

RESEARCH PAPER

Role of miR-27a, miR-181a and miR-20b in gastric cancer hypoxia-induced chemoresistance

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ABSTRACT

Despite the search for new therapeutic strategies for gastric cancer (GC), there is much evidence of progression due to resistance to chemotherapy. Multidrug resistance (MDR) is the ability of cancer cells to survive after exposure to chemotherapeutic agents. The involvement of miRNAs in the development of MDR has been well described but miRNAs able to modulate the sensitivity to chemotherapy by regulating hypoxia signaling pathways have not yet been fully addressed in GC. Our aim was to analyze miR-20b, miR-27a and miR-181a expression with respect to (epirubicin/oxaliplatin/capecitabine (EOX)) chemotherapy regimen in a set of GC patients, in order to investigate whether miRNAs deregulation may influence GC MDR also via hypoxia signaling modulation. Cancer biopsies were obtained from 21 untreated HER2 negative advanced GC patients, retrospectively analyzed. All patients received a first-line chemotherapy (EOX) regimen. MirWalk database was used to identify miR-27a, miR-181a and miR-20b target genes. The expression of miRNAs and of *HIPK2*, *HIF1A* and *MDR1* genes were detected by real-time PCR. *HIPK2* localization was assessed by immunohistochemistry. Our data showed the down-regulation of miR-20b, miR-27a, miR-181a concomitantly to higher levels of *MDR1*, *HIF1A* and *HIPK2* genes in GC patients with a progressive disease respect to those with a disease control rate. Moreover, immunohistochemistry assay highlighted a higher cytoplasmic *HIPK2* staining, suggesting a different role for it. We showed that aberrant expression of miR-20b, miR-27a and miR-181a was associated with chemotherapeutic response in GC through *HIF1A*, *MDR1* and *HIPK2* genes modulation, suggesting a possible novel therapeutic strategy.

Abbreviations: cHIPK2, cytoplasm *HIPK2*; DCR, disease control rate; EOX, epirubicin/oxaliplatin/capecitabine; GC, gastric cancer; MDR, multidrug resistance; nHIPK2, nuclear *HIPK2*; PD, progression disease

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Introduction

Gastric cancer (GC) is the sixth leading cause of cancer in Europe and the second leading cause of mortality worldwide.¹ 65% of patients with GC present an advanced or metastatic stage at diagnosis.² In this setting, Her2-neu positive GC patients receive first line therapy containing Trastuzumab associated with chemotherapy, achieving a median overall survival of 13.8 months. Conversely, Her2-neu negative patients who can be treated with chemotherapy alone have a shorter survival.³ Currently for this latter group of patients the gold standard includes treatment with the triplet formed by epirubicin, oxaliplatin, and capecitabine (EOX).^{4,5} Despite the search for new therapeutic strategies with targeted therapy in GC (future oncology), there is much evidence of progression under 12 months of diagnosis due to resistance to chemotherapy, the greatest obstacle to treatment success.⁶

Multidrug resistance (MDR) is the ability of cancer cells to survive after exposure to different chemotherapeutic agents and constitutes the most important obstacle for the effectiveness of

cancer treatment. Recently, the role of the hypoxic tumor micro-environment in chemotherapy failure has been increasingly considered. Emerging evidence has described the role of hypoxia inducible factor-1 (HIF-1 α) as a transcriptional regulator of P-glycoprotein (P-gp) expression.^{7,8} P-gp is a product of the *MDR1* gene and is known to play a role in the efflux of drugs from cancer cells.⁹ Among the mechanisms underlying MDR, the over-expression of P-gp seems to be the major form of resistance to chemotherapy. In the last years, increasing evidence has highlighted the contributions of homeodomain-interacting protein kinase-2 (*HIPK2*) in counteracting hypoxia-induced cancer chemoresistance. Besides its role as a regulator of p53 apoptotic function, *HIPK2* exerts antitumor activity by modulating the transcription activity of several transcription factors, including HIF-1 α . Inhibition of HIF-1 α activity by *HIPK2* reduces the expression of *MDR1* and stimulates drug-induced apoptosis in p53-dependent and -independent ways.¹⁰⁻¹² Recently, microRNAs (miRNAs), small non coding RNAs that act as post-transcriptional repressors of gene expression, have been suggested to

play essential roles in modulating chemotherapeutic tumor response.¹³⁻¹⁷ We selected some miRNAs that recent literature and bioinformatics tools have highlighted to be involved in *MDR1*, *HIF1A* and *HIPK2* gene modulation. Several studies have suggested the ability of miR-27a to modulate *MDR1*/P-gp expression¹⁸⁻²⁰ and the association of miR-181a deregulation with the modulation of *HIPK2*.²¹ Moreover, emerging evidence documented miR-20b as a direct regulator of *HIF1 α* expression.²² Given that hypoxia is one of the major factors that promotes MDR, we investigated whether miRNA deregulation may influence gastric cancer MDR by regulating the expression of the *MDR1* gene also via hypoxia signaling modulation. For this purpose, we analyzed the expression of miR-20b, miR-27a and miR-181a with respect to a first-line chemotherapy (epirubicin/oxaliplatin/capecitabine (EOX)) regimen in a set of GC patients, in order to investigate their eventual predictive role.

Results

Analysis of miR-27a, miR-181a and miR-20b and their target genes *HIF1A*, *MDR1* and *HIPK2*

The miRNA target analysis tool miRWalk²³ was used to screen validated targets of miR-27a, miR-181a and miR-20b: *HIF1A* was found to be a validated target of miR-20b, *MDR1* a validated target of miR-27a and *HIPK2* gene regulated by miR-181a and miR-27a. To confirm the targeting of *HIF1A*, *MDR1* and *HIPK2* by miR-27a, miR-181a and miR-20b, a correlation analysis was carried out. A negative correlation was found between each gene *HIF1A*, *MDR1* and *HIPK2* and the correlated miRNA (*HIF1A*/miR-20b: Spearman $r = -0.21$; $P = 0.34$; *MDR1*/miR-27a: Spearman $r = -0.5$; $P = 0.01$; *HIPK2*/miR-181a: Spearman $r = -0.4$; $P = 0.06$; *HIPK2*/miR-27a: Spearman $r = -0.66$, $P = 0.0009$) (Figs. 1A-D).

miRNAs and target genes expression with regards to clinico-pathological characteristics

The clinico-pathological characteristics of all advanced GC patients are summarized in Table 2. The expression level of miR-27a, miR-181a and miR-20b was evaluated with regards to different clinico-pathological features in order to highlight their eventual prognostic role in GC patients. Lower median expression levels of all 3 miRNAs resulted associated with wider nodal involvement, older age (>63 years) and male gender as reported in Table 2.

The expression of the 3 miRNA target genes *HIF1A*, *MDR1* and *HIPK2* was studied. Interestingly, an inverse trend of gene expression was highlighted compared to that of miR-27a, miR-181a and miR-20b. Higher median expression levels of *HIF1A*, *MDR1* and *HIPK2* were found in gastric N3 tumors and in males, while no difference was found in gene expression with respect to tumor size (T) or GC patient age.

Analysis of miR-27a, miR-181a and miR-20b and treatment response

To understand if miR-27a, miR-181a and miR-20b can regulate response to chemotherapy, patients were divided into those

with progression disease (PD) ($n = 15$) and those with disease control rate (DCR) ($n = 6$). The expression of miR-27a, miR-181a and miR-20b and of their target genes was measured with respect to the response.

The median levels of the 3 miRNAs were lower in GC patients with PD compared to those with DCR (miR-27a: 0.18 vs 2.04; $P = 0.07$; miR-181a: 0.63 vs 1.08; $P = 0.72$ and miR-20b: 0.21 vs 0.4; $P = 0.21$) (Fig. 2A). Also considering median expression level as cutoff, all 3 miRNAs resulted more frequently overexpressed in GC patients with DCR compared to those with PD (miR-27a: 83.3% vs 40%; $P = 0.14$; miR-181a: 66.7% vs 46.7%; $P = 0.63$ and miR-20b: 83.3% vs 40%; $P = 0.14$). Probably the low number of the enrolled cases prevented reaching a statistical significance (Fig. 2B).

We investigated the expression of the 3 genes in the treatment response GC subgroups. The median expression level of the 3 genes resulted higher in GC patients with PD compared to those with DCR (Fig. 2C). However, when considering the frequency of *HIF1A*, *MDR1* and *HIPK2* overexpression, a higher percentage of *HIF1A* and *MDR1* overexpression was observed in advanced GC with PD compared to those with DCR (*HIF1A*: 60% vs 33.3%; $P = 0.36$; *MDR1*: 66.7% vs 16.7%; $P = 0.06$). On the contrary, there was no difference in the frequency of *HIPK2* gene overexpression in the 2 GC subgroups (Fig. 2D). The role of the 3 genes in PD patients was stressed by the correlation among gene expression: a positive and significant correlation was observed between *HIF1A* and *HIPK2* (Spearman $r = 0.97$; $P < 0.0001$), between *HIF1A* and *MDR1* (Spearman $r = 0.63$; $P = 0.01$) and finally between *HIPK2* and *MDR1* (Spearman $r = 0.69$; $P = 0.003$) (Figs. 3A-3B). Conversely, a significant relationship between *HIF1A* and *MDR1* (Spearman $r = 0.94$; $P = 0.01$) was found in GC patients with DCR.

HIPK2 subcellular localization was explored by immunohistochemistry assay. In detail, cytoplasmic *HIPK2* (c*HIPK2*) (25%, range 0-60%) was much more expressed than nuclear *HIPK2* (n*HIPK2*) (3.7%, range 0-8%) ($P = 0.0009$) (Fig. 4).

Discussion

MDR, defined as a lack of sensitivity of cancer cells to different drugs, remains the major clinical obstacle to the effectiveness of chemotherapy. Recently, miRNAs have been suggested to play a key role in the development of MDR.²⁴ Through regulating gene expression, miRNAs can impact on different pathways associated with sensitivity or resistance to chemotherapeutic drugs in various tumors.¹³⁻¹⁷ Several miRNAs, such as miR-106a,²⁵ miR-508-5p²⁶ and miRNA-19a/b²⁷ have been reported to play a role in the development of MDR in GC. However, miRNAs able to modulate the sensitivity to chemotherapy by regulating genes involved in the hypoxia signaling pathways have not yet been fully addressed in GC. In the last years, the influence of hypoxia on chemotherapeutic effects has been well described,²⁸ thus becoming one of the main focuses of research in cancer treatment. In this study, we selected miR-27a, miR-181a and miR-20b because of literature and bioinformatics tools shown to be involved in *MDR1*, *HIF1A* and *HIPK2* genes modulation. With regard to treatment

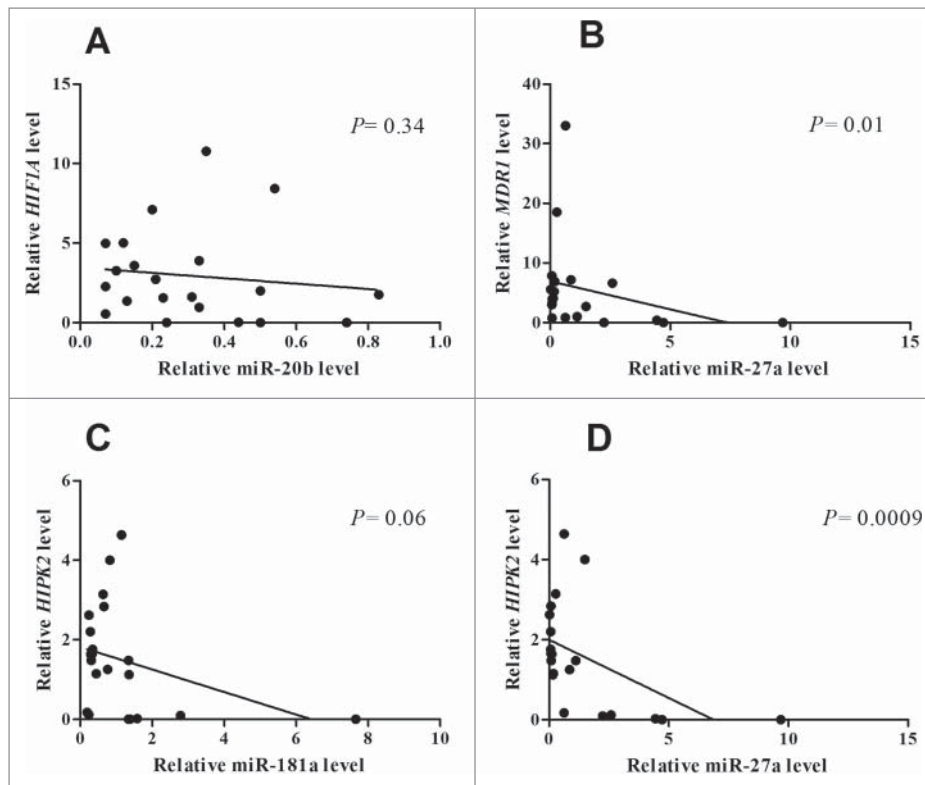


Figure 1. Correlation analysis between *HIF1A* and miR-20b expression (A) *MDR1* and miR-27a levels (B) and *HIPK2* and miR-181a (C) and *HIPK2* and miR-27a (D) in GC patients.

response, higher median values of *HIF1A* gene expression were observed in the PD subgroup. Interestingly, in line with the literature which reported the ability of miR-20b to target the *HIF1A* transcript,²² lower expression levels of miR-20b were found in the GC subgroup showing elevated amounts of *HIF1A* mRNA. Moreover, the negative correlation observed between miR-20b and *HIF1A* suggested a direct regulation of this miRNA on *HIF1A*. With regards to the impact of hypoxia on the MDR, mounting evidence has recently described the influence of increasing HIF-1 α levels on the expression and transport function of P-gp.^{7,8,29} P-gp is the product of the *MDR1* gene able to regulate the

bioavailability of drugs and its elevated levels represent the most highly frequent feature related to the MDR phenotype.³⁰ In addition to higher levels of *HIF1A* mRNA, patients with PD also showed a high expression of the *MDR1* gene, suggesting a close association between the 2 genes also confirmed by their positive correlation. Besides a transcriptional regulation of the *MDR1* gene by HIF1 α , we also supposed an epigenetic modulation of this gene. For this purpose, we explored the expression of miR-27a that both the literature and bioinformatics tools highlighted to target *MDR1*. In line with Feng et al's study³¹ in which miR-27a was inversely correlated with *MDR1* and transfection of exogenous miR-27a down-regulated *MDR1*, our results showed a significant and negative correlation between miR-27a and *MDR1* transcript. When miRNA expression was evaluated with respect to chemotherapy response, lower values of miR-27a and a higher expression of the *MDR1* gene were observed in patients with a PD. Both a direct and an indirect regulation on *MDR1* by miR-27a was described,³² although a downregulation of miR-27a with a decreased expression of P-gp was reported in GC.³³ Interestingly, bioinformatics software (mirWalk)²³ indicated *HIPK2* to be a validated target of miR-27a as well as of miR-181a. Recent studies have demonstrated the capacity of miR-27a to modulate *MDR1*/P-glycoprotein expression by targeting *HIPK2* in human ovarian cancer cells,¹⁸ highlighting a new player in hypoxia-mediated chemoresistance. *HIPK2* is a tumor suppressor gene involved in the activation of p53 pro-apoptotic function, but it also acts as a co-repressor for many transcription factors, such as HIF1 α .¹¹ Inhibition of HIF-1 α

Table 1. Characteristics of GC patients.

Response to chemotherapy	n
PD patients	15
DCR patients	6
Age	
≤63	11
>63	10
T	
II-III	15
IV	6
Lymph nodes	
N0-1-2	8
N3	13
Metastatic status	
M+	21
M0	0
Gender	
F	10
M	11

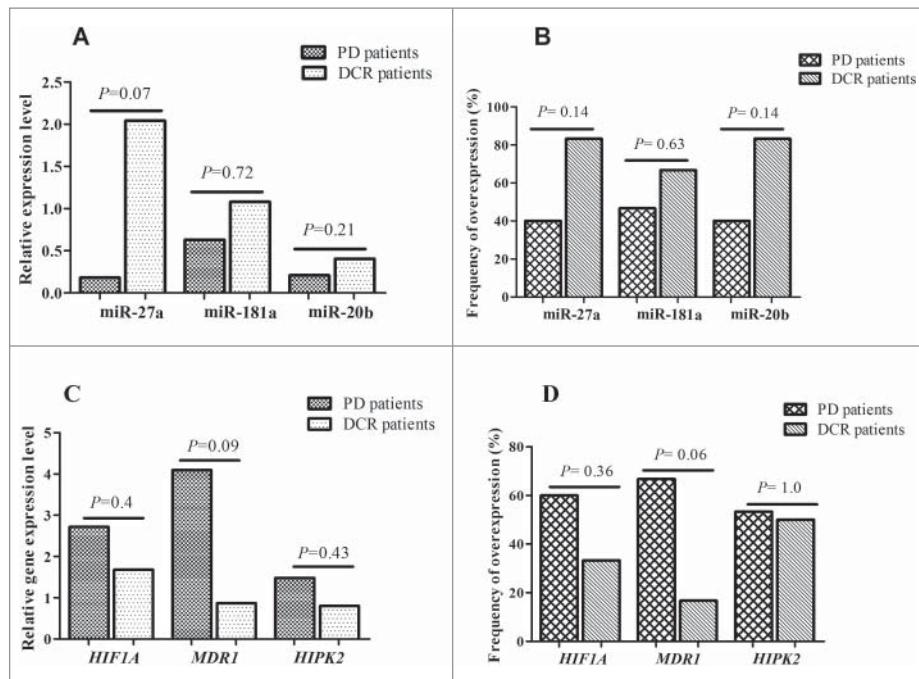


Figure 2. Median levels of miR-27a, miR-181a and miR-20b in GC patients with PD vs those with DCR (A). Percentage of miR-27a, miR-181a and miR-20b overexpression in GC patients with PD vs those with DCR (B). Median levels of *HIF1A*, *MDR1* and *HIPK2* in GC patients with PD vs those with DCR (C). Percentage of *HIF1A*, *MDR1* and *HIPK2* overexpression in GC patients with PD vs those with DCR (D).

activity by HIPK2 reduces *MDR1* gene expression and restrains HIF-1-induced tumor chemoresistance.¹² Despite the extensive knowledge on HIPK2 function in many tumors,³⁴ there are no reports about the role of HIPK2 in GC. Unexpectedly, in contrast to its tumor suppressor role, elevated levels of HIPK2 mRNA were observed in PD patients showing a lower expression of both miR-27a and miR-181a. A recent study reported a down-regulation of miR-181a with a subsequent modulation of HIPK2 after cisplatin treatment in neck squamous cell carcinoma,²¹ but in our study the role of HIPK2 in hypoxia-mediated MDR became less clear. Recently it has been reported that HIPK2 is not exclusively localized in the cell nucleus in which it exerts an pro-apoptotic role, but it can also be distributed into the cytoplasm^{35,36} where it was found associated to High-Mobility Group A1 (HMGA1) overexpression leading to inhibition of p53-mediated apoptosis.³⁷ A dual role depending on subcellular distribution has been recently described for HIPK2,³⁸ but it is still unclear whether altered HIPK2

localization could differently affect hypoxia-mediated chemoresistance. In order to verify the sublocalization of HIPK2 in patients of our cohort, an immunohistochemistry assay was performed. Interestingly, the immunohistochemistry assay revealed higher cytoplasm HIPK2 than nuclear dislocation in both the DCR and PD subgroups. No data are available on the link between cytoplasmic HIPK2 and HIF1 α or P-gp, but we speculated that the cytoplasm dislocation of HIPK2 could be a mechanism of tumors cells to contrast HIPK2 function on hypoxia-mediated MDR. Our results showed a significant and positive correlation of *HIPK2* with both *HIF1A* and *MDR1* genes only within PD in the GC subset, suggesting a close and diverse association of HIPK2 to hypoxia in non-responder GC patients.

In this study, we explored miRNA-mediated MDR by affecting hypoxia signaling in GC. We showed that aberrant expression of miR-20b, miR27a and miR-181a was associated with chemotherapeutic response in GC through *HIF1A*, *MDR1* and *HIPK2* gene modulation, suggesting a possible novel strategy

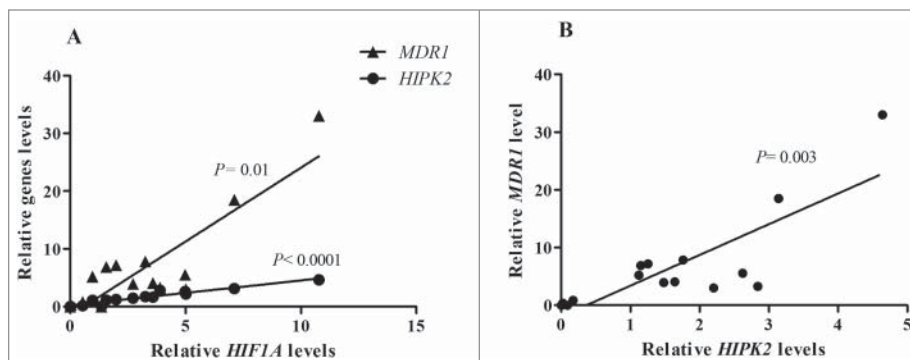


Figure 3. Correlation analysis between *HIF1A* and *HIPK2* levels (A) *HIF1A* and *MDR1* levels (A) and *HIPK2* and *MDR1* (B) in GC patients with PD.

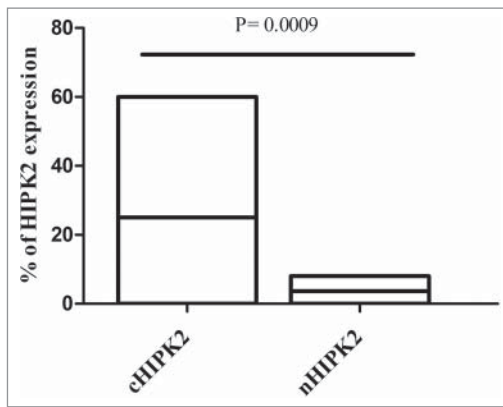


Figure 4. Percentage of HIPK2 protein expression in cytoplasm and nucleus of GC patients. Bars in the histogram represent percentage range and median value of HIPK2 protein expression.

for the reversal of the hypoxia effect on MDR in GC. These results merit confirmation in larger, prospective clinical series to better understand the correlation between miRNA and MDR associated genes.

Materials and methods

Patients and methods

Samples of cancer biopsy were obtained from 21 untreated HER2 negative advanced GC patients, retrospectively analyzed, from the tissue bank of the IRCCS, Istituto Tumori “Giovanni Paolo II” in Bari and of the Morgagni Pierantoni Hospital in Forlì (Italy). All patients received an intravenous bolus of epirubicin at a dose of 50 mg/m² (day 1) in combination with oxaliplatin at a dose of 130 mg/m² (day 1) and capecitabine at a twice-daily dose of 625 mg/m² (days 1→21) (EOX) in a cycle scheduled every 3 weeks. According to the Response Evaluation Criteria in Solid Tumors (RECIST) definition 1.1, all patients were evaluated after 3 cycles of treatment.³⁹ Information about clinical parameters such as sex, age, tumor size, lymph nodes status and response to chemotherapy were obtained from clinical records.

All patients were reviewed and the following data were collected (Table 1): gender (male versus female), age, tumor size, lymph nodes status and response to chemotherapy (CR:

complete response; PR: partial response; ORR, objective response rate: CR + PR; SD: stable disease; DCR, disease control rate: ORR + SD, PD: progression disease).

This study was approved by the Ethics Committee of the IRCCS Istituto Tumori “Giovanni Paolo II,” Bari, Italy (prot. n. 312/2015). All patients signed an informed consent and all data have been processed with respect for privacy and anonymity.

RNA extraction and miRNA detection

MiRNA expression analysis was performed on the set of GC samples stratified into 15 patients with PD and 6 with DCR.

Total RNA was extracted from formalin-fixed, paraffin-embedded GC and matched adjacent non-cancerous tissues by the RNeasy FFPE Kit (QIAGEN) according to the manufacturer’s protocol. Concentrations were estimated with the ND-8000 Spectrophotometer (NanoDrop Technologies). Briefly, for detection of miR-27a, miR-181a and miR-20b expression levels, 10 ng of total RNA were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit using miRNA specific primers according to the manufacturer’s protocol (Applied Biosystems). Real Time PCR analysis was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 μl of RT products in a reaction mixture containing TaqMan miRNA assay and the TaqMan Universal PCR Master Mix, according to the manufacturer’s instructions (Applied Biosystems). All PCR reactions were performed in duplicate. Relative quantities of each miRNA were calculated using the ΔΔCt method after normalization with endogenous reference RNU 48.

HIF1A, MDR1 and HIPK2 expression

Total RNA was extracted from FFPE GC tissues, as described above. The concentration of the isolated RNA was measured by a NanoDrop 8000 Spectrophotometer v2 1.0 (Thermo Scientific). Total RNA was reverse transcribed to single-stranded cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Reaction was performed using 500 ng of total RNA in a total volume of 20 μl according to the manufacturer’s protocol (Applied Biosystem). cDNA synthesis was performed at 25°C for 10 min, then the reaction was incubated at 37°C for 120min followed by 85°C for 5min. Quantitative real-time PCR was performed using 62.5ng of cDNA in a final

Table 2. Correlation between miR-27a, miR-181a, miR20b and relative target genes expression with clinicopathologic characteristics in GC patients.

	miR-27a median level		miR-181a median level		miR-20b median level		MDR-1 median level		HIF1A median level		HIPK2 median level	
Age												
≤63 (n = 11)	1.11	P = 0.13	1.14	P = 0.2	0.33	P = 0.41	3.02	P = 0.91	1.99	P = 0.75	1.25	P = 0.77
>63 (n = 10)	0.13		0.39		0.22		3.6		2.16		1.56	
T												
II-III (n = 15)	0.62	P = 0.87	0.66	P = 0.53	0.24	P = 0.45	3.94	P = 0.81	2.27	P = 0.61	1.25	P = 0.34
IV (n = 6)	0.69		0.72		0.27		1.83		1.68		1.56	
Lymph nodes status												
N0-1-2 (n = 8)	1.17	P = 0.36	0.79	P = 0.46	0.38	P = 0.27	0.80	P = 0.05	1.48	P = 0.32	0.71	P = 0.58
N3 (n = 13)	0.18		0.44		0.21		5.22		2.72		1.48	
Gender												
F (n = 10)	1.3	P = 0.34	1.08	P = 0.5	0.32	P = 0.37	1.83	P = 0.06	1.68	P = 0.54	1.3	P = 0.39
M (n = 11)	0.27		0.63		0.23		5.54		2.72		1.48	

volume of 20 μ l according to the manufacturer's instructions (Applied Biosystems), on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The ID assays used were the following: human HIF1A (Hs00153153_m1), human MDR1 (Hs00184500_m1), human HIPK2 (Hs00179759_m1). RN18S1 (Hs03928985_g1) was used as the endogenous reference. The samples underwent PCR analysis using the following cycling parameters: at 50°C for 2 min, at 95°C for 10 min, then at 95°C for 15 s and at 60°C for 1 min, for 45 cycles. Relative expression was calculated using the comparative Ct method. Each sample was tested in duplicate.

HIPK2 protein level and cellular dislocation were detected by immunohistochemistry. 4- μ m-thick slices were cut from formalin-fixed and paraffin-embedded histological blocks and these were immunohistochemically stained using standard immunoperoxidase techniques.⁴⁰ The primary antibody was mouse monoclonal anti-HIPK2 (ab57328; Abcam; 1:300 dilution). Human gastric tissues were used as positive control and the primary antibody was omitted and replaced by PBS pH7.6 for negative control.

Statistical analysis

Data analysis was performed using the GraphPad Prism statistics software package (GraphPad Prism 5.01). Statistical significance was determined using the Mann–Whitney U-test. The two-tailed Fischer's exact test was used to compare both miRNAs and gene frequency of overexpression between the GC subgroups. The cases were divided into 2 groups with the expression above and below the median expression of miRNAs or genes.

The Spearman correlation coefficient was used to analyze the correlations between gene expression or between miRNAs and their targets. A P value \leq 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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