Micro RNAs expressions in esophageal squamosus cell cancers and gastric adenocancers

Doctoral (PhD) thesis

Eva Stánitz MD

Head of theDoctoralSchool: Prof. József Bódis MD, PhD, DSC

Program Leader and Supervisor: Prof. István Kiss MD, PhD, DSC

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Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer-related mortality with 450,000 affected people worldwide. The incidence and histological subtypes of esophageal cancer vary by geographical areas. Esophageal squamosus 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.

Environmental, genetic and epigenetic factors can contribute the development of esophageal cancer. However there are differences in individual susceptibility to esophageal carcinoma due to polymorphisms in carcinogen-metabolising enzymes, the role of environmental exposure to tobacco smoking and alcohol is also highly dominant.

Gastric cancer is the fourth most frequently diagnosed cancer worldwide. Despite of the decreasing tendency of stomach cancer, about 1 million new cases occur each year. The high-incidence areas include Eastern Asia, Easter Europe and South-America. Adenocarcinoma is the most frequent histological subtype of this malignancy. The gastric carcinogenesis is a multistep process that involves the interference of many environmental, genetic and epigenetic factors.

In study our aim was to analyse the significance of altered microRNA expressions of esophageal (ESCC) and gastric cancer (GA) in relation to lifestyle, social, and lifestyle behaviour factors such as smoking and alcohol consumption.

Main aims of the study

1. Are there differences in the choosed microRNAs levels between normal and cancerous samples from ESCC and GA patients?

2. Are there differences in microRNAs expression levels of ESCC patients who are different in behaviour habits of smoking, alcohol consumption and living condition?

3. Are there differences in microRNAs expression levels of GA patients, who are different in behaviour habits of smoking, alcohol consumption and living condition?

4. Is there effect of two combined risks factors to expressions of microRNAs in ESCC?

5. Can the difference of social conditions change the microRNAs expression?

6. Is there correlation between microRNAs expression and survival of the patients with similar clinico-pahological stage of cancer?

Material and methods

Formalin-fixed paraffin embedded tumour samples were obtained from 39 patients diagnosed with gastric adenocarcinoma, and another 20 diagnosed with esophageal squamosus cell carcinoma.

They were observed between 2010 and 2014 at Oncoradiology Center of Markusovszky Hospital, Szombathely, in Hungary.

For further evaluation the cancer samples were classifield the patients' social status, living condition, drinking, smoking and paired habits. This data we collected from informations system of hospital, and from general practitioners of patients.

RNA extraction

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

Reverse transcription

cDNA was prepared from miRNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The 20μl reverse transcriptase mixture consisted of 2 μl RNA sample, 2μl random hexamer primer, 4μl transcriptor reverse transcriptase reaction buffer, 0.5μl transcriptor reverse transcriptase, 2μl deoxynucleotide mix, 0.5μl 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 transcriptase enzyme at 85 ˚C for 5 minutes.

Quantitative real-time polymerase chain reaction (qRT PCR)

The qRT-PCR was carried out using LightCycler SYBR Green kit (Roche Germany) on Light Cycler 480 system (Roche, Germany). 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), in ESCC' study. In GC study analyzed miR were themiR-21, miR-143, miR-34a, miR-93, miR-203, miR-205 and miR-223. The reaction mix was incubated in Light Cycler 480 Multiwell Plate 96 at 95 ˚C for 10min followed by 65 amplification cycle (at 95˚C for 10s, at 50˚C for 1min, at 72˚C for 10s).

Statistical analysis

The data were analysed by 18.0 and later 21.0 version of SPSS program (IBM Co., Armonk, NY, USA). Independent samples t-test was used to evaluate the expression results. The significant level was accepted at $p \leq 0.05$ value.

In the gastric carcinoma study for the correlation between the micro RNAs expression and survival rate we used log tank test.

Results

Quantitative real-time PCR values of six miRNAs (miR-21, miR-143, miR-196a, miR-203, miR-205 and miR-221) were compared between normal and esophageal cancer tissues. The tumorous patient data were classified according to social status, living condition, smoking and drinking habits, and were compared with miRNA expression levels.

ESCC

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 tissue compared to those with the normal. There was no significant difference between miR expression pattern of patients living in cities or in villages. miR-143 was decreased with 8.216-fold times (p=0.0001) in samples from patients with low social status. Reduced expression of miR-203 (p=0.001) was also detected in low social group.

	Tumour (mean±SD)	**Control (mean±SD)**	**Fold change**	**p-value**	**CI 95%**
hsa-miR-21	0.0002±0.0003	$1x10^{-5}$±$5x10^{-7}$	20.000	0.015	$4x10^{-5}$ - 0.0003
hsa-miR-143	0.0272±0.0118	0.0019±$5x10^{-5}$	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±$5x10^{-5}$	10.352	0.0001	0.0095 - 0.0221
hsa-miR-205	0.0345±0.0195	0.0069±$1x10^{-5}$	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 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

	Social status		Fold change	p-value	CI 95%
	low (Mean±SD)	normal (Mean±SD)			
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

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.

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

	Alcohol drinking habit		Fold change	p-value	CI 95%
	Drinkers (Mean±SD)	Non drinkers (Mean±SD)			
hsa-miR-21	0.0002±0.0003	$0.0001\pm4x10^{-6}$	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	$0.0026\pm2x10^{-5}$	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

Table III: 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.

	Smoking habit		Fold change	p-value	CI 95%
	Smokers (Mean±SD)	**Non smokers** (Mean±SD)			
hsa-miR-21	0.0002±0.0004	$6x10^{-5}$±$6x10^{-5}$	3.333	0.072	-$2x10^{-5}$- 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 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

Significant underexpression of miR-143 (p=0.012) and miR-203 (p=0.0001) was associated with combined of smoking and heavy drinking

Comparing patients with smoking and excessive alcohol consumption to smokers but non-drinkers, the expression of miR-143 (p= 0.012) and miR-203 (p=0.001) was significantly lower among subjects who didn't drink but smoked. 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

GC

The expression levels of some miR-s significantly differed in tumour and non-tumorous tissues. Up-regulation ofmiR-21 (p=0.001) miR-143(p=0.001) were found in gastric cancer tissues in comparison with control samples. In tumourous samples the expression level of miR-34a (p=0.001) was significantly lower than in normal tissues. We didn't found

significant difference between tumour and normal tissues in the expression level of miR-93, miR-203, miR-205 and miR-223.

	p value	Mean difference	std. error difference	CI 95%	
				lower	upper
miR-21	,001	,766269444	,123685142	,516292449	1,016246440
miR-34a	,001	-,931963611	,072517897	-1,078527747	-,785399475
miR-93	,137	,345581056	,227520379	-,114254783	,805416894
miR-143	,001	,657252781	,073024443	,509664877	,804840685
miR-203	,299	,106699167	,101415705	,098269620	,311667953
miR-205	,797	-,036902000	,142314295	-,324529919	,250725919
miR-223	,165	,047320956	,033486567	-,020357921	,114999832

Table V.: miR-expressions of normal and gastric-cancer-tissues

Higher expression of miR-21was detected in gastric cancer patients with smoking(p=0.001) and low social status(p = 0.049). In gastric cancer samples the expression of miR-143 was lower in smokers (p=0.004) related to non-smokers, while an opposite tendency was observed in patients with low social status (p=0.003).

	p value	Mean difference	std. error difference	CI 95%	
				lower	upper
miR-21	,001	1,14110000	,116770000	,898930000	1,383260000
miR-34a	,769	-,099516151	,334100106	-,792397363	,593365061
miR-93	,206	-,430372821	,330116469	-1,114992477	,254246834
miR-143	,004	-1,002503571	,307936354	-1,641124483	-,363882659
miR-203	,799	,031438270	,122255953	-,222105058	,284981597
miR-205	,526	,168821943	,262064633	-,374666841	,712310728
miR-223	,499	,025472269	,037064319	-,051394425	,102338962

Table VI.: miR expressions of gastric Adenocarcinoma samples according to the patients' smoking habit

	p value	Mean difference	std. error difference	CI 95%	
				lower	upper
miR-21	,049	,297840000	,14139000	,00079000	,594890000
miR-34a	,533	,226331832	,356218236	-,522054912	,974718575
miR-93	,545	,146569396	,237398671	-,352186705	,645325496
miR-143	,003	,856748901	,254623241	,321805322	1,391692480
miR-203	,939	,010169011	,131726047	-,266577145	,286915167
miR-205	,308	-,201619398	,192097307	-,605200865	,201196620
miR-223	,582	,023744744	,042399823	-,065333980	,112823467

Table VII.: miR-expressions of gastric adenocarcinoma samples according to the patients' social status

Significantly increased expression levels of miR-203 (p=0,000), miR-205 (p=0,003) and miR-223 (p=0,000) were identified in cancer tissues of patients with regular alcohol consumption. We detected significantly lower expression levels of miR-34a (p=0,032) and miR-143 (p=0,001) in samples of patients living in village than in city.

	p value	Mean difference	std. error difference	CI 95%	
				lower	upper
miR-21	,001	,84591000	,16587000	,49873000	1,193090000
miR-34a	,175	,576351905	,408979156	-,279651307	1,432355116
miR-93	,254	,455033190	,3386560820	-,354047903	1,264114284
miR-143	,828	,101692857	,4620944603	-,865481962	1,068867676
miR-203	,001	,635459714	,076620230	,475091730	,795827699
miR-205	,003	,790991714	,232196364	,304999139	1,276984289
miR-223	,001	,594559214	,059987380	,469004186	,720114243

Table VIII: miR expressions of gastric adenocarcinoma samples according to the patients' drinking habit

	p value	Mean difference	std. error difference	CI 95%.	
				lower	upper
miR-21	,698	,20822000	,532290000	-,87601000	1,292460000
miR-34a	,032	-1,631918333	,727892850	-3,112827472	-,151009194
miR-93	,969	,035298429	,907931313	-1,811901716	1,882498574
miR-143	,001	-2,544247625	,691385922	-3,950882860	-1,137612390
miR-203	,298	,797838038	,753544452	-,739026003	2,334702080
miR-205	,352	,263495657	,278202795	-,307329329	,834320642
miR-223	,562	-,018315167	,031230757	-,082096882	,045466549

Table IX.: miR-expression of gastric adenocarcinoma samples according to the patients' living conditions

In the gastric carcinoma study for the correlation between the micro RNA-s expression and survival rate we used log tank test. The correlation was significant atmiR-21, miR-34a and miR-205 values.

	Chi^2	df	Sig.
miR-21	4,934	1	0,026
miR-34a	7,955	1	0,005
miR-93	2,088	1	0,148
miR-143	2,769	1	0,096
miR-203	0,428	1	0,513
miR-205	3,898	1	0,048
miR-223	0,087	1	0,768

Table X: Connection between survival and miR expression in gastric adenocarcinoma

Summary

Our study's aim was to investigate the miRNA expression profile of gastric adenocarcinoma and esophageal squamosus cell carcinoma of patients with similar clinico-pathological stage of cancer but different environmental, lifestyle and social determinants such as smoking, alcohol consumption and social status from Northwest region of Hungary.

There are only a few international publications about correlation of patiens with ESCC or with GC and miRNAs expressions. The reaserch with these miRNAs were any more until 2010.

1. WefoundedhighlysignificentdifferenceinexaminedmiRNAsexpressionsbetweennormal and tumorousos tissues almost all in ESCC samples, and some in GC samples.

2. We detected significant differences in both type of malignancy in miR-203, miR-205, miR-221, miR-223 expressions, what caused alcohol.

3. We founded significant differences in miR-205, miR-21, miR-143 expressions connection with smoking effect, in ESCC and in GC.

4. The social status also caused significant differences in some miR expressions in ESCC and in GC, as well.

5. Our results showed significant differences between survival rate and miR-expressions in GC, in the miR-21, miR-34a, and -miR205 expressions levels.

Publications:

1. Stanitz E., Juhasz K., Gombos K., Gocze K., Toth C., Kiss I.
   Alteration of miRNA Expression Correlates with Lifestyle, Social and Environmental Determinants in Esophageal Carcinoma.
   ANTICANCER RESEARCH 35:(2) pp. 1091-1097. (2015)
   Impact factor: 1,872

2. Stanitz E., Juhasz K., Toth C., Gombos K., Natali PG., Ember I.
   Evaluation of MicroRNA Expression Pattern of Gastric Adenocarcinoma Associated with Socioeconomic, Environmental and Lifestyle Factors in Northwestern Hungary.
   ANTICANCER RESEARCH 33:(8) pp. 3195-3200. (2013)
   Független idéző: 9, függő idéző: 1, Összesen: 10

   Impact factor: 1,826

3. Juhász Krisztina , Stánitz Éva , Horváth Tibor , Ember István
   Humán nyelőcső és gyomor daganatok epidemiológiai és genomikai elemzése
   MAGYAR EPIDEMIOLÓGIA 8:(4) p. S. 55. (2011)

   Cumulative impact factor: 3,698

Other publications:

1. Stánitz Éva, Tompity Tünde, Ungvári Erika, Pászti Judit
Endémiás MRSA-kolonizáció felderítése időskorúak otthonában
LEGE ARTIS MEDICINAE 23:(3-4) pp. 198-202. (2013)

2. Stánitz Éva, Vollaint Mária, Pászti Judit
Endémiás MRSA kolonizáció időskorúak otthonában
MAGYAR EPIDEMIOLÓGIA 8: p. S. 48. (2011)

3. Schneider Ferenc, Stánitz Éva, Kalácska Judit, Tompity Tünde, Gábor Beáta
Pertussis egy szombathelyi középiskolában. Egy járvány tanulságai
ORVOSI HETILAP 150:(33) pp. 1557-1561. (2009)

4. Bognár E., Nádasi E., Dombi Zs., Végh Gy., Stánitz É., Fehér K., Szepes Farkas B., Ember I.
A bőrrák megelőzési lehetőségei magyar iskoláskorú populációban: Nyugat-Magyarországi vizsgálatok
MAGYAR EPIDEMIOLÓGIA 2:(1) pp. 29-36. (2005)

5. Bognár Enikő, Nádasi Edit, Dombi Zsuzsanna, Végh György, Stánitz Éva, Fehér Katalin, Szepes Éva, Farkas Beatrix, Mina Lomuscio, Ember István
A bőrrák megelőzési lehetőségei magyar iskoláskorú populációban: Nyugat-Magyarországi vizsgálatok
MAGYAR EPIDEMIOLÓGIA 2: p. 30. (2005)

6. Stánitz É., Hadarits F.
Funkcionális anatómia: Tanulási segédanyag egészségtantanár szakos hallgatóknak
Szombathely: Berzsenyi Dániel Főiskola, 2003. 134 p.
(ISBN 963 9290 94 7)