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Tumor Expression of *miR-10b, miR-21, miR-143* and *miR-145* Is Related to Clinicopathological Features of Gastric Cancer in a Central European Population

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Abstract. *Background/Aim: In Western countries, most patients with gastric cancer (GC) present in advanced stages. Therefore, there is imminent clinical need for novel diagnostic and prognostic biomarkers. Deregulation of microRNAs has been reported as a frequent event in GC development in a number of studies. Our study validated the potential of microRNAs to serve as diagnostic and prognostic biomarkers in patients with GC from the Central European population. Materials and Methods: Using quantitative real-time polymerase chain reaction, expression levels of six microRNAs (miR-10b, -21, -93, -107, -143, and -145) were examined in 67 tumor tissues and 67 paired adjacent gastric tissues, and correlated with clinicopathological features of GC patients. Results: Expression levels of miR-10b, miR-21, miR-93, and miR-107 were significantly higher in GC samples compared to non-tumor tissue. Furthermore, the expression levels of miR-10b, miR-143, and miR-145 positively correlated with advanced stages, and increased expression of miR-10b, miR-21 and miR-145 was significantly associated with worse prognosis of gastric cancer patients. Conclusion: Our results indicate that selected tissue microRNAs have the potential to serve as relevant diagnostic and prognostic biomarkers of GC in a central European population.*

Gastric cancer (GC) ranks as the fifth most commonly diagnosed cancer worldwide and the third leading cause of cancer-related death. For Europe in 2012, gastric cancer was estimated to lead to more than 60,000 deaths (1). Despite decreasing incidence observed in the last decade, the clinical outcome of patients with locally or metastatic cancer remains poor, with 5-year overall survival of only 20-30% (2). Introducing endoscopy as a population-based screening seems to be effective in reducing mortality from gastric cancer (3), however, this strategy is not applicable for most of Central and Western European countries demonstrating a low incidence of GC. Therefore, searching for new biomarkers and their definition is crucial for early GC diagnosis. Additionally, elucidation of mechanisms of treatment failure is essential for improving patient outcomes.

An increasing number of studies confirmed microRNAs (miRNAs) to be important regulators of gene expression, playing pivotal roles in development, progression and aggressiveness of virtually all human types of cancer (3, 4). miRNAs are highly conserved endogenous non-coding RNAs (18-24 nt in length) targeting protein-coding mRNAs at the post-transcriptional level. They can function as both oncogenes or tumor suppressors (5) and their deregulation contributes to tumorigenesis by having an impact on cancer cell proliferation, apoptosis, invasion and metastasis. Furthermore, in a wide range of cancer types, including GC, deregulation of miRNAs has been shown to correlate with clinicopathological features of the disease (6).

miR-10b, -21, -93, -107, -143, and *-145* were selected for our study, based on recent evidence (7-11), and their deregulation in tumor tissue and association with various clinicopathological features of GC were independently evaluated in a Central European population.

Materials and Methods

Patients and tissue samples. In this retrospective single-center study, 67 patients (39 males and 28 females) with histopathologically-confirmed GC were included. All patients underwent a radical or

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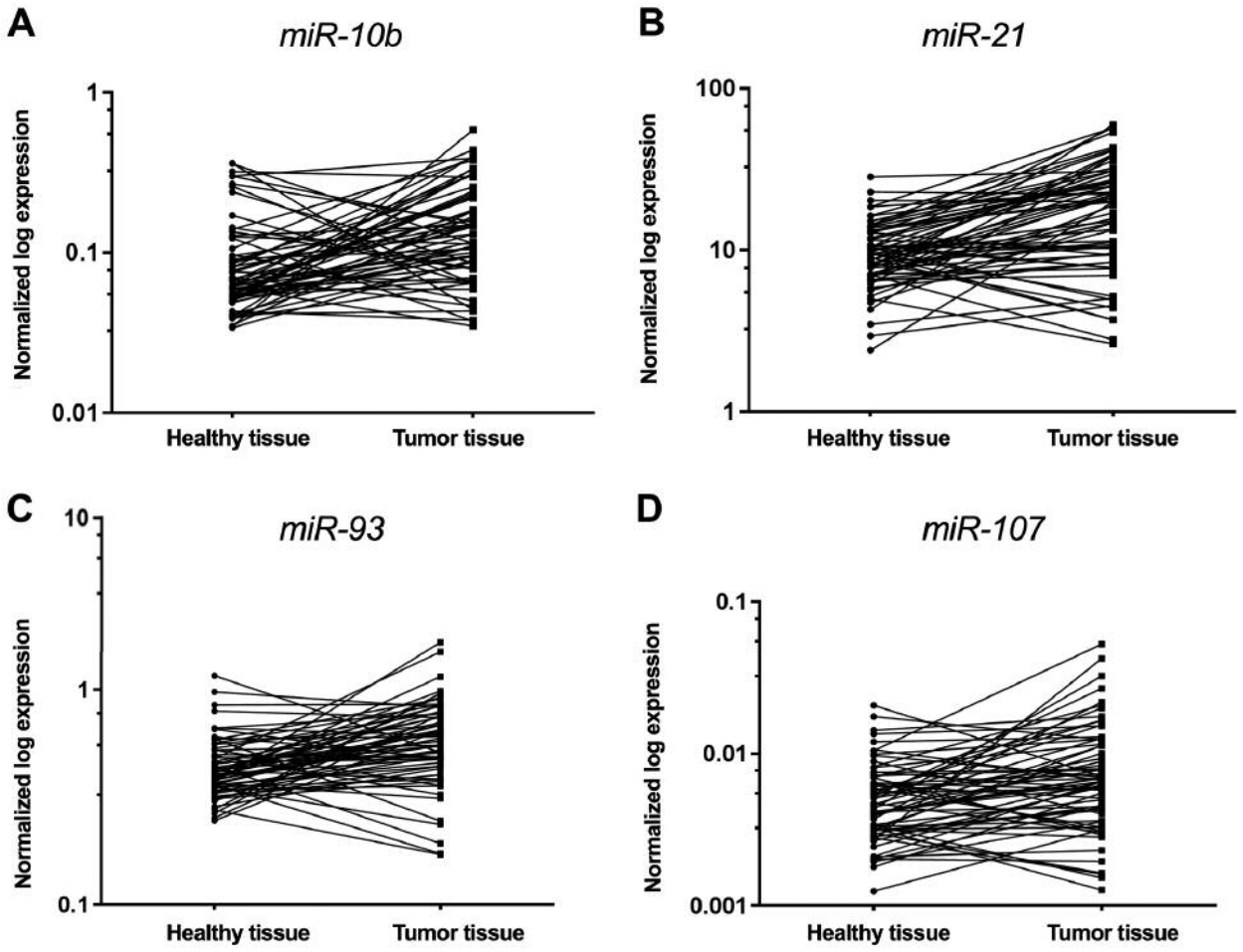

Figure 1. *Normalized expression of miRNAs analyzed in tumor tissue and adjacent non-tumor tissue of gastric cancer (GC) patients. A: miR-10b was increased in GC tissue compared to normal healthy tissue (p=0.0002). B: miR-21 was elevated in GC tissue compared to control tissue (p<0.0001). C: mir-93 was increased in GC tissue (p<0.0001). D: miR-107 was elevated in GC tissue compared to healthy control tissue (p=0.0002).*

palliative surgical procedure at Masaryk Memorial Cancer Institute (Brno, Czech Republic) between 2007 and 2014. Tumor tissue and paired control gastric tissue were collected during the surgery and immediately stored at −80˚C till further analysis. All patients were of the same ethnicity (Central European origin) with a median age of 68 years (range of between 36 and 85 years). Patient characteristics are summarized in Table I. Written informed consent was obtained from all patients and the study was approved by the local Ethics Committee at Masaryk Memorial Cancer Institute.

miRNA extraction. Isolation of total RNA enriched in small RNAs was performed using the mirVana miRNA Isolation Kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. RNA concentration and purity were determined by UV spectrophotometry (A260:A280 >2.0; A260:A230 >1.8) using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real-time quantification of miRNAs. cDNA was synthesized from total RNA using miRNA-specific primers according to the Taq-Man MicroRNA Assay protocol (Applied Biosystems, Foster City, CA, USA) using T1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Real-time polymerase chain reaction (PCR) was performed according to the standard protocol using TaqMan MicroRNA Assay Kit and an Applied Biosystems 7500 Sequence Detection System (both Applied Biosystems).

Data normalization and statistical analysis. The threshold cycle data were calculated by SDS 2.0.1 software (Applied Biosystems). All quantitative RT-PCR reactions were run in triplicates. The average expression levels of measured miRNAs were normalized using small nucleolar RNA, C/D box 48 (*RNU48*), (Applied Biosystems) and subsequently analyzed by the $2^{-\Delta\Delta Ct}$ method. Statistical differences between expression levels in paired tumor and adjacent non-tumor gastric samples were evaluated by Wilcoxon test. Statistical differences between clinicopathological parameters and miRNA

Table I. *Comparison of relative expression levels of miR-10b, miR-21, miR-93, miR-107, miR-143 and miR-145 in gastric cancer according to clinicopathological factors. Data are median relative expression, with 25th-75th percentile in parentheses.*

Characteristic	Sample size (n)	*miR-10b*	*p*-Value	*miR-21*	*p*-Value	miR-93	*p*-Value	*miR-107*	*p*-Value	*miR-143*	*p*-Value	*miR-145*	*p*-Value
Tumor tissue	67	0.130 (0.081-0.226)	0.0002	19.880 (9.344-29.580)	<0.0001	0.591 (0.450-0.766)	<0.0001	0.007 (0.004-0.012)	0.0002	7.995 (1.266-30.240)	0.057	4.642 (1.616-28.710)	0.169
Normal tissue	67	0.063 (0.053-0.102)		9.582 (7.003-12.870)		0.404 (0.356-0.510)		0.004 (0.003-0.007)		5.693 (2.766-14.540)		4.796 (3.660-12.510)	
Gender													
Female	28	0.122 (0.083-0.584)	0.965	21.440 (11.860-30.410)	0.285	0.515 (0.433-0.742)	0.198	0.006 (0.004-0.011)	0.515	7.177 (1.787-26.930)	0.806	4.262 (2.113-34.220)	0.815
Male	39	0.117 (0.07-0.242)		17.020 (7.747-27.090)		0.624 (0.471-0.842)		0.007 (0.004-0.012)		10.700 (1.024-31.360)		6.603 (0.807-27.280)	
Age													
<60 Years	19	0.149 (0.082-0.304)	0.220	22.100 (9.329-30.790)	0.539	0.591 (0.457-0.762)	0.831	0.007 (0.004-0.013)	0.961	12.640 (7.816-33.570)	0.059	12.860 (2.816-33.080)	0.083
≥60 Years	48	0.112 (0.079-0.182)		19.880 (9.407-26.990)		0.584 (0.433-0.789)		0.007 (0.004-0.011)		5.765 (0.920-29.010)		3.371 (0.557-25.700)	
TNM stage													
I+II	28	0.095 (0.065-0.140)	0.016	17.040 (8.662-24.630)	0.159	0.515 (0.382-0.668)	0.277	0.005 (0.003-0.011)	0.013	2.588 (0.745-13.130)	0.025	2.309 (0.284-7.221)	0.025
III	25	0.146 (0.091-0.207)		21.960 (12.240-29.080)		0.642 (0.393-0.846)		0.007 (0.005-0.008)		8.878 (5.218-82.120)		4.289 (3.001-21.950)	
IV	8	0.268 (0.103-0.382)		30.180 (14.480-38.960)		0.620 (0.494-0.791)		0.015 (0.007-0.017)		48.030 (5.218-82.120)		39.290 (1.860-89.790)	
NA	6	---		---		---		---		---		---	
Differentiation grade													
Low (1)	4	0.072 (0.041-0.103)	0.061	15.230 (6.494-20.690)	0.275	0.431 (0.271-0.567)	0.089	0.003 (0.002-0.006)	0.083	0.776 (0.530-2.035)	0.025	1.377 (0.200-2.771)	0.066
Middle + high (2+3)	51	0.117 (0.080-0.220)		21.100 (10.270-30.790)		0.624 (0.457-0.781)		0.007 (0.004-0.011)		8.174 (1.477-28.010)		4.235 (1.886-27.280)	
NA	12	---		---		---		---		---		---	
Laurén type													
Intestinal	37	0.095 (0.065-0.147)	0.0007	20.910 (10.360-30.140)	0.994	0.550 (0.448-0.754)	0.720	0.007 (0.003-0.010)	0.468	3.287 (0.504-15.730)	0.016	1.815 (0.173-7.983)	0.008
Diffuse	24	0.175 (0.110-0.310)		19.560 (9.392-30.480)		0.559 (0.388-0.701)		0.007 (0.004-0.011)		15.440 (6.688-50.660)		14.740 (4.425-44.800)	
Indeterminate	6	---		---		---		---		---		---	

NA: Not available, Wilcoxon test for paired samples, Mann–Whitney *U*-test between two groups and Kruskall–Wallis test for three or more groups.

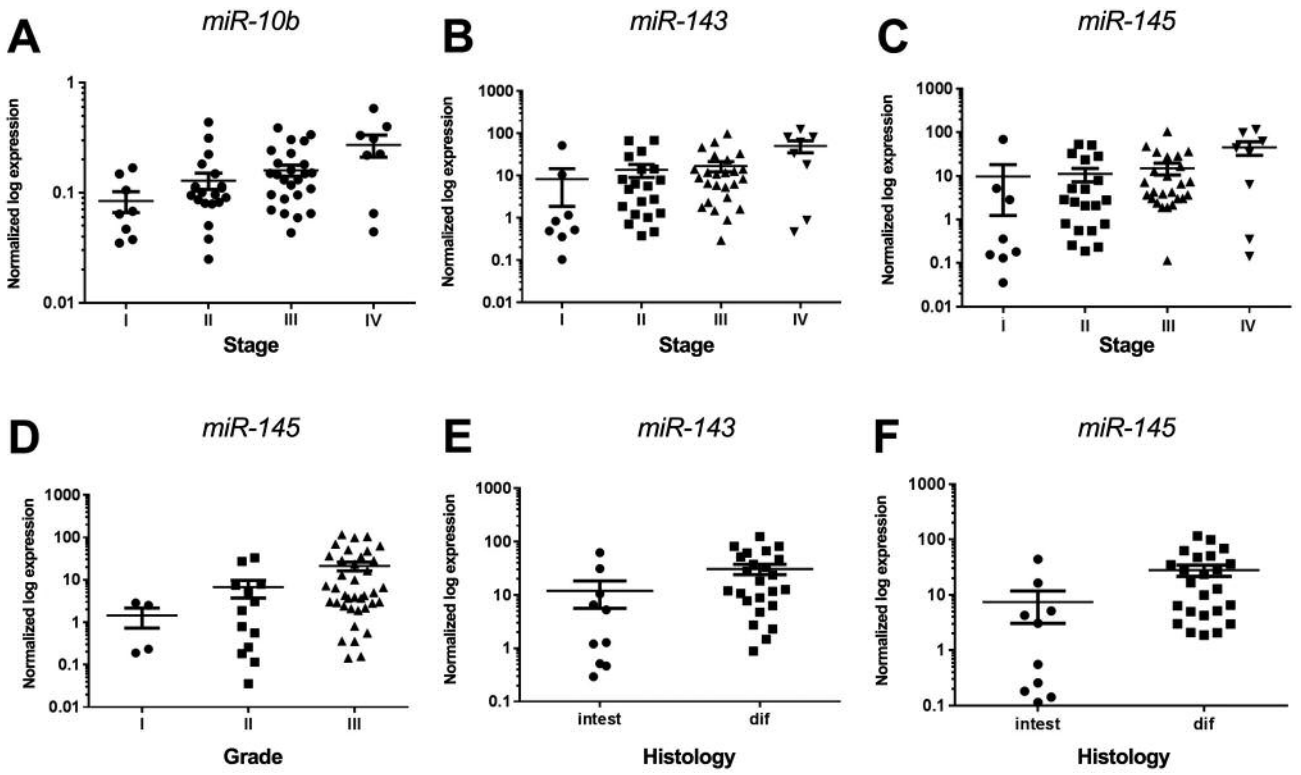

Figure 2. *Normalized expression of miRNAs analyzed in gastric cancer tissue from 67 patients according to tumor stage (A-C), grade (D), and histotype (E, F). Expression of miR-10b (p=0.0197) (A), miR-143 (p=0.0188) (B), and miR-145 (p=0.0294) (C) was positively correlated with tumor stage and that of miR-145 (p=0.0258) (D) with tumor grade. Expression of miR-143 (p=0.0164) (E) and miR-145 (p=0.0077) (F) was clearly increased in more aggressive diffuse (dif) GC than intestinal (intest) histotype of GC. Lines represent medians, and bars are 25th and 75th percentiles.*

expression levels were evaluated using non-parametric tests: the Mann-Whitney *U*-test between two groups and the Kruskal–Wallis test for three or more groups. Receiver operating curve (ROC) analysis was performed to identify cut-offs to distinguish patients with different prognoses. Disease-free (DFS) and overall (OS) survival analyses were carried out using the log-rank test and Kaplan–Meier plots. All calculations were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Differences with *p*-values of less 0.05 were considered statistically significant.

Results

In order to evaluate the diagnostic potential of six miRNAs (*miR-10b, miR-21, miR-93, miR-107, miR-143*, and *miR-145*), their expression levels in tumor tissue samples of 67 GC patients with 67 matched paired control gastric tissue samples were determined by quantitative RT-PCR (normalized to *RNU48*). Significantly higher levels of miR-10b (p=0.0001), *miR-21* (p<0.0001), *miR-93* (p<0.0001), and *miR-107* (p=0.0001) were observed in GC tumor tissue samples compared to control gastric tissue (Table I; Figure 1). There were no significant differences in expression levels of *miR-143* and *miR-145* in GC tumor and non-tumor tissues. Furthermore, tumor expression levels of *miR-10b* (p=0.0159), *miR-107* (p=0.0127), *miR-143* (p=0.0254), and *miR-145* (p=0.0247) significantly differed among groups of patients with different TNM stage, with expression levels progressively increasing with advancing TNM stage (Table I; Figure 2). Significantly different expression of miR-143 (p=0.0164) and *miR-145* (p=0.0077) was identified in different GC histological subtypes according to Lauren classification (Figure 2).

In order to evaluate the prognostic potential of analyzed miRNAs, Kaplan–Meier survival curves were generated and compared by log-rank test. High expression levels of *miR-10b* and *miR-21* were found to be significantly correlated with DFS in patients with non-metastatic GC in our cohort (Figure 3A and B). Considering the whole cohort, high *miR-10b, miR-21* and *miR-145* expression levels were significantly correlated with OS (Figure 3C-E). Regarding *miR-10b*, the median DFS in patients with low levels (cut-off=0.13) was 58 months, and was 17 months in those with high levels (HR=2.155, 95% CI=1.053-4.831; p=0.0379), with corresponding OS of 62 and 25 months, respectively (cut-

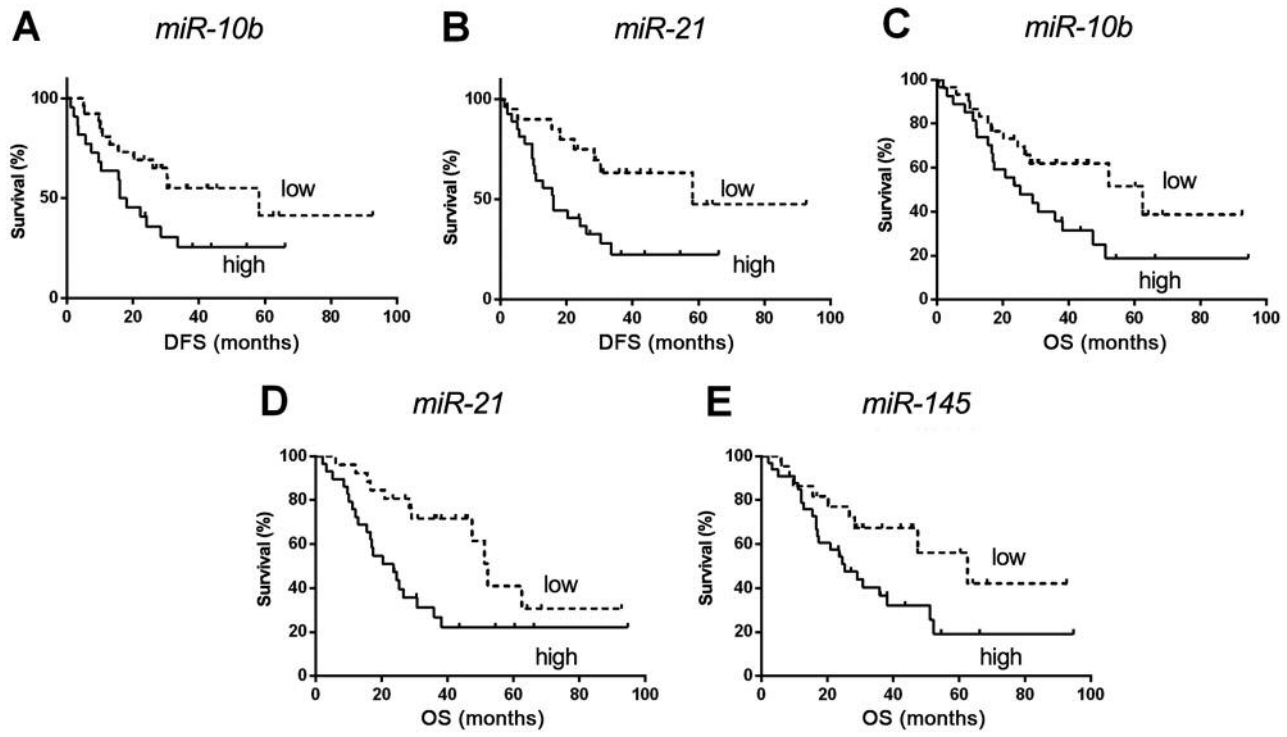

Figure 3. *Kaplan–Meier analysis of disease-free (DFS) and overall (OS) survival based on the expression of miRNAs in 67 patients with gastric cancer. Increased expression of miR-10b was associated with both shorter DFS (cut-off=0.13; p=0.0379) (A) and OS (cut-off=0.124; p=0.0490) (C). Increased expression of miR-21 was also associated with both shorter DFS (cut-off=16.34; p=0.008) (B) and OS (cut-off=20.56; p=0.0078) (D). Increased expression of miR-145 was associated with shorter OS (cut-off=3.342; p=0.0384) (E).*

off=0.124; HR=2.000, 95% CI=1.003-3.984; p=0.0490). Regarding *miR-21*, the median DFS in those with low levels (cut-off=16.34) was 58 months, and was 16 months in those with high levels (HR=2.841, 95% CI=1.323-5.848; p=0.008), while the corresponding OS durations were 52 and 23 months, respectively (cut-off=20.56; HR=2.608, 95% CI=1.287-5.286; p=0.0078). Regarding *miR-145*, the median OS in those with low levels (cut-off=3.342) was 62 months, and was 25 months in the cohort with levels higher than cut-off value (HR=2.096, 95% CI=1.041-4.219; p=0.0384).

Discussion

It has been documented that aberrant expression of miRNAs plays an important role in GC development (6-11). In this study, the utility of *miR-10b, miR-21, miR-93, miR-107, miR-143*, and *miR-145* as novel diagnostic and prognostic biomarkers of GC was evaluated. Based on previous results and consistently with other studies, statistically significant differences in expression were observed for *miR-10b* (8, 12), *miR-21* (7, 13, 14), *miR-93* (9, 15), and *miR-107* (10, 16), enabling the differentiation between tumor and non-tumor control gastric tissue.

miR-21 is the most frequently studied oncogenic miRNA in cancer, with overexpression repeatedly confirmed in gastric tumors (7, 13, 14). In agreement with previous observations, we recorded significantly higher expression levels in tumors in comparison to control gastric tissue. As well as confirming an increase in the level of *miR-107* in GC tissue, Inoue *et al*. reported significant association between *miR-107* level and the depth of tumor invasion, lymph node metastasis and stage. Furthermore, in the Cox multivariate analysis, they showed that *miR-107* expression in GC tissues was an independent prognostic factor for OS and DFS (16). Unfortunately, our results regarding *miR-107* expression levels and DFS and OS did not reach statistical significance. Correlation of the overexpression of *miR-10b* with Lauren classification and TNM stage in our cohort confirms the findings of Wang *et al*. (12), who proposed *miR-10b* as a useful molecular biomarker for assessing the risk of GC development. As diagnostic and prognostic factors beyond disease stage are clearly needed, histological type in combination with miRNAs could be proposed as a surrogate biomarker of disease biology (17, 18).

A second aim of this study was to identify miRNAs with the potential to differentiate between patients with good and

poor prognosis. We identified that increased levels of both *miR-10b, miR-21* and *miR-145* significantly were correlated with poor prognosis, *miR-10b* and *miR-21* with DFS and OS, and *miR-145* only with OS. Our findings regarding *miR-21* are in accordance with the results of Wang *et al*. (14) and Ren *et al*. (19), which showed that the survival times of patients in the group with high *miR-21* expression were significantly shorter than those of patients in the groups with normal or low expression. Zhang *et al*. described high expression of *miR-21* in GC as being regulated by phosphatase and tensin homolog (*PTEN*), which is associated with the growth and invasion of GC (20).

Taken together, our results indicate that miRNAs (*miR-10b, miR-21, miR-93, miR-107, miR-143*, and *miR-145*) have the potential to serve as relevant GC diagnostic biomarkers; moreover, *miR-10b, miR-21* and *miR-145* might also serve as molecular biomarkers predicting individual prognosis. After detailed and independent validation, they might provide potential value for the clinical decision-making process in GC

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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