Hypoxia-regulated MicroRNAs in Gastroesophageal Cancer

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Abstract. *Background/aim: The present study aimed to identify hypoxia-regulated microRNAs (HRMs) in vitro and investigate the clinical role of candidate HRMs in patients with gastroesophageal cancer (GEC). Materials and Methods: microRNA expression changes induced by hypoxia in human GEC cell lines were measured with microarrays and validated by quantitative real-time polymerase chain reaction. Candidate HRMs were measured in pre-therapeutic tumor samples from 195 patients with GEC. Results: Expression of miR-210 was shown to be significantly induced in esophageal squamous cell carcinoma (9.26-fold, p<0.001) and adenocarcinoma cell lines (4.95-fold, p<0.001) and miR-27a-star was significantly up-regulated in adenocarcinoma cell lines (4.79-fold, p=0.04). A weak but significant correlation between miR-210 expression and a 15-gene hypoxia signature was observed (Pearson r correlation: r=0.38, p<0.001). No significant associations of HRMs and clinical outcome in patients with GEC were identified. Conclusion: This study supports the involvement of hypoxia on miRNAs in vitro and confirms the role of miR-210 as being a universal HRM.*

Hypoxia is a common feature of the microenvironment of solid tumors. The exposure of cells to hypoxia leads to a variety of genetic and biological changes that allow them to adapt to the hypoxic conditions. Hypoxia-regulated genes are known to be involved in multiple biological processes, such as proliferation, angiogenesis, metabolism, immortalization and migration (1, 2) In addition to genomic instability and phenotypic aggressiveness (1, 3), the clinical effects of hypoxia include therapy failure and impaired survival (4, 5).

Hypoxia has been demonstrated to have regulatory effects on microRNA (miRNA) expression. miRNAs are short, non-coding RNAs (~22 nucleotides in length) that act as post-transcriptional regulators, inhibiting miRNA translation or targeting mRNA for degradation (6, 7). They function as tumor suppressors or oncogenes and are important regulators in pathological settings, including tumorigenesis (7, 8). Multiple miRNAs have been identified as hypoxia-regulated miRNAs (HRMs) *in vitro* and known hypoxia inducible HRMs include miR-21, miR-93, miR-181b, miR-210 and miR-373 (9-15). However, little overlap between hypoxia-induced miRNA profiles between *in vitro* studies have been reported. In addition, a large number of miRNAs have been shown to be down-regulated upon hypoxic exposure in a variety of cellular contexts, potentially through hypoxia-mediated down-regulation of Drosha and Dicer (16).

Hypoxia-inducible factors (HIFs) play an important role in the involvement of hypoxia and miRNA expression, regulating transcription factors targeting miRNAs or by direct binding to hypoxia-responsive elements (9, 10, 13). Conversely, recent *in vitro* studies have discovered the capability of miRNAs to regulate hypoxia-inducible factor 1 alpha (HIF1A) resulting in complex feedback loops (17, 18). However, also HIF2-dependent and HIF-independent regulation have been described (19, 20). Among the HRMs, miR-210 has emerged as the predominant and universal hypoxia-inducible miRNA across different cell types, including esophageal and gastric cancer cell lines, (9-15, 21), and has been shown to be associated with a poor prognosis in colorectal, breast and head and neck cancer (9, 11, 22), but with an improved prognosis in lung, soft-tissue sarcoma and renal cancer (20, 23, 24). The interaction between hypoxia and miRNAs is complex and may account for several events in tumorigenesis. However, the prognostic role of HRMs in gastroesophageal cancer (GEC) is yet to be elucidated.

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The aims of this study were to identify and validate HRMs *in vitro*, and to determine associations of HRMs with survival and treatment response in patients with GEC. Thus, an exploratory approach was undertaken to identify *in vitro*-derived HRMs from GEC cell lines (microarray). Validation of HRMs was performed with quantitative real-time polymerase chain reaction (qPCR) and candidate miRNAs were subjected to analysis for correlation with prognosis and treatment response in a retrospective cohort of 195 patients with locoregional GEC.

Materials and Methods

Patients. A total of 195 patients diagnosed with locoregional esophageal squamous cell carcinoma (ESCC) or esophageal, gastroesophageal junction or stomach adenocarcinoma (AC) with available formalin-fixed paraffin-embedded (FFPE) tumor specimens were included in the study. All specimens were pre-therapeutic, diagnostic biopsies from patients treated with curative intent in the period 1997 to 2013. Patients were recruited from three different cancer centers in Denmark (Department of Oncology, Aarhus University Hospital; Department of Oncology, Odense University Hospital; and Department of Oncology, Rigshospitalet, Copenhagen). Clinicopathological parameters were obtained from medical records and pathology reports. However, only TNM stages were available from the medical records and patients were therefore retrospectively staged by the Authors according to the 5th to 7th editions of The American Joint Committee on Cancer/Union for International Cancer Control staging guidelines (25-27).

The study was approved by The Central Denmark Region Committees on Health Research Ethics (M-20100204/1-10-72-38-13) and the Danish Data Protection Agency (J.nr. 2011-41-6731) and was conducted in accordance with the Helsinki declaration.

Overall, 129 patients were diagnosed with ESCC; this group of patients had received neoadjuvant or definitive chemoradiotherapy [concurrent 5-fluorouracil (5-FU) with/without cisplatin and 45-60 Gy). However, three patients received definitive radiotherapy (60 Gy) without chemotherapy due to age or comorbidity. AC of the esophagus, gastroesophageal junction or stomach was diagnosed in 66 patients who had received a regimen of perioperative chemotherapy (cisplatin, epirubicin and capecitabine), neoadjuvant or definitive chemoradiotherapy (concurrent 5-FU with/without cisplatin and 45-60 Gy). Two patients received definitive radiotherapy (60 Gy) alone due to age or comorbidity. Not all patients received full-dose chemotherapy.

As miRNAs have been shown to be aberrantly expressed and serve tumor- and histopathological-specificity, patients were stratified by histology and randomized into training and validation sets. Among the 129 patients with ESCC, 86 patients were allocated to the training set and 43 to the validation set. In the group of patients with AC, 44 were allocated to the training set and 22 to the validation set. Patient and tumor characteristics are summarized in Table I. No significant differences between clinicopathological variables, such as age, sex, tumor size, stage or treatment regimen, were identified between the training and validation sets for each type of histology.

Cell lines and hypoxia treatment. The human esophageal squamous cell line OE21 and the human gastroesophageal junction adenocarcinoma cell lines OE19 and OE33 were obtained from Sigma-Aldrich (St. Louis, MO, USA) and cultured in monolayer in 80-cm^2 tissue culture flasks. All cell lines were maintained at 37˚C in humified air balanced with 5% CO_2 in RPMI-1640 medium supplemented with GlutaMAX containing 15% foetal calf serum, 1% penicillin-streptomycin, 1% sodium pyruvate and 1% HEPES. Exposure of cell cultures to hypoxia was achieved by continually gassing the cells in an airtight chamber with 0% oxygen, 5% CO_2 and 95% nitrogen at 37˚C for 24 hours. Normoxic controls were gassed at 37˚C for 24 h in an identical airtight chamber with 95% atmospheric air and 5% CO_2. All experiments were performed in triplicate from independent cell cultures. Data with RNA from this experiment have previously been published (28).

RNA extraction. Cell lines: Cells were harvested immediately after removal from the airtight chamber: The medium was removed, cells washed with Dulbecco's phosphate-buffered saline and lysed with Qiazol Lysis Reagent (Qiagen, Hilden, Germany). Total RNA was extracted by using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quality was determined using a NanoDrop 1000 spectophotometer (NanoDrop Technologies, Thermo Scientific, Waltham, MA, USA).

Patient material: Isolation of miRNAs for microarray analysis was performed on 12 μm FFPE sections with the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions. Haematoxylin and eosin-stained slides were used to verify the presence of invasive carcinoma and confirmation of tumor presence was carried out by an experienced pathologist. The mean fraction of tumor area, defined as the area of invasive carcinoma compared to the total area of tissue, was estimated to be 63% (range=10-100%). A NanoDrop Spectophotometer was used to quantify total RNA quality.

MicroRNA microarray analysis. Cell lines: Analysis of miRNAs was performed using Affymetrix GeneChip miRNA 1.0 microarrays or Affymetrix GeneChip miRNA 2.0 microarrays (Affymetrix, Santa Clara, CA, USA). For each cell line, microarray analysis was performed in triplicates. miRNA was labeled using FlashTag™ HSR Biotin RNA Labeling Kit (Affymetrix) and hybridized to GeneChip miRNA version 1.0 or 2.0 microarrays (Affymetrix) according to the manufacturer's details. Arrays were washed and stained on an Affymetrix Fluidics Station 450 and scanned on an Affymetrix Scanner 3000 7G according to the manufacturer's instructions. Normalization of miRNA microarray data was performed with Affymetrix miRNAQCTool ver. 1.0.33 and Affymetrix Expression Console ver. 1.3.1 for version 1.0 microarrays and version 2.0 microarrays, respectively, using Robust Multi-array Average (RMA) for background correcting, summarizing and normalizing data from Affymetrix gene expression Genechips.

Patient material: miRNAs were labeled using FlashTag™ HSR Biotin RNA Labeling Kit (Affymetrix) and hybridized to Affymetrix GeneChip miRNA version 1.0 microarrays (Affymetrix) according to the manufacturer's details. Arrays were washed and stained on an Affymetrix Fluidics Station 450X and scanned on an Affymetrix G7 scanner according to manufacturer's instructions. Normalization of miRNA microarray data was performed in R using RMA.

Hypoxia-responsive miRNAs (microarray). After filtering for 'non-expressed' probe sets in the *in vitro* material, only human probes present in both cell lines and the clinical material were included for further analysis (N=229, hereof 128 miRNAs). As microarray analysis of the cell lines was carried out on two different Affymetrix

Table I. *Patient and tumor characteristics*.

Group	ESCC		AC	
	Training set N=86	Validation set N=43	Training set N=44	Validation set N=22
Treatment				
Neoadjuvant chemo-RT	61 (71)	29 (67)	1 (2)	1 (5)
Definitive chemo-RT	25 (29)	14 (33)	12 (28)	6 (27)
Perioperative chemotherapy			31 (70)	15 (68)
Gender - N (%)				
Male	56 (65)	29 (67)	36 (82)	19 (86)
Female	30 (35)	14 (33)	8 (18)	3 (14)
Median age (range), years	63 (37-81)	63 (47-78)	64 (32-86)	64 (42-79)
Median tumor size (range), mm	50 (10-150)	50 (10-120)	51 (15-100)	50 (10-100)
	n=72	n=35	n=36	n=17
Clinical TNM stage - N (%)				
I	10 (12)	3 (7)	6 (14)	4 (18)
II	26 (30)	12 (28)	19 (43)	10 (45)
III	47 (55)	26 (60)	18 (41)	6 (27)
IV	3 (3)	2 (5)	1 (2)	1 (5)
Unknown				1 (5)
Pathological response - N (%)	n=45	N=23	n=30	n=14
Complete response	18 (40)	10 (43)		
Non-complete response	27 (60)	13 (57)	30 (100)	14 (100)
Evaluation by CT or endoscopy - N (%)				
Complete response	15 (17)	9 (21)	7 (16)	2 (9)
Regression	35 (41)	18 (42)	20 (46)	8 (36)
Stable disease	22 (26)	10 (23)	8 (18)	10 (45)
Progressive disease	9 (10)	4 (9)	4 (9)	2 (10)
Unknown	5 (6)	2 (5)	5 (11)	
Median follow-up (range), months	25 (4-137)	21 (3-192)	22 (4-99)	27 (4-64)
OS (%) (95% CI)				
1 Year	66 (55-75)	70 (54-81)	77 (62-87)	77 (54-90)
3 Year	39 (28-50)	40 (25-55)	46 (30-60)	47 (24-67)

ESCC: Esophageal squamous cell carcinomas; AC: esophageal, gastroesophageal junction or stomach adenocarcinoma; CT: computed tomography; OS: overall surival; CI: confidence interval; RT: radiotherapy.

platforms and normalized separately, an additional normalization approach was needed in order to compare miRNA expression levels across platforms (see Appendix for details (http://pure.au.dk/portal/files/95109370/Appendix.pdf). After this normalization step, miRNAs with at least 1.5-fold up-regulation between hypoxic and normoxic samples for the ESCC cell line (OE21) and the two AC cell lines (OE19 and OE33) were chosen for further validation with qPCR. Fold changes were calculated by subtracting the mean (hypoxic) and mean (normoxic) log2-transformed expression levels for the ESCC cell line and the AC cell lines separately and then converting to absolute fold-change values. The cut-off value of 1.5 was based on previous miRNA profiling studies which have shown that small miRNA expression changes, such as a 1.5-fold difference, can have profound impact on the biology *in vitro* (29, 30) and is often used as cut-off for miRNA analysis (31).

Validation of hypoxia-responsive miRNAs with real-time qPCR. Validation of miRNA expression derived from microarray analysis was performed on RNA from the same experiment as had been used for microarray analysis using the Dynamic array IFC 48.48 on a Fluidigm Biomark Real-Time PCR system (Fluidigm Corporation, San Francisco, CA, USA) according to the manufacturer's instructions. Expression of the HRMs and selected reference genes were measured on the 18 cell line samples (three biological replicates of hypoxic samples and normoxic samples for each of the three cell lines) in one single qPCR experiment with assays loaded in duplicate. Two 8-point standard curves were prepared with universal reference RNA and RNA from one sample, respectively. Ct-values were calculated by use of Fluidigm qPCR analysis software v. 4.1.3. Fold changes in miRNA expression between hypoxic and normoxic samples were determined by the comparative ΔΔCt method (32), normalizing the results to the geometric mean of the internal reference genes. *p*-Values were calculated with a one-sided Student's *t*-test under the assumption of equal variance.

Calculation of stability of reference genes. In this study, geNorm and NormFinder (Real-Time Statminer – Intergromics, Madrid, Spain) were used for analysis of the stability of potential internal reference genes for normalization of qPCR data (see Appendix for details (https://goo.gl/Cky9yy).

Correlation of hypoxia-responsive miRNAs and hypoxic gene expression in ESCC. Correlation of HRMs with hypoxia was investigated in ESCC samples using a 15-gene hypoxia expression signature developed by Toustrup *et al*. (33). A hypoxia score was determined for 85 out of the 86 patients with ESCC in the training set for whom gene-expression data were available from two previous studies (34, 35). Based on the method described by Toustrup *et al*. (33), the hypoxia score was estimated in two steps. Firstly, the difference between the sum of gene expression levels of the 15 genes for each sample and the mean expression levels of the 15 genes for a predefined group of less hypoxic tumors and more hypoxic tumors, respectively, was calculated (Dless and Dmore). Next, the hypoxia score was generated by estimating the difference between Dless and Dmore.

Statistics. The primary outcome was overall survival (OS), defined as time from the date of histological diagnosis to death from any cause or last day of follow-up. Secondary outcomes were disease-specific survival (DSS), defined as time from diagnosis to death from or with GEC or last day of follow-up; pathological complete response (ypCR) was defined as no evidence of vital residual tumor cells remaining in the resected specimen, and radiographic response as complete response (CR) or regression of disease. Radiographic response was assessed in patients with treatment evaluation computed tomographic scans or esophagogastroduodenoscopy after completion of induction therapy prior to surgery or after completion of definitive therapy.

For each of the two training sets (ESCC and AC), survival analysis was carried out by dichotomizing the HRM expression values using the K-means clustering approach to allocate patients into groups of low and high miRNA expression based on the best discriminative value of the mean miRNA expression levels for the two groups. The generated cut-off values for each of the HRMs in the training sets were used in the validation sets for confirmation of results. Survival curves were generated using the Kaplan–Meier method and survival data was expressed as hazard ratios (HR) using univariate Cox proportional hazards model. The proportional hazards assumption was tested graphically using log-minus-log plots and verified with Schoenfeld's residuals. Correlation of miRNA expression with hypoxic status, and pathological or radiographic response was assessed using Spearman's rank test, chi-square statistical test or Fischer's exact test. Statistical analysis was performed using STATA, Version 12 (StataCorp, College Station, TX, USA). All *p*-values are two-sided with a 5% level of significance. Hazard ratios (HR) are presented with 95% confidence interval (CI).

Results

HRMs in vitro (microarray). In total, 128 human miRNAs were evaluated for HRM potency. Using a cut-off value of 1.5-fold difference between hypoxic and normoxic samples, seven miRNAs were found up-regulated and six miRNAs down-regulated in the ESCC cell line Table II. For the AC cell lines, 10 miRNAs were up-regulated and five miRNAs down-regulated (Table II). Interestingly, five miRNAs (miR-210, miR-193b-star, miR-200c-star, miR-30b-star and miR-1308) were up-regulated in both the ESCC and AC cell lines. Thus, 12 miRNAs (miR-210, miR-193b-star, miR-200c-star, miR-30b-star, miR-1308, miR-29b-1-star, miR-128, miR-27a-star, miR-21-star, miR-21, miR-1246 and miR-138-1-star) were subjected to validation by qPCR. However, a qPCR TaqMan assay for miR-1246 was not available and this miRNA was excluded from further analysis. Additionally, three HRMs from the literature, miR-93, miR-181b and miR-373, and miR-620, previously shown to be involved in radioresistance *in vitro* (36), were included in the qPCR analysis. miRNAs demonstrated to be down-regulated were not further investigated.

Confirmation of hypoxia-responsive miRNAs in vitro by qPCR. Only six (miR-21, miR-27a-star, miR-210, miR-93, miR-181b and miR128) out of the 15 miRNAs investigated by qPCR showed sufficient PCR efficiency for further analysis. Expression values for the HRMs were normalized to the geometric mean of the three internal reference genes (the two snoRNAs *RNU43* and *RNU44*, and miR-361) identified with geNorm and NormFinder (See Appendix Table I (https://goo.gl/Cky9yy)) and expressed as ΔCt values. For the ESCC cell line, up-regulation of miR-210 was confirmed by qPCR (fold change: qPCR: 9.26; microarray: 5.92) and this induction was significant ($p<0.001$). However, miR-128 was not differentially expressed between hypoxic and normoxic samples (fold change: qPCR: 0.82, $p=0.29$; microarray: 1.56). For the AC cell lines, a substantial and significant induction of miR-210 (fold change: qPCR: 4.95, $p<0.001$; microarray: 3.28) and miR-27a-star (fold change: qPCR: 4.79, $p=0.04$; microarray: 2.59) were demonstrated. miR-21 exhibited a fold-change of 2.21 (microarray: 1.85) but this up-regulation was not significant ($p=0.19$). Neither miR-181b nor miR-93, chosen from the literature, showed any significant differences in expression levels between hypoxic and normoxic samples in the ESCC or the AC group. Since only miRNAs that displayed an expression differing by at least 2-fold in the array data could be validated with qPCR, we chose to include the seven HRMs shown to be more than 2-fold up-regulated in the microarray data set for further analysis (miR-210, miR193b-star, miR200c-star, miR-30b-star, miR-1308, miR-27a-star and miR-21-star).

Correlation of hypoxia-responsive miRNAs and hypoxic gene expression in ESCC. To elucidate the possible correlation of HRMs with hypoxia gene-expression levels in ESCC samples, we determined a hypoxia score for 85 out of the 86 patients with ESCC in the training set for whom gene-expression data were available. A high positive score indicated a more hypoxic sample and a low negative score a less hypoxic sample. A weak but significant correlation between miR-210 expression and the hypoxia score was observed (Pearson r correlation: r=0.38, $p<0.001$; Figure 1). The expression levels of miR-193b-star, miR-200c-star, miR-30b-star and miR-1308 were not significantly correlated with the hypoxia score.

Table II. *Up- and down-regulated miRNAs according to histology (fold difference between hypoxic and normoxic in vitro samples). Microarray-data.*

ESCC		AC	
Up-regulated	Fold change	Up-regulated	Fold change
hsa-miR-210	5.92	hsa-miR-210	3.28
hsa-miR-193b-star	2.61	hsa-miR-193b-star	3.11
hsa-miR-200c-star	2.51	hsa-miR-27a-star	2.59
hsa-miR-30b-star	2.16	hsa-miR-1308	2.37
hsa-miR-1308	2.14	hsa-miR-21-star	2.29
hsa-miR-29b-1-star	1.75	hsa-miR-200c-star	2.09
hsa-miR-128	1.56	hsa-miR-30b-star	1.97
		hsa-miR-21	1.85
		hsa-miR-1246	1.76
		hsa-miR-138-1-star	1.71
Down-regulated	Fold change	Down-regulated	Fold change
hsa-let-7f	0.66	hsa-miR-183	0.64
hsa-miR-501-3p	0.65	hsa-miR-744	0.61
hsa-miR-1275	0.65	hsa-miR-1275	0.57
hsa-miR-18a-star	0.61	hsa-miR-23b-star	0.56
hsa-miR-21-star	0.54	hsa-miR-18a-star	0.49
hsa-miR-421	0.41		

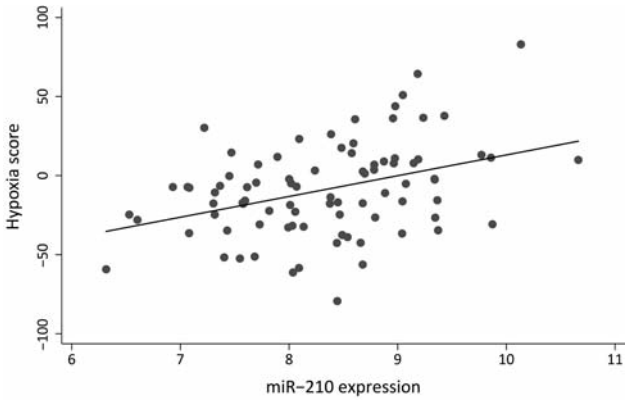

Figure 1. *Correlation between miR-210 expression values and the hypoxia score (Pearson r correlation: r=0.38, p<0.001). Hypoxia score: A high positive score indicates a more hypoxic sample and a low negative score a less hypoxic sample.*

Associations between HRMs and clinical outcome. As HRMs have been shown to serve prognostic significance in several types of cancer, the association between HRMs and outcome in the two study populations of ESCC and AC was evaluated. Using the K-means approach to establish HRM cut-off values, no significant correlations between expression levels of miR-210, miR-193b-star, miR-200c-star, miR-30b-star or miR-1308 and OS or DSS were observed for the ESCC training set, Table III. In the AC training set, miR-200c-star was significantly inversely associated with OS and DSS [OS: HR=2.76, 95% confidence interval (CI)=1.19-6.44; p=0.019 and DSS: HR=6.53, 95% CI=2.23-19.14; p=0.001] (Figure 2A and C). However, these results were not confirmed by the validation set of AC (Table III, and Figure 2B and D). In addition, no significant associations between miR-210, miR-193b-star, miR-1308, miR-27a-star or miR-21-star and survival were demonstrated in the AC training set (Table III). In the ESCC validation set, a significantly poorer OS and DSS was identified for patients with expression above the cut-off value for miR-200c-star (OS: HR=2.38, 95% CI=1.02-5.57; p=0.046) and DSS: HR=3.20, 95% CI=1.13-9.08; p=0.029) (Table III, and Figure 3B and D).

The correlation between HRMs and treatment response was investigated in the four study cohorts. No significant associations between HRMs and pathological or radiographic response in the ESCC training set were observed, nor between HRMs and radiographic response in the AC training set (correlation with pathological response was not assessed as no patients with AC achieved ypCR). In the validation set of ESCC, however, miR-193b-star expression below cut-off was identified to be significantly associated with ypCR (p=0.039).

Discussion

The first link between hypoxia and miRNA expression was demonstrated by Kulshreshtha *et al*. in 2007 (14) and since then multiple studies have identified HRMs *in vitro* and correlated these with patient outcome. However, the clinical role of HRMs is yet to be elucidated in GEC.

In this study, hypoxic regulation of miR-210 was demonstrated in three different GEC cell lines, showing a significant induction of miR-210 in hypoxic samples for both ESCC and AC cell lines. In support of this, miR-210 has been identified to be ubiquitously and robustly overexpressed in response to hypoxia in esophageal, gastric, head and neck squamous cell carcinoma (HNSCC), breast, ovarian and colon cell lines (9-15, 21) among others. The biological functions of miR-210 in tumorigenesis have been explored extensively and several miR-210 targets have been identified, indicating roles in cell-cycle regulation, differentiation, cell survival and angiogenesis (12, 14, 15, 37). In esophageal cancer cells, miR-210 was identified to induce cell apoptosis and cell-cycle arrest in G_1/G_0 and G_2/M phases (37). Consistent with these results, a study by Li *et al*. reported inhibition of ESCC proliferation by G_2/M cell-cycle arrest which was mediated by miR-210 targeting Polo like kinase 1 (PLK1) (15). These results indicate a tumor-suppressive function of miR-210 in esophageal cancer. In gastric cancer, however, miR-210 overexpression (transfection with miR-210 mimics) induced an epithelial–mesenchymal transition molecular profile with decreased expression of E-cadherin

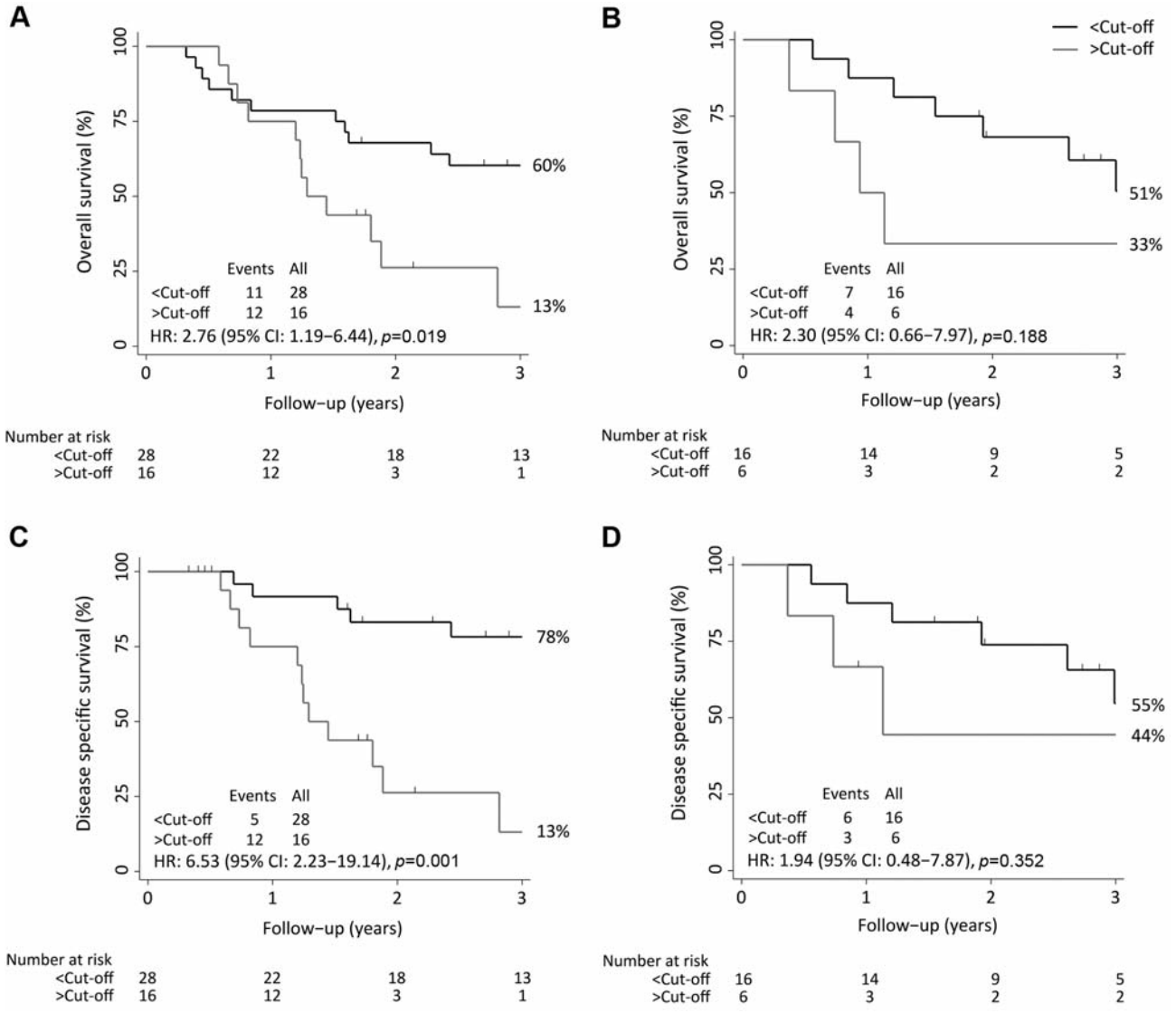

Figure 2. *Association of miR-200c-star and survival in patients with esophageal, gastroesophageal junction or stomach adenocarcinomas (AC). Overall survival for the training set (A) and validation set (B). Disease-specific survival for the training set (C) and validation set (D). Patients are stratified according to miR-200c-star expression levels; <cut-off represents low miR-200c-star expression and >cut-off represents high miR-200c-star expression. HR: Hazard ratio, CI: confidence interval.*

and increased vimentin and N-cadherin genes, indicating an oncogenic function of miR-210 in gastric cancer cells (21). This discrepancy in miR-210 function is interesting and might demonstrate tissue-type specificity of miRNAs.

In addition to miR-210 up-regulation, a significant induction of miR-27a-star expression was found in the AC cell lines of this study. Star miRNAs have been thought of as a processing byproduct of the forward miRNA sequence, considered to be degraded and not having functional effect in human cancer. However, emerging evidence indicates that star miRNAs are present in cells and exert a functional role (38). Studies on miR-27a-star are yet to be performed in GEC but miR-27a-star has been reported to exert tumor-suppressive effects in HNSCC, prostatic, pancreatic and breast cancer cell lines, in addition to reducing cell viability and expression of epidermal growth factor receptor, protein kinase B and mammalian target of rapamycin in HNSCC cells (39). However, a study by Salah *et al*. reported pro-metastatic functions of miR-27a-star in osteosarcoma cell lines *in vivo* (40), indicating an oncogenic function of this miRNA.

When comparing the seven miRNAs being more than 2-fold up-regulated in the microarray data set (miR-210, miR-

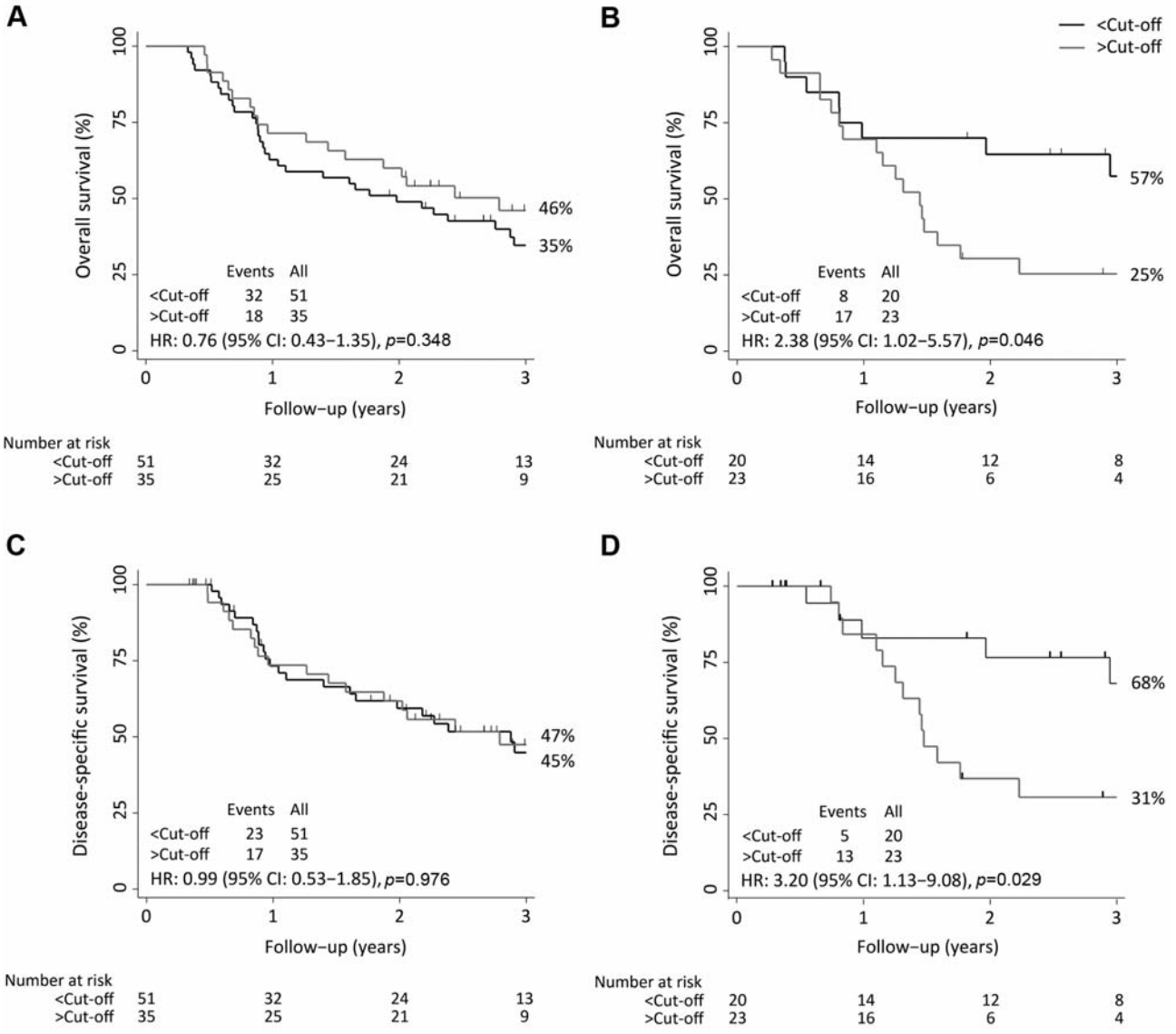

Figure 3. *Association of miR-200c-star and survival in patients with esophageal squamous cell carcinomas (ESCC). Overall survival for the training set (A) and validation set (B). Disease-specific survival for the training set (C) and validation set (D). Patients are stratified according to miR-200c-star expression level; <cut-off represents low miR-200c-star expression and >cut-off represents high miR-200c-star expression. HR: Hazard ratio, CI: confidence interval.*

193b-star, miR200c-star, miR-30b-star and miR-1308, miR-27a-star and miR-21-star), no overlap between these HRMs and previous reports on HRM profiles, except for miR-210, was identified (9-14, 41). The inconsistency of overlap between HRM profiles across studies is well known and may be attributable to the cellular context, variety of miRNA profiling platforms, normalization method, in addition to the duration and severity of oxygen deprivation among studies. Interestingly however, miR-210, miR-193b-star, miR200c-star, miR-30b-star and miR-1308 were up-regulated in both the ESCC and AC cell line of this study, indicating that these HRMs might be induced by common components of the hypoxia-mediated response.

In this study, correlation of HRMs with hypoxia was sought in ESCC samples using a 15-gene hypoxia expression signature developed by Toustrup *et al*. (33). Only a weak but significant correlation between miR-210 and the hypoxia score derived from the signature was shown. The 15 genes included in this signature have been shown to be hypoxia-inducible in several cancer cell lines, including an ESCC cell

Table III. *Univariate analyses of hypoxia-regulated miRNAs. Hazard ratio (HR) and confidence interval (CI). Cut-off values for each of the hypoxia-regulated miRNAs were calculated using the K-means approach in the training sets of esophageal squamous cell carcinomas (ESCC) and esophageal, gastroesophageal junction or stomach adenocarcinomas (AC). The generated cut-offs from the training sets were used as cut-off values in the validation sets.*

	ESCC				AC			
Univariate analyses	Training set (N=86)		Validation set (N=43)		Training set (N=44)		Validation set (N=22)	
High *vs.* low	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Overall survival								
miR-210	0.83 (0.48-1.45)	0.518	1.19 (0.53-2.70)	0.673	1.30 (0.57-2.96)	0.536	2.49 (0.75-8.25)	0.137
miR-193b-star	1.01 (0.57-1.77)	0.982	0.99 (0.45-2.22)	0.988	1.08 (0.42-2.74)	0.877	0.55 (0.16-1.88)	0.340
miR-200c-star	0.76 (0.43-1.35)	0.348	2.38 (1.02-5.57)	0.046	2.76 (1.19-6.44)	0.019	2.30 (0.66-7.97)	0.188
miR-30b-star	1.62 (0.92-2.83)	0.093	1.66 (0.75-3.67)	0.208				
miR-1308	0.99 (0.56-1.75)	0.960	0.95 (0.43-2.11)	0.894	0.47 (0.19-1.17)	0.105	2.14 (0.65-7.09)	0.214
miR-27a-star					1.98 (0.73-5.36)	0.178	1.07 (0.32-3.54)	0.910
miR-21-star					0.52 (0.23-1.20)	0.126	1.39 (0.40-4.81)	0.602
Disease-specific survival								
miR-210	0.92 (0.49-1.71)	0.786	1.36 (0.51-3.63)	0.539	1.43 (0.54-3.76)	0.469	2.27 (0.60-8.57)	0.228
miR-193b-star	1.26 (0.68-2.34)	0.471	0.58 (0.20-1.62)	0.295	1.75 (0.50-6.10)	0.381	0.63 (0.16-2.53)	0.513
miR-200c-star	0.99 (0.53-1.85)	0.976	3.20 (1.13-9.08)	0.029	6.53 (2.23-19.14)	0.001	1.94 (0.48-7.87)	0.352
miR-30b-star	1.39 (0.74-2.60)	0.301	2.11 (0.82-5.47)	0.123				
miR-1308	1.20 (0.64-2.25)	0.571	0.91 (0.35-2.34)	0.840	0.33 (0.10-1.02)	0.054	1.41 (0.37-5.34)	0.609
miR-27a-star					4.22 (0.96-18.52)	0.056	0.74 (0.20-2.79)	0.659
miR-21-star					1.27 (0.41-3.89)	0.679	1.22 (0.30-4.97)	0.777

line (28). This study included the same RNA as used in the present study, presenting a biological validation of the hypoxia treatment. In addition, the 15-gene hypoxia expression signature has shown prognostic significance in patients with HNSCC treated without hypoxic modification (42) but not in patients with ESCC (34, 35). In the past decade, several hypoxia gene-expression signatures have been developed (43), showing prognostic or predictive impact in patients with cancer. Interestingly, the studies by Champs *et al.* (9) and Gee *et al.* (11) demonstrated a correlation between miR-210 expression level and previously developed mRNA hypoxia signatures in breast cancer and HNSCC, indicating a link between miR-210 and hypoxia.

The prognostic value of *in vitro*-derived HRMs was herein investigated by use of the K-means approach to establish HRM's cut-off values. Only miR-200c-star showed significant associations with survival in the validation set of ESCC and training set of AC. To the best of our knowledge, no studies have addressed the prognostic impact of miR-200c-star in cancer. In the literature, the prognostic value of HRMs has been studied in a range of cancer types, with miR-210 standing out as the most investigated. However, opposing results have been reported with miR-210 being positively correlated to survival in renal cancer, soft-tissue sarcoma and non-small cell lung cancer (20, 23, 24) but serving as an adverse prognostic marker in colorectal, breast and head and neck cancer (9, 11, 22). In GEC, the prognostic impact of miR-210 is yet to be elucidated. However, expression levels of miR-210 have been reported in esophageal cancer, showing conflicting results. Significant down-regulation of miR-210 in ESCC compared with para-cancerous normal tissue was shown by Tsuchiya *et al.*, in addition to reduced miR-210 expression as the degree of tumor differentiation decreased (37). In contrast, a study by Li *et al.* observed an up-regulation of circulating miR-210 in patients with ESCC compared to healthy controls (15) and Liu *et al.* reported miR-210 overexpression in esophageal cancer tissue compared to para-cancerous normal tissue (44). These discordant results illustrate the complexity and heterogeneity of esophageal cancer. No HRMs showed any predictive impact in the ESCC or AC study cohorts.

To the best of our knowledge, this is the first study to elucidate the association of HRMs with clinical outcome in GEC. However, translating *in vitro*-derived HRMs to clinical outcome is challenging as gene expression in cell lines from *in vitro* cultures might not be representative of the hypoxic response observed in tumors *in vivo*. Thus, the lack of clinical impact of HRMs in this study might be explained by several factors: Cells from *in vitro* studies are grown in a controlled environment with culture media

containing nutrients and glucose, in addition to maintainence of correct pH. Moreover, cells in culture may no longer represent the cancer cell line of origin. The aspect of tumor heterogeneity in the microenvironment might also be considered. Lastly, clinical aspects might introduce further biases: The present study is retrospective, with heterogeneous cohorts in terms of treatment. Additionally, the study cohorts are small and might lack power to obtain statistical significance.

qPCR was chosen as the validation strategy for *in vitro* microarray results in this study. Proper normalization of qPCR data using stably expressed reference genes is essential to ensure reliable and accurate results when the comparative ΔΔCt method is used. Appropriate reference genes must exhibit constant expression levels independently of experimental conditions and be tissue-specific. Here, geNorm and NormFinder were used to select and validate candidate reference genes. Based on stability and function, *RNU43*, *RNU44* and *miR-361* were chosen as reference genes. By using two different algorithms, the strength of the method of reference gene selection was increased.

In conclusion, the present work provides support for the involvement of hypoxia on miRNAs *in vitro* and confirms the role of miR-210 as being a universal HRM. This study was not able to identify significant associations of HRMs and clinical outcome in patients with GEC. However, emerging evidence has shown both prognostic and predictive impact of HRMs and, thus, they may have the potential to serve as novel biomarkers and therapeutic targets. In addition, identification of HRMs as surrogate markers for hypoxia renders the opportunity to stratify patients according to hypoxic status (more and less hypoxic) and, ultimately, identify patients who may benefit from hypoxia-modulating therapy. Further studies are needed to enhance our understanding of the regulatory basis and function of HRMs in order to identify the translational potential of these miRNAs in GEC.

Conflicts of Interest

The Authors declare no conflicts of interest.

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