microRNA profiling of Iraqi patients with gastric cancer

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Research Article

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Abstract

Background: Gastric cancer has the fifth place commonly identified malignancy and third principal cause of cancer-related fatalities globally, with over 1 million cases globally. Since each miRNAs regulates multiple target mRNAs that participate in a variety of biological processes, including signal transduction, cell differentiation, apoptosis, and proliferation, totaling 30% of protein-coding genes. Thus, deregulation of miRNAs has a role in a variety of clinical diseases, including GC and characterize a developing field of cancer research. The goal of this study was to investigate the signature of miRNA profile in Iraqi patients with gastric cancer.

Methods: One-hundred tissue specimens include 50 cancerous tissues and 50 normal tissues from 50 patients were collected for miRNA profiling by microarray assay.

Results Our results exhibited that 37 miRNAs were up-regulated in patients with gastric cancer (P <0.05), and 40 miRNAs were down-regulated in patients with gastric cancer (P <0.05). Then, 3 highly overexpressed miRNAs (miR-221-3p, miR-106a-5 and miR-21-5p) were chosen for further validated by Real-Time polymerase chain reaction. In the validation set, the level of (miR-106a-5, miR-21-5p and miR-221-3p) were tested by qRT-PCR have significant up-regulation in GC related to normal tissues (P <0.05).

Conclusions: According to our present findings, the 3 miRNAs (miR-106a-5, miR-21-5p and miR-221-3p) strongly suggested involvement in gastric carcinogenesis, hence highlighting its serving as promising prognostic, diagnostic biomarkers or therapeutic targets for gastric cancer patients.

Introduction

The third leading cause of cancer-related fatalities globally is gastric cancer, with over 1 million cases globally and around 768,000 mortalities in 2020. It is also in fifth place commonly identified malignancy [1, 2]. Generally, gastric cancer has a wrong prognosis as it is frequently discovered at an advanced stage. Patients with locally advanced or metastatic disease may benefit from palliation, increased survival rates, and improved quality of life with systemic therapy [2]. According to GLOBCAN 202, In 2020, there will be a million or more new incidences of stomach cancer, which will result in predictable 769,000 fatalities (equivalent to one per 13 deaths worldwide), ranking fourth for global mortality and fifth for incidence. The accumulation of particular genetic abnormalities and a mix of environmental variables lead to gastric cancer. A number of variables, including family history, diet, alcohol intake, smoking, H. pylori infection, have been found to significantly enhance the risk of developing GC. Nearly all cases of non-cardia stomach cancer are thought to be caused by a chronic infection with the H. pylori [3, 4].

MicroRNAs are small, short and nc-RNAs that form a silencing complex with the protein Argonaute (AGO), it controls gene expression and is essentially engaged in all important biological processes by directly inhibiting target mRNAs after transcription. More than 18000 distinct miRNAs from 168 species are included in the miRBase database (www.mirbase.org). In humans, more than 2,500 miRNAs have been found, in addition to miRNAs controlling more than 60% of human's protein-coding genes [5–9]. Each
miRNAs regulates multiple target which take part in various biological processes, such signal transduction, cell differentiation, apoptosis, and proliferation, totaling 30% of protein-coding genes. Thus, deregulation of miRNAs has a role in a variety of clinical diseases, including GC. Additionally, GC has been found to express miRNAs aberrantly. Increasing amounts of evidence point to the potential importance of miRNAs in the drug resistance of GC [7, 8]. Theoretically, miRNA expression is dysregulated to some amount in all cancers. According to recent findings, greater than half of the miRNA-codifying genes in humans are in genomic regions that have been linked to dysregulated cancer growth [10, 11]. Tumor suppressor or oncogenic miRNAs (oncomiRs) are two categories for cancer-related miRNAs based on the function of their target mRNA. Based on the capacity of miRNA molecules to disrupt processes connected to carcinogenesis, such as those connected to cell migration and invasion, apoptosis and proliferation, this segregation is based.

**Materials And Methods**

**Patients and Specimens**

In the recent study, we analyzed 100 frozen cancerous, along with matching healthy para-cancerous tissues from 50 patients, who is having a surgery to resect a piece of gastric cancerous tissues at Nasiriyah general hospital, south of Iraq from November 2021 to June 2022. The medical ethical committee of MOH, IRAQ approved this study. No chemotherapy or radiotherapy therapy were taken by patients before the operation. Prior to participating, the patients' written informed permission was obtained. Then the specimens were immediately frozen in liquid nitrogen, following that, all samples were kept at -80°C. The tumor types and stages are confirmed by two pathologists, and the para-cancerous tissues were non-neoplastic. The basic information of the participants included in (Table 1).

**Table 1** Clinical and histological aspects of the research participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Gastric cancer patients with paired tissues (n = 50), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>31 (62%)</td>
</tr>
<tr>
<td>Females</td>
<td>19 (38%)</td>
</tr>
<tr>
<td>Ages (year)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>50-64</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>≥65</td>
<td>14 (28%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>39 (78%)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>11 (22%)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
</tr>
<tr>
<td>TNM1</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>TNM2</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>TNM3</td>
<td>31 (62%)</td>
</tr>
<tr>
<td>TNM4</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (14%)</td>
</tr>
<tr>
<td>No</td>
<td>43 (86%)</td>
</tr>
</tbody>
</table>

**Total RNA extraction**
Following the directions provided by the manufacturer, RNA was extracted from frozen tissues using the FavorPrep kit Tissue Total RNA (Favorgen Biotech Inc., Taiwan). RNA concentration was determined using a Nano-Drop 2000 Spectrophotometer (Thermo-Fischer Scientific, USA).

**miRNA expression profile array method**

We used affymetrix Gene-chip miRNA 4.0 array following the directions provided by the manufacturer. The samples had labels using the FlashTag™ Biotin HSR Labeling Kit (Affymetrix Inc., USA). Then, the labeled RNA products were prepared to chip hybridization according to the typical procedure. Afterwards, the labeled RNA was heated at 99°C for 5 mins and then at 45°C for another 5 mins. Fluidics Station 450 (Affymetrix Inc., USA) was used to wash and stain the chip after hybridization and according to instructions then fixed and scanned with a GCS 3000 microarray scanner (Affymetrix Inc., USA). The software for the Affymetrix® Gene-chip™ Command Console was used to determine signal values. Using the software given by AGCC, the Affymetrix data extraction procedure automatically extracted raw data. By species annotations on probes, microarray datasets were filtered. Fold change of $\geq 2$ (up-regulated) or $< 0.5$ (down-regulated), with a p-value of < 0.05. was used to define the miRNAs that are differently expressed in gastric cancerous tissues in comparison to the normal para-cancerous tissues.

**Quantitative Real time PCR**

SYBR Green qRT PCR assay was utilized as well as three potential microRNAs with an FC of $\geq 2$ or $< 0.5$, and a p-value of $< 0.05$ were chosen to confirm the accuracy of the miRNA microarray results. The sRNAPrimerDB web tool and the miRprimer2 program were used to create specific primers for the microRNAs by utilizing miRBase database to get sequences of microRNAs, and all the primers were synthesized by Macrogen Co., Ltd. (Macrogen, Korea), details of the primers are listed in (Table 2). Using the HyperScript™ kit (GeneAll Biotechnology, Korea), one to two micrograms of RNA were reverse-transcribed into cDNA. The RT reaction mixture consisted of $10 \mu l$ HyperScript TM 2X RT- Master mix, $1\mu l$ Random hexamer Primer, $7\mu l$ Nuclease free water and $1\mu l$ of $1-2 \mu g$ total RNA in a final volume of $20\mu l$ tubes. The reactions were incubated at $50^\circ C$ for 30–60 min. After the RT reaction, $5 \mu l$ of the cDNA product were added to a $20\mu l$ PCR master mix containing $10\mu l$ of 2X SYBR Green qPCR Master mix (RealAmpTM 2X SYBR qPCR Master mix, Geneall, Korea), $5\mu l$ of cDNA template, $1\mu l$ of forward primer, $1\mu l$ of reverse primer and $3\mu l$ of distilled water. Realtime PCR was performed in triplicate for each sample, with using of the U6 as an internal control to normalize the miRNAs expression. The PCR conditions were: enzyme initiation at $95^\circ C$ for 15 min and then 30 cycles of $95^\circ C$ for 20 sec, $55^\circ C$ for 20 sec and $72^\circ C$ for 30 sec. The data results for miRNA were analyzed using relative quantification method (fold change) by using of $2^{-\Delta\Delta Ct}$[12] to identify alterations in the target miRNAs' expression. A miRNA was deemed to be differentially expressed if its $2^{-\Delta\Delta Ct}$ value was $\geq 2$ or $< 0.5$. 

\[2^{-\Delta\Delta Ct} \geq 2 \text{ or } 0.5 \]
Table 2
Details about the primers employed in the study.

<table>
<thead>
<tr>
<th>Primers names</th>
<th>Primers sequences (5’-3’)</th>
</tr>
</thead>
</table>
| hsa-miR-221-3p | Forward: GCTACATTGTCTGCTGGGT  
Reverse: CTCAACTGGTGTCGTGGA |
| hsa-miR-106a-5p | Forward: AACCTCCAAAAGTGCTTACAGT  
Reverse: GTCGTATCCAGTGCAAGGGT |
| hsa-miR-21-5p  | Forward: ACCACCGTAGTTATCAGACT  
Reverse: GTCGTATCCAGTGCAAGGGT |
| U6            | Forward: ATTGGAACGATAACAGAG  
Reverse: GGAACGCTTCACGAATTT |

Bioinformatics analysis

Two databases include the TargetScan (www.targetscan.org) and microRNADB (www.mirdb.org) provided the lists of predicted target genes for the candidate miRNAs. To understand how miRNAs contribute to carcinogenesis and development, we used gene ontology (www.geneontology.org) to acquire pathway enrichment and DAVID database tool (www.david.ncifcrf.gov) for examine the KEGG pathways of candidate miRNAs. To map mRNA-miRNA network, the (Cytoscape 3.9.1) was applied to create biological network.

Statistical analysis

All Statistical tests were employed using SPSS version 21 (IBM Corporation, NY, USA). Paired t test was conducted to make comparative analysis of miRNAs expression among GC and normal para-cancerous tissues samples. GraphPad Prism 9.3.1 software (GraphPad Software, Inc., SanDiego, CA) employed to visualize of differentially expressed genes.

Results

The Differentially microRNA Expression in GC

Affymetrix Gene-Chip miRNA 4.0 array which contains 2578 capture probes human microRNAs, was used to screen the expression of miRNA profiles in the tested sample groups (normal adjacent and gastric cancer from paired matched tissues). With adjusting the fold change cut-off as ≥ 2-fold or < 0.5, 1365 miRNAs in total were differentially expressed in GC. Furthermore, only 77 miRNAs were found to have significant expression by statistical analysis. (Fig. 1a and 1b), of these there were 37 up-regulated miRNAs with a FC of ≥ 2 and a p-value of < 0.05: hsa-miR-191-5p, hsa-miR-221-3p, hsa-miR-25-3p, hsa-
miR-210-3p, hsa-miR-575, hsa-miR-32-3p, hsa-miR-1538, hsa-miR-6124, hsa-miR-601, hsa-miR-616-3p, hsa-miR-1248, hsa-miR-2355-3p, hsa-miR-181c-3p, hsa-miR-215-5p, hsa-miR-658, hsa-miR-331-3p, hsa-miR-103a-3p, hsa-miR-423-3p, hsa-miR-181a-2-3p, hsa-miR-301a-5p, hsa-miR-222-3p, hsa-miR-300, hsa-miR-449b-3p, hsa-miR-106a-5p, hsa-miR-106b-3p, hsa-miR-27a-3p, hsa-miR-223-5p, hsa-miR-191-3p, hsa-miR-21-3p, hsa-miR-3158, hsa-miR-222-5p, hsa-miR-520c-3p, hsa-miR-877-5p, hsa-miR-18a, hsa-miR-93-3p, hsa-miR-92a-1-5p and hsa-miR-21-5p.

While 40 miRNAs recorded a significantly downregulation with a FC of < 0.5 and a p-value of < 0.05: hsa-miR-195-5p, hsa-miR-218-5p, hsa-miR-518f-5p, hsa-miR-214-5p, hsa-miR-574-3p, hsa-miR-378c, hsa-miR-5000-3p, hsa-miR-939-3p, hsa-miR-140-3p, hsa-miR-451a, hsa-miR-873-5p, hsa-miR-422a, hsa-miR-29c-5p, hsa-miR-1225-5p, hsa-miR-1262, hsa-miR-146b-5p, hsa-miR-4316, hsa-miR-505-3p, hsa-miR-6165, hsa-miR-4290, hsa-miR-339-5p, hsa-miR-429, hsa-miR-506-3p, hsa-miR-203a-3p, hsa-miR-512-5p, hsa-miR-524-5p, hsa-miR-141-3p, hsa-let-7c-5p, hsa-miR-122-3p, hsa-miR-124-5p, hsa-miR-143-5p, hsa-miR-335-3p, hsa-miR-145-5p, hsa-miR-122-5p, hsa-miR-124-3p, hsa-miR-9-5p, hsa-miR-15b-5p, hsa-miR-143-3p, hsa-miR-16-5p and hsa-miR-497-5p (Supplementary file 1).

Quantitative RT-PCR assay to validate the results of miRNA profiling

Additionally, the findings of microarray data were validated by applying qRT-PCR to assess the microarray data's veracity and accuracy, the expression of three miRNAs candidates which might be associated with gastric cancer including miR-221-3p, miR-106a-5p and miR-21-5p were investigated by qRT-PCR in the same samples examined by the microarray. miR-106a-5, miR-21-5p and miR-221-3p expression were significantly up-regulated (FC = 12, P = 0.031; FC = 10.1, P = 0.024 and FC = 13.55, P = 0.001 respectively) in GC related to normal tissues. These results were in consistent with the miRNA microarray findings (Fig. 2).

Bioinformatics analysis

Prediction of a target gene

The possible prediction gene targets for these miRNAs were applied by utilizing of 2 databases (TargetScan and miRDB) in order to additional explore possible targets of miRNAs that are differently expressed, including (hsa-miR-221-3p, hsa-miR-106a-5p and hsa-miR-21-5p), we obtained putative genes from the two websites databases to improve the reliability of the prediction, and it has been established that each miRNA controls a number of target genes. Finally, a total of (1414) overlapped targets mRNAs was determined by combining the analyses of both databases in which 943, 189 and 282 were allocated to (miR-106a-5, miR-21-5p and miR-221-3p), respectively.

Then a (Cytoscape 3.9.1) was used to construct a network map between miRNAs and mRNAs, a total of 3 miRNAs and 41 strongly validated mRNAs were included in this network and the network analysis showed that miR-221-3p and miR-106a-5p had 2 common targets (RUNX1 and RB1), miR-221-3p and
miR-21-5p had 6 common targets (APAF1, SOCS1, ICAM1, TIMP3, RECK and PIK3R1), miR-106a-5p and miR-21-5p had 5 common targets (BCL10, TGFBR2, STAT3, E2F1 and VEGFA) (Fig. 3a).

**Gene function and Enrichment analysis**

Considering that target gene expression can be post-transcriptionally regulated by differentially expressed miRNAs and might be relevant for the physiology of gastric cancer, therefore enrichment analysis (KEGG Pathway) and (GO analysis) were explored to analyze the possible functions for overlapped 1414 target genes of the candidate miRNAs in the pathways and to determine their role in tumorigenesis and development. It was found that GO was highest enriched for miRNA-mediated gene silencing, GTPase binding and SMAD protein complex (Fig. 3b), whereas KEGG pathway indicated that target genes were significantly participate in different KEGG signaling pathways that are important for the progression and development of GC which includes pathways in cancer, gastric cancer and other oncogenic/tumor suppressor transduction systems including PI3K-Akt signaling pathway, p53 signaling pathway, mTOR signaling pathway, FoxO signaling pathway and MAPK signaling pathway (Fig. 3c).

**Discussion**

Numerous studies have directly evaluated the expression of miRNAs in gastric cancer. Though, there are no up-to-date and comprehensive reports on a miRNA profile of gastric cancer among the Iraqi population. In our study, a miRNA microarray platform was employed to show the gastric cancer and healthy tissue's miRNA patterns. Our findings demonstrate that GC may be distinguished from healthy gastric tissue through the miRNA expression pattern. As a result, microarray assay recorded 77 miRNAs that differentially expressed in gastric cancer. These miRNAs include, 37 significantly up-regulated miRNAs, while 40 miRNAs were significantly down-regulated. Then, 3 obviously upregulated miRNAs were chosen as candidate microRNAs, and qRT-PCR assay was applied to verified the expression in gastric cancer. The miRNA expression analysis of hsa-miR-221-3p, hsa-miR-106a-5p and hsa-miR-21-5p was significantly upregulated comparison with normal control and is in line with the miRNAs array. Many studies of cancer reported that miR-106a-5p was overexpressed in tumor and diminished GASL1's roles in cell proliferation and metastasis. Whereas GASL1 inhibited GC cell proliferation and diminished GASL1's roles in cell proliferation and metastasis via targeting and blocking PI3K/AKT ras/raf/MEK/ERK pathways [13–15] indicating role of miR-106a-5p as tumor oncogene. miR-106a-5p up-regulation is strongly correlated with invasion, lymphatic and distant metastasis, tumor stage, size, and differentiation [16]. miR-21-3p was also pointed to be upregulated in gastric cancer [17]. Furthermore, miR-21-3p was discovered to target SMAD7 and increase the proliferation of gastric cancerous cells [18]. Also, Yan et al reported that miR-21 specifically targets PIK3R1 and stimulates the PI3K/AKT signaling pathway, thus enhancing the migration, invasion and proliferation of breast cancer cells [19]. In addition, it has been proven that miR-221-3p is crucial for the development of GC. For example, according to Zhang et al miR-221-3p, regulate growth, invasiveness, and discovered that miR-221-3p was overexpressed in GC group as comparison to normal tissues [20]. Okamoto et al 2013 found miR-221-3p downregulated in cholangiocarcinoma [21], which is contrast to the current finding. Also, Tan et al conclusions about miR-221-3p revealed to target LIFR, thereby
enhanced HCC cell invasion, migration and proliferation [22]. Another research done by Zhou et al.
displayed that increased miR-221-3p expression in gastric cancer promotes the proliferation and inhibited
cell apoptosis by targeting SOCS3 [23]. These conflicting results can be explained in part by miR-221-3p
may have various roles in various cancers. In respect to above studies, it has been demonstrated that
(miR-106a-5, miR-21-5p and miR-221-3p) are more abundant in gastric cancer, indicating a potential role
of miR-221-3p, miR-106a-5p and miR-21-5p as oncogenic and a prognosis predictor in gastric cancer
which is consistent with our findings. Two widely used databases (TargetScan 7.1 and Micro-DB V6)
were applied for target genes prediction to investigate the possible contribution of 3 potential microRNAs
to the onset and progression of GC. The two databases discovered 1414 overlapping target genes, and it
was observed that numerous of these genes might have had a considerable impact on the occurrence,
growth, and prognosis of malignancies. This hypothesize that through regulating the expression of these
potential target genes, the progression of gastric cancer may be influenced by the differential expression
of microRNAs. In the current study, target genes that propose possible pathways in regard to the
candidate miRNAs were further functionally explored using Gene Ontology and KEGG pathway studies.
The GO findings demonstrated that potential targets may have a significant impact on many biological
processes that contribute to the occurrence of GC, such as miRNA-mediated gene silencing and ERBB
signaling pathway; for cellular component, such as focal adhesion and SMAD protein complex, for
molecular function, the role of nucleic acid binding and protein binding was considerably enriched in the
target genes. Furthermore, KEGG data were used to clarify the functions of potential targets in gastric
cancer, where microRNAs pathways in cancer, p53 signaling pathways and PI3K-Akt signaling pathways
considered to be the top three pathways and shown to have miRNA target genes that have been enriched
in them. The aforementioned findings show that the underlying targets of potential miRNAs are probably
related to these pathways, which have an impact on the development and progression of gastric cancer.
Additional investigation will be required in the future to validate the targets and enhanced signaling
pathways both in vivo and in vitro.

Conclusion

we successfully explored the profiling of miRNAs from fresh, frozen gastric tissues which in comparison
to healthy controls, are differentially expressed in GC patients. In contrast to normal controls, several
miRNAs exhibited deregulated expression in cancerous tissues. Among these, 3 miRNAs (miR-106a-5,
miR-21-5p and miR-221-3p) strongly suggested involvement in gastric carcinogenesis, hence highlighting
its serving as promising prognostic and diagnostic biomarkers for gastric cancer patients in Iraqi
population. As more miRNAs be discovered, the function of these aberrant miRNAs in the Iraqi population
will be clearly known. Recent study could be supportive in the future to detect possible gastric cancer
prognostic biomarkers.

Declarations

Acknowledgments
This research was self-funded and we are thankful to the staff of the laboratory, for technical support. The authors declare that they have no competing interests.

**Author contributions**

All authors analyzed, wrote and edited the manuscript. All authors read and approve the final manuscript.

**Compliance with Ethical Standards:**

**Funding:** The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

**Ethical approval:** All procedures performed in the study involving human participants were approved by the research committee of the medical ethics unit, ministry of health, Iraq.

**Informed consent:** Informed consent was obtained from all individual participants that gave a portion of their gastric cancer tissue specimens for including in the study.

**Conflicts of interest:** The authors have no relevant financial or non-financial interests to disclose.

**Data availability:** The data are available if requested by the Editor or reviewers.

**References**


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**Supplementary File**

Supplementary File 1 is not available with this version

**Figures**
A volcano plot diagram of the miRNA array illustrates that each miRNA's log2 fold change in expression between GC and normal is plotted against its -log10 p-value. The vertical two green lines shows that the thresholds in miRNA expression is FC $\geq 2$ or $<0.5$. The horizontal green line shows that p-value is $<0.05$. miRNAs that were significantly different in expression between GC and normal tissues presented by red and green dots (up- and down-regulated, respectively) (Fig. 1a). Hierarchical clustering heatmap showed differentially expressed miRNAs (Fig. 1b).
Figure 2

qRT-PCR validation results.
Figure 3

The mRNA and miRNA network map. The hexagon nodes indicate the microRNAs, while blue nodes indicate the mRNAs. The black lines indicate the network verified in miRtarBase (Fig. 3a). Analysis of miRNAs with differential expression, GO analysis of the candidate miRNAs genes (Fig. 3b). KEGG Pathways of the candidate miRNAs genes (Fig. 3c).