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Original Article

# miR-146a is deregulated in gastric cancer

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## ABSTRACT

**Background:** Gastric cancer is one of the most significant reasons for cancer-related death. miR-146a is one of the dysregulated factors associated with gastric tumorigenesis. However, deregulation of this microRNA (miRNA) has become controversial. Moreover, the inflammation-mediating role of this miRNA implies that miR-146a might be dysregulated by gastric cancer-related pathogens, such as *Helicobacter pylori*. However, the dysregulation of miR-146a in *H. pylori*-infected gastric tumors has not been widely studied.

**Objectives:** We aimed to analyze the expression level of miR-146a in gastric cancer tissues and then to assess any potential association between miR-146a and *H. pylori* infection and other clinical characteristics.

**Materials and Methods:** miR-146a expression level was quantitatively studied by reverse transcription quantitative polymerase chain reaction, in 144 fresh tissues including 44 normal and 100 gastric cancer samples.

**Results:** A dramatic overexpression of miR-146a was observed in primary gastric tumors. miR-146a showed lower expression in progressed tumors with greater stages and lymph node metastasis.

**Conclusion:** miR-146a is highly expressed in primary gastric tumor independent of *H. pylori* infection. It is highly expressed in the lower stages and lymph node-negative tumors. It might suggest the importance of upregulation and downregulation of this miRNA in the initiating/promoting and progressive steps of gastric tumorigenesis, respectively.

**KEY WORDS:** Gastric cancer, *Helicobacter pylori*, miR-146a, reverse transcription quantitative polymerase chain reaction

## INTRODUCTION

Gastric cancer is one of the most important reasons of cancer-related death (~10%) in the world.[1] This high death rate is associated with the lack of significant symptoms in the early stages of gastric cancer; consequently, many cases of gastric cancer are diagnosed at the progressed stages, showing poor prognosis.[2]

Gastric cancer is a multifactorial disease. There are many environmental and hereditary factors resulting in stomach tumorigenesis and also its progressions, including the genetic characteristics of the individuals, infectious agents such as *Helicobacter pylori*, and dietary habits such as alcohol consumption and smoking.[3,4] *H. pylori* infection has been shown to be associated with deregulation of some noncoding RNAs, known as microRNAs (miRNAs). miRNAs are single-stranded noncoding RNAs with approximately 20–25 nucleotides, playing important roles in regulating cellular processes such as growth, differentiation, cell death, and angiogenesis. All these functions have critical roles in the development of cancer, including gastric cancer.[5] In terms of cancer biology, oncogenic miRNAs are known as oncomiRs, while the rest are tumor-suppressor miRNAs. These molecules regulate gene expression posttranscriptionally, mainly via binding to the 3' untranslated region of their target mRNAs. This interaction can prevent protein biogenesis through either mRNA decay or translation inhibition.[6]

Several studies indicate that dysregulation of miRNAs has a direct impact on a wide range of pathological and physiological processes.[7] Recent evidence has demonstrated that miRNA

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**Cite this article as:** Adami B, Tabatabaeian H, Ghaedi K, Talebi A, Azadeh M, Dehdashtian E. miR-146a is deregulated in gastric cancer. J Can Res Ther 2019;15:108-14.

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dysregulation is tightly linked with different human cancers, such as breast, ovarian, prostate, and gastric cancer and hepatocellular carcinoma.[8-10] Among various miRNAs, miR-146a was found to be dysregulated in a majority of the human and mouse gastric adenocarcinomas and cervical, breast, pancreatic, and thyroid cancers.[11-15] However, there is a remarkable controversy regarding its expression level in gastric cancer in a way that some studies reported its overexpression,[12] while others reported its underexpression.[16,17]

Functionally, miR-146a is interestingly important due to its mediating activity in the inflammation process. This miRNA becomes upregulated by the inflammatory proteins such as interleukin 1 and tumor necrosis factor-alpha.[18] Therefore, miR-146a and *H. pylori* infection are both recognized to be involved in stomach inflammation and tumorigenesis, suggesting that *H. pylori* might impose its tumorigenic effect via dysregulating miR-146a. However, the association between miR-146a expression status and *H. pylori* infection, in the context of gastric cancer, has not intensively been studied and requires more investigations to be clarified more. Moreover, according to the controversial nature of findings about the expression status of miR-146a in gastric cancer, more quantitative studies are demanded. Hence, we first aimed to quantitatively evaluate the expression level of miR-146a in fresh gastric cancer tissues as compared to normal healthy cases, to know if miR-146a is either up- or down-regulated. Then, we statistically analyzed miR-146a expression level in relation to *H. pylori* infection and other clinicopathological features of studied patients.

In this study, we showed that miR-146a is overexpressed in gastric cancer, particularly in the primary steps of gastric tumorigenesis, in an *H. pylori*-independent manner. The higher expression of miR-146a in primary gastric tumors and also its higher expression in Stage 1, lymph node-negative tumors imply the importance of this miRNA in initiating steps of gastric tumorigenesis.

## MATERIALS AND METHODS

### Patient samples

A total of 144 fresh tissue samples including 44 normal controls and 100 gastric cancer cases were collected. The normal gastric epithelium samples were collected from the endoscopy candidates. Diagnosis of *H. pylori* positivity for healthy samples was tested with urease tubes.

Gastric tumor samples were excised from the patients during surgical operations. All samples were carefully investigated by pathologists to confirm either the normal or cancerous status. All the donors were informed precisely and clearly and also provided with written consent before surgery. After samples, all tissues were immediately stored in RNAlater (Ambion), frozen in liquid nitrogen, and kept at −80°C until RNA extraction.

**Table 1: Samples characteristics**

| Variable | Controls (*n*=44) | Cases (*n*=100) | *P* |
|---|---|---|---|
| Age (SD) | 49.73 (20.93) | 62.56 (12.06) | 0.002[a] |
| Sex | | | |
| Female | 16 | 20 | 0.14[b] |
| Male | 28 | 80 | |
| *Helicobacter pylori* infection | | | |
| No | 20 | 44 | 0.909[b] |
| Yes | 24 | 56 | |

[a]Independent *t*-Test, [b]Chi-square test. SD=Standard deviation

All data including age, sex, histologic grade, tumor size, tumor location, stage, metastasis, and lymphatic invasion were obtained from clinical and pathologic records [Table 1]. Patients received no chemotherapeutic agents or radiotherapy before the surgery.

### Ethics

*Ethics, consent, and permissions*

All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Islamic Azad University Ethics Committee, regarding to the criteria of Iranian Ministry of Health and Medical Education and with the 1964 Declaration of Helsinki.

*Consent to publish*

It is confirmed that we have received consent to publish from the participants to report individual patient data including age, sex, blood group, and clinicopathological characteristics.

### MicroRNA isolation and real-time reverse transcription polymerase chain reaction assay

Total RNA from fresh tissue samples was extracted by GeneAll Hybrid-RTM miRNA Kit (GeneAll, South Korea), according to the manufacturer's protocol. The quantity and purity of RNA were analyzed by NanoDrop (Thermo Scientific GmbH, Schwerte, Germany). Yielded total RNA was then stored at −80°C. cDNA synthesis for miR-146a was carried out by Universal cDNA Synthesis Kit (Exiqon, Denmark), based on a poly-A tailing method. miR-146a expression level was measured by ABI PRISM 7500 instrument (Applied Biosystems, USA) and $2^{-\Delta\Delta Ct}$ method.[19,20] miR-146a expression level was relatively normalized to U6 snRNA, as a reference gene.

### Statistical analysis

Statistical analyses were carried out using SPSS software (version 19, SPSS Inc., Chicago, IL, USA). Mann–Whitney U-test was recruited to compare the expression level of miR-146a between gastric cancer patients and healthy controls. Kruskal–Wallis test was also performed to study miR-146a expression among different stages, blood groups, histological types, and tumor locations. Chi-square test was used to investigate the association between gastric cancer incidence and sex and also *H. pylori* status of the patients. Independent *t*-test was used for comparing the age and gastric cancer incidence in the studied population. $P < 0.05$ was considered statistically significant.

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### Enrichment analysis

To find important miR-146a-related signaling pathways and also effective proteins, the miRWalk database was used to form the raw list of miR-146a targetome.[21-23] The miRTarBase database was recruited to identify the validated targets of miR-146a.[24] To figure out which genes are normally expressed in stomach tissue, we used UniGene database (http://www.ncbi.nlm.nih.gov/unigene/). Finally, the list of refined targets was imputed into DAVID databases to discover effective signaling pathways in gastric cancer.[25,26]

## RESULTS

### Upregulation of miR-146a in gastric tumors, independent of *Helicobacter pylori* infection

The expression level of miR-146a was evaluated by the reverse transcription quantitative polymerase chain reaction method, among normal and cancerous cases. miR-146a expression was normalized to the corresponding $C_t$ values of U6, as a control gene, in each sample. The specificity of primers for propagating miR-146a and U6 was evaluated by electrophoresis.

In overall, miR-146a was significantly overexpressed in gastric cancer tissues as compared to the normal tissues (Mann–Whitney test, $P < 0.001$), regardless of *H. pylori* status [Figure 1a]. Focusing on *H. pylori* infection, there was no significant difference between miR-146a expression level in *H. pylori*-positive and -negative samples, regardless of their pathological status (Mann–Whitney test, $P = 0.670$) [Figure 1b]. Likewise, there was not any difference between miR-146a expression level among *H. pylori*-positive and -negative normal samples (Mann–Whitney test, $P = 0.539$) [Figure 1c] and also gastric cancer cases (Mann–Whitney test, $P = 0.901$) [Figure 1d]. These data suggest the lack of association between miR-146a and pathogenicity of *H. pylori* in gastric cancer. Briefly, all this information showed the higher level of miR-146a in gastric cancer; however, this upregulation is independent of *H. pylori* infection.

### miR-146a is differentially expressed in clinicopathological groups of gastric cancer

As previously explained, all the data including tumor size, stage, lymph node invasion, and metastasis were obtained from the clinical and pathological department, and miR-146a

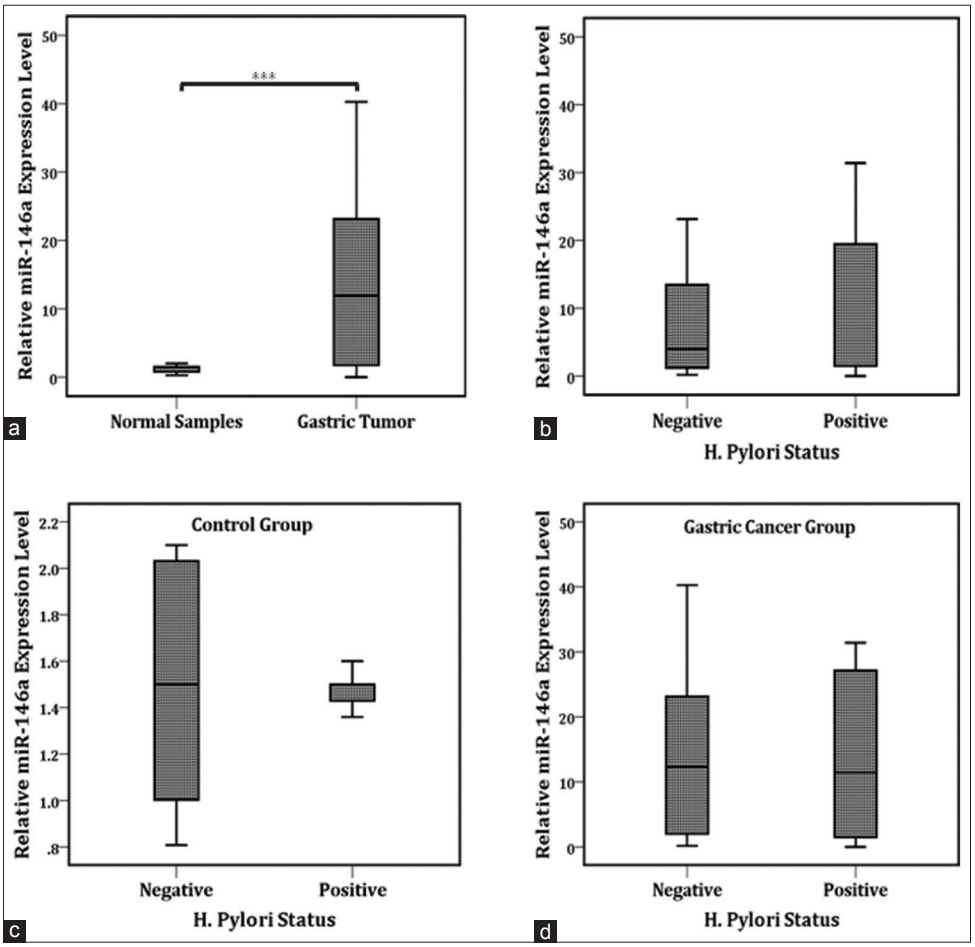


**Figure 1:** (a) miR-146a has significantly higher expression level in gastric cancer cases (normalized to U6 snRNA, ***$P < 0.001$). (b) In terms of *Helicobacter pylori* infection, miR-146a was not expressed differentially in all normal and gastric cancer samples and also separately within normal (c) and gastric cancer group (d)

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expression level was statistically analyzed within gastric cancer clinicopathological.

Statistically, miR-146a expression level was the same in gastric cancer patients with different blood groups, tumor size, metastasis, location of the tumor, and histological types [Figure 2a-e]. However, miR-146a expression was significantly higher in lymph node-negative gastric cancer patients (Mann–Whitney test, $P = 0.001$) [Figure 3a]. Moreover, the median values of miR-146a expression were not all the same for gastric cancer patients with Stages I, II, III, and IV (Kruskal–Wallis test, $P = 0.006$). Mann–Whitney test was recruited to know which group had different median values, describing that miR-146a was highly expressed in the patients with Stage I of gastric cancer as compared to the Stages III and IV ($P < 0.0001$ and $P = 0.017$, respectively) [Figure 3b]. Briefly, these data are listed in Table 2.

### *In silico* study of miR-146a in cancer

To investigate possible signaling pathways involved in the miR-146a-related development of stomach cancer, we first extracted the raw targetome of miR-146a from miRWalk and chose the genes with at least score of 5 out of 6 integrated algorithms. To find novel potential targets of miR-146a, the initial list was merged with the miRTarBase database to omit the repeated validated miR-146a targets of miRWalk.

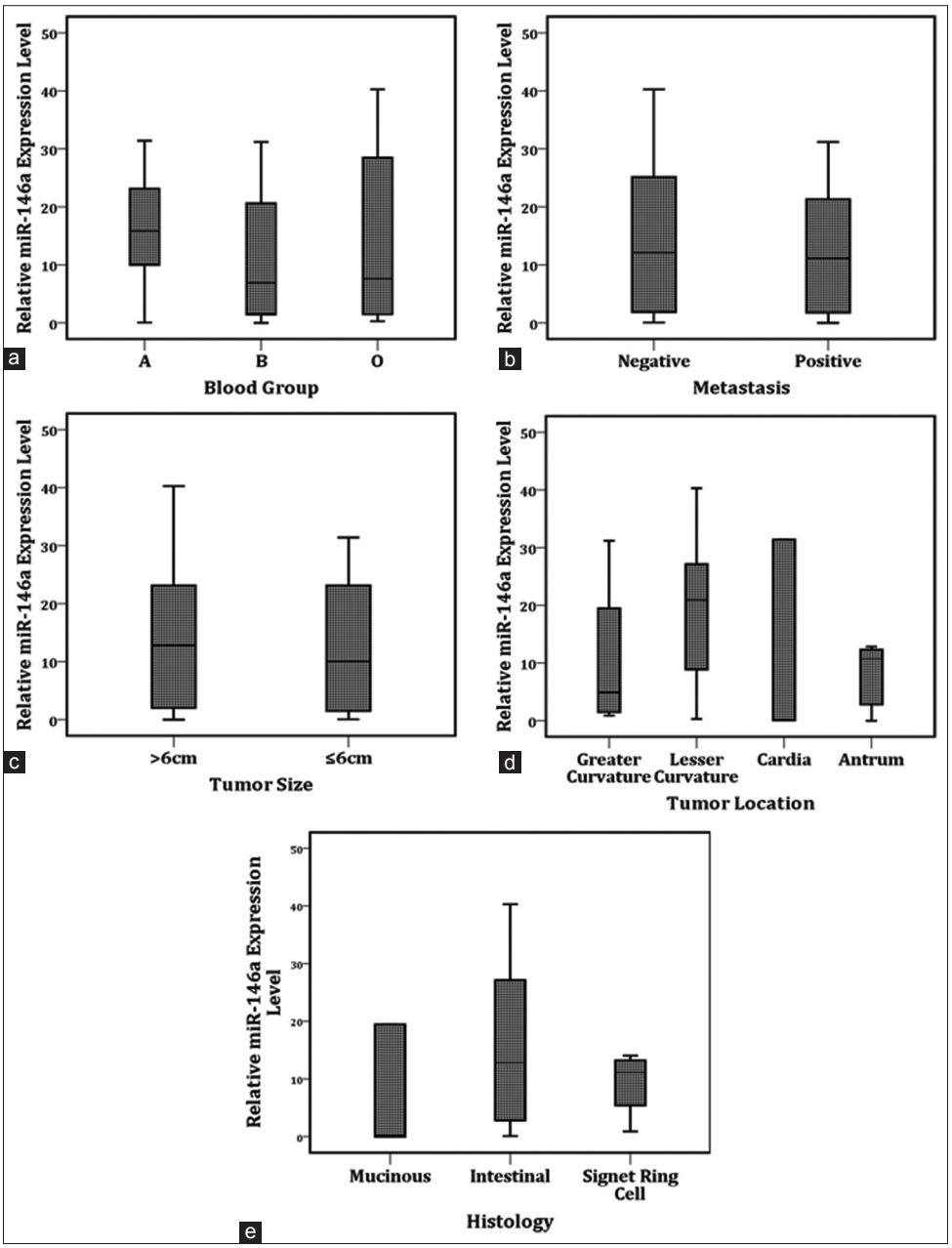


**Figure 2:** There was no significant difference between miR-146a expression levels in different (a) blood groups, (b) metastasis, (c) tumor size, (d) location of the tumor, and (e) histological types of gastric cancer. miR-146a is normalized to U6 snRNA

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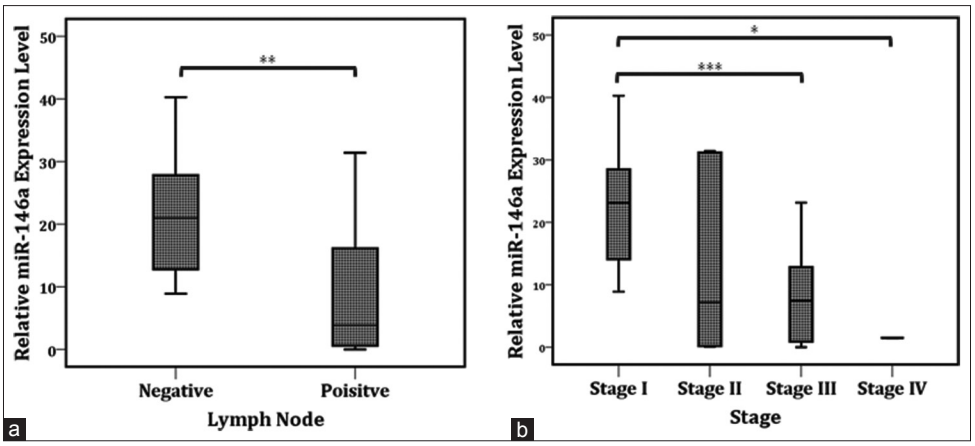


**Figure 3:** Statistically, miR-146a has higher expression level in lymph node-negative gastric tumors (normalized to U6 snRNA, $^{**}P < 0.01$, as compared to lymph node-positive cases (a), and also miR-146a is expressed more in gastric tumors with Stage I ($^{*}P < 0.05$, $^{***}P < 0.001$), as compared to Stages II and III (b)

**Table 2: The comparison between miR-146a expression and clinicopathological features of gastric cancer patients**

| Clinicopathological feature | miR-146a expression Median (range) | *P* |
|---|---|---|
| *Helicobacter pylori* | | |
| Negative | 2.47 (0.19-40.28) | 0.901[a] |
| Positive | 1.55 (0.01-31.38) | |
| Lymph node | | |
| Negative | 20.96 (8.88-40.28) | 0.001[a] |
| Positive | 3.85 (0.01-31.38) | |
| Blood group | | |
| A | 15.8 (0.08-31.38) | 0.596[b] |
| B | 6.88 (0.01-31.17) | |
| O | 7.57 (0.28-40.28) | |
| AB | 0 | |
| Tumor size (cm) | | |
| >6 | 12.82 (0.01-40.28) | 0.562[a] |
| ≤6 | 10 (0.038-31.38) | |
| Stage | | |
| I | 23.13 (8.89-40.28) | 0.006[b] |
| II | 7.16 (0.038-31.38) | |
| III | 7.43 (0.01-23.13) | |
| IV | 1.5 (1.5-1.5) | |
| Tumor location | | |
| Greater curvature | 4.86 (0.9-31.17) | 0.089[b] |
| Lesser curvature | 20.96 (0.28-40.28) | |
| Cardia | 0.19 (0.08-31.38) | |
| Antrum | 10.74 (0.01-12.82) | |
| Histological type | | |
| Mucinous adenocarcinoma | 0.19 (0.01-19.45) | 0.071[b] |
| Intestinal | 12.82 (0.08-40.28) | |
| Signet ring cell | 11.16 (0.9-14.08) | |
| Metastasis | | |
| Negative | 12.15 (0.08-40.28) | 0.694[a] |
| Positive | 11.16 (0.01-31.17) | |

[a]Mann-Whitney test, [b]Kruskal-Wallis test

Furthermore, we checked EST profile of nonvalidated genes in UniGene database to exclusively choose the ones which are normally expressed in stomach tissue. Finally, the ultimate gene list [Supplementary Table 1] was imputed in DAVID database to access the signaling pathways strikingly associated with cancer.

Interestingly, miR-146a ultimate targetome was shown to be potential in regulating some cancer-related signaling pathways, among which ERBB2, RET, ITGB, P21, FADD, and Fas were the most important ones. The main function of these genes is regulating proliferation and apoptosis in gastric cells [Figure 4]. Altogether, it might support the impact of miR-146a in gastric tumorigenesis.

## DISCUSSION

Commensurate with the World Health Organization, *H. pylori* has been categorized as a type 1 carcinogen.[27] These Gram-negative bacteria express a range of virulence factors such as VacA, CagA, and pathogenicity island, resulting in gastric tumorigenesis via dysregulation of host cell function and signaling pathways.[28-30] miRNAs might be one of the intracellular-affected molecules by *H. pylori* infection. These single-stranded noncoding RNAs have a key role in the regulation of cellular processes and growth through modulating gene expression posttranscriptionally.[31]

In this study, we analyzed the expression level of miR-146a in both control and gastric cancer fresh tissue samples to assess miR-146a expression level among different samples. Due to the role of miR-146a in mediating the inflammation process in human cells, we also hypothesized that *H. pylori* might disturb the miR-146a expression, which in turn causes gastric tumorigenesis. Therefore, we also analyzed the potential association between *H. pylori* infection and miR-146a dysregulation. To this aim, we quantitatively assessed miR-146a in 144 fresh tissue samples, including 44 normal controls and 100 gastric cancer cases.

Previously, Hou *et al*. indicated that miR-146a in significantly downregulated in gastric cancer tissues, suggesting that this miRNA may play a role as a tumor suppressor in the context of gastric cancer.[17] Similarly and through studying 90 samples, Kogo *et al*. reported the reduced expression of miR-146a in gastric cancer.[16] However, our data indicated that miR-146a is highly expressed in gastric tumors as compared

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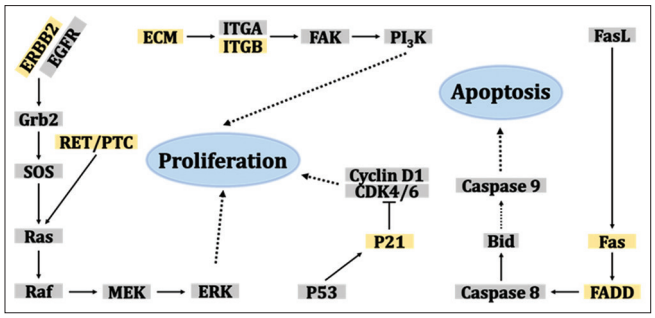


**Figure 4:** miR-146a is potential to target the still invalidated genes which regulate important intracellular processes such as proliferation and apoptosis. The invalidated predicted genes are illustrated by yellow color

to the healthy control samples [Figure 1a], supporting the oncogenic function of miR-146a in gastric tumorigenesis. This finding is consistent with Xiao *et al.*'s study, reporting that miR-146a is overexpressed in gastric cancer cases.[12] The discrepancy between various reports might be due to the use of the heterogeneous gastric samples, particularly in terms of histological subtypes, stages, and grades. Studying miR-146a expression in a larger population with enough samples in each group could be crucial to confirm either the oncogenic or the tumor-suppressor role of miR-146a in gastric cancer.

Functionally, Xiao *et al*. showed the regulatory role of miR-146a in apoptosis rate of gastric cancer cells via using flow cytometry method and caspase-3/7 activity assays. Their findings depicted that higher expression of miR-146a inhibits the apoptosis rate in gastric cancer cells, resulting in higher cell growth and proliferation.[12] They also reported that SMAD4 transcripts are the direct targets of miR-146a in gastric cancer. SMAD4 primarily works as the downstream component of transforming growth factor-beta signaling pathway, serving as a tumor-suppressor gene in gastric and colon carcinomas.[32] Moreover, TRAF6,[33] IRAK1, TLR4,[34] Stat1,[35] TBP,[36] and chemokine CCL8/MCP-2[37] are other validated targets of miR-146a. Using *in silico* analyses, we also showed that miR-146a might be capable of affecting the regulation of invalidated genes such as ERBB2, RET, ITGB, P21, FADD, and Fas. These genes can modulate the important cellular processes such as proliferation and apoptosis [Figure 4], which are tightly linked to the gastric tumorigenesis. These genes have not been validated yet as a direct target of miR-146a, and further, biochemical analyses are required to prove their probable interaction with miR-146a.

*H. pylori* infection is one of the main risk factors for gastric cancer via deregulating host innate immune system. Despite the inflammatory-mediating role of miR-146a, there was not any significant association between miR-146a and *H. pylori* infection in our study [Figure 1b-d]. Despite our results, Xiao *et al*. showed the higher expression level of miR-146a in gastric cancer cases,[12] reflecting the importance of further investigations to test the *H. pylori*–miR-146a relationship.

Our further analyses on the clinicopathological study demonstrated that miR-146a was highly expressed in the patients with Stage I of gastric cancer as compared to the Stages III and IV. The expression level of miR-146a was also significantly higher in lymph node-negative gastric cancer patients [Figure 3]. It supports the hypothesis that miR-146a is important for the initial steps of gastric tumorigenesis and is required to be downregulated for the later steps such as invasion and metastasis. Consistent with our results, Kogo *et al*. reported the lower expression level of miR-146a in lymph node-positive gastric cancer patients.[16] It might imply the importance of miR-146a in initiating steps of gastric tumorigenesis.

## CONCLUSION

Our study showed overexpression of miR-146a in the gastric tumor as compared to the healthy normal tissues, in an *H. pylori* infection-independent manner. However, lower expression of miR-146a was statistically linked to the higher stages of gastric cancer and also lymph node metastasis. It suggests that this miRNA is highly expressed in the initial steps of gastric tumorigenesis and is needed to be reduced upon metastasis.

### Acknowledgment

The staffs of Al-Zahra and Shahid Sadooghy Hospitals of Isfahan are highly appreciated for their contribution in collecting the samples.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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