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Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis

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Summary

Background—Analyses of microRNA expression profiles have shown that many microRNAs are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of various haematological and solid tumours. We aimed to assess the relation between microRNA expression and progression and prognosis of gastric cancer.

Methods—353 gastric samples from two independent subsets of patients from Japan were analysed by microRNA microarray. MicroRNA expression patterns were compared between non-tumour mucosa and cancer samples, graded by diffuse and intestinal histological types and by

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Contributors

All authors planned and implemented the investigation. TU, YS, MK, WY, HS, GAC, and CMC had the idea for and designed the experiments. TU, SN, NO, and KY obtained samples and clinical data. TU, HO, MS, HA, and C-gL undertook the experiments. SV, CT, SR, and TU did the statistical analysis. TU, SV, CT, GAC, and CMC wrote the report. All authors critically reviewed the manuscript and approved the final version.

Conflicts of interest

The authors declared no conflicts of interest.

progression-related factors (eg, depth of invasion, metastasis, and stage). Disease outcome was calculated by multivariable regression analysis to establish whether microRNAs are independent prognostic factors.

Findings—In 160 paired samples of non-tumour mucosa and cancer, 22 microRNAs were upregulated and 13 were downregulated in gastric cancer; 292 (83%) samples were distinguished correctly by this signature. The two histological subtypes of gastric cancer showed different microRNA signatures: eight microRNAs were upregulated in diffuse-type and four in intestinal-type cancer. In the progression-related signature, miR-125b, miR-199a, and miR-100 were the most important microRNAs involved. Low expression of let-7g (hazard ratio 2·6 [95% CI 1·3–4·9]) and miR-433 (2·1 [1·1–3·9]) and high expression of miR-214 (2·4 [1·2–4·5]) were associated with unfavourable outcome in overall survival independent of clinical covariates, including depth of invasion, lymph-node metastasis, and stage.

Interpretation—MicroRNAs are expressed differentially in gastric cancers, and histological subtypes are characterised by specific microRNA signatures. Unique microRNAs are associated with progression and prognosis of gastric cancer.

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Introduction

Gastric cancer is the fourth most common human malignant disease and the second most frequent cause of cancer-related death worldwide.[1] Improvement of diagnosis and treatment has resulted in good long-term survival for patients with early gastric cancer, whereas the outlook for individuals with advanced disease remains poor.[2] Advanced gastric cancer frequently recurs as nodal and haematogenous metastases and peritoneal dissemination. Although several types of non-surgical treatment have been assessed, surgical resection is still the primary curative treatment for localised gastric cancer.

Data from several studies show that various genetic alterations cause tumorigenesis and progression of gastric cancer.[3,4] Inactivation of runt-related transcription factor 3 (*RUNX3*) by methylation has also been reported.[5] Several groups have undertaken high-throughput analyses of gastric cancer expression profiles by DNA microarrays[4] and microdissection.[6] However, markers for tumorigenesis and progression of gastric cancer have not yet been discovered and specific therapeutic targets have not been identified.

A new class of small non-coding RNAs—microRNAs— has been discovered.[7] Mature microRNAs are composed of 19–25 nucleotides and are cleaved from 60–110-nucleotide hairpin microRNA precursors in the cytoplasm by the RNase III enzyme Dicer.[8] Single-stranded microRNAs bind mRNAs of potentially hundreds of genes at the 3′ untranslated region with imperfect complementarity, resulting in degradation of target mRNAs and inhibition of translation.[8] Several target-prediction programs have been developed, but very few targets have been proved experimentally.[9] MicroRNAs play a part in crucial cellular processes, including development, differentiation, stress response, apoptosis, and proliferation.[8,10] 475 human microRNAs have been reported to date (miRBase version 9.2; University of Manchester, Manchester, UK);[11] this number could reach 800–1000 through experimental confirmation of predicted microRNA genes.[12]

Microarray platforms have been developed for analysis of microRNA expression, and data show that several microRNAs are expressed aberrantly in various haematological and solid malignant diseases.[13–16] MicroRNAs act as novel oncogenes or tumour-suppressor genes.[17,18] We and others have noted that alterations in microRNA expression correlate highly with progression and prognosis of human tumours.[19–24] Thus, focusing on microRNAs in gastric cancer could yield new insights into the biological behaviour of this disease. For oncogenic microRNAs, antagomirs are a type of antisense oligonucleotide that inhibit microRNA function in vivo effectively;[25–27] for tumour-suppressive microRNAs, reconstitution with microRNA precursor sequences has an antitumour effect. Therefore, microRNAs are possible therapeutic targets for cancer.[22,28]

To ascertain whether microRNA expression signatures can differ between gastric cancer and non-tumour mucosa, we undertook genome-wide microRNA expression profiling in two sets of gastric tissues. With expression-profile results for these samples and associated clinical variables, we investigated the association between microRNAs and histological types, tumour progression, and prognosis of gastric cancer.

Methods

Tissue samples

For microRNA expression profiling, we obtained gastric tissue samples (cancer lesions and adjacent non-tumour mucosae) from patients who underwent gastrectomy between 2002 and 2005 at the University of Tokyo (group 1) and between 1998 and 2005 at Hiroshima University (group 2). We gathered all samples in the same manner, and they were snap-frozen immediately in liquid nitrogen and stored at −80°C until RNA and protein extraction could be done. Since microdissection is difficult to do in diffuse-type gastric cancer, for technical uniformity we used bulk tissue for all cases.

We obtained study approval from the ethics committee at the University of Tokyo and every patient from the University of Tokyo gave written informed consent for samples to be used. Because we did not obtain written informed consent for samples from Hiroshima University, for strict privacy protection, identification information was removed before analysis; this procedure is in accordance with ethical guidelines for human genome or gene research enacted by the Japanese Government and was approved by the ethics review committee of the Hiroshima University School of Medicine.

Panel: Patient cohorts and of analyses undertaken

STEP 1: MicroRNA expression patterns in gastric cancer (non-tumour mucosa *vs* cancer)

Samples

61 pairs in group 1 and 99 in group 2 were analysed independently

Statistical methods

1. Class comparison by BRB-ArrayTools; paired *t* test (p<0·01)

2. Class prediction by BRB-ArrayTools; paired class prediction by the leave-one-out cross-validation method

Samples

169 non-tumour mucosae (64 samples from group 1 and 105 from group 2) and 184 cancers (81 samples from group 1 and 103 from group 2) (unpaired condition)

Statistical methods

Average linkage clustering with centred Pearson correlation with 35 microRNAs

STEP 2: MicroRNA expression patterns and histological types (diffuse-type *vs* intestinal-type gastric cancer)

Samples

103 diffuse-type and 81 intestinal-type gastric cancer samples

Statistical methods

1. Class comparison by BRB-ArrayTools; two-sample *t* test ($p<0{\cdot}001$)
2. Average linkage clustering with centred Pearson correlation with the 19 most significant microRNAs ($p\leq 2\times 10^{-6}$)

STEP 3: MicroRNA expression and tumour progression correlation

Samples

- T3 and T4 *vs* T1 (101 *vs* 15 samples)
- Lymph-node metastasis (N) positive *vs* negative (126 *vs* 54 samples)
- Stage IV *vs* I (51 *vs* 37 samples)
- Peritoneal dissemination (P, CY) positive *vs* negative (33 *vs* 76 samples)
- Haematogenous metastasis (H, M) positive *vs* negative (12 *vs* 169 samples)

Statistical methods

1. Class comparison by BRB-ArrayTools; two-sample *t* test ($p<0{\cdot}01$, for haematogenous metastasis, $p<0{\cdot}05$)
2. Venn diagram of T, N, and stage
3. Significance analysis of microarrays (SAM) with rank-regression option for T and stage

STEP 4: MicroRNA expression and prognosis correlation

Samples

101 cases have information for disease outcome and underwent curative surgery. All 182 cases had surgery (curative or non-curative)

Overall survival

- Statistical methods

1. Univariate Cox proportional hazards regression in BRB-ArrayTools
2. Kaplan-Meier survival curves
3. Multivariable Cox proportional hazards regression analysis

Disease-free survival

- Statistical methods
  1. Univariate Cox proportional hazards regression in BRB-ArrayTools
  2. Kaplan-Meier survival curves
  3. Multivariable Cox proportional hazards regression analysis

Procedures

We did RNA labelling and hybridisation on microRNA microarray chips and undertook postprocessing, as described previously.[13,15,19–21] Briefly, 5 μg of total RNA from every sample was reverse transcribed with biotin end-labelled random-octamer oligonucleotide primers. Hybridisation of biotin-labelled complementary DNA was done on the Ohio State University custom microRNA microarray chip (OSU_CCC version 3.0; ArrayExpress [European Bioinformatics Institute, Cambridge, UK], array design A-MEXP-620), which contains nearly 1100 microRNA probes, for 326 human and 249 mouse microRNA genes, spotted in duplicates. We washed and processed the hybridised chips to detect biotin-containing transcripts with streptavidin Alexa Fluor 647 conjugate (Invitrogen, Carlsbad, CA, USA) and scanned them on a microarray scanner (4000B; Axon Instruments, Sunnyvale, CA, USA).

We analysed microarray images with GenePix Pro 6.0 (Axon Instruments). Average values of the replicate spots for every microRNA sample were background subtracted, normalised, and subjected to further analysis. Only probes for human mature microRNAs were used for analysis. We implemented quantile normalisation with the Bioconductor 1.8 package affy 1.1.2.

MicroRNAs were retained when they were present in at least 20% of samples and when they had changes of more than 1·5-fold from the gene median in at least 20% of samples. Absent calls (background-level signals on the microarray) were removed at a threshold of 4·5 ($\log_2$ scale) before statistical analysis. After the filtration, we included 237 microRNAs in further statistical analyses.

MicroRNA nomenclature is according to miRBase version 9.2.[11] The microarray dataset is deposited in ArrayExpress (experiment number E-TABM-341) according to MIAME (minimum information about a microarray experiment) guidelines.

Statistical analysis

The panel summarises the analyses. We identified differentially expressed microRNAs with BRB-ArrayTools version 3.5.0 (Biometric Research Branch, National Cancer Institute,

Bethesda, MD, USA),[29] and significance analysis of microarrays (SAM) version 3.0. The webappendix contains further descriptions of the methods used.

After filtration of microRNAs, we used the paired *t* test (level of significance, p<0·01) to independently analyse pairs of non-tumour mucosa and cancer samples from groups 1 and 2. We undertook class prediction with the leave-one-out cross-validation method, taking into account that samples were paired (eg, pairs of non-tumour mucosae and cancer lesions from the same patient).

We used hierarchical cluster analysis to generate a tree cluster showing the separation of every class. For hierarchical clustering, we used average linkage metrics and centred Pearson correlation of microRNAs identified between non-tumour mucosa and gastric cancer and between diffuse-type and intestinal-type gastric cancer (Cluster 3.0). For tree visualisation, we used Java Treeview version 1.1.1.

We identified microRNAs whose expression was related significantly to overall survival and disease-free survival of patients (endpoint of cancer-specific death and recurrence, respectively). We undertook univariate Cox proportional hazards regression in BRB-ArrayTools, and we judged microRNAs significant if p<0·05.

We used SPSS version 17.0.1 for Kaplan-Meier survival analysis and Cox proportional hazards regression. To generate survival curves, we converted continuous microRNA expression levels measured on microRNA array chips to a dichotomous variable, using the respective mean levels of expression as a threshold.[21] This procedure enabled division of samples into classes with high and low expression of microRNA. We compared survival curves by log-rank test and judged p<0·05 significant.

We examined the joint effect of covariates with Cox proportional hazards regression to ascertain whether microRNAs are independent prognostic factors. We censored data for three patients who died of other diseases; data for one patient were censored before the first event (death) in overall survival and were included in the Kaplan-Meier analysis, but were removed for Cox regression analysis in overall survival.

We regarded age as a continuous covariate. T was dichotomised on the basis of absence (T1, T2) versus presence (T3, T4) of serosal invasion of tumour. Stage was dichotomised on the basis of a more than 65% 5-year survival (stages I and II) versus a less than 50% 5-year survival (stages III and IV). For all microRNAs, patients were categorised into groups with high and low expression, with respective mean levels of microRNA expression as a threshold.

We undertook univariate Cox regression to examine the effect of every clinical covariate on patient's survival. We did multivariable analysis by stepwise addition and removal of covariates found to be associated with survival in univariate models (p<0·10). Conditions of the stepwise selection method were Score statistic (p<0·05 for addition) and Wald statistic (p<0·05 for removal). All stepwise addition models gave the same final models as did stepwise removal, and final models included only those covariates that were associated significantly with survival (Wald statistic, p<0·05). We tested proportional-hazard

assumption by the log-minus-log plot, and no covariate violated assumption. All p values reported are two-sided.

Role of the funding source

The sponsor had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

81 gastric cancer samples (from 79 patients; one patient had cancer in three regions) were obtained at the University of Tokyo (group 1) and 103 samples were gathered at Hiroshima University (group 2) for microRNA expression profiling. Corresponding non-tumour mucosae were available for analysis for 61 cancers in group 1 and 99 in group 2. We also obtained three additional samples of non-tumour mucosa in group 1 and six in group 2, making 353 samples in total— 184 cancers and 169 non-tumour mucosae.

Clinical features of patients and tumours are described in table 1 and the webappendix. Disease outcome was known for 101 patients who underwent curative surgery; 42 recurred and died of cancer within the follow-up period. The final follow-up date was Feb 25, 2007 (median follow-up 785 days [range 159–3070]). Most patients (disease stages IB–IV) were given anticancer drugs either orally or intravenously postoperatively as adjuvant chemotherapy. After disease recurrence, these individuals were given other anticancer drugs.

On microarray analysis, 35 microRNAs were expressed differentially in the paired non-tumour mucosa and cancer samples in groups 1 and 2 (table 2): 22 of these were upregulated and 13 were downregulated in cancer (designated as the gastric cancer signature). By paired class prediction, 97% of samples in group 1 and 94% in group 2 were classified correctly.

On the basis of the 35 microRNAs expressed differentially, cluster analysis with Pearson correlation of the 169 non-tumour mucosa and 184 cancer samples generated a tree showing good separation between non-tumour mucosa and cancer (page 4 of the webappendix). Despite the unpaired condition, 83% (292/353) of samples were classified correctly to non-tumour mucosa or cancer branches.

By quantitative reverse transcription-PCR (qRT-PCR), we analysed 24 pairs of samples investigated initially by microarray for miR-21 (upregulated) and miR-375 (downregulated). We compared the cancer:non-tumour mucosa expression ratio in qRT-PCR with that in the microRNA microarray. The microarray data were confirmed by qRT-PCR (page 5 of the webappendix).

The similarity of the microRNA signature in groups 1 and 2 enabled us to merge all samples (184 cancers) into one group for further analyses. 103 diffuse-type and 81 intestinal-type specimens were used to establish whether microRNAs are differentially expressed between histological subtypes. By class comparison, 78 microRNAs were selected (false-discovery rate ≤0·42%), designated as the histotype signature.

We used the 19 most significant microRNAs (page 9 of the webappendix) in the histotype signature and undertook cluster analysis on the 184 cancer samples. These molecules were selected because they were identified also by SAM in the same order according to the absolute value of the SAM score (data not shown). Even though the histological characteristics of gastric cancer are complex (including seven histological types and mixtures of types), 74% (137/184) of tumours were distinguished successfully by the expression pattern of these 19 microRNAs (page 6 of the webappendix). Cluster analysis indicated that miR-105, miR-100, miR-125b, miR-199a, miR-99a, miR-143, miR-145, and miR-133a are upregulated in diffuse-type gastric cancer, and miR-373*, miR-498, miR-202*, and miR-494 are upregulated in intestinal-type lesions. These microRNAs are those expressed most differentially, characterising diffuse-type and intestinal-type tumours.

Next, we investigated the correlation between microRNA expression and gastric cancer progression. To identify microRNAs related to progression for every clinical feature, class comparisons were undertaken. 65 microRNAs were selected for T, 17 for N, 14 for H and M, 15 for P and CY, and 38 for stage (figure 1A). False-discovery rate was 3·3% or less for T, 10·5% for N, 18·8% for P and CY, and 6·9% for stage. Because patients who have distant metastasis undergo surgery rarely, the sample number for positive H and M is just 12. This low number caused a reduction in power to detect microRNAs expressed differentially and a high false-discovery rate. However, six of 14 microRNAs were selected in T, N, or stage (shown in red in figure 1A), and miR-25, miR-106a, miR-20b, miR-181b, miR-181d, and miR-135a—which were upregulated in gastric cancer relative to non-tumour mucosa—were also chosen. To identify the most important microRNAs associated with progression, we chose T and N as representative progression features and compared them with stage. Ten microRNAs—miR-125b, miR-199a, miR-100, miR-107, miR-181b, miR-103, miR-494, miR-497, miR-126, and let-7f—correlated with these variables (figure 1A).

By SAM with rank-regression option, we selected 28 microRNAs whose expression was associated with progression from T1 to T4 and 47 microRNAs associated with progression from stage I to IV (data not shown). The q values in SAM of these microRNAs were 0% for T and 1·1% for stage. By comparison of these microRNAs with the ten identified in the previous step, we recorded miR-125b, miR-199a, and miR-100 as the most important microRNAs related to progression of gastric cancer. These three microRNAs showed increasing expression levels according to stage progression (figure 1B).

We investigated the correlation between microRNA expression profiles and prognosis to establish the microRNAs that might signify unfavourable prognosis (independent of clinical factors). We used samples from 101 patients who underwent curative surgery and their associated prognostic information. Univariate Cox proportional hazards regression indicated that ten microRNAs (let-7c, let-7e, let-7g, let-7i, miR-19a, miR-214, miR-410, miR-433, miR-452, and miR-495) were related to overall survival of patients with gastric cancer. Kaplan-Meier survival curves were generated for every microRNA, and five (let-7e [p=0·007], let-7g [p=0·002], let-7i [p=0·038], miR-214 [p=0·005], and miR-433 [p=0·015]) were associated significantly with survival.

Table 3 shows univariate Cox proportional hazards regression analysis of overall survival relative to clinical factors. T, N, and stage were associated significantly with overall survival, as were five microRNAs. To elucidate whether these microRNAs are independent prognostic factors, multivariable analysis was done. The dichotomised expression values of these five microRNAs were not associated with clinical factors (Fisher's exact test). Because T and N were associated highly with stage by Fisher's exact test, and the same microRNAs were chosen in the final model of multivariable analysis including stage and in the final model including T and N, we showed only the stage model (table 3). In the final multivariable model, let-7g, miR-214, and miR-433 were associated with overall survival independent of clinical covariates (table 3). Patients with low expression of let-7g (hazard ratio 2·6 [95% CI 1·3–4·9]), low expression of miR-433 (2·1 [1·1–3·9]), or high expression of miR-214 (2·4 [1·2–4·5]) had poorer survival than did patients with high expression of let-7g, high expression of miR-433, or low expression of miR-214 (figure 2).

We validated the results for let-7g and miR-214 by qRT-PCR. 12 samples selected from the low-expression group showed low expression of let-7g and miR-214 by qRT-PCR, and 12 samples selected from the high-expression group showed high expression (page 7 of the webappendix). We analysed three additional specimens by qRT-PCR that were not used in microRNA array analysis because of low RNA yield. One sample with an unfavourable outcome showed high expression of miR-214 (higher than the mean of 12 samples from the high-expression group), and two with a favourable outcome showed low expression of miR-214 (lower than the mean of 12 samples from the low-expression group), consistent with our results.

We undertook the same analyses for disease-free survival in 101 patients. By univariate Cox proportional hazards regression, 12 microRNAs (let-7b, let-7c, let-7d, let-7g, miR-19a, miR-196a, miR-220, miR-373, miR-410, miR-433, miR-452, and miR-495) were related to disease-free survival of patients with gastric cancer. By log-rank analysis, six microRNAs (let-7b [p=0·001], let-7g [p=0·001], miR-19a [p=0·031], miR-410 [p=0·015], miR-433 [p=0·011], and miR-495 [p=0·035]) were related to survival. On univariate analysis, T, N, stage, and these six microRNAs were associated significantly with disease-free survival (table 4). The dichotomised expression values of six microRNAs were not associated with clinical factors (Fisher's exact test). Because T and N were associated highly with stage by Fisher's exact test, and the same microRNAs were chosen in the final model of multivariable analysis including stage and in the final model including T and N, we showed only the stage model (table 4). In the final multivariable Cox regression model, let-7b, let-7g, miR-19a, and miR-495 were associated with disease-free survival independent of clinical covariates (table 4). In both overall survival and disease-free survival, let-7g was selected as an independent prognostic factor (tables 3 and 4).

101 patients were divided into two groups by histological type (intestinal and diffuse) and multivariable Cox proportional hazards regression analysis was undertaken in the same way. The selected microRNAs remained as independent prognostic factors (table 5).

Discussion

Aberrant microRNA expression patterns have been described in various haematological and solid cancers,[14–16,20–22] and alterations in microRNA expression correlate highly with progression and prognosis of human malignant diseases.[19–24] However, profiles of microRNAs differ and need to be investigated in every type of tumour. In this study, we recorded substantial associations between differential expression of specific microRNAs and progression and prognosis of gastric cancer.

Antiapoptotic miR-21 is upregulated in various solid cancers and is related to tumour growth.[15,30] In previous work, miR-21 was overexpressed in gastric cancer and in *Helicobacter pylori*-infected gastric mucosa.[30] *H pylori* is an important pathogen for gastric cancer, and data are already starting to suggest the molecular mechanism of evolution of normal mucosa to chronic gastritis, atrophic gastritis, and intestinal metaplasia. Our sample set contained no detailed information about *H pylori* infection status because pathologists recorded histological types, depth of invasion, and status of lymph-node metastasis to decide clinical stage of cases. Non-tumour mucosae were obtained during surgery from resected stomach that seemed to be normal macroscopically. Therefore, in this study we could not investigate the correlation between microRNA expression and *H pylori* or chronic gastritis; however, we will investigate this important area in further studies.

We identified 35 differentially expressed microRNAs without use of microdissection. This procedure is difficult to adapt to some diffuse-type gastric cancers because cancer cells are localised singly. In a previous report, we analysed by microarray 20 pairs of intestinal-type gastric cancer and non-tumour mucosa samples from a white population and noted 14 upregulated and five downregulated microRNAs in cancers.[31] All the upregulated microRNAs and three of those downregulated (60%) were similar to the molecules selected in this study, meaning that our method of using bulk samples of diffuse-type gastric cancer for microarray analysis can produce correct results, although they must be validated by in-situ hybridisation. This result also means that despite patients' different ethnic backgrounds in this and our previous study, the microRNA signature is linked to general mechanisms of gastric cancer tumorigenesis.

For some of the microRNAs we identified in gastric cancer samples, several targets have already been proven experimentally. We showed previously that molecules expressed differentially in the microRNA cluster miR-106b-25 are related to gastric cancer tumorigenesis,[31] suggesting that microRNAs have important roles in gastric cancer. Although gastric cancer is histologically complex and sometimes shows transition from differentiated to undifferentiated subtypes in the same tumour (ie, mixed type), we divided samples into diffuse and intestinal types and identified microRNAs expressed differentially, characterising these histological classes. A collaborator of ours reported that the Hedgehog signal is more active in diffuse-type than intestinal-type gastric cancer,[33] and glioma-associated oncogene homologue 1 (*GLI1*), a downstream target of the Hedgehog signal, is an in-silico target of miR-373*, which is downregulated in diffuse-type gastric cancer.

In this study, we identified microRNAs related to the progression of gastric cancer. In breast cancer, tumour invasion and metastasis are initiated by miR-10b,[24] which is one of the microRNAs associated with invasion in gastric cancer. miR-21 was selected in the progression signature of both T and stage, and it targets programmed cell death 4 (*PDCD4*) and maspin (*SERPINB5*), resulting in tumour invasion and metastasis.[24] Another group showed that miR-21 targets a tumour-suppressor gene, reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*), and that knockdown of miR-21 decreased invasion and migration of gastric cancer cells significantly.[30] The microRNAs that were related most significantly to progression of gastric cancer—miR-125b, miR-199a, and miR-100—were also upregulated in pancreatic adenocarcinoma in our previous study.[21] miR-125b is reportedly related to proliferation of differentiated cells[32] and downregulated in breast cancer[14] and thyroid anaplastic carcinoma,[32] suggesting that this microRNA functions differently in gastric cancer and pancreatic adenocarcinoma. Proapoptotic *BAK1* and *TP53* are proven targets of miR-125b in prostate cancer and neuroblastoma cells, supporting the oncogenic function of miR-125b.[34,35] Upregulation of miR-199a is associated purportedly with tumour cell growth in cervical carcinoma.[36]

We identified microRNAs associated with an unfavourable outcome (independent of clinical factors) in specimens from patients treated by curative surgery and adjuvant chemotherapy. Although our findings should be validated in an independent cohort, these microRNAs might help to identify individuals who are candidates for aggressive treatment because of their expression status and who could become candidates for therapeutic targets with antagomirs[25–27] or by reconstitution with microRNA precursor sequences.[28] Three microRNAs selected in the progression analysis were not chosen for the prognostic signature partly because they were associated highly with clinical factors. The difference of the selected microRNAs between overall and disease-free survival is probably caused by the effect of chemotherapy after disease recurrence.

We chose let-7g and let-7b as independent prognostic factors. The Ras family of oncogenes is regulated by the let-7 family in lung cancer,[37,38] and the high mobility group AT-hook 2 (*HMGA2*) oncogene is also targeted by this microRNA family.[37,38] *HMGA2* is regulated negatively by the let-7 family, and high expression of this gene correlates with tumour invasiveness and is an unfavourable prognostic factor in gastric cancer.[39] Additionally, in tumour-initiating cells of breast cancer (which have stem cell-like properties), let-7 regulates self-renewal (by silencing *HRAS*) and differentiation (by silencing *HMGA2*).[24] Administration of let-7 family members inhibits growth of lung cancer in mice.[37,38] A negative regulator of hedgehog signalling, suppressor of fused (*su[fu]*), is targeted by miR-214 in the development of zebrafish,[40] and activation of hedgehog signalling is involved in gastric cancer.[32] Recently, miR-214 was reported to induce cell survival and cisplatin resistance by targeting *PTEN* in ovarian cancer.[41] miR-433 targets growth factor receptor-bound protein 2 (*GRB2*) in gastric cancer.[42]

Further studies are needed to establish whether the microRNAs we selected in this study have full potential as either biomarkers or therapeutic targets in gastric cancer. Proving new targets and other biological experiments will clarify the functions and roles of microRNAs

in gastric cancer. However, we have shown already that microRNAs can meet criteria for ideal biomarkers and therapeutic targets.[22]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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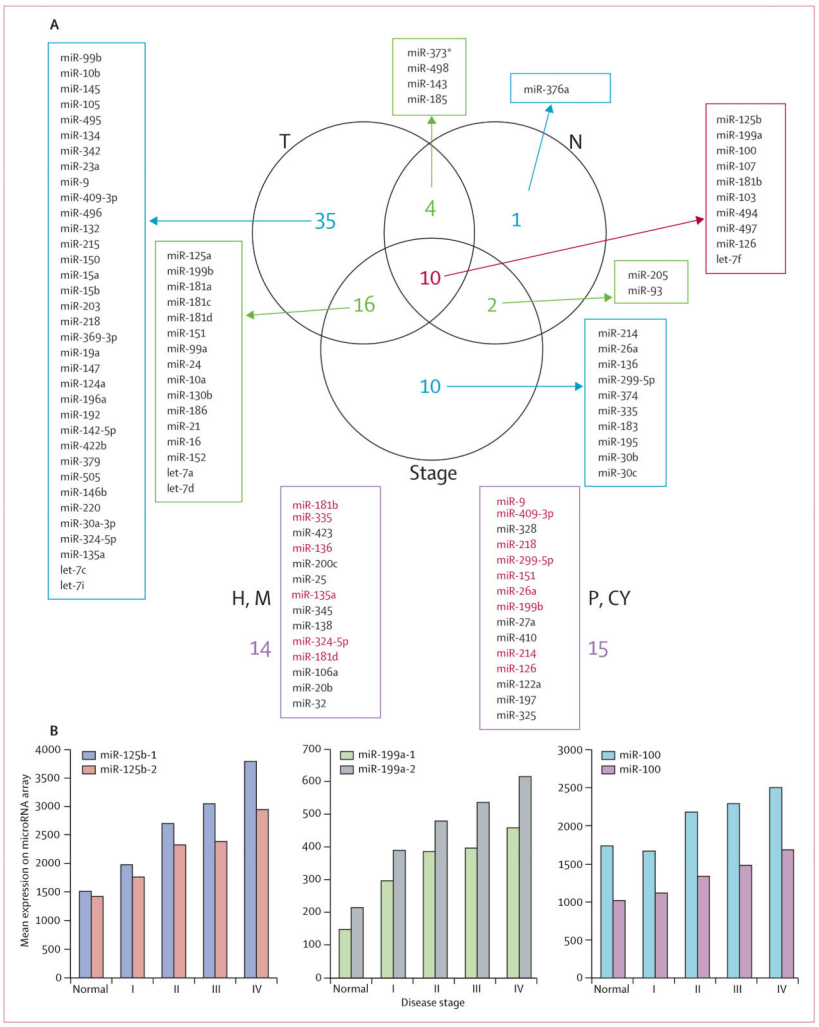

Figure 1. MicroRNAs associated with progression of gastric cancer

(A) Venn diagram of microRNAs related to T (depth of invasion), N (lymph-node metastasis), and stage. Listed microRNAs comprise the progression signature. Numerals indicate the number of microRNAs. Molecules corresponding to every part of the Venn diagram are shown. MicroRNAs in H and M (haematogenous metastasis) and P and CY (peritoneal dissemination) that are similar to those for T, N, or stage are shown in red. (B) Mean expression levels of miR-125b, miR-199a, and miR-100 on microRNA array according to progression in disease stage. Mean expression levels are shown as linear-scale data on microRNA array analysed with GenePix Pro 6.0; the calculation is based on the intensity (brightness) of each pixel on the microarray image. Mean expression levels of non-tumour mucosa (Normal) of group 1 are also shown. miR-125b-1 and miR-125b-2 are located on different chromosomes but the sequence of mature microRNA is the same; miR-199a-1 and miR-199a-2 are also the same. For miR-100, two probes were included on the microRNA array.

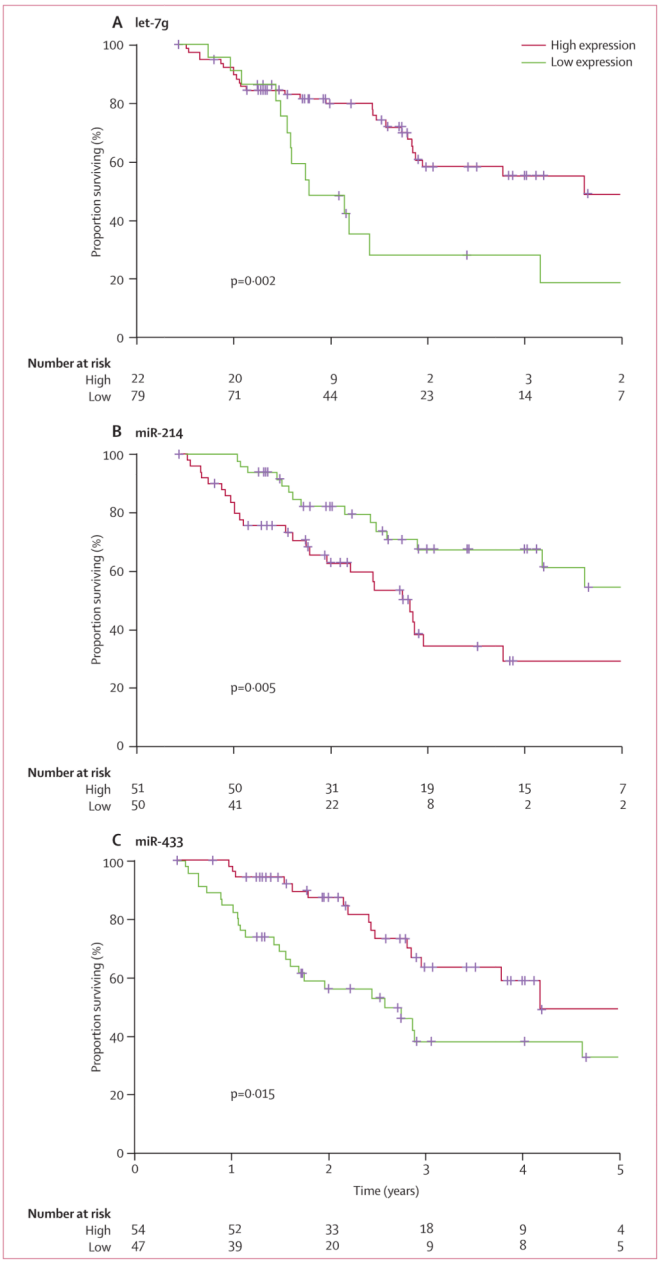

Figure 2. Kaplan-Meier curves of independent prognostic factors for overall survival

Curves are depicted with data for 101 patients. MicroRNA expression levels measured on the microarray were converted into discrete variables by division of samples into two classes (high and low expression), with the respective mean levels of microRNA expression as a threshold. Censored cases are shown on the curves. p values are log rank.

Table 1

Characteristics of patients and tissues

	Group 1 (n=79)	Group 2 (n=103)	p*	Total (n=182)
Age (years; mean [SD])	65·2 (9·8)	67·1 (11·6)	0·24	66·3 (10·9)
Sex			0·87	
Men	52/79 (66%)	66/102† (65%)		118/181 (65%)
Women	27/79 (34%)	36/102† (35%)		63/181 (35%)
Histological type‡			0·022	
Diffuse	53/81§ (65%)	50/103 (49%)		103/184 (56%)
Intestinal	28/81§ (35%)	53/103 (51%)		81/184 (44%)
Depth of invasion (T)‖			0·50	
T1	4/81§ (5%)	11/102† (11%)		15/183 (8%)
T2	29/81§ (36%)	38/102† (37%)		67/183 (37%)
T3	41/81§ (50%)	45/102† (44%)		86/183 (47%)
T4	7/81§ (9%)	8/102† (8%)		15/183 (8%)
Lymph-node metastasis (N)			0·028	
Negative (N0)	17/79 (22%)	37/101¶ (37%)		54/180 (30%)
Positive (N1–N3)	62/79 (78%)	64/101¶ (63%)		126/180 (70%)
Haematogenous metastasis (H, M)			0·69	
Negative	75/79 (95%)	94/102† (92%)		169/181 (93%)
Positive	4/79 (5%)	8/102† (8%)		12/181 (7%)
Peritoneal dissemination (P, CY)			<0·0001	
Negative	64/79 (81%)	12/30** (40%)		76/109 (70%)
Positive	15/79 (19%)	18/30** (60%)		33/109 (30%)
Stage††			0·13	
I	11/79 (14%)	26/102† (25%)		37/181 (21%)
II	14/79 (18%)	23/102† (23%)		37/181 (21%)
III	29/79 (37%)	27/102† (27%)		56/181 (30%)
IV	25/79 (31%)	26/102† (25%)		51/181 (28%)

Data are n (%) unless stated otherwise.

*Differences between groups calculated by *t* test for age and χ^2 test for all others.

†No information available for one patient.

‡Lauren's classification used for histological typing. Intestinal-type gastric cancer is almost the same as differentiated-type gastric cancer, and diffuse-type gastric cancer is almost the same as undifferentiated-type gastric cancer.

[§] One patient had cancer in three regions.

[||] Graded according to the International Union Against Cancer's TNM classification, 5th edn.

[¶] No information available for two patients.

[**] No information on intraoperative cytology available for 73 patients.

[††] Graded according to the Japanese Classification of Gastric Cancer, 2nd English edn. Clinical stage is decided by the factors T, N, H, M, P, and CY. Stages IA and IB are regarded as stage I, and stages IIIA and IIIB as stage III.

Table 2

Frequent differentially expressed microRNAs (gastric cancer signature)*

	p†	FDR (%)‡	Fold change	Chromosomal location	Gastric signature§	Proved targets‖	Cancer involvement¶
MicroRNAs upregulated in cancer							
miR-181d	$<1\times10^{-7}$	<0·01	2·3	19p13.12	Progression	*CDX2*, *GATA6*, *NLK*	Pancreas
miR-181a-1, miR-181a-2	$<1\times10^{-7}$	<0·01	2·2	1q31.3, 9q33.3	Progression	*HOXA11*, *BCL2*, *CD69*, *TRA*α, *PTPN11* (*SHP2*), *PTPN22*, *DUSP5*, *DUSP6*, *KAT2B* (*PCAF*), *CDKN1B*, *CDX2*, *GATA6*, *NLK*	Breast, pancreas, liver, thyroid, uterus, brain
miR-181c	$<1\times10^{-7}$	<0·01	2·1	19p13.12	Progression	*CDX2*, *GATA6*, *NLK*	Lung, pancreas, liver, thyroid, uterus, brain
miR-181b-1, miR-181b-2	$<1\times10^{-7}$	<0·01	2·0	1q31.3, 9q33.3	Progression	*TCL1A*, *VSNL1*, *GRIA2*, *KAT2B* (*PCAF*), *AICDA (AID)*, *CDX2*, *GATA6*, *NLK*	Breast, colon, pancreas, prostate, stomach, thyroid, uterus, brain, CLL
miR-21	$<1\times10^{-7}$	<0·01	2·0	17q23.2	Histotype, progression	*PTEN*, *TPM1*, *PDCD4*, *SERPINB5*, *BMPR2*, *BTG2*, *CDK6*, *IL6R*, *SOCS5*, *NFIB*, *SPRY2*, *RECK*, *TIMP3*, *TP63* (*TP73L*), *DAXX*, *HNRNPK*, *TOPORS*, *TP53BP2*, *JMY*, *TGFBR2*, *TGFBR3*, *APAF1*, *PPIF*, *SPRY1*, *MTAP*, *SOX5*, *TGFB1*, *NCAPG*, *RTN4*, *DERL1*, *PLOD3*, *BASP1*, *MARCKS*, *IL12A*, *JAG1*, *LRRFIP1*	Breast, colon, lung, pancreas, prostate, stomach, liver, thyroid, uterus, ovary, brain, CLL, lymphoma
miR-25	$<1\times10^{-7}$	<0·01	1·7	7q22.1	Progression	*BCL2L11*, *KAT2B* (*PCAF*), *CDKN1C*	Pancreas, prostate, stomach, liver, thyroid, uterus, oesophagus, brain, AML
miR-92-1, miR-92-2	$<1\times10^{-7}$	<0·01	1·7	13q31.3, Xq26.2	··	*MYLIP*, *HIPK3*, *BCL2L11*, *VHL*, *ITGA5*, *TP63 (TP73L)*	Colon, pancreas, prostate, stomach, thyroid, CLL, AML
miR-93	$<1\times10^{-7}$	<0·01	1·6	7q22.1	Progression	*E2F1*, *CDKN1A*, *VEGFA*, *KAT2B* (*PCAF*), *STAT3*, *TP53INP1*, *TUSC2*	Colon, pancreas, prostate, stomach, ovary, AML
miR-17-5p	2×10^{-7}	<0·01	1·7	13q31.3	··	*E2F1*, *NCOA3* (*AIB1*), *RUNX1* (*AML1*), *RBL2*, *CDKN1A*, *PTEN*, *BCL2L11*, *TIMP1*, *VEGFA*, *HIF1A*, *CCND1*, *MAPK9*, *MAP3K8*, *PKD1*, *PKD2*, *PPARA*, *RBL1*, *STAT3*, *TSG101*, *KAT2B* (*PCAF*), *CRK*, *GAB1*, *MYCN*, *IRF1*, *NR4A3*, *RNF111*, *TP53INP1*, *APBB2*, *BRCA1*, *APP*, *RASSF2*,	Breast, colon, lung, pancreas, prostate, stomach, bladder

	p[†]	FDR (%)[‡]	Fold change	Chromosomal location	Gastric signature[§]	Proved targets[‖]	Cancer involvement[¶]
						TNFSF12, *MAPK14*, *FN1*, *FNDC3A*, *BCL2*, *MEF2D*, *MAP3K12*	
miR-106a	3×10^{-7}	<0·01	1·7	Xq26.2	Progression	*RB1*, *RUNX1* (*AML1*), *ARID4B* (*RBP1L1*), *MYLIP*, *HIPK3*, *CDKN1A*, *VEGFA*, *APP*, *IL10*	Colon, lung, pancreas, prostate, stomach, liver, AML
miR-20b	4×10^{-7}	<0·01	1·9	Xq26.2	Progression	*ARID4B* (*RBP1L1*), *MYLIP*, *HIPK3*, *CDKN1A*, *VEGFA*	··
miR-135a-1, miR-135a-2	7×10^{-7}	<0·01	2·1	3p21.1, 12q23.1	Progression	*APC*, *SMAD5*, *JAK2*	Colon, prostate, thyroid, uterus, AML, lymphoma
miR-425-5p	1×10^{-6}	<0·01	2·2	3p21.31	··	··	··
miR-106b	1×10^{-6}	<0·01	1·6	7q22.1	··	*E2F1*, *CDKN1A*, *VEGFA*, *KAT2B* (*PCAF*), *ITCH*, *APP*, *STAT3*, *MAPK14*	Colon, stomach, AML
miR-20a	3×10^{-6}	<0·01	1·8	13q31.3	··	*E2F1*, *E2F2*, *E2F3*, *TGFBR2*, *RUNX1* (*AML1*), *CDKN1A*, *ZBTB7A* (*LRF*), *VEGFA*, *HIF1A*, *CCND1*, *STAT3*, *MYF5*, *APP*, *MAPK14*, *BCL2*, *MEF2D*, *MAP3K12*	Colon, pancreas, prostate, uterus, ovary, AML
miR-19b-1, miR-19b-2	5×10^{-6}	<0·01	1·7	13q31.3, Xq26.2	Histotype	*THBS1* (*TSP1*), *MYLIP*, *HIPK3*, *SOCS1*	Prostate
miR-224	2×10^{-5}	0·02	2·2	Xq28	··	API5	Pancreas, liver, thyroid, ovary, AML
miR-18a	5×10^{-5}	0·04	1·7	13q31.3	··	*CTGF*, *CDKN1A*, *NR3C1* (*GR*), *THBS1* (*TSP1*), *ESR1*, *RUNX1* (*AML1*)	Pancreas, liver, AML
miR-135b	5×10^{-5}	0·04	1·6	1q32.1	··	APC	Uterus
miR-19a	0·0008	0·5	1·5	13q31.3	Histotype, progression, prognostic	*PTEN*, *THBS1* (*TSP1*), *SOCS1*	Uterus, CLL
miR-345	0·001	0·5	1·5	14q32.2	Progression	··	Prostate, thyroid
miR-191	0·002	1·0	1·3	3p21.31	··	··	Breast, colon, lung, pancreas, prostate, stomach
MicroRNAs downregulated in cancer							
miR-148a	$<1\times10^{-7}$	<0·01	0·2	7p15.2	··	*NR1I2* (*PXR*), *DNMT3B*, *TGIF2*	Lung, pancreas, prostate
miR-148b	$<1\times10^{-7}$	<0·01	0·3	12q13.13	Histotype	DNMT3B	Colon, lung, pancreas, prostate
miR-375	$<1\times10^{-7}$	<0·01	0·3	2q35	··	*JAK2*, *MTPN*, *C1QBP*, *USP1*, *ADIPOR2*, *PDK1*, *AIFM1*, *RASD1*, *EEF1E1*, *GPHN*, *ELAVL4*, *CADM1*, *PLAG1*	Pancreas

	p[†]	FDR (%)[‡]	Fold change	Chromosomal location	Gastric signature[§]	Proved targets[‖]	Cancer involvement[¶]
miR-29b-1, miR-29b-2	1×10^{-6}	<0·01	0·7	7q32.3, 1q32.2	Histotype	*TCL1A*, *DNAJB11*, *SFPQ*, *MCL1*, *DNMT3A*, *DNMT3B*, *INSIG1*, *CAV2*, *BACE1*, *COL1A1*, *COL1A2*, *COL3A1*, *FBN1*, *ELN*, *YY1*, *PIK3R1* (*p85-ALPHA*), *CDC42*, *COL4A2*, *COL5A3*, *HDAC4*, *TGFB3*, *ACVR2A*, *DUSP2*, *CTNNBIP1*	Breast, colon, lung, pancreas, prostate, thyroid, uterus, AML
miR-29c	1×10^{-5}	0·01	0·7	1q32.2	Histotype	*DNMT3A*, *DNMT3B*, *INSIG1*, *CAV2*, *COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL4A2*, *COL15A1*, *SFRS13A*, *LAMC1*, *SPARC*, *TDG*, *YY1*, *PIK3R1* (*p85-ALPHA*), *CDC42*	Breast, pancreas, liver, thyroid, oesophagus, nasopharyngeal
miR-152	1×10^{-5}	0·01	0·7	17q21.32	Histotype, progression	..	Pancreas
miR-218-2	2×10^{-5}	<0·01	0·6	5q34	Histotype, progression	*LAMB3*, *MAFG*	Lung, pancreas, prostate, stomach, liver, uterus
miR-451	6×10^{-5}	<0·01	0·4	17q11.2	..	*GATA2*, *ABCB1* (*MDR1*), *MIF*	..
miR-30d	7×10^{-5}	<0·01	0·7	8q24.22	Histotype	..	Lung, pancreas, thyroid, uterus
miR-30a-5p	7×10^{-5}	0·06	0·7	6q13	..	*NOTCH1*, *BDNF*, *BECN1*	Lung, pancreas, prostate, thyroid
miR-30b	8×10^{-5}	0·06	0·7	8q24.22	Progression	..	Pancreas, prostate, uterus, lymphoma
miR-30c-1, miR-30c-2	0·0003	0·2	0·7	1p34.2, 6q13	Histotype, progression	*CTGF*, *RUNX1* (*AML1*), *UBE2I*	Breast, colon, pancreas, prostate
miR-422b	0·0008	0·5	0·7	5q32	Progression	..	..

FDR=false discovery rate. AML=acute myeloid leukaemia. CLL=chronic lymphocytic leukaemia.

[*] These microRNAs were used in the clustering of webfigure 1.

[†] Paired class comparison.

[‡] 1% FDR predicts that this list is 99% accurate.

[§] Similarities in gastric cancer signature and other (histotype, progression, and prognostic) signatures.

[‖] Information obtained from Tarbase (http://diana.cslab.ece.ntua.gr/tarbase), miRecords (http://mirecords.umn.edu/miRecords), and previous reports.[15,17,18,24,29,30,31]

[¶] Information obtained from previous reports.[14–16,18–23,32]

Table 3

Univariate and multivariable Cox regression analysis of overall survival*

	Univariate analysis		Multivariable analysis†	
	Hazard ratio (95% CI)	**p**	**Hazard ratio (95% CI)**	**p**
Age	1·0 (0·9–1·0)	0·47	··	··
Sex				
Men	1·0 (reference)	0·33	··	··
Women	1·3 (0·7–2·5)		··	
Histological type				
Intestinal	1·0 (reference)	0·63	··	··
Diffuse	1·1 (0·6–2·1)		··	
T				
T1–T2	1·0 (reference)	0·001	··	··
T3–T4	3·0 (1·5–6·0)		··	
N				
Negative	1·0 (reference)	<0·0001	··	··
Positive	6·0 (2·3–15·5)		··	
Stage				
I–II	1·0 (reference)	<0·0001	1·0 (reference)	<0·0001
III–IV	5·2 (2·5–10·6)		4·3 (2·0–9·2)	
let-7g expression				
High	1·0 (reference)	0·003	1·0 (reference)	0·002
Low	2·6 (1·3–4·9)		2·9 (1·4–6·0)	
miR-214 expression				
Low	1·0 (reference)	0·007	1·0 (reference)	0·004
High	2·4 (1·2–4·5)		2·7 (1·3–5·6)	
miR-433 expression				
High	1·0 (reference)	0·015	1·0 (reference)	<0·0001
Low	2·1 (1·1–3·9)		3·4 (1·7–6·6)	
let-7e expression				
High	1·0 (reference)	0·009	··	··
Low	2·2 (1·2–4·2)		··	
let-7i expression				
High	1·0 (reference)	0·039	··	··
Low	1·9 (1·0–3·5)		··	

*One patient was censored before first event (patient's death) and these data were removed.

[†]For the final model of multivariable analysis, stage, let-7g, miR-214, and miR-433 were included.

Table 4

Univariate and multivariable Cox regression analysis of disease-free survival*

	Univariate analysis		Multivariable analysis†	
	Hazard ratio (95% CI)	**p**	**Hazard ratio (95% CI)**	**p**
Age	1·0 (0·9–1·0)	0·63	··	··
Sex				
Men	1·0 (reference)	0·31	··	··
Women	1·3 (0·7–2·5)		··	
Histological type				
Intestinal	1·0 (reference)	0·67	··	··
Diffuse	1·1 (0·6–2·1)		··	
T				
T1–T2	1·0 (reference)	0·001	··	··
T3–T4	3·1 (1·5–6·1)		··	
N				
Negative	1·0 (reference)	<0·0001	··	··
Positive	5·5 (2·1–14·2)		··	
Stage				
I–II	1·0 (reference)	<0·0001	1·0 (reference)	<0·0001
III–IV	4·5 (2·2–9·2)		5·2 (2·4–11·2)	
let-7b expression				
High	1·0 (reference)	0·003	1·0 (reference)	0·001
Low	2·7 (1·4–5·4)		3·2 (1·6–6·6)	
let-7g expression				
High	1·0 (reference)	0·002	1·0 (reference)	0·042
Low	2·7 (1·4–5·2)		2·0 (1·0–3·9)	
miR-19a expression				
High	1·0 (reference)	0·032	1·0 (reference)	<0·0001
Low	2·0 (1·0–3·6)		3·3 (1·7–6·5)	
miR-495 expression				
Low	1·0 (reference)	0·035	1·0 (reference)	0·007
High	1·9 (1·0–3·6)		2·4 (1·2–4·7)	
miR-410 expression				
Low	1·0 (reference)	0·016	··	··
High	2·2 (1·1–4·3)		··	
miR-433 expression				

	Univariate analysis		Multivariable analysis†	
	Hazard ratio (95% CI)	**p**	**Hazard ratio (95% CI)**	**p**
High	1·0 (reference)	0·011	··	··
Low	2·1 (1·1–4·0)		··	

*No patients were censored before first event (disease recurrence).

†For the final model of multivariable analysis, stage, let-7b, let-7g, miR-19a, and miR-495 were included.

Table 5

Multivariable Cox regression analysis of disease-free survival and overall survival of patients with intestinal-type and diffuse-type gastric cancer

	Hazard ratio (95% CI)	p
Disease-free survival		
Intestinal type (n=45)		
Stage, III–IV *vs* I–II*	3·2 (1·1–9·1)	0·032
let-7g expression, low *vs* high*	2·8 (1·0–7·8)	0·043
miR-19a expression, low *vs* high*	7·5 (2·3–24·6)	0·001
miR-495 expression, high *vs* low*	4·9 (1·7–14·3)	0·004
Diffuse type (n=56)		
Stage, III–IV *vs* I–II*	5·5 (1·9–15·7)	0·001
let-7b expression, low *vs* high*	2·6 (1·1–6·2)	0·031
Overall survival*		
Intestinal type (n=45)		
Stage, III–IV *vs* I–II*	5·7 (2·0–16·0)	0·001
miR-433 expression, low *vs* high*	4·4 (1·6–12·2)	0·004
Diffuse type (n=55)		
Stage, III–IV *vs* I–II*	6·3 (2·1–18·9)	0·001
miR-214 expression, high *vs* low*	2·7 (1·0–7·3)	0·048
miR-433 expression, low *vs* high*	2·4 (1·0–5·6)	0·050

* Reference group. For all microRNAs, patients were categorised into high-expression and low-expression groups with the same cutoff values of microRNA expression used in tables 3 and 4. Multivariable analysis was undertaken by stepwise addition and removal of covariates found to be associated with survival in tables 3 and 4. Only final models are shown.

* In overall survival of diffuse-type gastric cancer, one patient was censored before first event (patient's death) and these data were removed.