Original Article

Validation of salivary microRNA 301a as a potential non-invasive diagnostic biomarker in gastric carcinoma

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Abstract

Context: Early detection of cancer is key to good prognosis and improved survival rate. Gastric cancer (GC) is fatal and presents with poor prognosis as it is usually diagnosed only at advanced stages. Saliva is emerging as a preferred diagnostic tool due to its advantages of being non-invasive, easy to collect, and cost-effective. Salivary microRNAs (miRNA) are more reliable due to their stability, resistance to degradation and its abundant involvement in cancer progression.

Aim: To estimate and validate the potential of salivary miRNA 301a in the diagnosis of Gastric cancer.

Methods and Materials: This Cross-sectional study comprised of 60 GC patients (Group I) and 60 normal controls (Group II). Fold change (FC) values of serum and salivary miRNA301a levels were estimated using the Real Time-Polymerisation Chain Reaction (RT-PCR) and compared between the study groups. Correlation between the serum and salivary miRNA301a levels was also evaluated. MiRNA301a levels were compared and correlated, with the clinical stage and histopathological grades of GC.

Results: The mean FC of serum (Mean ± SD = 2.62 ± 0.75, Mean Rank = 90.5) and salivary (Mean ± SD = 2.03 ± 0.56, Mean Rank = 90.5) miRNA301a was significantly higher in Gastric cancer patients compared to controls (Mean ± SD = 0.99 ± 0.004, Mean Rank = 30.5). Salivary miRNA301a levels exhibited significant positive correlation with serum miRNA301a in gastric cancer patients (r = 0.941). The mean FC of serum and salivary microRNA 301a exhibited significant correlation with the clinical stages and histopathological grades of GC.

Conclusion: Salivary miRNA301a is a potential reliable diagnostic tool for early screening of Gastric cancer.

Keywords: Biomarker, gastric carcinoma, microRNA, non-invasive, saliva, serum

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INTRODUCTION

Gastric cancer (GC) is the third leading cause of malignancy-related deaths globally with over 1 million annual incidences and approximately 7,83,000 associated deaths.[1] GC is associated with increased mortality and poor prognosis. The fatality is often due to the delayed diagnosis as the early symptoms are non-specific and the disease is usually in advanced stage at the time of diagnosis. The 5-year survival rate of GC diagnosed at advanced stage is only 30% but the survival rate is 70%–90% when diagnosed at early stage.[2]

The most common currently available diagnostic tool for Gastric cancer is upper endoscopy. Despite being a reliable method with a sensitivity of 70%–92%, the limitations of invasiveness, cost, and anaesthetic requirements makes it the least preferred method.[3] The second available alternative to endoscopy is radiography but the major disadvantage of this modality is its low sensitivity and high negative predictive value.[4] Hence, the need of the hour is an effective early screening tool for GC.

'Liquid Biopsy' has made rapid progress and a plethora of biomarkers have been discovered in blood. Conventional serum biomarkers have been reported to be ineffective in early screening of GC.[5] Alternative biomarkers such as trefoil factor three and pepsinogen have limited sensitivity and specificity.[6] Saliva has recently emerged as a promising diagnostic tool for various systemic diseases including cancers and is gaining momentum over other body fluids, such as blood as saliva collection is easy, safe, non-invasive and cost-effective.

Numerous salivary biomarkers have been detected in gastric cancer, including cell free DNA, cell free RNA, microRNA, proteins and exosomes.[7] Among these biomarkers, Salivary microRNAs has been reported to be more reliable due to their stability, resistance to degradation and also a preferred biomarker as it is abundantly involved in regulation of numerous oncogenic and tumour suppressor genes.[8]

MicroRNAs are small, endogenous, non-coding single-stranded RNAs (17–25 nucleotides) that bind to complementary sequences of target mRNAs promoting its degradation or translational repression.[9] MicroRNAs are classified as Oncomirs (Oncogenic microRNAs) and TSmirs (Tumor Supressor microRNAs) based on their role and expression pattern in the cancer progression.[10] Numerous microRNAs have been reported to show aberrant expression in serum and saliva of gastric cancer patients. Significant alterations in salivary microRNA301a have been reported in gastric cancer.[11] Various *in-vitro* studies have analysed the role of miRNA301a in gastric cancer cell lines.[12-15] However, there is a paucity of studies in Indian population. Hence, the current study was undertaken to determine and evaluate the potential of salivary microRNA 301a as a potential diagnostic marker in gastric cancer.

MATERIALS AND METHODS

The study population included 120 individuals (N = 120) divided into two study groups: Group I comprises of 60 patients with Gastric cancer and Group II comprises of 60 normal healthy individuals. Healthy individuals without any co-morbid condition, such as diabetes or hypertension or deleterious habits like smoking or alcohol consumption were included as Controls in group II. The study was approved by the Institutional Ethical Committee and informed consent was obtained from all the study participants.

Demographic patient data, clinical history, clinical stage and histopathological tumour grade was recorded for group I individuals. Collection of serum samples were done using Venipuncture method from the study participants. Blood samples were centrifuged for 10 minutes at 1500 RPM and the supernatant serum was frozen at −80ºC until further use. Unstimulated salivary samples were collected by spit technique from groups I and II. The samples were centrifuged at 2600 RPM for 15 min at 4ºC. This cell-free salivary supernatant sample was stored at −80ºC until further use.

MicroRNA 301a was extracted from serum and salivary samples using mirVana miRNA extraction kit. Complementary DNA (cDNA) synthesis was conducted using Revert Aid First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Primer sequences were prepared [Table 1]. U6 small nuclear RNA was used as the reference

Table 1: Primer sequences used in the study

Name	Sequence 5'—>3'
miR-301a-5p RT	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTAGT 3'
miR-301a-5p forward	5'-GCTCTGACTTTATTGCACTACT- 3'
URP	5'- CCAGTGCAGGGTCCGAGGTAT -3'
U6 RT	5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATG−3'
U6 forward	5'- GCGCGTCGTGAAGCGTTC-3'
U6 reverse	5'- GTGCAGGGTCCGAGGT -3'

gene for normalizing the data. Detection of miRNA 301a and U6 gene expression in saliva and serum of cases and controls was done using Reverse Transcriptase PCR (RT PCR) with TaqMan probes.

RESULTS

The study included a total of 120 patients divided into two groups. Group I comprised of 60 patients with Gastric cancer and Group II consisted of 60 normal controls. The mean age of study participants in Gastric cancer group was 58.87 ± 6.5 years and the mean age of individuals in the control group was 64 ± 7.6 years. The study sample (N = 120) included a total of 78 males and 42 females. There were 39 males (65%) and 21 females (35%) in Gastric cancer group. The normal controls included 39 males (65%) and 21 females (35%).

The mean fold change (FC) for Serum miRNA301a levels were higher in the gastric cancer group (Mean ± SD = 2.62 ± 0.75, Mean Rank = 90.5) compared to the mean fold change for Serum miRNA301a levels in the control group (Mean ± SD = 0.99 ± 0.004, Mean Rank = 30.5). The difference was highly statistically significant (P = 0.000) [Table 2].

The mean fold change (FC) for Salivary miRNA301a levels were higher in the gastric cancer group (Mean ± SD = 2.03 ± 0.56, Mean Rank =90.5) compared to the mean fold change for Salivary miRNA301a levels in the control group (Mean ± SD = 0.99 ± 0.004, Mean Rank = 30.5). The difference was highly statistically significant (P = 0.000) [Table 2].

Table 2: Comparison of Serum and Salivary miRNA301a Fold change (FC) levels between the study groups

Sample	Study Group	Mean	Standard Deviation	Std Error Mean	Mean Rank	*P*
Serum	Group I	2.62	0.75	0.09	90.5	0.000*
	Group II	0.99	0.004	0.0005	30.5	
Saliva	Group I	2.03	0.56	0.07	90.5	0.000*
	Group II	0.99	0.004	0.0005	30.5	

*Highly Significant, Mann-Whitney test

Pearson's correlation analysis revealed a positive correlation between the mean fold change for Serum miRNA301a levels with the mean fold change for Salivary miRNA301a levels (r = 0.941) and the correlation was statistically significant (P = 0.000*). A similar significant positive correlation was observed in the control group also [Table 3].

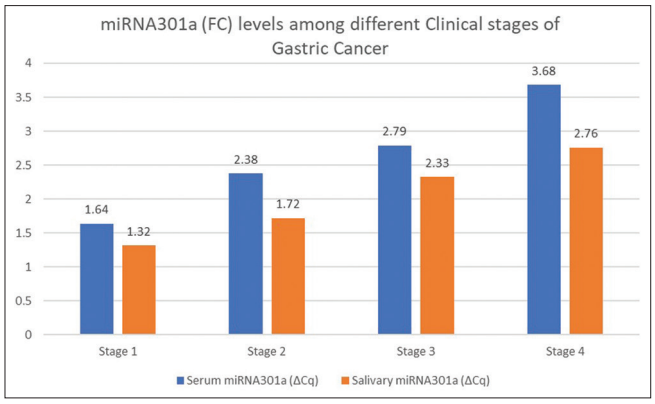
The mean FC of serum and salivary microRNA 301a in group I was 2.62 ± 0.75 and 2.03 ± 0.56 respectively. The mean FC of serum and salivary microRNA 301a exhibited significant correlation with the clinical stages of gastric cancer [Kruskal-Wallis H – 54.39 (Serum), 55.57 (Saliva), df =3, P value = 0.000**, Graph 1]. Pairwise comparison revealed that both serum and salivary miRNA301a levels correlated with clinical stages of gastric cancer (Kruskal-Wallis H – 54.39 (Serum), 55.57 (Saliva), df =3, P value = 0.000**). Pairwise comparison revealed that both serum and salivary miRNA301a levels were significant and highly reliable in differentiating clinical stages I-III, I-IV and II-IV of Gastric cancer (P = 0.000*). However, miRNA301a did not show a significant efficacy in differentiating clinical stages I-II (P = 0.11), II-III (P = 0.10) and II-IV (P = 0.11).

The distribution of histopathological grades of GC in group I was Well-differentiated (WD, n = 16), Moderately differentiated GC (MD, n = 17) and poorly, differentiated (PD, n = 27) GC. The mean FC of serum and salivary microRNA 301a exhibited significant correlation with the histopathologic grades of gastric cancer [Kruskal-Wallis H – 45.29 (Serum), 47.04 (Saliva), df =2, P value = 0.000**, Graph 2]. Pairwise comparison revealed that serum miRNA301a levels were significant and highly reliable in differentiating Well-differentiated GC from Poorly differentiated GC (P = 0.000*); Well-differentiated and Moderately-differentiated GC (P = 0.01*) and between Moderately differentiated and

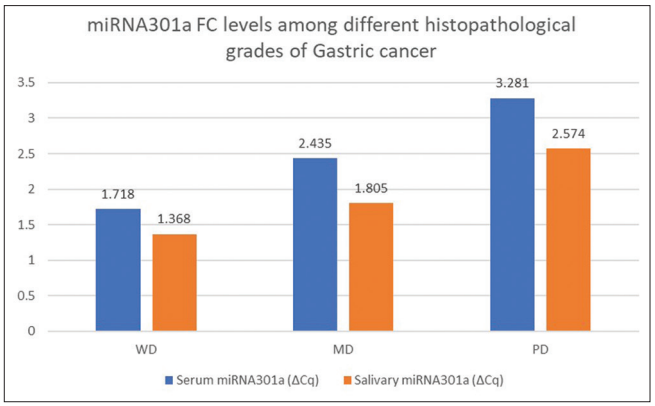
Table 3: Correlation between Serum and Salivary miRNA301a (FC) levels in the study groups

Study group	Sample		Serum miRNA301a (FC)	Salivary miRNA301a (FC)
Gastric cancer	Serum miRNA301a (FC)	Pearson Correlation	1	0.941**
		Sig. (2-tailed)		0.0.000
		n	60	60
	Salivary miRNA301a (FC)	Pearson Correlation	0.941**	1
		Sig. (2-tailed)	0.000	
		n	60	60
Normal control	Serum miRNA301a (FC)	Pearson Correlation	1	1.000**
		Sig. (2-tailed)		0.000
		n	60	60
	Salivary miRNA301a (FC)	Pearson Correlation	1.000**	1
		Sig. (2-tailed)	0.0000	
		n	60	60

*P<0.05, Statistically significant, Pearsons's correlation analysis



Graph 1: Comparison of Serum and Salivary miRNA301a (FC) levels with the clinical staging in the Gastric cancer study group



Graph 2: Comparison of Serum and Salivary miRNA301a (FC) levels with the histopathological grading in the Gastric cancer study group

poorly differentiated GC ($P = 0.001$*). Pairwise comparison revealed that salivary miRNA301a levels were significant and highly reliable in differentiating Well-differentiated GC from Poorly differentiated GC ($P = 0.000$*); Well-differentiated and Moderately-differentiated GC ($P = 0.04$*) and the between Moderately differentiated and poorly differentiated GC ($P = 0.000$*).

DISCUSSION

Gastric cancer ranks among the top five most common cancers in India.[16] GC remains the fifth most common cancer among males and seventh most common among females in India.[17] Considering the occult nature of GC due its anatomic location, asymptomatic clinical presentation in early stages and invasive endoscopy is the only currently available screening tool, it is essential that a reliable, easy, cost-effective, non-invasive tool must be devised to improve the prognosis of these patients. Saliva being an excellent diagnostic liquid biopsy tool with all these advantages and microRNAs being stable and abundant biomarkers in saliva,[11] this study aims to assess the potential of salivary miRNA301a in early screening of GC.

Numerous miRNAs in human blood are usually derived from lysis of apoptotic or necrotic cells and secretion of specific cells. The miRNAs remain stable by binding to Argonaut 2 protein or through the auto specific hairpin structure. This prevents degradation by ribonuclease in the blood and facilitates quantitative detection.[18] In 2008, Chen X *et al.* first reported the quantitative detection of miRNA in serum samples, proving the feasibility for the approach.[19]

The diagnostic potential of salivary microRNAs in different cancer types, its implication in therapeutics has been elucidated by Rapado-González O *et al.*[7] A systematic review by Setti *et al.*[8] aiming at analysing if salivary microRNAs are reliable biomarkers for diagnosis of cancer and systemic diseases has revealed that salivary miR 140-5p and miR301a are promising diagnostic markers for Gastric cancer.

The current study has proved that the expression of salivary miRNA301a is increased in Gastric cancer patients and the difference is statistically significant. Our findings are in concordance with those by Xu *et al.* who reported an upregulation of miRNA301a in GC tumour tissues and cell lines using RT-PCR.[12] He assessed the effects of miRNA301a on clone formation, migration and invasion of HGC-27 and SGR 7901 post transfection of an miRNA301a inhibitor. The study concluded that a significant correlation exists between miRNA301a expression and TNM staging and prognosis. Li *et al.* discovered and validated 3mRNA (SPINK 7, PPL and SEMA4B) and 2 miRNAs (MIR140-5p and MIR301a) in unstimulated saliva as potential biomarkers for early screening of GC.[11]

Our study revealed a correlation between the miRNA301a expression levels and the stage and grade of GC substantiating the role of this microRNA in evaluating the prognosis of GC. Similar findings were reported by Xu *et al.*[12]

Guo *et al.*[20] proved that exosome miR301a-3p could serve as a non-invasive biomarker for Trastuzumab resistance and a novel potential therapeutic target. Numerous *in-vitro* studies have elucidated the downstream targets and pathway of action of miRNA301a in tumour progression of GC. Huang Y *et al.* performed bioinformatic analysis of miRNA301a and its target mRNA. The expression levels of miR301a-5p and Scinderin (SCIN) were detected using RT-PCR and concluded that increased expression of miRNA301a promoted malignant phenotype by targeting SCIN in GC.[13] Liu *et al.* identified a negative correlation

between miR301a-3p (increased expression in GC) and CX 43 (decreased expression in GC) using RT-PCR and Dual-Luciferase reporter assay.[14] Xu X *et al*. reported that an upregulated miRNA301a promoted tumour progression in GC by supressing NKRF and activation of NF-KB signalling.[15]

Salivary miRNA301a can serve as a potential non-invasive diagnostic biomarker for early diagnosis of GC. Also, the correlation of these biomarker expressions with clinical stage and histopathological grade of GC will establish the prognostic potential of the validated biomarkers, thereby improving the survival rate of GC patients.

Future research can focus on profiling the saliva of GC patients and identifying a discriminatory panel of miRNAs exhibiting differential expression in GC. Discovery and validation of salivary microRNAs as early screening biomarkers for GC will produce a significant paradigm shift in current clinical practice serving as an effective, reliable, particularly non-invasive alternative diagnostic modality, thereby improving the prognosis and survival rate in these patients.

Validation of a discriminatory panel of serum and salivary microRNAs will be a significant development in patient care as liquid biopsy will overcome the limitations of the existing screening modalities of Gastric cancer. Successful validation of a reliable, effective, discriminatory panel of salivary miRNA biomarkers in early detection of GC can form the basis for further progress towards fabrication of a chairside screening device as an early diagnostic tool for GC. Detection of significantly altered miRNAs in GC patients will facilitate further exploration of its therapeutic implications using small molecular inhibitors of miRNAs (SMIR) approach and miRNA mimics.

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Conflicts of interest

There are no conflicts of interest.

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