Alteration of miRNA Expression Correlates with Lifestyle, Social and Environmental Determinants in Esophageal Carcinoma

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Abstract. *Background/Aim: Esophageal cancer (EC) is the eighth most common cancer with a highly aggressive potency. Considering the poor survival of esophageal carcinoma there is a need for useful molecular biomarkers for prevention and early detection. Our aim was to determine the significance of altered microRNA (miRNA) expression in esophageal cancer, in relation to lifestyle, social and environmental factors. Materials and Methods: The relative expression levels of the following miRNAs: miR-21, miR-143, miR-196a, miR-203, miR-205 and miR-221 were monitored in control and esophageal squamous-cell carcinoma (ESCC) samples using real-time polymerase chain reaction (RT-PCR). miRNA expression pattern of tumor tissues were evaluated according to patients' social status, living condition, smoking and drinking habits alone and in combinations. Results: miR-21, miR-143, miR-203, miR-205 and miR-221 were over-expressed in esophageal cancer compared with normal tissues. Increased expression of miR-205 was related to smoking, while excessive alcohol consumption showed a correlation with under-expression of miR-143, miR-203 and miR-205 in tumor samples. Significant associations were detected between reduced expression of miR-143, miR-203 and low social status, and combination of smoking and heavy drinking. Conclusion: Alterations of miRNA expression in ESCC can be correlated with the presence of common risk factors. The altered expression of certain miRNAs could be used as novel molecular markers of esophageal carcinoma.*

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Esophageal cancer (EC) is the sixth leading cause of cancer-related mortality with 450,000 affected people worldwide (1). The incidence and histological subtypes of esophageal cancer vary by geographical areas. Esophageal squamous-cell carcinoma (ESCC) has the highest incidence in "Asian esophageal cancer belt", such as Turkey, Northeast Iran, Kazakhstan, North and Central China. High-risk regions are also Japan, India, South and East Africa (2).

Environmental, genetic and epigenetic factors can contribute to the development of esophageal cancer (3). However, there are differences in individual susceptibility to esophageal carcinoma due to polymorphisms in carcinogen-metabolizing enzymes; the role of environmental exposure to tobacco smoke and alcohol are highly dominant (4). In a cohort study by Fan *et al*., the effect of tobacco and alcohol alone and in combination was investigated and they found a synergic effect of cigarette smoking and alcohol consumption on ESCC development (5). The low social status, poor oral hygiene, nutritional deficiencies as vitamins, minerals, fruits, vegetables, ingestions of hot and spicy foods and liquids, as well as infection by human papillomavirus are also associated with a higher risk of ESCC (6, 7). Patients with esophageal cancer are diagnosed often at later stage, which can lead to a poor prognosis with a 5-year overall survival rate of 15% to 25% (8).

The dysregulation of micro-RNAs (miRNAs) in malignancies of the gastrointestinal tract was investigated by several researchers (9). Previously, we demonstrated significant associations between certain risk factors of gastric cancer, including smoking, alcohol consumption, social status, demographic condition and miRNA expression pattern of gastric adenocarcinoma samples (10). In the present study, we analyzed the expression of miRNAs showing strong correlation with development of esophageal squamous cancer.

The investigated miRNAs (miR-21, miR-143, miR-196a, miR-203, miR-205 and miR-221) were selected based on literature data. MiR-21 can induce cell proliferation in Eca109 cells through activation of the ERK1/2/MAPK

signaling pathway (11). Li *et al*. reported that up-regulation of miR-21 is involved in progression of esophageal cancer through inhibition of the expression of phosphatase and tensin homolog gene (*PTEN*) (12). Increased expression of Fas and its natural ligand (FASL), tissue inhibitor of metalloproteinase-3 (TIMP3) and reversion-inducing-cysteine-rich protein with kazal motifs (RECK) was observed by Wang *et al*. after transfection of miR-21 inhibitor into EC9706 and EC-1 cells (13). Ni *et al*. demonstrated that over-expression of miR-143 can suppress cell proliferation and induce apoptosis in esophageal carcinoma cell lines (14). Additionally, miR-143 and miR-145 are involved cell migration and invasion in ESCC (15). Luthra *et al*. found an inverse correlation between expression level of miR-196a and Annexin A1 (ANXA1) in esophageal cancer (16). Zhang *et al*. showed that miR-203 induces cell apoptosis, suppresses cell proliferation, migration, invasion and down-regulates miR-21 in esophageal squamous cell carcinoma cell lines (17). When Yuen *et al*. transfected esophageal carcinoma cell lines with miR-203, they observed an anti-proliferative effect of miR-203 targeting ΔNp63 (18). MiR-205 inhibits epithelial to mesenchymal transition (EMT) enhancing E-cadherin expression through Zinc finger E-box-binding homeobox 2 (ZEB2) (19). MiR-221 is a frequently reported oncogenic miRNA in squamous cell carcinomas regulating apoptosis, proliferation and invasion of cancer cells (20).

To evaluate the potential application of miRNAs as markers for environmental exposures in gastroesophageal cancers, our research group analyzed the correlation between alteration in expression of selected miRNAs and the presence of risk factors. Herein, we focused on the correlation between common risk factors of esophageal carcinoma and alteration in expression of some miRNAs.

Materials and Methods

Patients and tissue samples. A total of 28 patients (23 men and 5 women) diagnosed with ESCC were included in the study. Tissue samples were obtained from each patient during radical resection at the Oncoradiology Center of Markusovszky County Hospital (Szombathely, Hungary) between 2005 and 2010. Patients' mean age was 61.5, while the median age at diagnosis was 59 . All were diagnosed at advanced stages of ESCC in stage III and stage IV. Normal tissues of the esophagus were collected for the control sample. All patients in the study gave informed consent and the study was carried out in accordance with the ethical guidelines of the Institutional Review Board of the Hospital. The patients' data about their environmental, lifestyle and social factors, such as social status, living conditions, smoking and drinking habits were collected from the hospital information system and the patients' general practitioners. According to smoking status, the patients were classified into two categories: smokers and non-smokers. Patients with smoking at least 10 years who used at least 15 pieces/day before the diagnosis, were defined as smokers. Based on alcohol consumption, subjects were divided into two groups, those who regularly drank at least two units of alcohol a day and those who did not. Patients were also assigned in two groups according to their social status: patients with high and low social status. The low social group included persons who were unemployed and disability pensioners. Between the investigated groups there were no significant difference according to age and stages of disease.

RNA extraction. Ten μm horizontal slices of formalin-fixed paraffin-embedded blocks were examined. Following de-paraffinization, miRNA was prepared using the High Pure FFPE RNA Micro Kit (Roche, City, Germany). The concentration and purity of the isolated miRNA fractions were checked by absorption photometry at 260/280 nm.

Reverse transcription. cDNA was prepared from miRNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The 20 μl reverse transcriptase mixture consisted of 2 μl RNA sample, 2 μl random hexamer primer, 4 μl transcriptor reverse transcriptase reaction buffer, 0.5 μl transcriptor reverse transcriptase, 2 μl deoxynucleotide mix, 0.5 μl protector RNase inhibitor and 9 μl H_2O. The reaction mix was incubated at 55˚C for 30 min, followed by inactivation of the reverse transcription enzyme at 85 ˚C for 5 min.

qRT-PCR analysis. The quantitative real time polymerase chain reaction (qRT-PCR) was carried out using the LightCycler SYBR Green kit (Roche) on a Light Cycler 480 system (Roche). The 20 μl PCR mixture contained 5 μl template cDNA, 2 μl sequence-specific primer, 10 μl Master Mix and 3 μl H_2O. The miR-specific primers (miR-21, miR-143, miR-196a, miR-203, miR-205, miR-221) and 5s rRNA were synthesized by TIB Molbiol (Berlin, Germany). Primer sequences were as follows: *miR-21* forward: 5'-GCTTATCAGA CTGATGTTGACTG-3', reverse: 5'-CAGCCCATCGA CTGGTG-3'; *miR-143* forward: 5'-TGAGGTGCAGTGCTGCATC-3', reverse: 5'-GCTACAGTGCTTCATCTCAGACTC-3'; *miR-196a* forward 5'-TAGGTAGTTTCATGTTGTTGGG-3', reverse: 5'-ATCGGGTGGT TTAATGTTG-3'; *miR-203* forward: 5'-TCCAGTGGTTCTTAACA GTTCA-3', reverse: 5'-GGTCTAGTGGTCCTAAACAT TTC-3'; *miR-205* forward: 5'- CCTTCATTCCACCGGAGT-3', reverse: 5'-GAACTTCACTCCACTGAAATCTG-3'; *miR-221* forward: 5'-CCTG GCATACAATGTAGATTTCTG-3', reverse: 5'-AAACCCAGC AGACAATGTAGCT-3', *5s rRNA* forward: 5'-ACGC GCCCGATC TCGTCTGAT-3', reverse: 5'-GCCTACAGCACCCGG TATTCCC-3'. The reaction mix was incubated in a Light Cycler 480 Multiwell Plate 96 at 95 ˚C for 10 min followed by 65 amplification cycles (at 95˚C for 10 s, at 50˚C for 1 min, at 72˚C for 10 s). Relative quantification was performed in relation to 5S rRNA expression by the application of the 2^{-dCp} method.

Statistical analysis. The data were analyzed by the SPSS program (21.0 version; IBM Co., Armonk, NY, USA). Independent samples *t*-test was used to evaluate the expression results. We accepted significant levels at a value of $p \leq 0.05$.

Results

Quantitative real-time PCR values of six miRNAs (miR-21, miR-143, miR-196a, miR-203, miR-205 and miR-221) were compared in normal and esophageal cancer tissues. The tumorous samples, compared with miRNA expression levels,

Table I. *Differential expression of miRNAs in normal and esophageal cancer tissues. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Tumor (Mean±SD)	Control (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0003	$1\times10^{-5}\pm5\times10^{-7}$	20.000	0.015	4×10^{-5}-0.0003
hsa-miR-143	0.0272±0.0118	$0.0019\pm5\times10^{-5}$	14.315	0.0001	0.0197-0.0308
hsa-miR-196a	0.0059±0.0124	0.0065±0.0005	1.101	0.831	–0.0064-0.0052
hsa-miR-203	0.0176±0.0135	$0.0017\pm5\times10^{-5}$	10.352	0.0001	0.0095-0.0221
hsa-miR-205	0.0345±0.0195	$0.0069\pm1\times10^{-5}$	5.000	0.0001	0.0185-0.0368
hsa-miR-221	0.6005±0.5608	0.1081±0.0069	5.555	0.001	0.2303-0.7551

Table II. *Differential expression of miRNAs according ESCC patients' social status. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Social status				
	Low social status (Mean±SD)	High social status (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0001±0.0001	0.0002±0.0005	2.000	0.432	–0.0004-0.0002
hsa-miR-143	0.0037±0.0023	0.0304±0.0093	8.216	0.0001	–0.0328-(–0.0205)
hsa-miR-196a	0.0041±0.0057	0.0082±0.0177	2.000	0.475	–0.0160-0.0077
hsa-miR-203	0.0034±0.0026	0.0205±0.0136	6.029	0.001	–0.0262-(–0.0087)
hsa-miR-205	0.0329±0.0196	0.0366±0.0204	1.112	0.684	–0.0227-0.0151
hsa-miR-221	0.4386±0.3453	0.914±0.6564	2.083	0.052	–0.9545-0.0038

were classified according to social status, living condition, smoking and drinking habits.

Significant up-regulation of miR-21 (p=0.015), miR-143 (p=0.0001), miR-203 (p=0.0001), miR-205 (p=0.0001) and miR-221 (p=0.001) was observed in esophageal tumorous tissues compared to those in normal tissues (Table I).

There was no significant difference between miRNA expression pattern of patients living in cities and in villages (Table III). MiR-143 was decreased with a 8.216-fold change (p=0.0001) in samples from patients with low social status. Reduced expression of miR-203 (p=0.001) was also detectable in the low-social group (Table II).

Significantly lower expression of miR-143 (p=0.002), miR-203 (p=0.0001), miR-205 (p=0.016) was characteristic for cancerous tissues of heavy alcohol drinkers compared to non-drinkers, while the expression of miR-205 was more than two-times (p=0.009) up-regulated in smokers than in non-smokers (Tables IV-V). Significant under-expression of miR-143 (p=0.012) and miR-203 (p=0.0001) was associated with combination of smoking and heavy drinking (Table VI).

Comparing patients with smoking and excessive alcohol consumption to smokers but non-drinkers, it was found that the expression of miR-143 (p=0.012) and miR-203 (p=0.001) was significantly lower in subjects who did not drink but smoked (Table VII). The combined effect of alcohol and cigarette smoking was analyzed and more than four-times higher expression of miR-205 (p=0.001) was found in patients' tissues with smoking and drinking alcohol relative to alcohol drinkers but non-smokers (Table VIII).

Discussion

Clarifying the role of molecules related to environmental factors has a great importance in the pathogenesis of gastroesophageal malignancies and their potential application in prevention and early detection. In the present study, we evaluated expression alterations of miRNAs playing an important role in esophageal carcinogenesis even more according to common risk factors of esophageal carcinoma. The alteration of miRNA expression in EC has been investigated by several researchers. According our results, the expression levels of miR-21, miR-143, miR-203, miR-205 and miR-221 were significantly higher in ESCC compared to controls. Liu *et al*. also detected an elevated expression level of miR-143 in esophageal cancer compared to that in normal tissues (21). The over-expression of miR-21 was demonstrated by several researchers in esophageal tumors (22-24). Analyzing the expression level of miRNAs in ESCC and HNSCC patients' samples, Kimura *et al*. found an over-expression of miR-205 besides an elevated level of miR-21 (25), while in a study conducted by Feber *et al*., four-to-five-fold lower levels of miR-203 and miR-205 were

Table III. *Differential expression of miRNAs according ESCC patients' living condition. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Living condition				
	City (Mean±SD)	Village (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	$6\times10^{-5}\pm4\times10^{-5}$	0.0002±0.0004	3.333	0.117	-0.0004-5×10^{-5}
hsa-miR-143	0.0251±0.0060	0.0281±0.0133	1.119	0.482	–0.0114-0.0056
hsa-miR-196a	0.0147±0.0094	0.0063±0.0148	2.333	0.088	–0.0013-0.0182
hsa-miR-203	0.0094±0.0122	0.0164±0.0132	1.744	0.167	–0.0171-0.0031
hsa-miR-205	0.0495±0.0195	0.0342±0.0196	1.447	0.051	-4×10^{-5}-3.1×10^{-5}
hsa-miR-221	0.3377±0.2893	0.7002±0.6328	2.073	0.064	–0.0748-0.0222

Table IV. *Differential expression of miRNAs according ESCC patients' smoking habits. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Smoking habit				
	Smokers (Mean±SD)	Non-smokers (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0004	$6\times10^{-5}\pm6\times10^{-5}$	3.333	0.072	-2×10^{-5}- 0.0004
hsa-miR-143	0.0259±0.0133	0.0231±0.0076	1.121	0.51	–0.0057-0.0112
hsa-miR-196a	0.0073±0.0148	0.0019±0.0007	3.842	0.192	–0.0031-0.0141
hsa-miR-203	0.0164±0.0125	0.0091±0.0148	1.802	0.196	–0.0037-0.0176
hsa-miR-205	0.0358±0.0193	0.0145±0.0204	2.468	0.009	0.0057-0.0367
hsa-miR-221	0.6535±0.6125	0.3583±0.3698	1.823	0.135	–0.0976-0.6881

Table V. *Differential expression of miRNAs according ESCC patients' drinking habits. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Drinking habit				
	Alcohol users (Mean±SD)	Non-alcohol users (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0003	$0.0001\pm4\times10^{-6}$	2.000	0.421	–0.0001-0.0002
hsa-miR-143	0.0264±0.0127	0.0381±0.0049	1.443	0.002	–0.0185-(–0.0047)
hsa-miR-196a	0.0065±0.0134	$0.0026\pm2\times10^{-5}$	2.500	0.249	–0.0032-0.0108
hsa-miR-203	0.0153±0.0134	0.0327±0.0011	2.137	0.0001	–0.0231-(–0.0104)
hsa-miR-205	0.0327±0.0207	0.0463±0.0009	1.415	0.016	–0.0242-(–0.0028)
hsa-miR-221	0.6133±0.5905	0.8811±0.0043	1.436	0.08	–0.5713-0.0358

observed in squamous cell carcinoma (SCC) compared to normal esophageal tissues (26).

Environmental exposures, such as diet, smoking, alcohol, stress, infectious agents and environmental carcinogens, play important roles in the pathogenesis of cancerous diseases through epigenetic modifications (27). MiRNAs and their deregulation is frequently investigated field of epigenetics. There are several observations concerning dietary factors and changes in miRNA expression. High-fat diet led to increased expression of miR-143 in the mesenteric fat of mice (28). Deficiency of micronutrients, such as vitamin E and folate also linked to altered miRNA expression in human and animal models. Marsit *et al*. demonstrated a global elevation in miRNA expression in response to folate deficiency (29). Decreased expression of miR-122a and miR-125b was detected by Gaedicke *et al*. in rat liver cells after a six-month vitamin E-deficient diet (30). Long-term ethanol feeding induced up-regulation of miR-34a, miR-103, miR-107, miR-122 and down-nregulation of miR-19b, miR-200b in rat liver (31). Tang *et al*. reported an elevated level of miR-212 in

Table VI. *Differential expression of miRNAs according ESCC patients' smoking and drinking habits. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Smoking and drinking habits				
	Alcohol users and smokers (Mean±SD)	Non-alcohol users and non-smokers (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0004	0.0001±3×10-6	2.000	0.328	–0.0001-0.0003
hsa-miR-143	0.0255±0.0138	0.0373±0.0038	1.462	0.012	–0.0200-(–0.0029)
hsa-miR-196a	0.0077±0.0153	0.0026±1×10-5	2.961	0.253	–0.0041-0.0143
hsa-miR-203	0.0149±0.0123	0.0327±0.0009	2.194	0.0001	–0.0252-(–0.0102)
hsa-miR-205	0.0352±0.0203	0.0463±0.0004	1.315	0.068	–0.0232-0.0009
hsa-miR-221	0.6373±0.6338	0.8819±0.0015	1.383	0.189	–0.6270-0.1384

Table VII. *Differential expression of miRNAs according ESCC patients' smoking and drinking habits. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Smoking and drinking habits				
	Alcohol users and smokers (Mean±SD)	Smokers and non-alcohol users (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0004	$0.0001\pm3\times10^{-6}$	2.000	0.403	–0.0001-0.0003
hsa-miR-143	0.0255±0.0138	0.037±0.0038	1.450	0.012	–0.020-(–0.002)
hsa-miR-196a	0.0077±0.0153	$0.0026\pm3\times10^{-5}$	2.961	0.251	–0.0041-0.0143
hsa-miR-203	0.0149±0.0123	0.0307±0.0003	2.060	0.001	–0.023-(–0.008)
hsa-miR-205	0.0352±0.0200	0.0439±0.0003	1.247	0.145	–0.0207-0.0034
hsa-miR-221	0.6373±0.6338	0.8710±0.0038	1.366	0.208	–0.6167-0.1493

Table VIII. *Differential expression of miRNAs according ESCC patients' smoking and drinking habits. The expression values were calculated according to the 2nd derivative maximum method and normalized to 5s rRNA.*

	Smoking and drinking habits				
	Alcohol users and smokers (Mean±SD)	Alcohol users and non-smokers (Mean±SD)	Fold change	*p*-Value	95% CI
hsa-miR-21	0.0002±0.0004	$2\times10^{-5}\pm5\times10^{-5}$	10.000	0.061	-1×10^{-5}-0.0004
hsa-miR-143	0.0255±0.0138	0.0215±0.0071	1.186	0.362	–0.0053-0.0131
hsa-miR-196a	0.0077±0.0153	0.0017±0.0007	4.529	0.186	–0.0033-0.0152
hsa-miR-203	0.0149±0.0123	0.0051±0.0121	2.921	0.051	–0.0001-0.0198
hsa-miR-205	0.0352±0.0203	0.0087±0.0164	4.045	0.001	0.0116-0.0413
hsa-miR-221	0.6373±0.6338	0.2617±0.3108	2.435	0.067	–0.0285-0.7797

intestinal epithelial cells in alcoholic liver disease patients (32). Smoking-modulated miRNAs were determined in human lung adenocarcinoma tissues by Seike *et al*. where 36 miRNS, including miR-29b, miR-30b, miR-96, miR-145, miR-148a, miR-155, miR-191, miR-193b, miR-204, miR-208, miR-210 and miR-223 were associated with smoking history (33). Avissar *et al*. investigated the relationship between alcohol consumption and the miRNA expression in human head and neck cancer (34). They found that higher miR-375 expression was related to increasing alcohol exposure, while over-expression of miR-21 correlated with poor prognosis in HNSCC patients.

Yang *et al*. evaluated the risk of esophageal carcinoma according to aberrant expression of miRNAs. Additionally,

they analyzed the associations between miRNA expression and patients' demographic data, epidemiological behavior, including dietary, smoking and drinking habits (35). According to their results, a significantly increased risk correlated with reduced expression of miR-139-5p, miR-338-3p and increased expression of miR-21, miR-183, miR-574-5p and miR-601. They also found a significantly higher expression of miR-21 in heavy drinking patients. Our analysis revealed no significant associations between miR-21 levels and alcohol consumption. On the other hand, there was a significantly lower expression of miR-143, miR-203 and miR-205 detectable in patients with alcohol use. A reduced expression level of miR-143 and miR-203 was significantly associated with low social status. These results seem to be linked to an under-expression of miR-143 and miR-203 in tumor tissues from patients who smoked and were heavy drinkers. Since, however, drinking alcohol was often combined with cigarette smoking, we also investigated subjects who met the criteria for either smoking or drinking. The patients from groups of smokers or drinkers were compared with those who lived with using both, evaluating the combined effect of alcohol and tobacco use at miRNA expression level. The combination of smoking and heavy drinking resulted in reduced levels of miR-143 and miR-203 compared to drinkers but non-smokers and elevated level of miR-205 in relation to smokers but non-drinkers.

Our data demonstrate the different miRNA expression pattern between normal and esophageal squamous cancer tissues. Analyzing the expression of tumor samples, we revealed that different miRNA levels can be detected in esophageal carcinoma depending upon the presence of major risk factors and their combinations. These results indicate that miRNA expression related to the presence of common risk factors of ESCC and could have a potential to become new biomarkers for prevention and early diagnosis for esophageal carcinoma.

MicroRNAs can regulate the pathways in human carcinogenesis by their altered expression. These different patterns also relate to various types of lifestyle and environmental encounters. However, the final results still need more extensive and precise research, in larger cohorts of patients.

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