Evaluation of MicroRNA Expression Pattern of Gastric Adenocarcinoma Associated with Socioeconomic, Environmental and Lifestyle Factors in Northwestern Hungary

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Abstract. *Aim: Gastric cancer is among the most common causes of cancer-related death worldwide. Since microRNAs (miRNAs) represent an emerging field of cancer research, there is an increasing interest regarding the miRNA responses to environmental and lifestyle exposures. The aim of our study was to analyze whether social status, living conditions and lifestyle behaviours, such as cigarette smoking and alcohol consumption, are associated with specific miRNA expression patterns of gastric adenocarcinoma. Materials and Methods: Thirty-nine formalin-fixed paraffin-embedded primary gastric adenocarcinoma and nine non-tumourous samples were analyzed by real-time polymerase chain reaction. The expression levels of miR-21, miR-34a, miR-93, miR-143, miR-203, miR-205, miR-223 were compared in gastric adenocarcinoma samples of patients with different demographical characteristics, social status, drinking and smoking habits. Results: Overexpression of miR-21, miR-143 and underexpression of miR-34a were observed in gastric cancer samples relative to the controls. Elevated expression of miR-21 was detected in patients with low social status. Smokers showed higher expression of miR-21 and lower expression of miR-143. Up-regulation of miR-203, miR-205 and miR-223 was identified in patients with regular alcohol consumption. Patients living in an urban environment had elevated expression levels of miR-143 and miR-34a. Discussion: Differential miRNA expression patterns of gastric adenocarcinoma of the same histopathology from a small geographic region's population show homogenous correlations with the existence of common risk factors.*

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Gastric cancer is the fourth most frequently diagnosed cancer worldwide. Despite the decreasing incidence of stomach cancer, approximately one million new cases occur each year (1). The high-incidence areas include Eastern Asia, Eastern Europe and South America (2). Gastric adenocarcinoma is the most frequent histological subtype of this malignancy. Gastric carcinogenesis is a multistep process that also involves many environmental, genetic, epigenetic, and other factors (3). It was previously demonstrated that a low socioeconomic status, *Helicobacter pylori* infection, smoking, alcohol consumption and certain demographic features are associated with a high risk of gastric cancer (4, 5). There are data available on the role of genetic factors involved in the molecular pathogenesis of gastric tumour. Zhang *et al*. identified frequent occurrence of glutathione S-transferase pi 1 (*GSTP1*) Val allele in patients with gastric cancer who smoked, consumed alcohol and had *Helicobacter pylori* (6). In another study, the X-ray repair complementing defective repair in Chinese hamster cells 1 (*XRCC1*) and X-ray repair complementing defective repair in Chinese hamster cells 3 (*XRCC3*) gene polymorphisms showed association with smoking, drinking habits and cancer outcome (7).

MicroRNAs (miRNAs) are 18-23-nucleotide length molecules. Through binding to 3' untranslated region of their target mRNA, they can inhibit the translation of proto-oncogenes and tumour suppressors, thus regulating signal transduction and cell-cycle processes (8). An increasing number of studies have provided evidence of the involvement of miRNAs in human tumours (9). Previous studies have reported on the association between gastric cancer development and aberrant expression of miRNAs (10-12). Based on recent evidence, miRNAs seem to have a close relationship with environmental exposures. Associations were

Table I. *Demographical characteristics of the study groups.*

	Social status		Living conditions		Smoking status		Alcohol consumption	
Groups	Low social status	High social status	Village	City	Non-smokers	Smokers	Non-alcoholic	Alcoholic
Number of individuals (n)	6	13	14	21	8	14	6	14
Median age (years)	63.66	68.38	66.71	67.66	66.71	65.21	69.6	66.07
Gender (M/F)	4/2	11/2	4/10	14/7	6/2	12/2	3/3	14/0

found between miRNA de-regulation and lifestyle behaviours such as smoking and alcohol consumption, stress, infection and radiation (13). The correlation between the altered miRNA expression and the presence of such factors is essential for the better understanding of molecular mechanisms of gastric cancer development and for developing new preventative methods.

Our aim was to investigate the miRNA expression profile of gastric adenocarcinoma of patients with similar clinico-pathological stages of cancer but different environmental, lifestyle and social determinants, such as smoking, alcohol consumption and social status, in the Northwestern region of Hungary.

Materials and Methods

Formalin-fixed paraffin-embedded (FFPE) tumour samples were obtained from 39 patients diagnosed with gastric adenocarcinoma between 2005 and 2010 at the Oncoradiology Center of the Markusovszky County Hospital, Szombathely, Hungary. The gastric adenocarcinoma samples were selected from pathological archives at the Markusovszky County Hospital according to International Classification of Diseases (ICD) between 1500-1690 codes. Samples were classified according to the patient's social status, living conditions, drinking and smoking habits, which data were collected from the patient's medical records existing in E-MedSolution System of Markusovszky County Hospital (Table I). The median age of the patients was 62.12 years and the cohort consisted of 25 males and 14 females. The clinical stages of gastric tumour samples were the following: stage II in ten patients, stage III in nineteen, and stage IV in ten patients. Written informed consent was obtained from each patient. The study was carried out in accordance with the ethical guidelines of the Institutional Review Board.

FFPE blocks were cut into 10 μm horizontal slices. The samples were deparaffinized using xylene and 95% ethanol extraction followed by Proteinase K treatment. We isolated miRNA from tissues using High-Pure FFPE RNA Micro Kit (Roche) according to the manufacturer's protocol. RNA concentration and purity were assessed by absorption photometry at 260/280 nm. Optical density of the RNA was between 1.9 and 2.1. miRNA was reverse transcribed into cDNA using Transcriptor First-Strand cDNA Synthesis Kit (Roche). The reaction mix (20 μl) contained 2 μl template miRNA, 9 μl H_2O, 2 μl of random hexamer primer, 4 μl transcriptor reverse transcriptase reaction buffer, 0.5 μl protector RNase inhibitor, 2 μl deoxynucleotide mix and 0.5 μl transcriptor reverse transcriptase. The reaction mix was heated to 80˚C for 5 min to denature the RNA, followed by incubation for 30 min at 55˚C. Quantitative real-time polymerase chain reaction was carried out in a Light Cycler 480 system (Roche) using Light Cycler 480 SYBR Green kit. The following primers were used: *miR-21* forward: 5'-GCTTATCAGACTGATGTTGACTG-3', reverse: 5'-CAG CCCATCGACTGGTG-3'; *miR-34a* forward: 5'-TGGCAGTGTCTT AGCTGGTTG-3', reverse 5'-GGCAGTATACTTGCTGATTGCTT-3'; miR-93 forward 5'-AAGTGCTGTTCGTGCAGGT-3', reverse: 5'-CTCGGGAAGTGCTAGCTCA-3'; *miR-143* forward: 5'-TGAG GTGCAGTGCTGCATC-3', reverse: 5'-GCTACAGTGCTTCATCTCA GACTC-3'; *miR-203* forward: 5'-TCCAGTGGTTCTTAACAGTTCA-3', reverse: 5'-GGTCTAGTGGTCCTAAACATT TC-3'; *miR-205* forward: 5'-CCTTCATTCCACCGGAGT-3', reverse: 5'GAACTTCA CTCCACTGAAATCTG-3'; *miR-223* forward: 5'-CCGTGTATTT GACAAGCTGAGT-3', reverse: 5'-TGGGGTATTTGACAAA CTGACA-3'. The PCR reaction mixtures included 5 μl template cDNA, 3 μl H_2O, 2 μl sequence-specific primer, 10 μl Master Mix. The reaction mixtures were incubated in LightCycler 480 Multiwell Plate 96 at 95˚C for 5 min, followed by 55 three-step amplification cycles (at 95˚C for 10 s, at 55˚C for 20 s and at 72˚C for 15 s). The concentration of each miRNA was determined by absolute quantification. Independent two-sample t-test was performed for comparison between miRNA expression levels and *p*-Values were calculated. *p*-Values less than 0.05 were considered statistically significant. Values were expressed as the mean±SD. The calculation was performed using the Statistical Program for Social Science 19.0 (SPSS) software (IBM, Armonk, NY, USA).

Results

The expression levels of some miRNAs significantly differed in tumour and non-tumorous tissues. Up-regulation of *miR-21* (p=0.001) and *miR-143* (p=0.001) was found in gastric cancer tissues in comparison with control samples. In tumour samples, the expression level of *miR-34a* (p=0.001) was significantly lower than in normal tissues. We found no significant difference between tumour and normal tissues in the expression levels of *miR-93*, *miR-203*, *miR-205* and *miR-223* (Figure 1).

Higher expression of *miR-21* was detected in patients with gastric cancer who smoked (p=0.001) and low social status (p=0.049) (Figures 2 and 3). In gastric cancer samples, the expression of *miR-143* was lower in smokers (p=0.004) compared to non-smokers, while it was higher in patients with low social status (p=0.003) (Figures 2 and 3). Significantly increased expression of *miR-203* (p<0.001),

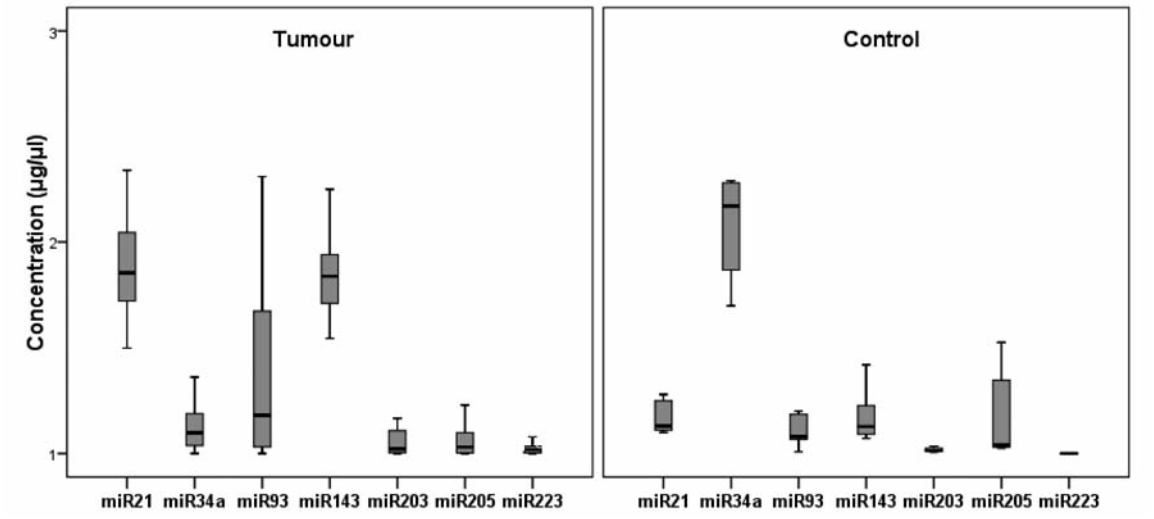

Figure 1. *Columns represent miRNA expressions of normal and gastric cancer tissues with absolute quantification in µg/µl.*

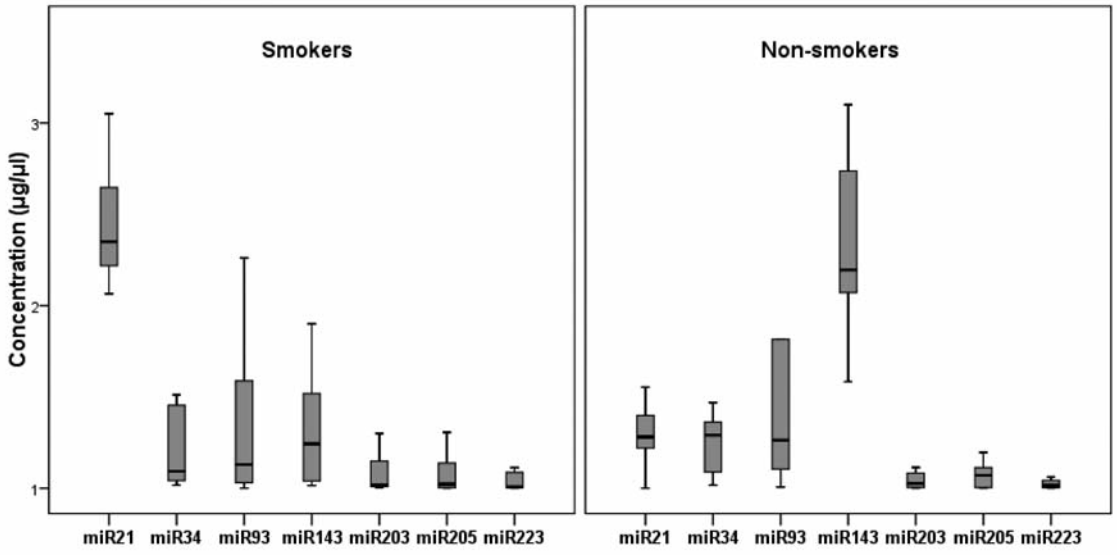

Figure 2. *miRNA expressions of gastric adenocarcinoma samples according to the patients' smoking habit. Columns represent miRNA expression with absolute quantification in µg/µl.*

miR-205 (p=0.003) and *miR-223* (p<0.001) was found in cancer tissues of patients who regularly consumed alcohol (Figure 4). We detected significantly lower expression levels of *miR-34a* (p=0.032) and *miR-143* (p=0.001) in samples of patients living in villages rather than in the cities (Figure 5).

Discussion

The expression profiles of some miRNAs, including *miR-21, miR-34a, miR-93, miR-143, miR-203, miR-205* and *miR-223*, of tumours and normal gastric tissues were determined as a first step of the analysis, then we focused on patients with gastric cancer, where the correlation between miRNA expression pattern and difference in living condition, lifestyle behaviour and social status were investigated.

By targeting oncogenes and tumor suppressor genes, miRNAs have a crucial role in carcinogenesis (14). Aberrant expression of *miR-34a* was found in several types of cancer such as of the colon, pancreas, breast, lung, kidney, bladder and prostate (15). *miR-34a* can inhibit B-cell lymphoma 2 (*BCL2*) and *Sirtuin* 1 genes, and seems to be a direct target of *p53* gene (16, 17). *miR-21* is known as an oncomiR, directly inhibiting

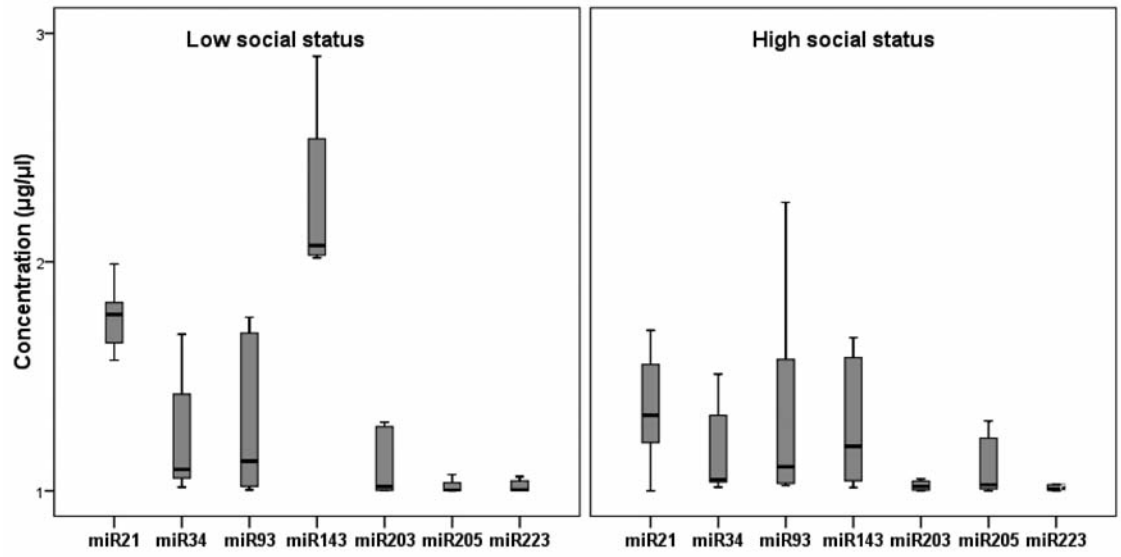

Figure 3. *miRNA expressions of gastric adenocarcinoma samples according to the patients' social status. Columns represent miRNA expression with absolute quantification in µg/µl.*

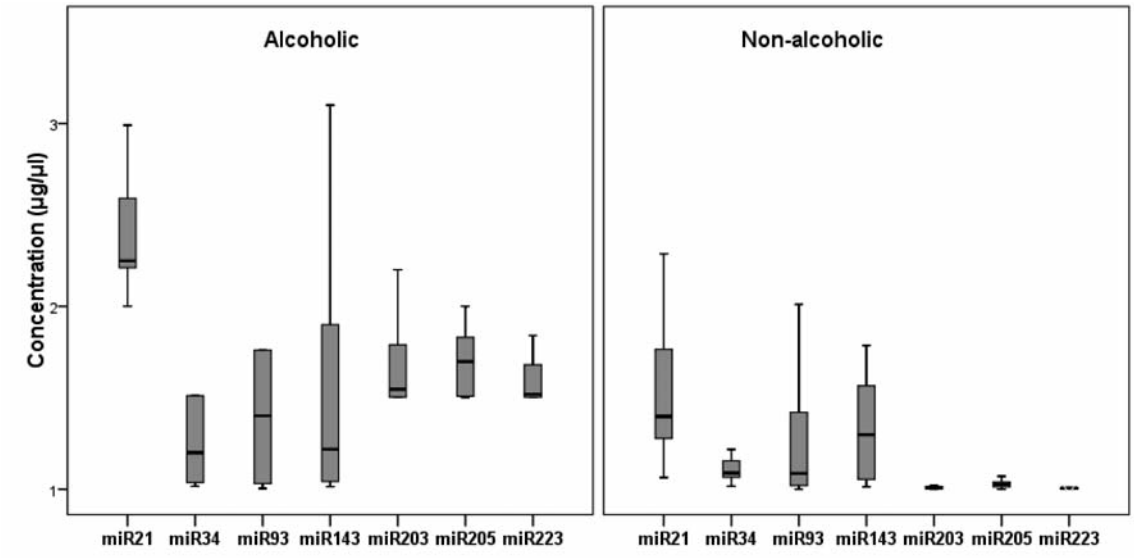

Figure 4. *miRNA expressions of gastric adenocarcinoma samples according to the patients' drinking habits. Columns represent miRNA expression with absolute quantification in µg/µl.*

the expression of phosphatase and tensin homolog (*PTEN*), tropomyosin 1 (alpha) (*TPM1*), programmed cell death 4 (*PDCD4*), sprouty homolog 1 (*SPRY1*) and sprouty homolog 2 (*SPRY2*) genes (18). miR-21 is involved in the pathogenesis of several types of human cancer (19). Zhang *et al*. demonstrated the role of *miR-21* in the pathogenesis and progression of gastric cancer through regulating reversion-inducing cysteine-rich protein with kazal motifs (RECK) tumour suppressor gene in gastric cell lines. Additionally, they found overexpression of *miR-21* in association with *Helicobacter pylori* infection in gastric cancer (20). *miR-93* is a member of the miR-106B-25 cluster, which can promote tumour growth and angiogenesis in human malignancies (21). Tumor protein p53-inducible nuclear protein 1 (*TP53INP1*) tumour suppressor gene serves as a direct target of *miR-223* (22). *miR-143* was reported to have lower expression in colorectal and oesophageal tumours by inhibiting Kristen rat sarcoma 2 viral oncogene homolog (*KRAS*) and DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*) genes

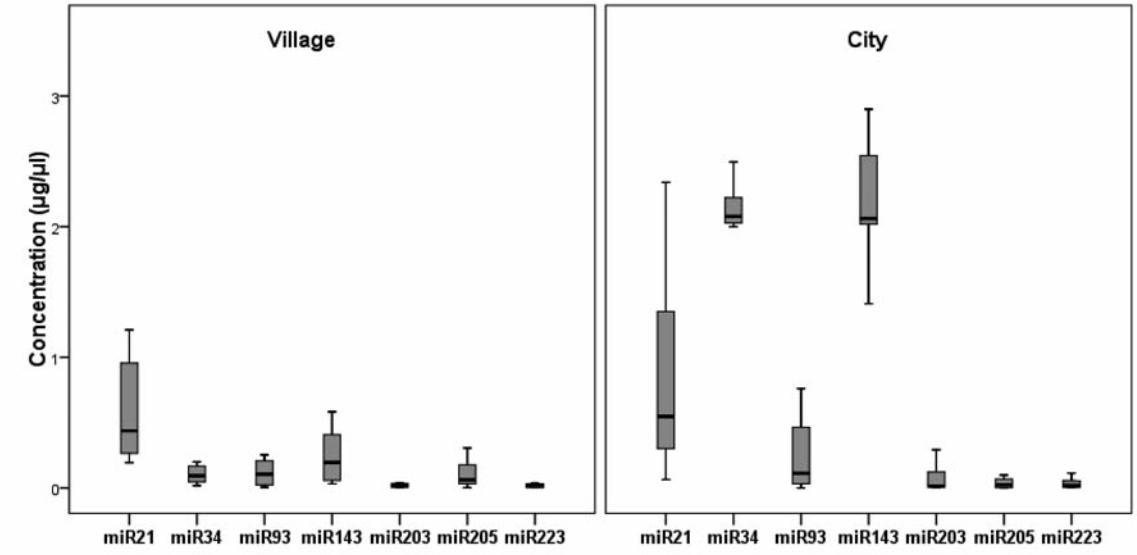

Figure 5. *miRNA expression of gastric adenocarcinoma samples according the patients' living location. Columns represent miRNA expression with absolute quantification in µg/µl.*

(23-25). Deregulation of *miR-223* was reported first in myeloid differentiation then further investigations identified lower expression of *miR-223* in different types of tumours including colorectal cancer, hepatocellular carcinoma and stomach cancer, where stathmin 1 (STMN1) is a putative target of *miR-223* (26). *miR-203* is also a frequently studied miRNAs, which target p63 responsible for development and maintenance of normal epithelial structures and functions (27). Altered expression of *miR-203* was described in gastro-oesophageal, hepatocellular carcinoma, brain tumour, lung cancer, T-cell lymphoma, and pancreatic, breast, bladder and ovarian cancer (28). There are diverse data concerning the role of *miR-205* in tumourigenesis. *miR-205* can function as a tumour suppressor in oesophageal carcinoma, melanoma and breast cancer by suppressing expression of zinc finger E-box binding homeobox 1 (*ZEB1*) gene, E2F1 transcription factor 1 and erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*) genes. *miR-205* can promote tumour initiation, and progression by targeting *PTEN*, cysteine-rich angiogenic inducer 61 (*CYR61*) and connective tissue growth factor (*CTGF*) genes in nasopharyngeal carcinoma, cervical and endometrial cancer (29).

In the literature, there is an increasing number of studies reported on the deregulation of miRNAs in gastric cancer. Osawa *et al*. analyzed the expression of 885 miRNAs by miRNA-oligo chip in FFPE gastric samples, and found up-regulation of *miR-21* in tumours, which is consistent with our study (30). They also demonstrated the overexpression of *miR-34* and down-regulation of *mir-143* in cancer samples related to the normal tissues, which is in contrast with our results. Two other studies by Zhang *et al*. and Guo *et al*. emphasized the importance of *miR-21* as a potential biomarker in gastric cancer (31, 32). Up-regulation of *miR-223* is frequently reported; however, we found no significant alteration in *miR-223* expression between control and tumour samples (26, 33).

To understand how environmental and other factors influence the miRNA regulation in gastric cancer, we compared the expression levels of the same miRNAs in different groups of patients with gastric cancer and showed that differences in lifestyle, demographic features and social status can influence miRNA regulation. Higher expression of *miR-203, miR-205* and *miR-223* were found in gastric cancer samples from patients who regularly consumed alcohol. In samples obtained from patients who smoked and had a low social status, the expression level of *miR-21* was markedly higher than in non-smokers and patients with high social status. Patients with gastric cancer living in urban areas were characterized by higher expression of *miR-143* and *miR-34a*.

According to our results, there are considerable differences in miRNA expression patterns in gastric cancer of the same histopathological entity with respect to lifestyle behaviour, social status and demographic characteristics. This finding can contribute to a better understanding of the exact role of such factors in gastric tumourigenesis and emphasise the role of miRNAs as new molecular markers for diagnosis and prognosis of this human malignancy.

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