Association between *Helicobacter pylori* Infection and Stomach Tumors in Sudan Using Polymerase Chain Reaction

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Abstract: This study was aimed to determine the association between *H. pylori* infection and stomach cancer using PCR technique. Stomach samples were collected from 60 patients suffering from stomach tumors using endoscopes, each sample was divided into two specimens, one specimen was fixed in 10% formalin, prepared in paraffin block and stained with Heamatoxilin and Eosin (H&E) for general morphology; another specimen was fixed in 10% normal saline and prepared for Polymerase Chain Reaction (PCR) to detect *H. pylori*. The age of the patients ranged from 35-75 years. Their mean age was 54.9+11.6 year. Male to female ratio was about (1:1) Histopathologically, 30(50%) cases were diagnosed as malignant tumor, 22 (73.3%) of cases were diagnosed as well differentiated adenocarcinomas, 6 (20.0%) were poorly differentiated adenocarcinomas, while 2 (6.6%) were carcinoid tumors. The other 30(50%) cases were diagnosed as polypoid (benign) tumors. *H. pylori* was detected in malignant cases only. This bacterium was seen in 9(30.0%) sections stained with H&E. While this bacterium was seen in 19 (63.3%) cases when using conventional PCR technique. The study indicates that stomach adenocarcinoma is more frequently encountered than other types and *H. pylori* infection associated with stomach adenocarcinoma.

Key words:

INTRODUCTION

Stomach cancer, or gastric cancer, refers to cancer arising from any part of the stomach. It causes about 800,000 deaths worldwide per year. Stomach cancer is the fourth most common cancer worldwide (Garcia *et al*., 2007). It is a disease with a high death rate making it the second most common cause of cancer death worldwide after lung cancer. It is more common in men and in developing countries (Parkin, 2002).

Metastasis occurs in 80-90% of individuals with stomach cancer, with a six month survival rate of 65% in those diagnosed in early stages and less than 15% of those diagnosed in late stages. Many studies show that less than 5% of stomach cancers occur in people under 40 years of age with 81.1% of that 5% in the age-group of 30 to 39 and 18.9% in the age-group of 20 to 29 (Abrams and Gerald, 2008).

In Sudan stomach cancer is the sixth most common cancer in 2011 (Alsir and Ibnouf, 2011). Infection by Helicobacter pylori is believed to be the cause of most stomach cancer while autoimmune atrophic gastritis, intestinal metaplasia and various genetic factors are associated with increased risk levels. The Merck Manual states that diet plays role in the genesis of stomach cancer. However, the American Cancer Society lists the following dietary risks, and protective factors, for stomach cancer: "smoked foods, salted fish and meat, and pickled vegetables .Nitrates and nitrites are substances commonly found in cured meats. They can be converted by certain bacteria, such as *H. pylori,* into compounds that have been found to cause stomach cancer in animals. On the other hand, eating fresh fruits and vegetables that contain antioxidant vitamins such as A and C appears to lower the risk of stomach cancer (Rustgi, 2007).

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Karry, 1993). The aim of this study was to determine the association between *H. pylori* infection and stomach cancer using PCR technique.

Methodology:
Sample Collection:
Sixty stomach biopsies were collected from 60 patients suffering from stomach tumors using endoscopes, patient particulars (age and sex,) were recorded and each patient well informed about the study and signed a written ethical consent form approved by the ethical committee, College of Medical Laboratory Research Board; Sudan University of Science and Technology, Sudan.

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Sample Preparation and Staining:

Two samples were taken from each patient by surgeon using endoscopes one sample were fixed in 10% formalin and stained with hematoxylin and eosin (H&E) described by (Bancroft *et al*,2002) for general histopathology; another sample were fixed in 10% normal saline for molecular biology.

DNA Extraction:

DNA extraction was performed from gastric tissues using DNA Extraction Kit (Qiagen, USA). The entire extracted DNA was stored at -20º C until PCR.

Polymerase Chain Reaction:

Samples were amplified using PCR machine (PeQLab, advanced primus 96). The samples were amplified by *Helicobacter* genus-specific 16S rRNA primers (designated HC). The forward (C97) and the reverse (C98) primer amplified a product of approximately 400 bp. The protocol for the PCR was that, amplification consisted of initial denaturation at 94°C for 4 min, followed by denaturation at 94°C for 1 min, primer annealing at 55°C for 1.5 min, and extension at 72°C for 2 min. The samples were amplified for 35 cycles, with a final extension step at 72°C for 10 min. PCR were carried out in 25μl containing master mixture of the following components, 2.5μl of buffer, 17μl of H_2O, 0.75μl of $MgCl_2$, 0.5μl of DNTPs 2μl of primer and 0.25μl of Taq polymerase was added to the mixture and 2μl of DNA were taken from each sample (Nilsson *et al*, 2000). The sequences of the primers were as followed:
Forward primer: GCGCAATCAGCGTCAGGTAATG
Reverse primer: GCTAAGAGAGCAGCCTATGTCC

Gel Electrophoresis:

Five μl of product was added to 2 μl of gel loading buffer (0.25% (w/v) xylene cyanol, 30% (v/v)glycerol, 100 mM EDTA, pH 8.0),then run in electrophoreses on a 2% agarose gel containing Ethidium bromide (0.5 μg/ml), visualized under ultraviolet illumination, and photographed. A 100-bp DNA ladder was used as a size marker.

Statistical Analysis:

Data was analyzed using SPSS computer program. Frequencies, cross tabulation and chi-square were calculated.

Results:

Sixty patients suffering from stomach tumors were diagnosed, half of cases were stomach adenocarcinomas distributed as followed 22 (36.7%) were well differentiated adenocarcinoma,6 (10%) were poorly differentiated adenocarcinoma and 2 (3.3%) were carcinoid tumors. The other half of cases diagnosed as polypoid (benign) tumors (table 1). The patient age was ranged between 35-75 years. Their mean age was 54.9+11.6 year, 25 patients (41.7%) were within the age group 56-65 years and 16 (26.6%) patients were within the age group 35-45 years. Thirty two (53.3%) of the study population were male, while twenty eight (46.7%) were female (table 3).

Table 1: Frequency of Histopathological results among study population.

Histopathological results	Frequency	Percent
Well differentiated adonocarcinoma	22	36.7
Poorly differentiated adonocarcinoma	6	10.0
Carcinoid tumors	2	3.3
Benign tumors	30	50.0
Total	60	100.0

Table 2: Distribution of *H. pylori* among study population.

Presence of *H. pylori*	Stomach cancer		Benign tumors		Total	
	N	%	N	%	N	%
PCR positive	19	31.7	10	16.7	29	48.3
PCR negative	11	18.3	20	33.3	31	51.7
Total	30	50	30	50	60	100.0

The frequency of cancer was highest in the age group 56-65 years, 25 (41.6%) ,13 (52.0%) were male and 12 (48.0%) were female and the lowest in the age group 66-75 years 5 (15.0%) were male and 4 (3.3%) were female, 35-45 were 16 patients, 7 (43.7%) were male and 9 (56.3%) were female, 46-55 were 10 patients, 7 (70.0%) were male and 3 (30.0%) were female (table 3).

PCR technique for detection of *H. pylori* revealed positive results in 19 (31.6) cases of malignant stomach tumor distributed as followed 15 (25.0%) cases were well differentiated adenocarcinoma, 4(6.7%) cases were poorly differentiated adenocarcinoma and not detected in carcinoid tumor, while in benign tumor *H. pylori* revealed positive results in 10 (16.7) cases .(table 2 and 4).

Table 3: Different types of stomach tumors in relation to age and sex of patients.

Age and sex of patients		Histopathological results									
		Malignant						Benign tumors		Total	
		Well differentiated Adonocarcinoma		Poorly differentiated Adonocarcinoma		Carcinoid tumors					
		N	%	N	%	N	%	N	%	N	%
35-45	Male	2	12.5	2	12.5	0	0.0	3	18.8	7	43.7
	Female	4	25.0	1	6.3	0	0.0	4	25.0	9	56.3
46-55	Male	1	10.0	1	10.0	0	0.0	5	50.0	7	70.0
	Female	2	20.0	0	0.0	0	0.0	1	10.0	3	30.0
56-65	Male	6	24.0	1	4.0	1	3.3	5	20.0	13	52.0
	Female	5	20.0	0	0.0	1	3.3	6	24.0	12	48.0
66-75	Male	1	11.1	1	11.1	0	0.0	3	33.3	5	55.6
	Female	1	11.1	0	0.0	0	0.0	3	33.3	4	44.4
Total		22		6		2		30		60	100.0

Table 4: Distribution of *H. pylori* between different types of stomach tumors by PCR technique.

Presence of *H.pylori*	Histopathological results									
	Malignant						Benign tumors		Total	
	Well differentiated Adonocarcinoma		Poorly differentiated Adonocarcinoma		carcinoid tumors					
	N	%	N	%	N	%	N	%	N	%
PCR positive	15	25.0	4	6.7	0	0.0	10	16.7	29	48.3
PCR negative	7	11.7	2	3.3	2	3.3	20	33.3	31	51.7
Total	22	36.7	6	10.0	2	3.3	30	50.0	60	100.0
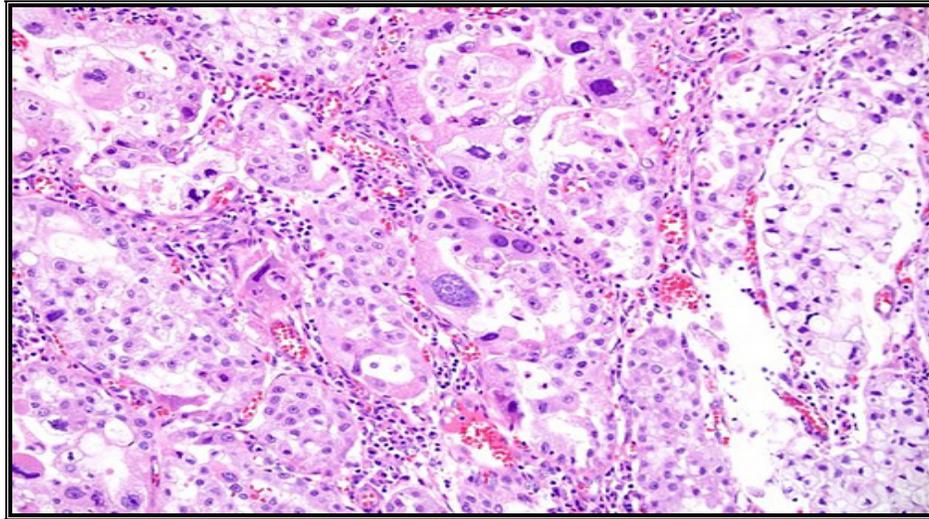
Photo 1: Stomach section taken from 70 years old male showing poorly differentiated adenocarcinoma with scattered giant tumor cells and signet ring cells (H&E 40x).
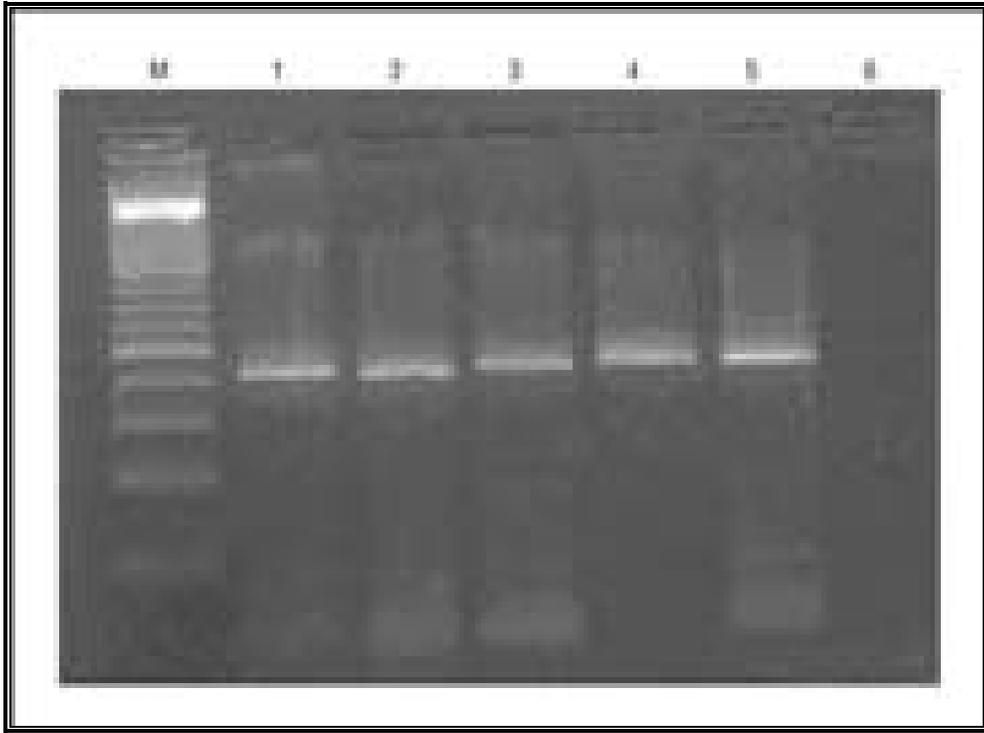
Photo 2: The 400-bp fragments were analyzed by 2% agarose gel electrophoresis. for confirming *H. pylori* in gastric biopsy specimens. (M): molecular ladder of 100 bp marker (100-1000bp, Fermentas, Germany), Lane1was positive control, lanes 2, 3, 4 and 5 *were H. pylori* positive samples and lane 6 was *H. pylori* negative sample.

Discussion:

Gastric cancer incidence and mortality have fallen dramatically over the past 70 years (Parkin *et al*, 1993). Despite its recent decline, gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide (Parkin *et al*., 2001). Its one of the commonest GIT problems (Stewart and Kleihues, 2003) (Parkin *et al*., 2004).

In the present study 60 patients suffering from stomach tumors were included. Their mean age was 54.9+11.6 year. Forty seven percent of cases occurred in age group 56-65, and this seems to agree with a study of Guamer *et al*,(1993), reported that the mean age of the overall population was 52.9 ± 13.5 years. Male to female ratio was about (1:1) suggesting that male may be more prone to stomach cancer than female. Such sex difference has also been indicated by Myint (2000).

Histopathological evaluation of stomach samples showed that (73.3%), (20.0%) and (6.6%) of patients had well differentiated adenocarcinomas, poorly differentiated adenocarcinoma and carcinoid tumours respectively, indicating that most type of cancer is adenocarcinomas. This may be supported by Carlo *et al*., (2006) , who reported about 90% of stomach tumors are adenocarcinomas, which are subdivided into two main histological types: well-differentiated and undifferentiated.

The presence of *H. pylori* in stomach sections detected here using PCR (63.3%) suggests that leakage and growth of bacteria inside the stomach is the cause of cancer. These findings disagreed with a study by Lars-Erik *et al*., (1996), who found that the incidence of gastric cancer was significantly lower than expected. After the second year of follow-up, the standardized incidence ratio was only 0.6 and remained stable there after, he conclude that gastric ulcer disease and gastric cancer have etiologic factors in common. A likely cause of both is atrophic gastritis induced by *H. pylori.* By contrast, there appear to be factors associated with duodenal ulcer disease that protect against gastric cancer. In this study detecting of *H. pylori* by H&E is less sensitive than PCR. This result agreed with Kolts *et al*., (1993), who found that The H&E stain interpreted by the rotating pathology staff was the least sensitive method and one of the least specific tests that were studied. The PCR product revealed 4 fragments with different molecular size. This observation might suggest different strains of the microbial pathogen might be associated with malignancy level and/or benign tumor of the study population and exert insight into strain specific virulence. In this regard this observation supported by the work done recently in Malasia by Alfiza *et al*., (2012). In this study there is no significant differences between male and

female whom infected by *H. pylori*, this finding supported by Koqa *et al.*,(2002), who reported no differences between male and female infected by *H. pylori*.

Conclusion:

The study indicates that stomach adenocarcinoma is more frequently encountered than other types and *H. pylori* infection associated with stomach cancer. 56-65 years was the most frequently affected age group with stomach cancer.

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