Integrated MicroRNA Network Analyses Identify a Poor-Prognosis Subtype of Gastric Cancer Characterized by the miR-200 Family

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Abstract

**Purpose:** Our aim was to investigate whether microRNAs can predict the clinical outcome of patients with gastric cancer. We used integrated analysis of microRNA and mRNA expression profiles to identify gastric cancer microRNA subtypes and their underlying regulatory scenarios.

**Experimental Design:** MicroRNA-based gastric cancer subtypes were identified by consensus clustering analysis of microRNA profiles of 90 gastric cancer tissues. Activated pathways in the subtypes were identified by gene expression profiles. Further integrated analysis was conducted to model a microRNA regulatory network for each subtype. RNA and protein expression were analyzed by RT-PCR and tissue microarray, respectively, in a cohort of 385 gastric cancer cases (including the 90 cases for profiling) to validate the key microRNAs and targets in the network. Both *in vitro* and *in vivo* experiments were carried out to further validate the findings.

**Results:** MicroRNA profiles of 90 gastric cancer cases identified two microRNA subtypes significantly associated with survival. The poor-prognosis gastric cancer microRNA subtype was characterized by overexpression of epithelial-to-mesenchymal transition (EMT) markers. This gastric cancer “mesenchymal subtype” was further validated in a patient cohort comprising 385 cases. Integrated analysis identified a key microRNA regulatory network likely driving the gastric cancer mesenchymal subtype. Three of the microRNAs (miR-200c, miR-200b, and miR-125b) targeting the most genes in the network were significantly associated with survival. Functional experiments demonstrated that miR-200b suppressed ZEB1, augmented E-cadherin, inhibited cell migration, and suppressed tumor growth in a mouse model.

**Conclusions:** We have uncovered a key microRNA regulatory network that defines the mesenchymal gastric cancer subtype significantly associated with poor overall survival in gastric cancer. *Clin Cancer Res;* 20(4); 878–89. ©2013 AACR.

Introduction

Gastric cancer is a highly aggressive and life-threatening malignancy. It is the second leading cause of cancer-related deaths worldwide, accounting for nearly 10% of all cancer deaths. More than half of the gastric cancer–related deaths occur in East Asia, mainly in China (1). The prognosis for patients with gastric cancer is heterogeneous, and the 5-year overall survival rate is only approximately 20% (2). Surgery is the mainstay of treatment, but the results are often disappointing. The lack of successful treatment strategies has led researchers to comprehensively measure genomic and epigenomic abnormalities of gastric tumors to identify gastric cancer microRNA subtypes and their underlying regulatory scenarios (3).

Accumulated evidence shows that microRNAs play important roles in gastric cancer development and progression (4). MicroRNA expression patterns can be especially rich in biologic information, as variations in expression of hundreds of protein-coding genes may, to an extent, be captured in the expression patterns of one or a few microRNAs that...
Translational Relevance
Our observations on the role of the miR-200 family in regulating epithelial-to-mesenchymal transition (EMT) enhance our understanding of the microRNA regulatory pathways influencing the clinical progression and prognosis of gastric cancer, potentially opening up a new avenue for therapeutic intervention in patients with localized primary gastric cancer.

MicroRNA Network Predicts Gastric Cancer Survival

MicroRNA expression profiling. GeneChip microRNA arrays (Affymetrix) containing 2,202 probe sets unique to pre-microRNA were analyzed according to Affymetrix protocols. Microarray processing procedures were conducted as described in the Affymetrix GeneChip Expression Analysis Manual.

mRNA expression profiling. Genechip HT HG-U133+ PM 96-array plates from Affymetrix, containing probe sets for more than 47,000 transcripts, were analyzed according to Affymetrix protocols. Sample labeling and processing, GeneChip hybridization, and scanning were conducted using the GeneTitan Instrument (Affymetrix) as the protocol described. Total RNA was isolated from liquid nitrogen–frozen gastric cancer tissues (n = 90) and normal adjacent tissues (n = 10). The total RNA was extracted and purified with TRIzol reagent (Invitrogen) and ethanol precipitation according to the instructions of the manufacturer. RNA quality and concentration were determined by NanoDrop-8000.

Statistical analysis. In the profiling phase, cluster analyses were conducted to look for natural groupings in the microRNA and mRNA expression profiles. Consensus clustering was conducted as in previous studies (9, 10). Increasing values of K (2 through 6, inclusive) were used to identify optimal segregation. For each K, 1,000 random iterations were conducted to characterize the clusters. The Benjamini–Hochberg correction was used to estimate the false discovery rate when multiple testing was applied. Consensus k-mean clustering (11) of the 90 tumor samples identified 2 robust clusters with clustering stability decreasing for k = 2–6 (Supplementary Fig. S1). Cluster significance was evaluated using SigClust (12) with 1,000 times simulation. The class boundary was statistically significant (P < 10\(^{-16}\)).

To validate the association between gastric cancer survival and expression of the candidate microRNAs and epithelial-to-mesenchymal transition (EMT) markers, the correlation of the expression of candidate microRNAs by microarray and by quantitative RT-PCR (qRT-PCR) analysis was determined by the Spearman rank test and was statistically significant. Representative qRT-PCR results are shown in Supplementary Fig. S2. For survival analysis, we used univariate and multivariate Cox proportional hazards models to estimate the HR between patients with high expression and those with low expression of candidate microRNAs and EMT markers. Variables included in the multivariate model were patients’ sex, age, smoking status, and alcohol consumption and disease characteristics, including pathologic type, differentiation, location, stage, and treatment. The Kaplan–Meier method was used to estimate the survival curves.

The following approach was used for separation of the patients into 2 groups according to relative expression levels of candidate microRNAs and EMT markers. For microRNAs, the lowest quintile values of the expression data were used as the cutoffs. For the EMT markers, values around the median expression were used as the cutoffs. Survival was
defined as the interval from the date of diagnosis until date of death from gastric cancer, date of death from other cause, or the end of follow-up (May 31, 2012), whichever came first. Patients lost to follow-up were censored at the date of last follow-up contact. Statistical analyses were conducted using R 2.10.0 (R Foundation). All P values were 2-tailed and are reported as significant when $P < 0.05$.

Results

Clinical characteristics of gastric cancer patients

A total of 385 patients with pathologically confirmed gastric cancer were included in this study. Their demographic and clinical characteristics are summarized in Supplementary Table S1. The male:female ratio was 2.7:1. The mean age of the participants at diagnosis was $60.5 \pm 9.3$ years. The median follow-up interval was 35 months (range, 1–112 months), and 180 patients died of gastric cancer during this period.

Identification of two gastric cancer subtypes with distinct prognoses

To identify microRNA subtypes of gastric cancer, consensus clustering was applied to the microRNA expression profile of 90 gastric tumors, on the basis of the most variable 50% of microRNAs across all samples. The analysis identified 2 clusters with distinct microRNA expression patterns (Fig. 1A). Cluster 1 comprised 31 gastric cancer cases that overexpressed 43 microRNAs. Cluster 2 comprised 59 gastric cancer cases that overexpressed 54 microRNAs. Survival analysis revealed that patients in cluster 1 had significantly shorter overall survival and progression-free survival than those in cluster 2 ($P = 0.050$ and $P = 0.022$, respectively; Fig. 1B and C). These microRNA subtypes remained strong predictors of survival in a multivariate Cox regression model that included sex, age, disease grade, and metastasis status (yes or no; $P = 0.015$ and $P = 0.006$ for overall survival and progression-free survival, respectively).

Functional characterization of the two gastric cancer subtypes

To determine whether the 2 gastric cancer subtypes were functionally distinct, we identified signature genes and pathways that were specifically altered in each subtype. Using the genome-wide protein-coding gene expression data on the 90 tumors, we identified 1,245 and 965 signature genes for clusters 1 and 2, respectively (Fig. 2A).
Pathway analysis of the signature genes showed that mesenchymal phenotype–related pathways, including EMT, regulation of EMT, and regulation of mesenchymal cell proliferation, were activated in cluster 1, the poor-prognosis subtype (Fig. 2B). The biosynthetic- and metabolic-related pathways were upregulated in cluster 2, the favorable-prognosis subtype (Fig. 2B). Specific investigation of the mesenchymal and epithelial markers in the 2 subtypes showed that mesenchymal markers, such as N-cadherin, vimentin (VIM), ZEB1, ZEB2, and Slug were significantly upregulated in cluster 1 compared with cluster 2 (P < 0.001, Fig. 2C). Epithelial markers, such as E-cadherin and cytokeratin were significantly downregulated in cluster 1 compared with cluster 2.

Identification of key microRNAs regulating the mesenchymal and epithelial subtypes. To predict candidate key microRNAs that play driving roles in the mesenchymal and epithelial subtypes, the MIRACLE algorithm (13) was used to identify microRNAs whose expression was significantly upregulated in one subtype compared with the other subtype and normal tissue (Supplementary Methods). This analysis revealed 24 key microRNAs for the mesenchymal subtype and 15 key microRNAs for the epithelial subtype. We next integrated the microRNA and protein-coding gene expression data to predict the potential targets for each microRNA. These analyses revealed 19 microRNAs targeting 269 genes for the mesenchymal subtype and 10 microRNAs targeting 288 genes for the epithelial subtype. Among the 39 key microRNAs identified in our analyses, 10 were predicted to regulate 79.2% (411 of 557) of all targets. Besides having binding sites on the 3'-untranslated regions (UTR) of their predicted targets, expression levels of these 10 microRNAs were inversely correlated with the expression levels of their predicted targets.

Three key microRNAs associated with gastric cancer survival. Among the 10 key microRNAs with the most targets, 6 showed significant upregulation in the mesenchymal subtype compared with both the epithelial subtype and normal tissues (Fig. 3A). Specifically, miR-125b was upregulated by more than 4-fold in the mesenchymal subtype, and its overexpression was significantly associated with poor prognosis (P = 0.01).
downregulated in the mesenchymal subtype, 3 (miR-200a, miR-200b, and miR-200c) belong to the miR-200 family (Fig. 3A). In our analysis, miR-200a \( (P = 0.05) \) and miR-200b \( (P = 0.02) \) were both associated with good gastric cancer prognosis and were predicted to target \( \text{ZEB1/2} \) and other targets (Fig. 3B and C). Detailed information about the key microRNA identification can be seen in Supplementary Table S2.

**Validation of the mesenchymal and epithelial subtypes in an independent population.** We identified an independent dataset, a genome-wide gene expression profile comprising 200 gastric cancer cases from Singapore, with which to evaluate the validity of the mesenchymal and epithelial subtypes and the microRNA regulatory network. Consensus clustering using the 164 genes in our microRNA regulatory network segregated the 200 gastric cancers into 67 mesenchymal cases and 133 epithelial cases. Consistently with our previous observation, the mesenchymal cases had significantly shorter progression-free survival \( (P = 0.02) \) than the epithelial cases (Supplementary Fig. S3).

**Validation of the association between key microRNA expression and gastric cancer survival.** We further validated the association between the expression of key microRNAs and gastric cancer prognosis among the 385 gastric cancer cases from the Tianjin Medical University Cancer Institute and Hospital. On the basis of their association with survival in the first phase of the analysis, 3 microRNAs (miR-200a, miR-200b, and miR-125b) were selected for validation. miR-200c was also selected because it is a member of the miR-200 family. Among these 4 microRNAs, 3 were significantly associated with gastric cancer survival. Interestingly, the associations of miR-200a and miR-200b with survival were significant only in women: women with higher expression of either miR-200a or miR-200b had a more favorable prognosis \( (P = 0.027 \text{ and } P = 0.048, \text{ respectively; Supplementary Fig. S4A and S4B}) \). The association of miR-125b with gastric cancer survival was significant overall: patients with higher miR-125b expression had poor prognosis \( (P = 0.005) \). Again, however, the association was significant in women \( (P = 0.002) \) but not in men \( (P = 0.1348; \text{ Supplementary Fig. S4C}) \). The associations between the expression

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**Figure 3.** Integrated analysis identifies key microRNAs that play driving roles in the mesenchymal and epithelial subtypes. A, expression of the 10 key microRNAs was compared among the mesenchymal subtype, the epithelial subtype, and the adjacent normal tissue. Four microRNAs showed significantly lower expression in the mesenchymal subtype, whereas 6 microRNAs showed significantly higher expression in the mesenchymal subtype. B, integrated analysis revealed the functional targets of the 10 key microRNAs. C, the 10 key microRNAs were ranked by number of targets; 3 microRNAs showed significant association with gastric cancer survival.

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of miR-200c and gastric cancer survival were not statistically significant. Detailed results on the associations between the expression of the 4 key microRNAs and overall survival and progression-free survival of gastric cancer are shown in Supplementary Table S3.

Validation of the association between expression of EMT markers and gastric cancer survival. To further evaluate the relationship between expression of EMT markers and gastric cancer survival, we conducted immunohistochemical analysis for 11 EMT markers in 364 gastric tumor tissues assembled on a tissue microarray. Representative cases are shown in Fig. 4A and B. Among the 11 EMT markers, 5 were associated with gastric cancer survival. Expression of E-cadherin, cytokeratin, or β-catenin was significantly associated with longer survival (log-rank test). Expression of ZEB1 or Twist2 was associated with shorter survival (log-rank test). Expression of N-cadherin was borderline associated with gastric cancer survival.
Figure 5. Overexpression of miR-200b in gastric cancer cells induces epithelial phenotype. A, changes in microRNA and mRNA levels in MGC-803 and SGC-7901 cells transfected with miR-200b or control miRNA (miR-Ctrl) as measured by real-time RT-PCR (TaqMan). Two independent time course experiments were carried out; the average ± SE (indicated by the error bars) of the 2 experiments are shown. B, MTT assay in MGC-803 and SGC-7901 cells transfected with miR-200b or miR-Ctrl. (Continued on the following page.)
with longer survival ($P < 0.0001$, $P = 0.0148$, and $P = 0.0467$, respectively, log-rank test; Fig. 4C). Expression of ZEB1 or Twist2 was associated with poor survival ($P = 0.0405$ and $P = 0.0466$, respectively, log-rank test; Fig. 4C). Expression of N-cadherin was borderline associated with gastric cancer survival ($P = 0.0627$, log-rank test; Fig. 4C). Expression of vimentin was associated with poor progression-free survival of gastric cancer (Supplementary Table S4). The associations between expression of Twist1, Sip1, Slug, or Snail and gastric cancer survival were not statistically significant. Details of the associations between the 11 EMT markers and overall survival and progression-free survival of gastric cancer are shown in Supplementary Table S4. Tumors with low E-cadherin expression exhibited a more mesenchymal phenotype, with elongated tumor cells and looser connections between tumor cells, whereas those with high E-cadherin expression exhibited more of an epithelial phenotype, such as a papillary structure that was covered by the typical cobblestone cell features, characterized by aggregated cells with typical cobblestone structure; immunofluorescence staining revealed that E-cadherin protein was localized on the membrane at cell–cell junctions, indicative of epithelial cells (Fig. 4A and B).

**miR-200b promoted the epithelial phenotype in vitro.** To determine whether forced expression of miR-200b can promote the epithelial phenotype, we transfected gastric cancer cells MGC-803 and SGC-7901 with either miR-200b mimic (miR-200b) or a scrambled negative microRNA control (miR-Ctrl). miR-200b overexpression significantly increased the expression of the epithelial marker E-cadherin in both cell lines (Fig. 5A). In addition, the growth-inhibitory effect of miR-200b was detected by MTT assay (Fig. 5B). These results suggested that cells overexpressing miR-200b gained an epithelial signature characterized by induction of E-cadherin expression and suppression of mesenchymal markers.

To further confirm these results, we conducted immunofluorescence staining to directly visualize the effect of miR-200b on E-cadherin expression, localization, and cell morphology. As shown in Fig. 5C (left), miR-200b–transfected MGC-803 and SGC-7901 cells showed epithelial cell features, characterized by aggregated cells with typical cobblestone structure; immunofluorescence staining revealed that E-cadherin protein was localized on the membrane at cell–cell junctions, indicative of epithelial cells (Fig. 5C, left). In addition, F-actin distribution was rearranged to a cortical pattern, another hallmark of the epithelial phenotype (Fig. 5C, left). In contrast, the cells transfected with miR-Ctrl showed a mesenchymal phenotype, indicated by an absence of E-cadherin on the cell membrane and rearrangement of F-actin from a cortical to a stress-fiber pattern (Fig. 5C, left). Consistently, forced miR-200b expression decreased ZEB1 expression and markedly decreased expression of mesenchymal markers vimentin and N-cadherin (Fig. 5C, right; Supplementary Fig. S5). In a Transwell invasion assay, miR-200b expression significantly decreased invaded cell numbers compared with miR-Ctrl (Fig. 5D, left). In addition, ectopic miR-200b expression decreased cell migration compared with miR-Ctrl in a wound-healing assay (Fig. 5D, right).

**Systematic delivery of miR-200b suppressed tumor growth, inhibited ZEB1, and induced E-cadherin expression in vivo.** We established a gastric cancer transplantation mouse model in BALB/C nude mice by administering a subcutaneous injection of MGC-803 cells (see Supplementary Methods for details). For this model, we used in vivo JetPEI (Polyplus Transfection) as a carrier for delivery of miR-200b, and this resulted in significant reduction in tumor volumes ($P = 0.013$; Fig. 6A and B) compared with miR-Ctrl. We conducted immunohistochemical staining of E-cadherin, N-cadherin, vimentin, and ZEB1 in the tumors to determine whether systemic delivery of miR-200b affected the expression of these EMT markers. Representative sections stained for these markers are shown in Fig. 6C. Compared with miR-Ctrl, miR-200b treatment significantly suppressed the expression of N-cadherin ($P < 0.05$; Fig. 6D), vimentin ($P < 0.05$; Fig. 6D), and ZEB1 ($P < 0.05$; Fig. 6D) and significantly induced E-cadherin ($P < 0.05$; Fig. 6D).

**Discussion**

Using integrated approaches, we have uncovered a key microRNA regulatory network that reproducibly defines the mesenchymal gastric cancer subtype significantly associated with poor overall survival. Tissue microarray validation in 385 gastric cancer cases solidified our discovery at the protein level that patients with tumors showing the mesenchymal phenotype had a poor prognosis in comparison with patients whose tumors were of the epithelial phenotype. This study is a major step forward from current approaches for predicting gastric cancer outcome in that it reveals regulatory mechanisms associated with the subtypes. In particular, our integrated analysis highlights the important role of a microRNA regulatory network consisting of 10 key microRNAs for the mesenchymal gastric cancer subtype. Notably, three of the top key microRNAs (miR-200c, miR-200b, and miR-125b) were associated with survival in both microarray discovery patients and PCR validation patients, suggesting their essential role in gastric cancer progression. Our extensive functional studies consistently validated miR-200b as a potent EMT inhibitor that may have therapeutic potential in gastric cancer, one of the most aggressive cancer types among women. To the best of
our knowledge, this is the first integrated analysis of microRNA, mRNA, and protein expression data in a study on gastric cancer survival.

The integrated profiling method has been used successfully in studies on cancer outcome. However, previous studies using microRNA profiling have identified few consistent and repeatable prognostic markers for gastric cancer (14–17). This may be due partly to population heterogeneity. Selection of markers based solely on statistical association and neglecting functional context may have made the results less reliable. The miR-200a/b identified in our study, although never reported as gastric cancer prognostic markers in previous microRNA profiling studies, is functionally related to gastric cancer.

The miR-200 family consists of 5 members organized in 2 clusters: miR-200a, miR-200b, and miR-429 on chromosome 1 and miR-200c and miR-141 on chromosome 12. So far, no population study has demonstrated an association between the miR-200 family and gastric cancer survival, whereas an in vitro study found that miR-200b has the potential to regulate metastasis in gastric cancer (18). In fact, members of the miR-200 family have been used as prognostic markers for several cancer types (19–24). The predominant function of the miR-200 family in cancer progression is...
suppression of EMT, the initiating step of metastasis. The miR-200 family has been recognized as a master regulator of the epithelial phenotype by targeting transcriptional repressors of the cell adherence gene (25, 26). Each of the 5 family members has been shown to inhibit EMT and cell migration.

EMT plays a key role in invasion and metastasis during carcinogenesis. One of the molecular hallmarks driving EMT is functional loss of E-cadherin, a cell adhesion protein and a major constituent of adherens junctions that acts as a suppressor of migration and invasion during carcinoma progression (27). The mechanism for E-cadherin transcriptional silencing during EMT has been proposed to be direct inhibition by transcriptional repressors such as ZEB, Twist, and Snail. During EMT, gastric cancer cells with fibroblastic morphologic changes show increased migration and invasiveness as a result of decreased cell–cell adhesion, and the cells then acquire a spindle-shaped, highly motile fibroblast phenotype (28). Several studies have reported associations between EMT-related proteins and tumor metastasis and prognosis in gastric cancer (29–31). Generally, loss of epithelial proteins (such as E-cadherin and cytokeratin) and/or acquisition of mesenchymal proteins [such as β-catenin (nuclear) and N-cadherin] are associated with poor tumor differentiation, advanced stage, and poor outcome in gastric cancer (30), consistent with our findings.

Target screening and luciferase assays have linked the miR-200 family with ZEB1 and ZEB2 (32). Several studies demonstrated direct binding sites for the miR-200 family on the 3’-UTR of ZEB (33, 34). It has been reported that upregulation of miR-200 reduced the expression of ZEB and increased the expression of E-cadherin in the plasma membrane. Increased expression of miR-200 in gastric cancer cells was associated with a change in their morphology to more epithelial-like and with inhibition of cellular invasion, migration, and proliferation (18). Our data suggest that miR-200a/b may negatively regulate EMT and thus result in better prognosis in gastric cancer. There was a significant inverse correlation between miR-200a/b and ZEB expression. Our study, for the first time, shows the association from a population view between the miR-200/EMT regulatory network and gastric cancer prognosis. Our study extends previous studies in identifying miR-200a/b as a prognostic marker of gastric cancer, thus capturing the biologic information of the complex EMT regulatory network in a single microRNA.

In our validation, significant association between miR-200a/b expression and gastric cancer survival was observed mainly in women, not in men. We cannot absolutely rule out chance findings in this study, although there are several studies indicating that the miR-200 family may be related to hormones (35, 36). Gastric cancer is a hormone-related cancer. Treating male mice with estrogen dramatically lowers their rates of gastric cancer (37). A population-based Swedish cohort study, designed to detect possible effects of estrogen in the etiology of gastric cancer, revealed a reduced risk of gastric cancer among a cohort of patients with prostate cancer, most of whom had received estrogen treatment (38). Male and female gastric cancers differ in their etiology, and it is possible that the miR-200 family is functionally dependent on estrogen and affects gastric cancers more in women than in men.

The expression of miR-200c was not associated with gastric cancer prognosis in our study. The members of the miR-200 family largely target a common subset of genes that includes ZEB, and members from each cluster are co-expressed. However, the expression of miR-200 family members in the 2 gastric cancer clusters does not appear to be highly correlated (32, 39). Expression of miR-200a and miR-200b was highly correlated, but their expression was not significantly correlated with miR-200c. More often than not, no synergy is shown between the two clusters of the miR-200 family. Hur and colleagues investigated the role of miR-200 members in the pathogenesis of metastatic colorectal cancer and found that miR-200c, but not miR-200a/b, plays an important role in mediating EMT and metastatic behavior in the colon (40). In a similar study on ovarian cancer, researchers found that low-level expression of the miR-200a/b cluster predicts poor survival (19).

This study has several limitations. First, miR-125b, as a well-known oncomiR, has been associated with poor survival in many cancer types, including gastric cancer. Ueda and colleagues identified miR-125b as the most important progression-related signature of gastric cancer among 237 microRNAs analyzed (41). miR-125b may act as an oncogene in gastric cancer by dysregulating gastric cell proliferation and apoptosis (42). A recent study found miR-125b expression correlates inversely with HER2 status, and dysregulation of miR-125b and HER2 is an early event in the gastric (intestinal-type) oncogenesis (43). In our integrated data analysis, no cancer-related regulatory network was constructed specifically for miR-125b. The underlying mechanism for its association with gastric cancer survival has yet to be explored. Second, we focused on EMT, so other functional pathways such as nucleotide metabolism and transcription regulation were not explored. Although EMT is closely related to both the miR-200 family and gastric cancer progression, other pathways may also hold great insight into the differential survival of the 2 subtypes of gastric cancer.

Our observation on the role of the miR-200 family (miR-200a/b) in regulating EMT through ZEB1 and E-cadherin enhances our understanding of the microRNA regulatory pathways influencing the clinical progression and prognosis of gastric cancer, especially in women. The miR-200 family may serve as a good prognostic marker for gastric cancer, potentially opening up a new avenue for therapeutic intervention in patients with localized primary gastric cancer. Further studies are warranted to replicate our findings in different populations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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