Validation of miRNAs as Gastric Cancer Biomarkers in Serum and Effect of Chemotherapy on miRNA Expression in a Gastric Cancer Cell-Line

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***Abstract*— Gastric cancer (GC) is one of the most prevalent and lethal cancers. Its asymptomatic nature results in late diagnosis, and thus, poor prognosis. Recent literature[1] has suggested that miRNAs are promising diagnostic biomarkers for GC. The first part of the study aims to validate 4 miRNAs (miR-101-3p, miR-486-5p, miR-107, miR-27a-3p) as diagnostic GC biomarkers. 4 miRNAs were identified to be upregulated or downregulated in serum of GC patients. A combined index comprising these 4 miRNAs was highly significant in discriminating between GC and control samples (p=0.01). The results confirm our hypothesis that these 4 miRNAs can distinguish between GC and non-cancer. Recent literature has also shown that miRNAs influence numerous cancer-relevant processes such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism.[2] The second part of the study aims to find out if miRNA (intracellular and extracellular) expression in gastric cells is influenced by chemotherapeutics. Individual miRNAs were identified to be specifically and highly secreted within and/or outside of the cell, and could potentially serve as drug response biomarkers to evaluate clinical benefit. miRNAs play an active role in drug treatment, and could potentially serve as prognostic biomarkers for chemotherapy, as well as therapeutic targets for gene therapy, given their close relationship. The findings are very promising, albeit with substantial limitations due to small sample size. Further studies with expanded sample sizes are needed to further evaluate the clinical usefulness of miRNAs as biomarkers for GC. The involvement of miRNAs in cancer-relevant processes could also be investigated.**

Keywords- gastric cancer, miRNAs, biomarkers, chemotherapy

I. INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and one of the leading causes of cancer-related deaths in the world.[3] GC is either asymptomatic or causes nonspecific symptoms[4], and is therefore often diagnosed at late stages, resulting in poor prognosis. A definitive diagnosis of GC requires a gastroscopic or surgical biopsy[4], which can provide accurate diagnosis but is invasive and costly as a screening tool. Better methods are needed for early diagnosis of GC, for improving and predicting patient outcomes. Biomarkers for early diagnosis and for predicting survival and response will be clinically useful. Carcinoembryonic antigen (CEA), CA19-9, CA72-4[5], pepsinogen and *Helicobacter pylori* antibodies were studied as potential biomarkers of GC. However, relatively low sensitivity and specificity in the diagnosis and prognosis of GC limit their further use. Therefore, searching for more specific and more sensitive novel markers to GC is urgently required for the establishment of more accurate screening strategies. In recent years, miRNAs have been identified as potential diagnostic biomarkers for GC. miRNAs are small non-coding RNA molecules, about the length of 22 nucleotides, which functions in transcriptional and post-transcriptional regulation of gene expression.[6] Recent data[7] has suggested that miRNAs are promising biomarkers for GC, including disease susceptibility, diagnosis, prognosis, response to therapy, and overall survival. Previous studies have shown that 4 miRNAs (miR-101-3p, miR-486-5p, miR-107, miR-27a-3p)[8] are potential GC biomarkers. One of two objectives of this study was to test the hypothesis that these 4 miRNAs may serve as potential blood-borne diagnostic GC biomarkers in a new, independent set of 5 GC and 5 control samples. Recent studies have also shown that miRNAs influence numerous cancer-relevant processes such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism.[9] Given that miRNA has profound impact on pathogenesis of a variety of cancers, it is likely that chemotherapy could influence the expression levels of these miRNAs, by modifying the regulation of physiological processes. The other objective of this study was to investigate the effect of chemotherapeutics on the expression of miRNA (intracellular and extracellular) in gastric cells. The results indicate the possibility of the use of miRNAs as a predictor of tumour response to conventional chemotherapy.

II. HYPOTHESIS

The 4 miRNAs (miR-486-5p, miR-101-3p, miR-27a-3p, miR-107) can serve as biomarkers for GC, if there are significant difference between GC and high-risk samples. Another hypothesis is that these miRNAs are expressed in gastric cancer cells and the expressions (intracellular and extracellular) are influenced by chemotherapeutics.

III. Materials and Methods

A. Validation of miRNAs as Gastric Cancer Biomarkers in Serum

1) Clinical Samples

Serum miRNAs were isolated from 5 GC samples, 5 control high risk samples and 2 reference samples using the miRNeasy Serum/Plasma Kit. The GC samples consist of 1 Stage I GC sample, 2 Stage II GC samples and 2 Stage III GC samples. The control samples belong to patients with high risk of GC with some form of gastric disease and the reference samples belong to healthy patients. Before experimentation, all samples were randomized and split into two different groups which experiments were conducted by two different operators.

2) Total RNA Extraction

RNAs were isolated from serum sample using QIAGEN® miRNeasy Serum/Plasma Kit, following the Quick-Start Protocol: A reagent containing QIAzol® and Isolation Spike-in incl MS2 Carrier RNA was added to each clinical sample. After vortexing and incubation at room temperature, chloroform was added. The samples were then centrifuged for 15 minutes at 12000g at 4ºC. The aqueous phase was then removed and purified RNA was isolated from the aqueous phase in the QIAcube after undergoing a series of washing and spin-down.

3) RT-PCR and qPCR

The RNA containing miRNA was mixed with conformational constrained reverse transcription primers in appropriate reaction buffer conditions. A reverse transcriptase was then added to the RNA samples to reverse transcribe RNA into cDNA to be used in real-time polymerase chain reaction (qPCR). qPCR was then used to amplify and simultaneously quantify miRNAs with different miRNA specific primer sequences to obtain the ct values that will be used in statistical analysis. All qPCR assays and workflows were provided by MiRXES, Singapore.

4) Statistical Analysis

Ct values of each duplicate were obtained from qPCR graphs and averaged to minimize random error, and standard deviation was calculated to access accuracy. The significance of plasma miRNA level was determined using established data analysis models: standard deviation, arithmetic average, geometric average and Student's t-test.

B. Effect of Chemotherapy on miRNA Expression in a Gastric Cancer Cell-Line

1) Clinical Samples

GC cells (YCC-16), originally established an advanced gastric cancer patient (provided by Singapore Gastric Consortium), were cultured and treated over 72 hours, in 24-well plates. Wide concentration screening was done before finalising on six conditions for the dose response trial. For Fluorouracil (5-FU), the concentrations in millimole are 0 (Control), 0.00025, 0.0005, 0.001, 0.01 and 0.1; for Doxorubicin, the concentrations in micromole are 0 (Control), 0.0001, 0.001, 0.01, 0.05 and 0.25. The treated cells and supernatant, which contains excreted RNA, were collected in separate tubes. The cells were lysed with TRI Reagent® to ensure that all the expressed RNA are released and measured later.

2) Total RNA Extraction, RT-PCR and qPCR

Identical to Validation of miRNAs as Gastric Cancer Biomarkers in Serum.

3) Augmentation, cDNA Dilution

cDNA is augmented with Applied Biosystems® to ensure that there are sufficient copies for amplification in qPCR. cDNA dilution was conducted to obtain a suitable concentration of cDNA to be used in qPCR.

4) Statistical Analysis

Raw qPCR data was normalised against the geometrical mean spike-ins and analysed*. A miRNA secretion profile was plotted to distinguish between miRNAs secreted intracellularly and extracellularly, and identify highly secreted miRNAs. Heat maps were plotted to observe the global change in (intracellular and extracellular) miRNA expression levels with increasing concentration of drugs (5-FU/Dor) administered. Two dose response curves were plotted to illustrate the effect of increasing concentration of drugs administered on the percentage of live cells relative to control, as well as to compare the effectiveness of the two drugs using the half maximal inhibitory concentration (IC_{50}). Graphs for individual miRNAs, which were significantly upregulated or downregulated intracellularly and/or extracellularly were plotted for further analysis.

*Graphs and heat maps were generated by an established researcher, Dr Zou Ruiyang, with an original algorithm.

IV. Results and Discussion

A. Validation of miRNAs as Gastric Cancer Biomarkers in Serum

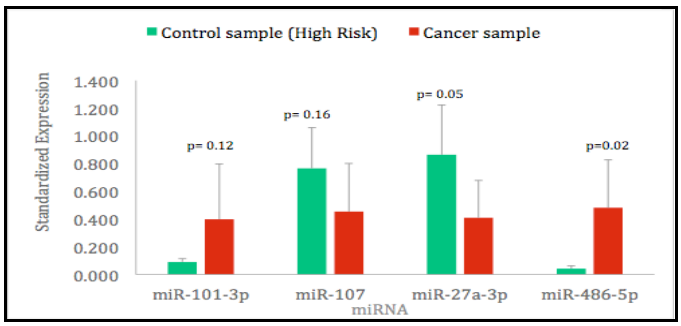

Figure 1. miRNA Expression in High Risk and Cancer Patients

As seen from Figure 1, miR-101-3p from the GC serum was up-regulated as compared to that of the high risk sample. miR-486-5p was also up-regulated, and the up-regulation of this miRNA was more significant than that of miR-101-3p (p=0.02 and p=0.12 respectively). miR-107 and miR-27a-3p were down-regulated, though the down-regulation of miR-27a-3p was more notable than that of miR-107 (p=0.05 and p=0.16 respectively). The more overexpressed or underexpressed the miRNAs are, the more specific and sensitive they are. The error bars suggest that the values range quite a bit, and thus,

there was uncertainty in the measurement. However, this can be attributed to the limited sample size, as only 5 samples were used.

Type of sample	Sample ID	Score = 107*27a-3p / (101-3p * 486-5p) * 100
Control(High Risk)	N1264/CSW	0.66
Control(High Risk)	N1302/YHC	0.66
Control(High Risk)	N1339/LSH	0.67
Control(High Risk)	N1347/TKS	0.53
Control(High Risk)	N1494/LYL	0.52
Stage I GC	NGCII005/K-D	70.83
Stage III GC	NGCII068/J-W	183.46
Stage III GC	NGCII255/LBH	196.29
Stage II GC	TGCII014/LSC	152.05
Stage II GC	TGCII089/YPH	7.11

Type of sample	Standard Deviation	Mean
Control (High Risk)	0.076	0.609
Cancer	80.65	121.949
P-value		0.01

TABLE I. 4-MIR GC BIOMARKER PANEL

Next, an attempt was made to test the hypothesis that a multivariate panel of biomarkers (using the 4 miRNAs) may provide a simple and accurate means to distinguish GC and high-risk samples. The score for each serum sample is calculated by dividing the product of the 2 miRNAs that are up-regulated by the 2 miRNAs that are downregulated. (Table 1) The scores of the control (high risk) and GC samples were then averaged to obtain the mean respectively. As seen from Table 1, the GC mean was significantly higher than that of the control, 121.949 as compared to 0.609. The standard deviation of the control (high risk) samples was small, of 0.076, which showed minimal technical error. However, standard deviation of the GC samples was large, of 80.65, as the GC samples were of different stages, which resulted in a difference in expression of the miRNAs. This is graphically represented by the box and whisker plot (Figure 2). Interestingly, a combined index comprising these 4 miRNAs was highly significant (p=0.01) in discriminating between GC and control samples.

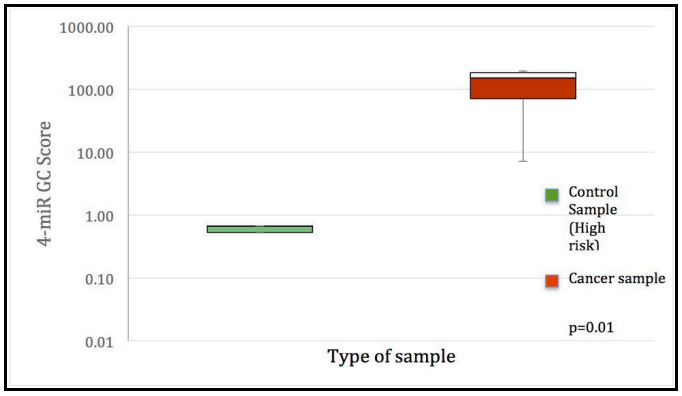

Figure 2. Multi miR-GC Classifer

It is evident that the 2 boxes representing GC samples and control samples respectively are very far apart on the score axis, the GC samples having a significantly higher score (p=0.01). Hence, we can confidently distinguish the 2 populations of GC and control using the 4-miRNA panel.

B. Effect of Chemotherapy on miRNA Expression in a Gastric Cancer Cell-Line

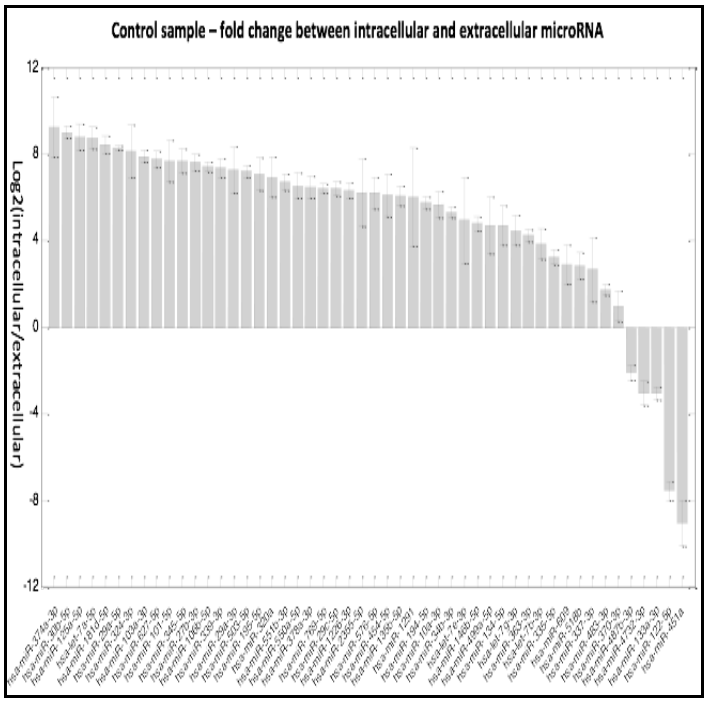

Figure 3. miRNA Secretion Profile

Next, the hypothesis that miRNAs are expressed in GC cells and that chemotherapeutics may affect the expression was tested. The expression levels of most miRNAs are higher intracellularly than extracellularly, as seen by the vast majority of miRNAs with positive fold changes. This suggests that these miRNAs are biosynthesized intracellularly, while miRNAs with negative fold changes are secreted extracellularly. These fold changes were acquired by dividing intracellular miRNA expression levels by extracellular expression levels, represented on a log2 scale, to clearly distinguish between miRNAs secreted intracellularly and extracellularly. The representation of miRNA copy numbers in a logarithmic scale prevents the values on the y-axis from approaching 0, especially if extracellular miRNA expression levels are extremely high. Thus, a positive value will be obtained for miRNAs that are secreted more intracellularly, while a negative value will be reflected for miRNAs that are secreted more extracellularly. As seen from Figure 1, the last five miRNAs are highly secreted extracellularly. Given the vast amounts of these miRNAs secreted, it is likely that these miRNAs were secreted specifically, rather than randomly, as a result of cell-to-cell communication or other functions. These highly secreted miRNAs present in serum are circulatory miRNAs, and could potentially serve as good biomarkers.

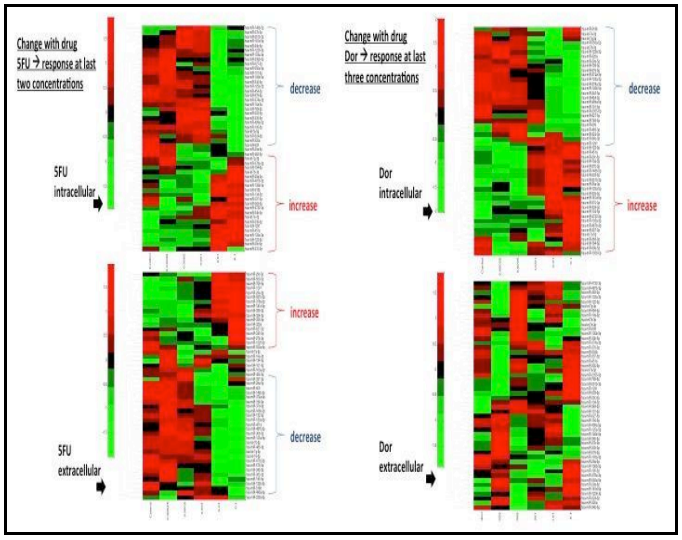

Figure 4. Heat Map Representation of Drug Response (5-FU and Dor)

The mean of all 6 replicates was taken to plot the above heat maps to allow for more accurate and reliable results. The coloured bar on the left of the heat maps represents the range of fold changes of miRNA expression levels against the control, represented on a log2 scale. Each colour on the scale corresponds to a fold change, where the shades of red are positive (indicating upregulation) while the shades of green are negative (indicating downregulation). From Figure 4, there is a drastic change in the global profile of the miRNAs expressed intracellularly and extracellularly when treated with 5-FU at a concentration of 0.01mM onwards. Half of the miRNAs were downregulated both intracellularly and extracellularly at 0.01mM, while the other half were upregulated. There is also a drastic change in the global profile of the miRNAs expressed intracellularly when treated with Doxorubicin at a concentration of 0.001uM onwards. Half of the miRNAs were downregulated both intracellularly and extracellularly at 0.001uM, while the other half were upregulated. There is no notable trend for the miRNAs expressed extracellularly when treated with Doxorubicin, as the change in miRNA expression levels is not very significant. The response by the cells to Doxorubicin is inconsistent and asystematic, which resulted in wide error bars, indicating unreliability and inaccuracy. However, the general miRNA profiles suggest that miRNA expression levels are affected by the treatment of drugs (5-FU and Doxorubicin). Extracellular miRNA expression may have been affected by released miRNA during lysis or cell apoptosis. Across increasing drug concentrations, no noticeable trend is observed, due to the lack of a gradual colour change from red (upregulation) to green (downregulation), or green to red.

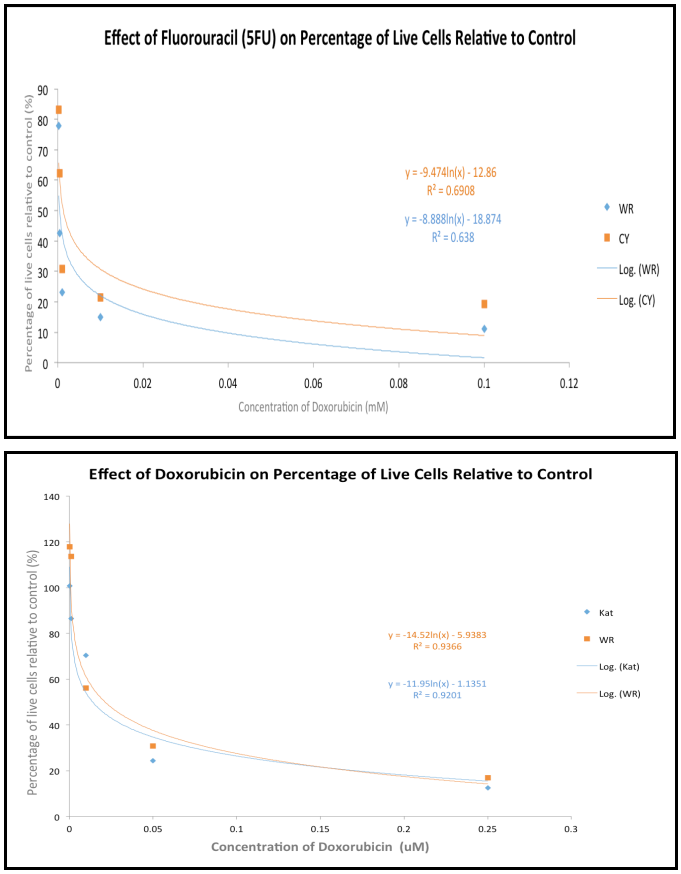

Figure 5. Effect of Fluorouracil (5-FU) and Doxorubicin (Dor) on Percentage of Live Cells Relative to Control

Based on Figure 4, since the change in miRNA expression occurred at a lower concentration for Doxorubicin as compared to the 5-FU drug, it might suggest that Doxorubicin may be more effective in inhibiting the growth of or killing GC cells as compared to 5-FU. This is supported by the above dose-response curves, where the half maximal inhibitory concentration (IC_{50}) of Doxorubicin is 0.016uM, as compared to the lower IC_{50} of 5-FU, of 0.53uM. It is evident from the above graphs that the higher the concentration of drug administered, the lower the percentage of live cells relative to control. Thus, the IC_{50} is a good indicator of the concentration at which there is 50% cell death/inhibited growth. Following the drug treatment, there is a drastic change in miRNA expression levels intracellularly and extracellularly at various concentrations. This indicates that miRNAs play an active role in drug treatment, and thus by observing miRNA profiles, miRNAs can potentially serve as predictive biomarkers for chemotherapy, given their high correlation. This also implies that miRNAs can possibly serve as a therapeutic target for gene therapy, given its high correlation with treatment, both intracellularly and extracellularly. Evidently, miRNAs secreted are functional in various ways, including cell-to-cell communication[9]. Since cells secrete these miRNAs in response to the drugs, it is hypothesised that the removal of miRNAs from media might hinder cell-to-cell communication, and consequently, increase the susceptibility of cancer cells to

the drugs, thereby increasing the effectiveness of chemotherapeutic drugs against cancer cells. On the other hand, an increase in miRNA expression levels intracellularly could be a biological response of the cell to the drug treatment. Biological activity in some parts of the cell may be increased or inhibited due to the change in miRNA expression levels exerting a positive or negative feedback on regulatory pathways in the cell, hence increasing the resistance of the cell to the drugs.

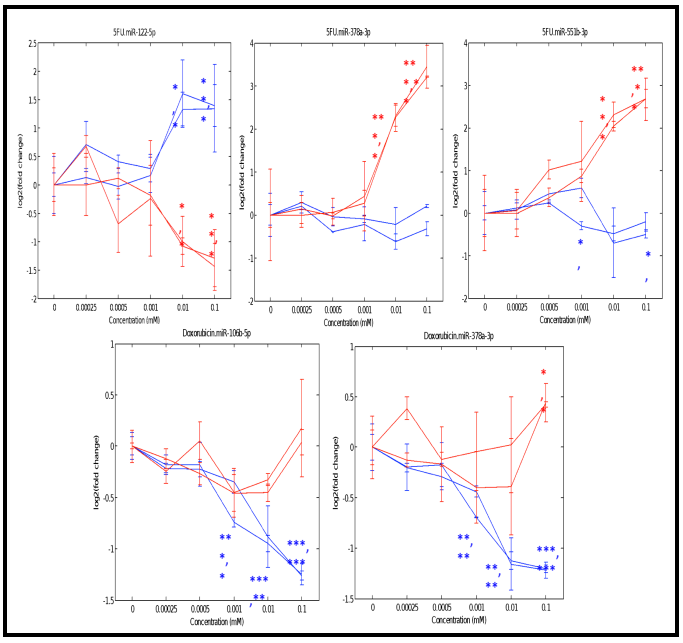

Figure 6. Drug Response Profile of Individual miRNAs

Figure 5 contains the drug response profiles of various individual miRNAs (5FU.miR-122-5p, 5FU.miR-378a-3p, 5FU.miR-551b-3p, Doxorubicin.miR-106b-5p, Doxorubicin.miR-378a-3p) with significant changes in their expression levels intracellularly (red line) and extracellularly (blue line). Technical normalization and global normalization were carried out before fold change as compared to control is plotted against the various drug concentrations. The p-values are indicated by the asterisks. These individual miRNAs are specifically and highly secreted within and/or outside of the cell due to their involvement in logical functions such as cell-to-cell communication. They can potentially serve as drug response biomarkers to evaluate clinical benefit.

V. Conclusion and Recommendations for Future Work

The results confirmed our hypothesis that these 4 miRNAs can distinguish between GC and non-cancer. These results will prove extremely useful in acquiring a cut off point to allow clinicians to distinguish between GC patients and high risk patients. Recent studies showed that miRNAs are potentially good biomarkers in various types of cancer. Consistent with these studies, we found that the 4 miRNAs examined are collectively good biomarkers for GC. Statistically, the separation between GC and control samples is quite distinct, thus these 4 miRNAs can clearly distinguish between GC and non-cancer. However, the limited sample size, of 5, resulted in wide error bars, suggesting that the values range quite a bit, and thus, uncertainty in measurement. This might compromise the accuracy of the serum miRNA test, as GC only affects 1 in 111 people[9], hence misdiagnosis cannot be afforded. The results also suggest that miRNA (intra and extracellular) expression in gastric cells are influenced by chemotherapeutics. Some miRNAs are specifically and highly secreted, and could potentially serve as good biomarkers for GC due to their presence in serum as circulatory miRNAs. Response to drugs proved that the higher the concentration of drug administered, the lower the percentage of live cells relative to control and that Doxorubicin may be more effective in inhibiting the growth of or killing GC cells as compared to 5-FU. Individual miRNAs that are specifically and highly secreted within and/or outside of the cell due to their involvement in logical functions such as cell-to-cell communication can potentially serve as drug response biomarkers to evaluate clinical benefit. It is evident that miRNAs play an active role in drug treatment, and thus by observing miRNA profiles, miRNAs can potentially serve as prognostic biomarkers for chemotherapy, given their high correlation. This also indicates that miRNAs can possibly serve as a therapeutic target for gene therapy, given its high correlation with treatment, both intracellularly and extracellularly. The findings were very promising, albeit with substantial limitations due to small sample size. Further studies with expanded sample size are needed to further evaluate miRNAs' clinical usefulness as potential biomarkers for GC. However, if proven sensitive and specific, serum miRNA tests will be affordable and practical, especially in screening large numbers of patients. Furthermore, the interesting effects of chemotherapeutics on miRNA expression levels may serve to provide a novel approach to evaluate the effects of drugs on GC cells. The mechanisms of individual miRNAs can be explored to identify therapeutic targets for gene therapy.

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