto amine-reactive slide substrates GE CodeLink (Amersham Biosciences). Each microarray contained four spot replicates for each probe. Small RNA (<200 nucleotides) was purified from 100 μg of total RNA using FlashPAGE fractionation gels (Ambion) and FlashPAGE Clean-up kit (Ambion) was used for further purification and concentration of the product. The synthetic sequence control 1 (Ambion) was used for the control of the labeling process. It was spiked for each normoxic and hypoxic sample in a ratio of 1:10, respectively. Five hundred nanograms of small RNA from each sample was labeled using NCode miRNA labeling kit (Invitrogen) and two-color hybridizations were done. Twelve independent samples (six hypoxic and six normoxic) were used in two series of three hybridizations including dye-swapping between both series of experiments. Hybridization signals were detected and quantified using a GenePix 4000B scanner and the GenePix Pro 4.0, respectively (both

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from Axon Instruments, Molecular Devices). The median values of each spot were background-subtracted by using a local background subtraction method (GenePix Pro 4.0).

**Analysis of microarray data.** The Bioconductor implementation of Limma (30) was used to analyze the data. Limma is an established general linear regression algorithm that uses empirical Bayes methods to gain power when a large number of predictors (i.e., probes) are present with a relatively small number of cases (i.e., arrays). Loess normalization was done and average values were calculated for within-array replicate spots. To estimate fold changes between hypoxic and normoxic conditions, and relative standard errors, a linear model was fit to each probe in the array; dye-swaps were indicated in the design matrix for this fit. Empirical Bayes smoothing was then applied to the standard errors and the Benjamini and Hochberg method was used to correct for multiple testing. In addition, data was filtered based on fold-changes (using 2-fold as a threshold). Control 1 was detected according to the expected spike concentration. We used the miRNA nomenclature according to the miRNA Registry (hsa-miRBase) at Sanger Institute, except for Ambion-predicted miRNA sequences (designed as amb-miR#).

**Small interfering RNA treatment of MCF7 cells.** RNA oligonucleotides (Ambion) were used for siRNA suppression of individual HIF family members (HIF-1α; sense, CIGAGUAAGCCACCCACGAA; antisense, UACAGUUGGUUCGGUGUUGA). A set of oligonucleotides (Ambion) against a sequence from the Drosophila melanogaster HIF (D-HIF; sense, CACGUACUIUAGUCCUUUAU; antisense, UCUAGCUGUGGUGUGGUGA). The Bioconductor implementation of Limma (30) was used to analyze the data. Limma is an established general linear regression algorithm that uses empirical Bayes methods to gain power when a large number of predictors (i.e., probes) are present with a relatively small number of cases (i.e., arrays). Loess normalization was done and average values were calculated for within-array replicate spots. To estimate fold changes between hypoxic and normoxic conditions, and relative standard errors, a linear model was fit to each probe in the array; dye-swaps were indicated in the design matrix for this fit. Empirical Bayes smoothing was then applied to the standard errors and the Benjamini and Hochberg method was used to correct for multiple testing. In this case, the fractional rank of the expression was considered, that is, the patients were ranked using the miRNA expression levels and the ranks were normalized between 0 and 1. Statistical analyses were done using SPSS and R.

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**Results**

**miRNA expression profile in hypoxic MCF7.** We chose the breast cancer line MCF7 for study of miRNA regulation by hypoxia as we had previously characterized the expression of the components of the HIF system in that cell line and undertaken an extensive study of the gene expression profile in response to hypoxia, a prolyl hydroxylase inhibitor dimethyl-oxalylglycine, and HIF-α isoform manipulations (10). MCF7 breast cancer cells were grown under conditions of either normoxia (21% oxygen) or hypoxia (1% oxygen) for 16 hours. Among the 377 miRNAs included in our microarray, 4 miRNAs were significantly up-regulated between hypoxic and normoxic conditions (multiple test–corrected P < 0.05 using Limma; Supplementary Table S2). Nevertheless, only three miRNAs (hsa-miR-210, amb-miR-7105, and mmu-miR-322-3p) showed >2-fold induction in response to hypoxia (see Supplementary Table S2). No significantly down-regulated miRNAs were found in our analysis after multiple test correction of P values.

**Confirmation of the hypoxic induction of hsa-miR-210 in MCF7 cells by quantitative-PCR.** We elected to confirm hypoxic regulation of hsa-miR-210 by analyzing eight independent pairs of RNA samples obtained from normoxic and hypoxic MCF7 cells using quantitative-PCR (Q-PCR). In agreement with our previous data, we found a substantial and significant induction of hsa-miR-210 expression by hypoxia (4.11 ± 0.92-fold; P < 0.001). To exclude the possibility that hypoxia produced an artifactual change in miRNA recovery, we also examined the expression of hsa-miR-93, a miRNA that was not regulated by hypoxia based on our microarray assays. This miRNA did not show significant regulation by hypoxia (0.92 ± 0.22) when assayed by Q-PCR. We also examined the hypoxic induction of hsa-miR-21, reported to be enhanced in a wide range of cancers (32) and induced by hypoxia at 24 hours in microarray studies (24). However, under our experimental conditions, we were unable to detect any significant regulation previously (10). The cyclin D1 gene was used as a reference. The following primers were used: RASF7 (forward, 5’ CACTAGGCCCCAGGAAAGTGGCC 3’; reverse, 5’ ACATCGCTGCAAGGTTGAGTCTG 3’; annealing temperature, 60°C), AK093839 (forward, CCGGACGACGGCTGTG 3’; reverse, 5’ AAGGGGAGGATCACCTGAG 3’; annealing temperature, 59°C), and cyclophilin (forward, 5’ ATGGCGGCTGGCAAAATCAACG 3’; reverse, 5’ CCGTCTTAGGGCAACAGCTG 3’; annealing temperature, 58°C).

**Statistical methods.** Correlation of hsa-miR-210 and hsa-miR-21 with clinical and pathologic variables was assessed using Pearson and Spearman’s rank tests. Univariate survival analysis was done by applying the log-rank test to hsa-miR-210 and hsa-miR-21 expression levels stratified by median value and in four quartiles. Both disease-specific overall survival (called overall survival in the rest of this article) and disease-free survival were considered as outcome. Multivariate Cox survival analyses were also done in which the miRNAs were introduced as a continuous variable together with other relevant clinical factors. In this case, the fractional rank of the expression was considered, that is, the patients were ranked using the miRNA expression levels and the ranks were normalized between 0 and 1. Statistical analyses were done using SPSS and R.

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4 http://www.bioconductor.org/
5 http://miromaps.sanger.ac.uk/sequences/
6 http://www.r-project.org/
of hsa-miR-21 by hypoxia when assayed either by microarray or by Q-PCR.

In order to further explore the regulation of hsa-miR-210 by hypoxia, we examined the time course of hsa-miR-210 induction by hypoxia. MCF7 cells were cultured in hypoxia (1% oxygen) for 1, 2, 4, 8, 16, 32, and 48 hours. The expression of hsa-miR-210, hsa-miR-93, and hsa-miR-21 were analyzed by Q-PCR. Induction of hsa-miR-210 by hypoxia was discernable at 8 hours and showed a progressive increase in expression, becoming significant at 16 hours (3.08 ± 0.34, P < 0.001) and being maximal at the latest (48 hours) time point (4.78 ± 0.32, P < 0.0001), whereas expression of the hsa-miR-93 and hsa-miR-21 were again unaffected by exposure to hypoxia (Fig. 1A).

To study the oxygen dependence of the regulation of hsa-miR-210, further experiments were undertaken in cells exposed to a range of differing oxygen tensions (MCF7 cells were cultured for 16 hours in 0.1%, 1%, 3%, 5%, and 21% oxygen). A significant induction of hsa-miR-210 was seen at 0.1% oxygen (2.56 ± 0.53, P = 0.011) with more modest regulation at 3% and 5% oxygen, but the greatest induction occurred with exposure to 1% oxygen (3.08 ± 0.33, P = 0.001) (Fig. 1B). Again, the expression of hsa-miR-21 and hsa-miR-93 were unaffected by hypoxia.

**hsa-miR-210 hypoxic induction in MCF7 cells is HIF-1α dependent.** The time course and oxygen sensitivity of the hsa-miR-210 induction by hypoxia was similar to that seen for the regulation of many HIF-dependent, hypoxically regulated genes. Therefore, we undertook experiments to examine the role of HIF-1α and HIF-2α in the hypoxic induction of hsa-miR-210. We did a siRNA-mediated suppression of either HIF-1α or HIF-2α in MCF7 cells under hypoxic conditions of 1% oxygen for 16 hours, including controls as previously described (10). Three independent cell cultures were examined for each condition. The siRNA-mediated suppression of HIF-1α produced a striking abrogation of the hypoxic response of hsa-miR-210 (Table 1). Although this was the only statistically significant change compared with the untreated control (P = 0.001), siRNA oligonucleotides targeting HIF-2α and a control sequence produced a modest reduction in the induction of hsa-miR-210 by hypoxia, suggesting a nonspecific effect of siRNA transfection on miRNA expression. In agreement with this explanation, comparable small reductions in the expression of hsa-miR-93 and hsa-miR-21 were also observed with all siRNA oligonucleotide treatments (Table 1). These results suggest that the hypoxic induction of hsa-miR-210 is mediated at the transcriptional level by HIF-1α.

Further support for this was obtained from an examination of microarray gene expression data from MCF7 cells exposed to hypoxia, a HIF prolyl hydroxylase inhibitor DMOG, and similar suppression with HIF targeting siRNA oligonucleotides (10). hsa-miR-210 is an intronic miRNA contained within the sequence of the predicted transcript AK123483 (Supplementary Fig. S1). This transcript also showed HIF-1α–dependent hypoxic regulation in keeping with the coordinate transcription of intronic miRNAs and their host genes that has been seen for many intronic miRNAs (33). Putative HIF binding sites can be found 5′ of this gene (Supplementary Fig. S1). Interestingly, the adjacent gene RASSF7 (3′ of AK123483 in tail to tail orientation; Supplementary Fig. S1) also showed modest hypoxia and DMOG induction in an HIF-1α–dependent fashion when determined by the microarray experiments.

In order to confirm the hypoxic regulation of the expression level of AK123483 and adjacent gene products, we undertook

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**Table 1.** hsa-miR-210 expression is regulated by HIF-1α

<table>
<thead>
<tr>
<th>HIF-1α siRNA</th>
<th>hsa-miR-210</th>
<th>hsa-miR-93</th>
<th>hsa-miR-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6.10 ± 1.69*</td>
<td>-1.53 ± 0.32</td>
<td>-1.69 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>HIF-2α siRNA</td>
<td>-2.14 ± 0.33</td>
<td>-2.36 ± 0.86</td>
<td>-2.04 ± 0.11</td>
</tr>
<tr>
<td>DHIF siRNA</td>
<td>-2.53 ± 0.88</td>
<td>-2.02 ± 1.04</td>
<td>-1.38 ± 0.76</td>
</tr>
</tbody>
</table>

NOTE: The level of expression of hsa-miR-210, hsa-miR-93, and hsa-miR-21 relative to the RNU43 control was measured in MCF7 cells treated with siRNA oligonucleotides targeting HIF-1α, HIF-2α, or a control sequence (DHIF) after exposure to 1% hypoxia for 16 h (three replicates/treatment). Results are expressed as the average fold induction (±SD) between the indicated HIF targeting siRNA oligonucleotides and the transfection reagent Oligofect-AMINE alone. A negative value indicates suppression of expression relative to that seen in the untreated control. Significant differences compared with the untreated control were established using ANOVA followed by Scheffe’s post hoc test.

*P = 0.001.
Q-PCR assays of the expression of RASSF7 and AK093839 (all within 8 kb 3’ of AK123483; Supplementary Fig. S1) in MCF7 cells under hypoxia. These assays confirmed significant induction of RASSF7 in hypoxic MCF7 cells (3.70 ± 0.61), but not of the AK093839 (1.18 ± 0.15), all consistent with previous data (10). However, these results do suggest that the hypoxic regulation of hsa-miR-210 might form part of a wider area of regulation of transcripts at this locus.

**VHL dependence of hsa-miR-210 hypoxic induction in RCC4 cells.** Mutations affecting the function of the VHL tumor suppressor ablate proteolytic regulation of HIF-1α and HIF-2α subunits, leading to constitutive activation of hypoxic pathways in renal cell carcinoma. To examine whether the hypoxic regulation of hsa-miR-210 was seen in other cell types and to examine for the VHL dependence of regulation, we examined the VHL-defective RCC4 cell line (RCC4) and RCC4 cells stably transfected with VHL (RCC4 + VHL) for the expression of hsa-miR-210 (Table 2). In the RCC4 cells with VHL function restored (RCC4 + VHL), there was a substantial (5-fold) induction of hsa-miR-210 expression by hypoxia, thus showing its hypoxia regulation in a different cell type. Furthermore, cells lacking VHL function showed a very substantial increase in hsa-miR-210 expression (16-fold), again supporting the action of the HIF/VHL system in its regulation. When the expression of hsa-miR-93 and hsa-miR-21 was tested, no significant VHL dependence of hsa-miR-210 hypoxic induction in RCC4 and those in which VHL expression has been reintroduced (RCC4 + VHL). Significant differences compared with the respective controls were established using ANOVA followed by Scheffe’s post hoc test.

**Table 2. hsa-miR-210 expression in a range of cancer cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>hsa-miR-210 F</th>
<th>hsa-miR-93 F</th>
<th>hsa-miR-21 F</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC4 (hypoxia/normoxia)</td>
<td>1.14 ± 0.29</td>
<td>1.48 ± 0.51</td>
<td>1.36 ± 0.77</td>
</tr>
<tr>
<td>RCC4 + VHL (hypoxia/normoxia)</td>
<td>5.62 ± 0.32*</td>
<td>1.55 ± 0.14</td>
<td>2.01 ± 0.59</td>
</tr>
<tr>
<td>RCC4/RCC4 + VHL (normoxia)</td>
<td>16.26 ± 2.64</td>
<td>1.43 ± 0.39</td>
<td>1.63 ± 0.81</td>
</tr>
<tr>
<td>HeLa (hypoxia/normoxia)</td>
<td>6.45 ± 0.22</td>
<td>1.11 ± 0.10</td>
<td>1.03 ± 0.36</td>
</tr>
<tr>
<td>Hep3B (hypoxia/normoxia)</td>
<td>4.73 ± 0.65</td>
<td>0.65 ± 0.12</td>
<td>0.58 ± 0.20</td>
</tr>
</tbody>
</table>

NOTE: The level of expression of hsa-miR-210, hsa-miR-93, and hsa-miR-21 relative to the RNU43 control was measured in several different cell lines (RCC4, RCC4+VHL, HeLa, and Hep3B) in either normoxic or hypoxic (1% oxygen for 16 h) conditions. Results are expressed as mean fold induction (±SD) between hypoxic and the parallel normoxic control. miRNA expression is also compared between normoxic cells lacking functional VHL (RCC4) and those in which VHL expression has been reintroduced (RCC4 + VHL). Significant differences compared with the respective controls were established using ANOVA followed by Scheffe’s post hoc test.

*P = 0.03.
†P < 0.001.
‡P = 0.002.

**Table 3. Summary statistics of miRNA expression level distributions in 219 samples of breast cancer**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Mean</th>
<th>Minimum</th>
<th>Quartile 1</th>
<th>Median</th>
<th>Quartile 3</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-210</td>
<td>5.48</td>
<td>0.08</td>
<td>1.20</td>
<td>2.80</td>
<td>6.35</td>
<td>67.81</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>1.31</td>
<td>0.009</td>
<td>0.52</td>
<td>0.92</td>
<td>1.82</td>
<td>6.63</td>
</tr>
</tbody>
</table>

NOTE: The expression levels statistics in the table are expressed as a fold change of hsa-miR-210 and hsa-miR-21 in breast cancer in comparison to averages from 10 control samples.
hsa-miR-21 did not show a significant correlation (Spearman’s $\rho = 0.14$, $P_{\text{two-tailed}} = 0.24$) with the hypoxia score (shown in Fig. 2A). We analyzed the fold change distribution of hsa-miR-210 (Fig. 2B) and hsa-miR-21 (Fig. 2C) for samples with positive (hypoxia score < 0) and negative hypoxia score (hypoxia score > 0). The mean (and median) of the two sample groups are different for hsa-miR-210, but not for and hsa-miR-21. One of the 99 genes included in the hypoxia signature is vascular endothelial growth factor, which plays a key role in induced angiogenesis by hypoxia. Therefore, we studied the correlation of vascular endothelial growth factor microarray expression data with miRNA expression levels, finding a significantly positive correlation with hsa-miR-210 ($P = 0.03$) but not with hsa-miR-21 (see Supplementary Table S3). All these results support the hypothesis that hsa-miR-210 expression is regulated by hypoxia in breast cancers.

**hsa-miR-210 is a prognostic marker in breast cancer.**

Furthermore, we hypothesized that the expression of the hypoxically regulated miRNA, hsa-miR-210, would not only reflect the degree of tumor hypoxia but would also be correlated with tumor aggressiveness and patient disease-free and overall survival. To test this hypothesis, we examined the relationship between the expression of hsa-miR-210 in tumor specimens and patient outcome in 219 patients with long-term follow-up of early breast cancer and with other prognostic variables. hsa-miR-210 expression levels showed a highly significant inverse correlation with disease-free and overall survival in univariate analysis when considered as a continuous variable, as a binary variable divided by median value (Fig. 3A), as quartiles (Fig. 3B), or by fold change (data not shown). Patients with hsa-miR-210 levels below the median have a survival of 10 years (77.1%), whereas for patients with hsa-miR-210 levels above the median, the percentage is only 53.6% (see Fig. 3A). More importantly, hsa-miR-210 expression levels also showed a significant inverse correlation with both disease-free and overall survival in a Cox multivariate analysis ($P = 0.003$ and $P < 0.001$, respectively, when considered as a continuous variable; Table 4), indicating that hsa-miR-210 expression levels are an independent prognostic marker. We also obtained a significant correlation of ER status, an accepted prognostic marker included in the Cox multivariate analysis, with both overall ($P = 0.03$) and disease-free survival ($P = 0.002$; Table 4). No significant interaction effect was observed between ER and hsa-miR-210 levels or between nodal status and hsa-miR-210 levels for overall or disease-free survival.

We examined for a correlation of hsa-miR-210 expression levels with clinicopathologic variables (for details, see Supplementary Table S1) and found an association of hsa-miR-210 expression levels with tumor size ($P = 0.04$; Table 4). However, hsa-miR-210 expression levels were not significantly correlated with ER status, nodal status, or tumor grade (Table 4).

In order to exclude any artifactual effect that might account for these striking correlations, and knowing that the expression of hsa-miR-21 has been found altered in a wide range of tumors including breast cancer, we studied the correlation of hsa-miR-21 expression levels in the same sample collection with the clinical variables and patient outcome. The expression levels of hsa-miR-21 and hsa-miR-210 were significantly correlated (Spearman’s $\rho = 0.44$, $P < 0.001$); however, hsa-miR-21 expression did not correlate with any of the clinical variables. Furthermore, although hsa-miR-21 levels showed significant correlation with both overall survival and disease-free survival in a univariate analysis ($P = 0.021$ and 0.037, respectively; data not shown), it was not a significant independent predictor in a
Cox multivariate analysis including the other clinical variables (data not shown).

**Discussion**

This work provides support for the involvement of certain miRNAs in the regulation of gene expression by hypoxia. For one specific miRNA, hsa-miR-210, regulation by hypoxia was seen in several different cell lines and responses to hypoxia, and manipulations of HIF and VHL, suggest that regulation is mediated transcriptionally via HIF-1α. In support of this, during the course of this work, Kulshreshtha and colleagues also showed hsa-miR-210 induction by hypoxia in several cell lines (24). Furthermore, they also implicated the transcriptional role of HIF in the hypoxic regulation as hsa-miR-210 expression could be enhanced by increasing the levels of a degradation-resistant form of either HIF-1α or HIF-2α and dynamic recruitment of HIF-1α to the putative hsa-miR-210 promoter was seen by chromatin immunoprecipitation. Therefore, the data we present here are complementary to the Kulshreshtha et al. report and provide additional evidence that HIF-1α, but not HIF-2α, induces hsa-miR-210 expression. However, further work will be required to precisely analyze the location of the HIF DNA-binding sites conferring this response, particularly given the hypoxic regulation of adjacent transcripts by hypoxia we detected in this work, although both indirect or epigenetic effects on gene expression remain possible. The importance of the hypoxic regulation of hsa-miR-210 has been further highlighted by the recent observation of enhanced placental levels of hsa-miR-210 in patients with preeclampsia, a disease in which hypoxia seems to play a central role (34). Although we focused our studies on the regulation of one highly overexpressed miRNA, the regulation of other miRNA sequences are also likely to be important in the control of gene expression by hypoxia. Indeed, one interesting observation is that five of the down-regulated miRNAs (hsa-miR-424, hsa-miR-193, hsa-miR-182, hsa-miR-520h, and hsa-miR-502) have putative binding sites within the 3'-untranslated region of the vascular endothelial growth factor, and their down-regulation is consistent with a miRNA-mediated regulation of vascular endothelial growth factor protein levels, as suggested by Hua and colleagues (35).

The occurrence of hypoxia in many tumors and the enhanced action of HIF suggests this as the most probable mechanism for the up-regulation of hsa-miR-210 we have observed in breast cancer, particularly given the correlation with other markers of hypoxia. However, other potential mechanisms for increased expression include the increased copy number at this locus seen in certain cancers (20). The role of HIF in regulating hsa-miR-210 expression and its enhanced levels in tumors is further emphasized by the striking increase (>16-fold) of expression observed in renal cancer cells lacking functional VHL.

The effects of alterations in hsa-miR-210 levels remain to be established. Resultant changes in target gene expression seem probable but the relative effects on mRNA transcription, mRNA stability, and translation remain unclear. The computational prediction of mRNA targets for hsa-miR-210 binding and action generates a very large number of potential targeted genes.
hsa-miR-210 Expression, Hypoxia, and Breast Cancer Prognosis

Table 4. hsa-miR-210 expression in breast cancer samples: multivariate Cox analysis and correlation with clinicopathologic variables

<table>
<thead>
<tr>
<th>Multivariate Cox analysis</th>
<th>P</th>
<th>HR</th>
<th>95.0% CI for HR Lower</th>
<th>95.0% CI for HR Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease-free survival</td>
<td>0.003</td>
<td>4.07</td>
<td>1.7</td>
<td>9.75</td>
</tr>
<tr>
<td>hsa-miR-210</td>
<td>0.02</td>
<td>1.8</td>
<td>1.12</td>
<td>2.93</td>
</tr>
<tr>
<td>Positive nodes</td>
<td>0.002</td>
<td>0.46</td>
<td>0.28</td>
<td>0.75</td>
</tr>
<tr>
<td>Overall survival</td>
<td>&lt;0.001</td>
<td>11.38</td>
<td>4.1</td>
<td>31.65</td>
</tr>
<tr>
<td>hsa-miR-210</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.001</td>
<td>1.28</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Positive nodes</td>
<td>0.09</td>
<td>1.6</td>
<td>0.94</td>
<td>2.74</td>
</tr>
<tr>
<td>ER status</td>
<td>0.03</td>
<td>0.56</td>
<td>0.33</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Correlation with clinicopathologic variables

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>Z statistics</th>
<th>P</th>
<th>Continuous variable</th>
<th>Spearman’s ρ</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal status</td>
<td>-1.69</td>
<td>0.09</td>
<td>Tumor size (cm)</td>
<td>0.14</td>
<td>0.04</td>
</tr>
<tr>
<td>Grade</td>
<td>5.63</td>
<td>0.06</td>
<td>ER ELISA</td>
<td>-0.09</td>
<td>0.19</td>
</tr>
</tbody>
</table>

NOTE: The reduced model following backwards likelihood elimination (entry P = 0.05, elimination P = 0.10) are shown. Variables included in the analysis were hsa-miR-210 as a continuous variable (expression levels were ranked between 0 and 1), age (premenopausal, ≤50; postmenopausal, >50), tumor size (small, <1.5 cm; large, ≥1.5 cm), positive nodes (negative, 0; positive, >0), ER status (negative ELISA, <5; positive ELISA, ≥5), grade (1-3).

(15 targets predicted using PicTar,7 1,034 using miRanda,8 and 21 using TargetScan9) with a myriad of functions. It has been shown that the repression of hsa-miR-210 gives rise to an increase of apoptosis in cell cultures (24, 36) suggesting an antipapoptotic effect when overexpressed in cancer. A more exact understanding of the precise function of hypoxic induction of hsa-miR-210 and indeed all miRNAs will require further direct, experimental confirmation of their targets. However, HIF-1α-dependent regulation of hsa-miR-210 suggests that the dominant effects of HIF-1α in hypoxic gene regulation (9–12) may not all be mediated directly via the transcriptional action of HIF but also via the intermediary of alterations in specific miRNA levels.

The correlation of hsa-miR-210 levels with breast cancer mortality is a striking one. This could be due to a direct effect of hsa-miR-210 on tumor biology, perhaps through its antipapoptotic action and/or because it is acting as a sensitive marker of tumor hypoxia which has been linked to tumor aggression in many studies (8). The dependence of hsa-miR-210 induction on HIF-1α and VHL and the correlation of hsa-miR-210 levels with hypoxia score in breast cancer samples seen in this work strongly support the latter possibility.

The role of miRNAs in cancer and their potential utility as prognostic markers has emerged recently (37). Increased levels of hsa-miR-210 and hsa-miR-21 have been detected in several solid tumors including breast cancer (19, 32, 38), and the expression levels of hsa-miR-21 have been shown to correlate with tumor stage. In our analysis, the levels of expression of hsa-miR-21 showed significant correlation only with overall and disease-free survival in univariate analysis, whereas hsa-miR-210 expression levels were an independent predictor of disease-free and overall survival. Furthermore, we have not found any evidence that hsa-miR-21 is induced by hypoxia or that its expression is controlled by HIF-1α, HIF-2α, or VHL despite previous reports (24). These findings are further supported by the lack of correlation between hsa-miR-21 expression and hypoxia score in breast cancer samples and suggest the specificity of hsa-miR-210 regulation by hypoxia in cancer pathology.

The correlation of hsa-miR-210 levels with survival in breast cancer which we describe here adds a further dimension to the recent work reporting differences in miRNA abundance in cancer. It complements the recent descriptions of expression levels of multiple miRNAs that are predictive of clinical outcome in breast cancer (for example, refs. 39–41) and indicates that the single hsa-miR-210 assay has strong potential as an independent prognostic factor in this disease.

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References

hsa-miR-210 Is Induced by Hypoxia and Is an Independent Prognostic Factor in Breast Cancer

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