Tissue microRNAs as predictive markers for gastric cancer patients undergoing palliative chemotherapy

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Abstract. MicroRNAs have the potential to become valuable predictive markers for gastric cancer. Samples of biopsy tissue, routinely taken from gastric cancer patients undergoing palliative chemotherapy, constitute suitable material for microRNA profiling with the aim of predicting the effect of chemotherapy. Our study group consisted of 54 patients, all of whom underwent palliative chemotherapy based on 5-fluorouracil (5-FU) or 5-FU in combination with platinum derivatives between 2000 and 2013. The expression of 29 selected microRNAs and genes BRCA1, ERCC1, RRM1 and TS, in gastric cancer tissue macrodissected from FFPE tissue samples, was measured by quantitative RT-PCR. The relationship between gene expression levels and time to progression (TTP) and overall survival (OS) was analysed. From the set of the 29 microRNAs of interest, we found high expression of miR-150, miR-342-3p, miR-181b, miR-221, miR-224 and low levels of miR-520h relate to shorter TTP. High levels of miR-150, miR-192, miR-224, miR-375 and miR-342-3p related to shorter OS. In routinely available FFPE tissue samples, we found 6 miRNAs with a relation to TTP, which may serve as predictors of the effectiveness of palliative treatment in gastric cancer patients. These miRNAs could also help in deciding whether to indicate palliative chemotherapy.

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Introduction

Gastric cancer rates as the fourth most common malignancy worldwide and is the third most common cause of death from a malignant disease, after lung and liver cancer (1). Eastern Asia accounts for more than 50% of cases registered globally. A gradual decrease in the number of cases has been observed in regions such as the United States, Western and Central Europe (2,3).

In 2012, the incidence of gastric cancer in the Czech Republic was 14.6 cases per 100,000 inhabitants. Most cases are diagnosed in the late stages of the disease and only palliative treatment remains possible. Chemotherapy is often embarked upon as part of palliative treatment and most patients receive the same medications, overall survival varies greatly from patient to patient, however. One possible cause of this variability could be the gene expression changes occurring in cancer tissue, which may alter the effect of cytostatics. Evaluating the expression of specific genes including genes for microRNA (miRNA) could help single out chemotherapy efficacy predictors in gastric cancer patients undergoing palliative treatment (4,5). By identifying patients with chemoresistant tumors, we hope to spare them the strain of inefficient chemotherapy.

The tissue samples were the same as those used by the pathologists when making the diagnoses, to measure the levels of chemotherapy response predictors. The use of efficacy predictors makes up an important part of the development of new targeted therapy drugs and their introduction into clinical practice; this is no less the case for conventional chemotherapeutics.

The ability of cancer cells to overcome the effects of chemotherapy by changing the expression of repair genes, enzymes partaking in nucleic acid metabolism and genes involved in apoptosis limits the success of chemotherapy. Eukaryotic cells react to DNA damage by activating repair mechanisms with these key functions: DNA damage detection, stopping replication of damaged DNA, repair of damaged area if possible

Table I. Primer sequences for quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the probes of Universal Probe Library.

Symbol	Gene name	Function of the product of this gene	Primer sequence 5'-3'	UPL probe
Reference genes				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Enzyme of glycolysis.	AGCCACATCGCTCAGACAC GCCCAATACGACCAAATCC	60
HPRT	Hypoxanthine guanine phosphoribosyltransferase	Purine salvage pathway.	TGACCTTGATTTATTTTGCATACC CGAGCAAGACGTTCAGTCCT	73
Predictors of treatment response				
ERCC1	Excision repair cross-complementary group 1	Nucleotide excision repair pathway, catalyzes the 5' incision in the process of excising the DNA lesion. Studied as a predictor of platinum chemotherapy drugs.	GAAATTTGTGATACCCCTCGAC GATCGGAATAAGGGCTTGG	79
RRM1	Ribonucleotide reductase subunit M1	Subunit of an enzyme essential for the production of deoxyribonucleotides needed for DNA synthesis. Studied as a predictor of vinorelbine.	AAGCACCCTGACTATGCTATCC GTTATAGAGGTCTTCCATCACATCAC	71
BRCA1	Breast cancer 1	Protein involved in DNA repair of double-stranded breaks, and recombination. Studied as a predictor of platinum drugs.	TTGTTGATGTGGAGGAGCAA CAGATTCCAGGTAAGGGGTTC	11
TS	Thymidylate synthase	Methylation of deoxyuridylate to deoxythymidylate needed for DNA synthesis. Studied as a predictor of 5-fluorouracil and folate antimetabolites.	CCCAGTTTATGGCTTCCAGT GCAGTTGGTCAACTCCCTGT	43

or else the induction of apoptosis. Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and homologous recombination repair (HRR) belong to the most important repair mechanisms of eukaryotic cells (6,7). Damage to these mechanisms means that mutations are passed on to the next cell generation. Impaired function of DNA repair mechanisms is thus linked to both ageing and cancerogenesis. However, increased activity of these mechanisms can hinder chemotherapy by forestalling further effective damage to DNA and thereby preventing the activation of apoptosis in proliferating tumor cells (8).

Cisplatin acts cytotoxically by creating adducts, which participate in crosslinking DNA, and in so doing activates programmed cell death. Decrease in the scale of damage to DNA, whether as a result of fewer adducts being created (in lower drug dosage, for instance) or because of their repair, can lead to a decrease of efficacy of this chemotherapeutic substance (9).

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutics in gastric cancer treatment regimens (10). The primary site of action for 5-FU is thymidylate synthase (TS), an essential enzyme in *de novo* biosynthetic pathway of deoxythymidylate (dTMP). Thymidylate synthase catalyzes the reductive methylation of dUMP (deoxyuridine-5-prime monophosphate or deoxyuridylate) to dTMP (deoxythymidine-5-prime monophosphate or deoxythymidylate) using 5,10-methylenetetrahydrofolate as a cofactor. Maintaining a dTMP pool is crucial for DNA replication and repair.

We measured the levels of mRNA of the excision repair cross-complementary group 1 gene (ERCC1), ribonucleotide reductase subunit M1 gene (RRM1), breast cancer 1 gene (BRCA1) and TS, all of which participate in repair to damaged DNA. We determined their relationship to time to progression (TTP), using the definition of progression according to RECIST (response evaluation criteria in solid tumours) criteria (11), and overall survival (OS). Table I lists the basic characteristics of the genes of interest.

In addition to genes whose products are directly involved in DNA repair and nucleic acid metabolism, we also focused on gene products that seem to affect the expression of genes

involved in the above mentioned processes. MicroRNAs (also known as miRNAs or miRs), small non-coding RNA molecules 18-23 nucleotides in length, make up an immense group of regulatory molecules involved in carcinogenesis. The human genome may encode over 2,500 miRNAs, which may target ~60% of mammalian genes and are abundant in many human cell types (see miRNA database available online at www.mirbase.org). MicroRNAs participate in the post-transcriptional regulation of gene expression controlling development and maintain diverse cellular processes including proliferation, apoptosis, differentiation, motility and morphogenesis.

The effect of microRNA regulatory networks in cancer tissue can be oncogenic (by targeting tumor suppressor genes) or tumor-suppressive (by post-transcriptional repression of oncogenes) (12). However, the final effect of any particular miRNA is time and tissue dependent (13).

Many studies have described changes in expression of miRNAs and their involvement in carcinogenesis, tumor progression, invasion, metastasis and the effects of treatment in gastric cancer tissue. MicroRNAs may become valuable diagnostic markers and therapeutic targets for gastric cancer (14). Based on our research of published literature, we chose 29 miRNAs, which have a potential role in carcinogenesis or drug metabolism and therefore could be expected to influence the efficacy of treatment. A list of the main characteristics of miRNAs of interest is shown in Table II.

Materials and methods

Ethics statement. The present study was approved by the ethics committee of the University Hospital in Pilsen (decision from 11.7.2012 to the grant NT14227). Anonymised data were used to conduct this study.

Patients. This was a retrospective study. The patients were treated at the Complex Oncology Center of the University Hospital in Pilsen between 1st January 2000 and 30th June 2013. The inclusion criteria of this study were: patients with gastric cancer, with no gastric resection treatment, who underwent palliative chemotherapy only. We evaluated nearly 1,300 cases, all of which were treated at the Complex Oncology Center, but our inclusion criteria meant only 54 cases could be used in the present study. Stage of disease was determined using the TNM (tumor-node-metastasis) system of the International Union Against Cancer (IUCC, 7th edition) (15). All patients were in the fourth stage of the disease. Each diagnosis of gastric cancer was verified by a pathologist.

Tissue samples. Biopsy tissue samples, gathered, using endoscopy, from gastric cancer patients for diagnostic purposes prior to chemotherapy, were processed by standard laboratory techniques at the Institute of Pathology of the University Hospital in Pilsen, Czech Republic. FFPE tissue samples were stored at room temperature until use. Paraffin sections (4-μm thick) were stained with hematoxylin and eosin (H&E), microscopically verified by pathologists and examined in order to identify sites with cancer cells and sites of adjacent non-cancerous epithelial tissue suitable for macrodissection. Areas selected for expression analysis were highlighted manually.

RNA isolation. Total RNA (including microRNA) was extracted from 10-μm FFPE sections following macrodissection of tumor tissue and adjacent non-cancerous tissue using the miRNeasy FFPE kit (Qiagen, Hilden, Germany) as we previously described (16). The paired samples (tumor and adjacent non-cancerous tissue) were only available from 18 patients. The 10-μm sections corresponded to H&E representatives, on the areas highlighted for macrodissection.

Quantitative estimation of protein coding gene expression. Quantitative estimation of mRNA of selected genes (BRCA 1, ERCC1, RRM1 and TS) was performed by a real-time RT-PCR method with Universal Probe Library (UPL) probes (Roche, Mannheim, Germany). Reverse transcription was performed on 50 ng of total RNA with Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamers as primers. The sequences of primers and corresponding UPL probes generated by ProbeFinder software (Roche) are shown in Table I. The primers were synthesized by East Port Praha (Prague, Czech Republic). The quantitative estimation was performed in technical duplicates on Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA). The 20-μl PCR reactions included 1.0 μl of RT product, FastStart TaqMan Probe Master (Roche), 2.5 μl of each primer and 2.5 μl of UPL probe. The reactions were incubated in 96-well plates at 95˚C for 10 min and then followed by 48 cycles of 95˚C for 10 sec and 60˚C for 30 sec.

All the samples were also assessed for the expression of reference genes *GAPDH (*glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine-guanine phosphoribosyltransferase). Due to generally low HPRT expression and small yield of RNA isolated from FFPE tissue (small tissue samples), we were unable to measure the expression of HPRT in all of our samples. Therefore, we did not use HPRT data for normalization along with GAPDH and total RNA.

Quantitative estimation of microRNA expression. A quantitative estimation of selected microRNAs (Table II) was performed by a RT real-time PCR method using TaqMan® MicroRNA assays (Applied Biosystems, Foster City, CA, USA) in technical duplicates on Stratagene Mx3005P apparatus (Agilent Technologies). A two-step protocol requires reverse transcription with a miRNA-specific primer, followed by a real-time PCR with TaqMan® probes. The assays target only mature miRNAs, not their precursors. We used RNU6B (U6snRNA) as a normalizer.

Processing of real-time PCR data. Samples were assessed in technical duplicates. The Ct values were corrected using calibrators to eliminate differences between the individual runs of the real-time PCR apparatus. In cases where there was a discrepancy between the results obtained from both technical duplicates, the sample assessment was repeated. The results are presented as normalized values as a ratio of the number of copies of the given gene to that of the reference gene. To obtain gene expression data we used the $\Delta\Delta$Ct approach ($2^{-\Delta\Delta Ct}$ algorithm).

Statistical analysis. The statistical analysis was performed using SAS 9.3 software (SAS, Institute Inc., Cary, NC, USA).

Table II. The analysed microRNAs and their involvement in the cancer process.

Symbol	miRBase accession no.	Role in gastric cancer/Prediction potential	Ref.
miR-15b	MIMAT0000417	Regulates cisplatin resistance and metastasis by targeting PEBP4 in lung adenocarcinoma cells	(40)
		Modulates multidrug resistance by targeting BCL2 in human gastric cancer cells	(41)
miR-16	MIMAT0000069	Associated with chemosensitivity in gastric cancer	(42)
		Modulates multidrug resistance by targeting BCL2 in human gastric cancer cells	(41)
miR-21	MIMAT0000076	Stimulates gastric cancer growth and invasion by inhibiting many tumor suppressors (PTEN, PDCD4)	(43)
		Confers cisplatin resistance in gastric cancer cells by regulating PTEN	(44)
miR-27a	MIMAT0000084	Functions as an oncogene in gastric adenocarcinoma by targeting prohibitin	(45)
		Potential biomarker for predicting resistance to fluoropyrimidine-based chemotherapy	(46)
miR-34a	MIMAT0000255	Inhibits the growth, invasion and metastasis of gastric cancer by targeting PDGFR and MET expression	(47)
		Regulates cisplatin-induced gastric cancer cell death by modulating PI3K/AKT/survivin pathway	(48)
miR-99a-3p	MIMAT0004511	Predicts fluoropyrimidine-based chemotherapy response in patients with advanced colorectal cancer	(49)
miR-101	MIMAT0000099	Downregulated in gastric cancer and involved in cell migration and invasion	(50)
		Enhances cisplatin sensitivity in bladder cancer cells	(51)
miR-106a	MIMAT0000103	Confers cisplatin resistance by regulating PTEN/Akt pathway in gastric cancer cells	(52)
		Induces multidrug resistance in gastric cancer by targeting RUNX3	(53)
miR-107	MIMAT0000104	Significantly dysregulated in gastric adenocarcinoma tissues	(54)
		Regulates cisplatin chemosensitivity of A549 non-small cell lung cancer cell line by targeting cyclin dependent kinase 8	(55)
miR-141	MIMAT0000432	Inhibits tumor growth and metastasis in gastric cancer	(56)
		Overexpression of miR-141 results in enhanced resistance to cisplatin in gastric cancer cells	(57)
miR-143	MIMAT0000435	Suppresses gastric cancer cell growth and induces apoptosis	(58)
		Involved in cisplatin resistance of gastric cancer cells via targeting IGF1R and BCL2	(59)
miR-145	MIMAT0000437	Suppress invasion-metastasis cascade in gastric cancer	(60)
		Reverses 5-FU resistance in tumor xenograft models	(61)
miR-150	MIMAT0000451	Promotes gastric cancer proliferation by negatively regulating the pro-apoptotic gene EGR2	(22)
		Reduces cisplatin chemosensitivity and promotes invasiveness of muscle-invasive bladder cancer cells	(62)
miR-181b	MIMAT0000257	Aberrantly overexpressed in gastric cancer cells and primary gastric cancer tissues	(26)
		Prognostic significance in gastric cancer patients treated with S-1/oxaliplatin or doxifluridine/oxaliplatin	(25)
miR-192	MIMAT0000222	miR-215/192 significantly upregulated in gastric cancer tissues from gastrectomy	(28)
		miR-192/miR-215 influence 5-FU resistance in colorectal cancer cell lines	(63)
miR-193a-3p	MIMAT0000459	Associated with precancerous lesions of gastric cancer	(64)
		Regulates the multi-drug resistance of bladder cancer	(65)
miR-202	MIMAT0002811	Inhibits the expression of γ-catenin and BCL-2; miR-202 has decreased expression in gastric cancer	(66)

Table II. Continued.

Symbol	miRBase accession no.	Role in gastric cancer/Prediction potential	Ref.
miR-206	MIMAT0000462	Suppresses gastric cancer cell growth and metastasis	(67)
		Inhibition of gastric cancer progression through the c-Met pathway	(68)
miR-211	MIMAT0000268	Associated with gastric cancers as potential biomarkers for gastric cancer diagnosis and treatment	(69)
		Downregulation of ribonucleotide reductase	(70)
miR-218	MIMAT0000275	Inhibits invasion and metastasis of gastric cancer	(71)
		Regulates cisplatin chemosensitivity in breast cancer by targeting BRCA1	(72)
miR-221	MIMAT0000278	miR-221/222 are encoded in tandem and they have the same seed sequence;	(33)
miR-222	MIMAT0000279	miR-221 and miR-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN	
miR-224	MIMAT0000281	Promotes chemoresistance of lung adenocarcinoma cells to cisplatin	(73)
		5-FU chemosensitivity is significantly increased in miR-224 knockdown cells	(74)
miR-342-3p	MIMAT0000753	Upregulation is associated with chemosensitivity in gastric cancer	(42)
miR-372-3p	MIMAT0000724	Maintains oncogene characteristics by targeting TNFAIP1 and affects NF-κB signaling in human gastric carcinoma cells	(75)
miR-375	MIMAT0000728	Downregulated in gastric cancer, inhibits cell migration and invasion by targeting JAK2	(76)
		Predictive for response for non-small cell lung cancer treated with cisplatin-vinorelbine A	(77)
miR-509-3p	MIMAT0002881	Inhibits cell proliferation and migration by targeting CDK2, Rac1, and PIK3C2A	(78)
miR-575	MIMAT0003240	Significantly upregulated in gastric cancer	(79)
miR-520h	MIMAT0002867	Downregulates histone deacetylase 1 and so contributes to the chemotherapeutic effect of doxorubicin	(39)
		Controls ABCG2 level and thereby anticancer drug response	(80)

The statistical results were calculated by a Wilcoxon non-parametric two sample test. For the maximum hazard ratio (OS, TTP) the Cox regression hazard model was used. After finding an 'optimal cut off' given by the lowest P-value of log-rank test for the examined markers, the Kaplan-Meier survival distribution functions determined by the 'optimal cut off' in given groups and subgroups were generated.

Results

Prior to our analysis of the relationship between gene expression and TTP and OS, we compared gene expression in cancer and adjacent non-cancer epithelial gastric tissue. We found the expression of miR-221 in cancer tissue to be significantly higher, while the expression of miR-202 and miR-509 proved to be lower compared to healthy tissue (P-value 0.013, 0.011 and 0.018, respectively). However, when drawing conclusions from this analysis, we have to take into account the low number of paired samples, caused by the lack of non-cancerous tissue in some of the FFPE samples.

The Cox regression hazard model was used to determine the relation of marker level to OS or TTP. Results for all markers are summarized in Table III. From the set of the 29 microRNAs of interest, we found high expression levels of miR-150, miR 342-3p, miR-181b, miR-221, miR-224 and low levels of miR-520h related to shorter TTP. High levels of miR-150, miR-192, miR-224, miR-375 and miR-342-3p related to shorter OS. For markers with statistically significant results, optimal cut off values were chosen and Kaplan-Meier survival distribution functions for OS and TPP were generated.

The treatment regimen of all patients included 5-FU. We noted a definite correlation between high levels of miR-150 and miR-342-p in cancerous tissue and shorter TTP as well as OS. High expression of miR-224, however, only proved to have a relation to shorter OS (Table IV and Fig. 1).

In the subgroup of patients receiving 5-FU as the only treatment, we recorded a relation between high levels of miR-181b and shorter TTP and between high levels of miR-150, miR-192 and miR-342-p and shorter OS. In the subgroup treated with both 5-FU and cisplatin, we noted that high levels of miR-221, miR-224 and low levels of miR-520 related to shorter TTP and high levels of miR-221, miR-224 and miR-375 to shorter OS (Table V and Fig. 2).

Discussion

The present study focused on patients in advanced stages of gastric cancer. Therefore, these patients could not have the

Table III. Relation between level of given marker and TTP or OS (Cox regresion hazard model).

	All patients				5-FU alone				5-FU/cisplatin			
	OS		TTP		OS		TTP		OS		TTP	
Marker	P-value	HR	P-value	HR	P-value	HR	P-value	HR	P-value	HR	P-value	HR
ERCC1	0.9896	1.000	0.6452	0.547	0.2203	1.083	0.1464	1.103	0.2740	*	0.0679	*
RRM1	0.8615	*	0.8486	*	0.7395	*	0.7137	*	0.4886	*	0.1703	*
BRCA1	0.1658	17.340	0.4511	4.261	0.1881	*	0.2095	*	0.9996	*	0.4076	*
TS	0.3422	*	0.4118	*	**0.0524**	**0.985**	0.4693	*	0.5148	*	0.3000	*
miR-15b	0.3772	1.247	0.5953	1.199	0.2288	2.749	0.7050	1.380	0.2717	1.468	0.4768	1.297
miR-16	0.4178	1.003	0.7514	1.001	0.4816	1.003	0.6070	1.002	0.1216	1.053	0.1344	1.050
miR-21	0.5122	1.001	0.7040	1.001	0.5362	1.001	0.5916	1.001	0.4965	1.007	0.3586	1.011
miR-27a	0.1592	1.105	0.3201	1.072	0.1169	1.315	0.4536	1.141	0.1402	1.387	0.0716	1.568
miR-34a	0.5279	1.014	0.8185	1.005	0.5976	1.019	0.8735	1.006	0.0567	1.536	0.0806	1.626
miR-99a-3p	0.1951	*	0.7863	2.705	0.4441	*	0.7846	5.008	0.4712	*	0.7964	5.091
miR-101	0.3734	4.040	0.6222	2.142	0.3617	4.703	0.6637	2.110	0.9744	1.299	0.0966	*
miR-106a	0.4283	1.034	0.5075	1.028	0.4467	1.038	0.6843	1.022	0.4066	1.106	0.7731	1.039
miR-107	0.4980	1.829	0.6703	1.438	0.6056	1.713	0.9425	0.928	0.1013	*	0.1739	*
miR-141	0.2236	1.241	0.1042	1.295	0.0646	1.946	0.1968	1.609	0.7076	1.134	0.2205	1.460
miR-143	0.5144	1.002	0.8042	1.001	0.5847	1.002	0.6593	1.002	0.0619	1.395	0.0537	2.322
miR-145	0.4063	1.001	0.8176	1.000	0.5373	1.001	0.6376	1.001	0.0727	1.057	0.1059	1.121
miR-150	**0.0494**	**1.004**	**0.0056**	**1.006**	**0.0438**	**1.039**	0.0743	1.034	0.1311	1.004	0.2351	1.046
miR-181b	0.1406	1.061	0.0882	1.063	0.0564	1.130	**0.0333**	**1.777**	0.3327	1.185	0.2270	1.082
miR-192	0.7227	1.011	0.8569	1.006	**0.0233**	**1.200**	0.3684	1.080	0.8814	0.992	0.5269	0.959
miR-193a-3p	0.2323	4.798	0.7265	1.577	0.2401	7.129	0.5706	2.464	0.3158	*	0.0606	*
miR-202	0.3803	7.022	0.6739	0.320	0.4932	6.787	0.6010	4.384	0.1694	*	0.0946	*
miR-206	0.0594	*	0.4452	*	0.1810	*	0.2963	*	0.2441	*	0.1440	*
miR-211	0.1548	*	0.2151	*	0.1155	*	0.3240	*	0.5506	*	0.4309	*
miR-218	0.0639	*	0.6789	2.981	0.4176	*	0.0687	*	0.0961	*	0.0847	*
miR-221	0.1934	1.051	0.3627	1.034	0.6144	1.032	0.7147	1.023	**0.0160**	**2.438**	**0.0371**	**2.099**
miR-222	0.5086	1.001	0.6742	1.000	0.5679	1.001	0.6445	1.001	0.2627	1.012	0.2698	1.012
miR-224	**0.0175**	**7.609**	0.2724	2.620	0.1441	4.532	0.3603	2.602	**0.0283**	**322.120**	**0.0367**	**436.694**
miR-342-3p	**0.0286**	**1.261**	**0.0144**	**1.383**	**0.0443**	**2.516**	0.1531	2.077	0.1383	1.272	0.1641	1.692
miR-372-3p	0.6113	1.000	0.1651	7.159	0.5731	*	0.5128	*	0.2737	6.344	0.2863	6.080
miR-375	0.1968	*	0.9422	1.000	0.4974	1.001	0.9324	1.011	**0.0427**	**1.362**	0.8672	1.000
miR-509-3p	0.2446	3.507	0.6196	*	0.6909	*	0.5540	*	0.1246	*	0.1607	*
miR-575	0.1999	*	0.4849	*	0.4778	*	0.5664	*	0.6211	*	0.5531	*
miR-520h	0.1712	*	0.1190	*	0.3799	*	0.2106	*	0.0977	*	**0.0483**	**0.584**

*Clinically unrealistic hazard ratio (HR) values due to the coincidence of extreme marker level and extreme time to event in some cases.

tumors surgically removed and underwent palliative treatment only. The main clinical concern in such cases is deciding which chemotherapeutic regimen is indicated. The question was whether we could predict the effect of chemotherapy and thereby determine, if the aggressive chemotherapeutic treatment, which inevitably decreases quality of life, would prolong survival. If a low effect of treatment is predicted, it would be appropriate to offer to those patients inclusion in new ongoing studies.

In our laboratory assessment we used tissue samples taken gastroscopically for routine diagnostic purposes and macrodissected a sample of cancerous tissue, verified by a pathologist from FFPE sections, to analyse the expression of genes influencing the effects of therapy. This approach can be easily translated into clinical practice. We conducted expression analysis from a large number of tumor cells. Cancerous tissue is heterogeneous by nature, and originates in the process of clonal evolution, and therefore examining the collective

Table IV. Relation between level of given microRNA and TTP or OS (Kaplan-Meier estimation).

Marker	No. of patients	Cut-off	Patients below cut-off		Patients above cut-off		P-value
			N	Median (days)	N	Median (days)	
Time to progression (TTP)							
miR-150	40	45	37	113	3	26	0.0016
		6.7	23	138	17	90	0.0232
miR-342-3p	42	2.7	40	106.5	1	12	0.0006
		0.6	31	113	10	66	0.0997
Overall survival (OS)							
miR-150	41	45	38	215	3	69	0.0020
		6	21	424	20	172.5	0.0145
miR-342-3p	42	0.45	25	304	17	170	0.0319
miR-224	41	0.048	15	494	26	175	0.0090

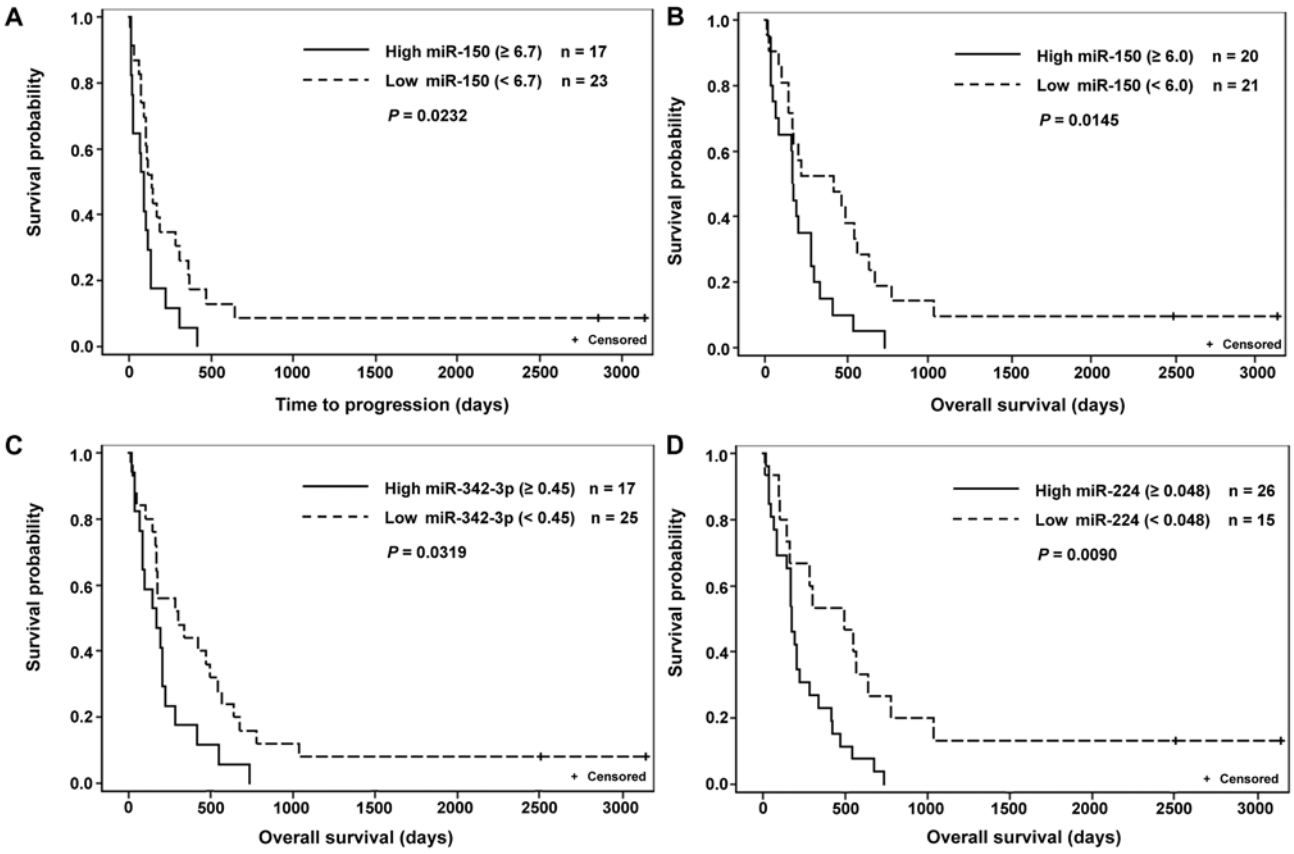

Figure 1. Relation of microRNA expression to time to progression (TTP)/overall survival (OS) in all gastric cancer patients (Kaplan-Meier curves).

changes in expression of a variety of cancer cell types (gathered by macrodissection) provides a more complex insight regarding prognosis. We set out to ascertain the prognostic potential of chosen miRNAs, which influence apoptosis and cell proliferation and in so doing interact with the mechanism of chemotherapy indicated in the cases we examined. However, we could not leave out monitoring protein coding genes frequently investigated as possible treatment outcome predictors.

Many studies have noted the prognostic value of low ERCC1 expression in gastric cancer patients undergoing chemotherapy; a meta-analysis published in 2015 concluded ERCC1 may be a useful prognostic factor for gastric cancer and furthermore that low mRNA levels of ERCC1 appear to be associated with a significant OS benefit to patients treated with platinum-based chemotherapy (17). However, the predictive value of the ERCC1 gene for survival and response to platinum-based chemotherapy in gastric cancer remains

Table V. Relation between level of given marker and TTP or OS based on the treatment (Kaplan-Meier estimation).

Marker	No. of patients	Cut-off	Patients below cut-off		Patients above cut-off		P-value	Relation to OS/TTP
			N	Median (days)	N	Median (days)		
Treatment								
5-fluorouracil								
TS	14	0.008	11	282	3	547	0.0226	OS
miR-150	23	6.300	12	424	11	170	0.0099	OS
miR-181b	21	0.260	2	13.5	19	147	0.0038	TTP
miR-192	24	2.300	16	339	8	39	0.0001	OS
miR-342-3p	24	0.600	18	282	6	62.5	0.0141	OS
5-fluorouracil/cisplatin								
miR-221	10	0.600	4	732	6	129.5	0.0038	OS
	10	1.500	4	262.5	6	67	0.0356	TTP
miR-224	9	0.150	5	684.5	4	84	0.0049	OS
	9	0.150	5	108	4	43	0.0027	TTP
miR-375	9	26.000	5	637	4	76.5	0.0027	OS
miR-520h	10	40.000	8	88	2	563.5	0.0265	TTP

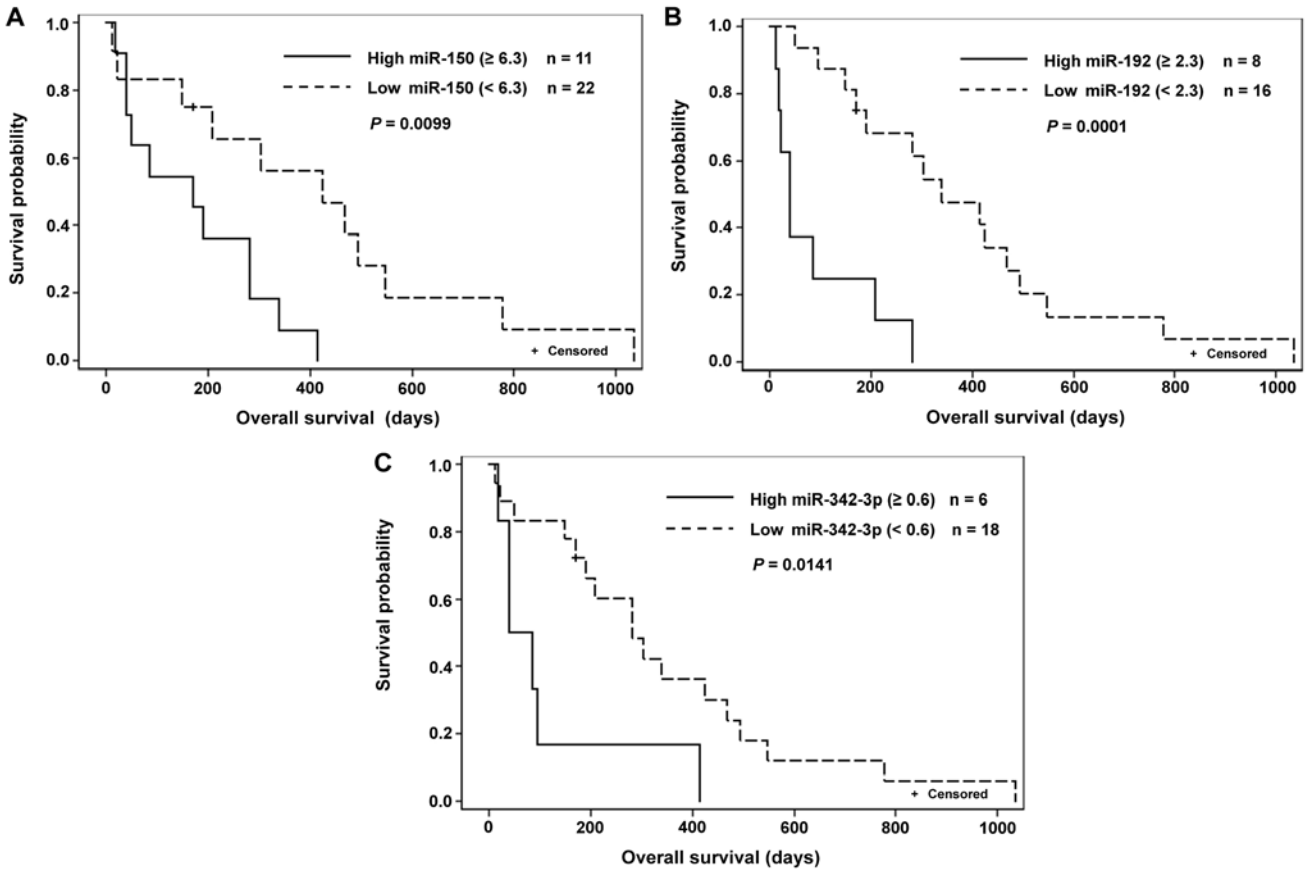

Figure 2. Relation of microRNA expression to overall survival (OS) in the subgroup of gastric cancer patients receiving 5-fluorouracil (5-FU) as the only treatment (Kaplan-Meier curves).

controversial (18). We found no statistically significant relation between the levels of ERCC1 and survival. It is possible our results reflect our test group, as in all cases we examined 5-FU was part of the treatment regimen but platinum-based

chemotherapy was only used in a subgroup, whose analysis was affected by the small number of patients.

The very nature of the TS enzyme makes its expression the most commonly examined in relation to 5-FU chemotherapy. We determined high levels of TS predict longer OS (Table V). Similar results were published by Wei *et al* (19), who conducted their analysis on a group of patients treated in the same way and also used RT-PCR to assess TS expression. On the other hand, some studies have shown high levels of TS to have the opposite effect (20).

From the set of the 29 microRNAs of interest, we found that high expression levels of miR-150, miR-181b, miR-192, miR-221, miR-224, miR 342-3p, miR-375 and low expression of miR-520h relate to unfavourable outcomes for patients (shorter TTP or OS) Table III.

MicroRNAs have the potential to become accurate, easily measurable biomarkers, with features fortuitous for diagnostic testing methods, such as stability in FFPE tissue, blood and perhaps other bodily fluids (21).

Wu *et al* (22) found miR-150 was overexpressed in gastric cancer cell lines and tissue samples and demonstrated overexpression of miR-150 could promote proliferation and growth of cancer cells by targeting the tumor-suppressor EGR2. In undifferentiated gastric cancer, higher miR-150 levels appeared to be associated with shorter postoperative patient survival, however, miR-150 was deemed to be an insufficiently independent prognostic factor in these cases (23). In the study of Chen *et al* (24), miR-150 showed decreased expression in gastric cancer patients compared to healthy test subjects. In the present study, higher levels of miR-150 showed a relation to shorter TTP and OS (Table IV; Figs. 1A and B and 2A).

Compared to normal gastric tissue samples, there is an overexpression of miR-181b in gastric tumors. Lower levels of miR-181b relate to longer OS of patients on regimens based on 5-FU and platinum derivatives (25). Furthermore, overexpression of miR-181b was found to downregulate the tissue inhibitor of metalloproteinases 3 (TIMP3) (26). Our results hint at the association of higher miR-181b levels to shorter TTP of patients treated with 5-FU (Table V). The results of another study show the ambiguous nature of the effects of certain miRNA; Chen *et al* (27) observed miR-181b was downregulated in human gastric adenocarcinoma tissue samples compared to adjacent normal gastric tissue and also described how miR-181b could suppress tumor cell proliferation by downregulating the expression of cAMP responsive element binding protein 1 (CREB1).

Our analysis of miR-192 levels showed its high expression related to shorter OS in the group of patients treated with 5-FU (Table V and Fig. 2B). To the best of our knowledge, no other study dealing with the predictive value of miR-192 in gastric cancer patients treated with 5-FU has been published. Xu *et al* (28) found miR-192 to be upregulated in gastric cancer tissue samples obtained by gastrectomy. The upregulation of both miR-192 and miR-215 was related to clinical characteristic such as lymph node metastases, while the inhibition of miR-215 or miR-192 significantly decreased gastric cancer cell invasion. The results reported by Chen *et al* (29) demonstrate that elevated circulating miR-192 has the potential to improve the early detection of distant metastases of GC.

Recently published studies show that miR-221 is an oncogenic microRNA involved in several malignancies (30,31). We found higher miR-221 expression in tumor samples in comparison to adjacent noncancerous tissue. This is in accordance with Liu *et al* (32) who found miR-221 was upregulated in 88% of gastric cancer tissue samples. Moreover, we observed a relation of high miR-221 expression to shorter TTP and OS in 5-FU monotherapy treated patients (Table V). The influence of miR-221 on the effect of chemotherapy is corroborated by published experiments conducted on the human gastric cancer cell line SGC7901 showing the knockdown of miR-221 inhibited cell growth and invasion and increased the radiosensitivity of the cells (33).

We found higher levels of miR-224 indicate shorter OS (Table IV and Fig. 1D). Mao *et al* (34) concluded that miR-224 is overexpressed in human gastric cancer cells. Reducing the expression of miR-224 can effectively inhibit growth and promote apoptosis of gastric cancer cells. These results are also supported by the study of Liu *et al* (35) who investigated the expression of miR-224 in the human gastric cancer cell line SGC-7901. In examining the effects of miR-224 mimics, they observed miR-224 could negatively regulate the expression of Raf-1 kinase inhibitor protein (RKIP). RKIP contributes to the suppression of proliferation and invasion of gastric cells.

We determined higher levels of miR-342-3p correlated to shorter TTP and OS in 5-FU monotherapy treated patients (Table V and Fig. 2C); similar results were described in colorectal cancer. High levels of miR-342-3p were associated with shorter survival time (36). Kim *et al* (37) screened miRNAs associated with response to chemotherapy using microarrays and found miR-342-p belongs to the miRNAs, whose upregulation is associated with chemosensitivity in gastric cancer.

Our observations of the relation of high miR-375 expression to shorter OS in 5-FU monotherapy treated patients (Table V) could be explained by the findings of Liu *et al* (38), who showed that miR-375 downregulated p53 expression through an interaction with the 3' UTR region of p53. In addition, they observed the expression of miR-375 desensitized cells to ionizing radiation and etoposide.

We demonstrated that higher levels of miR-520h correlated to longer TTP in 5-FU and cisplatin therapy treated patients (Table V). Shen *et al* (39) found that miR-520h downregulates histone deacetylase 1 and, thus, contributes to the chemotherapeutic effect of doxorubicin.

Summarizing the aforelisted results, amongst the miRNAs we examined, we found six miRNAs (miR-150, miR-181, miR-221, miR-224, miR-342-p and miR-520h) with a relation to TTP, which could serve as predictors of the effectiveness of treatment. These results merit multifactorial analysis, which we were, however, unable to perform due to the limited number of samples.

In our experience, microRNAs can generally be assessed with more precision and ease than mRNA of coding genes. This is essentially due to the fact that miRNA analysis is less demanding in terms of both quality and quantity of isolated RNA, features problematic in samples of RNA extracted from FFPE tissue. FFPE tissue samples are routinely taken and analysed during standard gastric cancer management and

that is why we believe microRNAs could become clinically applicable predictors of the effectiveness of palliative treatment in gastric cancer patients.

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