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Circulating Micro RNA 21 Levels in Gastric Cancer Patients in Sri Lanka and Role of *Helicobacter pylori* as a Risk for Development of Gastric Cancer

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Abstract

Background: Gastric cancer is the second leading cause of cancer-related mortality and the fourth most common cancer globally. This study was conducted to evaluate the usefulness of miR-21 as a less invasive screening and prognostic marker in Gastric cancer patients from Sri Lanka. Further the *H. pylori* status was investigated.

Methods: Twenty patients who were diagnosed as having gastric cancer were enrolled along with the age sex matched healthy controls in this study. Specimens and clinicopathologic features of the patients were collected at 2 teaching hospitals. Circulating miR-21 levels in serum were quantified by real time PCR using miR-16 as a normalization control. The serum of each patient with gastric cancer was tested for the presence of IgG antibody to *H. pylori*.

Results: The age of the patients ranged between 45 to 79 years, with a median of 59 years. The peak incidence of tumors was seen in the fifth decade of life. Majority of the patients 60% (12/20) presented with non-cardia gastric cancer and 70% (14/20) of patients had intestinal type according to Lauren's classification. The median miR-21 expression of gastric cancer group was found to be higher (0.79) than the healthy controls (0.586), although not significant. Proportion of *H. pylori* infection was high in gastric cancer patients (85%). No significant difference in expression levels of miRNA-21 was observed in gastric cancer patients who were positive or negative serologically for *H. pylori*.

Keywords: Gastric cancer, miRNA-21, *Helicobacter pylori*

Introduction

The etiology of gastric cancer can be multifactorial where host genetic factors such as familial susceptibility, obesity, pernicious anemia, BRCA1 and BRCA2, socio-economic background, *H. pylori* infection, diet, alcohol and tobacco may have a potential role in triggering its development [1-4]. Among these risk factors infection by *H. pylori* has been reported as a significant risk factor [3,5].

Early detection of gastric cancer is a challenge to physicians due to the nonspecific clinical presentations associated with symptoms of dyspepsia [6]. As a consequence, gastric cancer patients present late resulting in high mortality. Therefore, there is an urgent need for early identification techniques. The use of biomarkers for detection and monitoring of gastric cancer patients is an expanding field of research with unlimited potential.

MicroRNAs are endogenous non-coding regulatory sequences that play a vital role in cell proliferation, metastasis, differentiation, development and apoptosis [7]. Aberrant expression of micro RNA has been correlated with the development of cancer and have been studied as potential markers for diagnosis and cancer definition [8,9].

MicroRNA-21 (miR-21) is a conserved mammalian miRNA encoded by the MIR21 gene [10]. It has been reported to be up regulated in various malignant and non-malignant conditions. Several studies have reported that expression of miR-21 is significantly higher in gastric cancer cells compared with non-cancerous cells [11-13]. *H. pylori* infected gastric tissue also showed higher expression of miR-21[11]. However, these results have not been validated as different study groups have published conflicting results [14,15].

In this study, the expression levels of miR-21 were compared between gastric cancer patients and healthy controls. Further the *H. pylori* status and miR-21 expression was investigated in these two groups to determine the usefulness of miR21 as a biomarker in detection of gastric cancer.

Materials and Methods

This was carried out as a comparative, descriptive study at the Faculty of Medical Sciences, University of Sri Jayewardenepura, Department of Microbiology and Department of Pathology. Specimens were collected from Endoscopy unit, Colombo South Teaching Hospital and National Cancer Institute, Maharagama Sri Lanka. This study was approved by the ethical Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura.

A blood sample (5 ml) was collected from twenty gastric cancer patients, dyspeptic patients and healthy volunteers after obtaining written consent. The blood specimen was transported to the laboratory and serum was separated and aliquoted into sterilized cryovials for storing under -800°C till use.

Extraction of microRNA from serum and real time PCR

Micro RNA was extracted from serum using miRNeasy Serum/ Plasma kit (Promega) and cDNA was synthesized according to the

manufacturer's instructions. Synthesized cDNA was amplified using Taqman PCR Master Mix using gene specific stem-loop primers and a gene-specific probe on a 7500 Real-Time PCR system (Applied Biosystems). This procedure was carried out using Taqman miRNA assays with predesigned primers and probe (MGB probe; FAM dye and a non-fluorescent quencher) for miR-21 and miR-16.

First, miR-16 and miR-21 amplification curves were analyzed to check the validation of the current real time quantitative PCR (qPCR). The cDNA template was mixed with the master mix and RNase-free water and was placed in a PCR real-time plate and specified thermal conditions for the reaction to occur was provided by 7500 Real-Time PCR system (Applied Bio-systems). Each sample was done in duplicate. The relative expression of miR-21 in each sample was calculated using the comparative threshold cycle method $\Delta\Delta Ct=\Delta Ct$ (miR-21)-ΔCt (miR-16) using Applied Biosystems 7500 software v2.0.6.

Data analysis

Mann-Whitney U test was carried out to compare the expression of miR-21 in gastric cancer patients and *H. pylori* positive dyspeptic patients with the expression of miR-21 in healthy group as a control. A two-sided p value less than <0.05 was taken as significant. Data analysis was carried out using SPSS 16.0 (SPSS Inc., Chicago, USA).

Detection of *H. pylori* specific IgG in serum

The serum of gastric cancer patients and healthy controls were tested for the presence of IgG antibodies using a *Helicobacter pylori* IgG ELISA kit (Bioactivadiagnostica, Germany) following the manufacturer's instructions. Serum separated from blood was diluted with sample diluent and ELISA procedure was carried out. The absorbance was measured at 450 nm. The results were interpreted using GraphPad Prism version 6.

Results

Demographic and clinicopathological features of gastric cancer patients

Twenty gastric cancer patients were enrolled in the study. The age of the patients ranged between 45 to 79 years, with a median of 59 years. The peak incidence of tumors was seen in the fifth decade of life. Number of 13 gastric cancer patients, were males. Majority of Gastric Cancer patients (70%) had low BMI. Seventeen patients were identified as *H. pylori* positive in this group while in the control group 15 were found to be positive for *H. pylori*. Although data on food habits, smoking and alcohol were gathered comparison could not be made due to the small sample size in *H. pylori* negative Gastric cancer group.

The clinicopathological features of the gastric cancer group are described in Table 1. Majority of the patients 60% (12/20) presented with non-cardia gastric cancer. Histological examination revealed 70% (14/20) of patients had intestinal type according to Lauren's classification. TNM classification of tumors revealed that 10% of the population had T_1 and T_2 whilst 35% was classified as T_3 and 45% as T_4. Lymph node metastasis was detected in 25% of patients for each N_0, N_1, N_2 and N_3 classification types. Distal metastasis was detected in 20% of patients while it could not be assessed in 25%. When staging according to the TNM classification, majority were found to be in stage III (No of patients 12) while 3 patients were in each stage I and II. Only one patient was graded in stage IV.

Clinicopathological Feature	Number of Patients (%) (N=20)
Cancer site	
Cardia	08 (40%)
Non- cardia	12(60%)
Histology Type	
Intestinal	14 (70%)
Diffuse	07 (30%)
Duration of Gastric cancer	
=<6 months	13 (65%)
>6 months	07 (35%)
TMN classification	
Tumor invasion in gastric wall	
T_1	2 (10%)
T_2	2 (10%)
T_3	7 (35%)
T_4	9 (45%)
Lymph node involvement	
N_0	5 (25%)
N_1	5 (25%)
N_2	5 (25%)
N_3	5 (25%)
Metastasis	
M_0	5 (25%)
M_1	4 (20%)
No data	11 (55%)
Neoajuvant Therapy	
Given	11 (55%)
Not given	9 (45%)

Table 1: Clinicopathological Features of gastric cancer patients enrolled in this study.

miR-21 levels in serum

Circulating miR-21 levels in serum were quantified by real time PCR using miR-16 as a normalization control. Following Real Time PCR validation, the miR-21 levels in serum were measured from gastric cancer patients (n=20), and 20 age and sex matched healthy controls. Median values were obtained as the data was not normally distributed. The median miR-21 expression of gastric cancer group was

found to be higher (0.79) than the healthy controls (0.586), although not significant. The miR-21 expression in the Gastric cancer group ranged between 0.02 to 3.301 whereas the healthy controls ranged from 0.05 to 2.05. There were 3 patients who exceeded the highest miR-21 expression level of the healthy control group. There was no difference in miR-21 expression with gender and mean age in the gastric cancer patients.

The median miR-21 expression was higher in diffuse type (1.3215) when compared to the intestinal type (0.5). Non-cardia gastric cancer patients also had higher expression 0.909 (median) compared to cardia type (median 0.492). The median miR-21 expression levels for each cancer stage I, II, III and IV were 1.395, 0.999, 0.478 and 3.3.01 respectively.

Out of 20 patients 3 had developed metastasis (M_1) while 7 patients had no metastasis (M_0) and in other patients the status was unknown. The relative miR-21 expression in M_1 patients had a higher range 0.329–2.635 (median 0.473) compared to M_0 patients who had a range of 0.02–1.85 (median 0.5).

Proportion of *H. pylori* infection was high in gastric cancer patients (85%). No significant difference in expression levels of miRNA-21 was observed in gastric cancer patients who were positive or negative serologically for *H. pylori*. The mean miR-21 expression in *H. pylori* positive was 0.958 while it was 1.835 in the *H. pylori* negative Gastric cancer patients.

When histology type revealed based on Lauren's criteria, the intestinal type (70%;14/20) was seen to be the most prevalent. *H. pylori* infections were detected in 92.8% and 66.6% of intestinal type and diffuse type respectively. The median miR-21 expression was 0.847 in the *H. pylori* positive patients with intestinal type cancer while in *H. pylori* positive patients with diffuse type cancer the expression was found to be 1.921.

Discussion

The diagnosis of gastric cancer and its progression is hindered by the lack of early diagnostic markers. miRNAs have been suggested to be expressed in certain tumors and their expression as circulating miRNAs have been reported from patients with gastric cancer. Studies suggest that MiR-21 expression is significantly elevated in a variety of solid tumor tissues compared to normal tissues and was associated with local invasion and lymph node metastasis [16]. Among the miRNAs studied in patients with gastric cancer, miR-21 expression has been investigated by several groups [10,17] and has been found to have a potential for diagnostic value with moderate sensitivity and specificity.

The role of miR-21 as a circulating biomarker among patients with gastric cancer in Sri Lanka is yet not addressed. This study evaluated the usefulness of miR-21 as a less invasive screening and prognostic marker in Gastric cancer patients from Sri Lanka. In this study group the mean miRNA-21 expression in gastric cancer patients were higher compared to healthy age and sex matched controls although statistical significance could not be observed which may be due to the small sample size which is a limitation of this study. Several groups have reported a significant elevation of miR-21 expression in gastric tumors among patients from various geographical regions. [11,14,18,19] Further a study by Guo-Jian et al report a significant reduction of miR-21 in post-operative gastric cancer patients supporting its usefulness as a marker of GC [13].

A systematic meta-analysis suggests that patients with elevated miR-21 expression had poor prognosis. In the present study majority of the patients (12/20) were found to be in stage III of the TNM classification. One patient classified as stage IV reported the highest miR-21 relative expression (3.301). Further in this study, patients with distal metastasis had elevated expression of miR-21 compared to patients with no metastasis (M_0) supporting previous findings which showed that high expression of miR-21 was associated with high tumor differentiation, lymph node metastasis and TNM stage [20]. The miR-21 acts as an oncogene and regulates cellular processes and thus has a role in increasing cell proliferation, colony formation and cell migration of tumor cells [21-23].

Conclusion

Higher expression of this marker has not been reported among the Sri Lankan gastric cancer population previously and hence this study suggests that miR-21 may be a good candidate for screening for gastric cancer and in determining the prognosis. In the present study the relative miR-21 expression was found to be higher in both stages II and III as opposed to stage I of TNM classification. Further based on literature it is also suggested to be promising in post-operative prognosis. Although a limitation of this study was the limited sample size, this study suggests a future direction in gastric cancer management in the local setting and needs further investigation.

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