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The Investigation of *p53* and *Ki-67* Gene Mutations in Relation with *Helicobacter pylori* Infection in Patients with Gastric Cancer

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The aim of this study was to evaluate the role of *Helicobacter pylori* infection in relation with *p53* and *Ki-67* mutations in a case-control study in Iran, an endemic region for *H. pylori* infection. Sixty nine cases with gastric cancer and 96 cancer-free control subjects with either gastric ulcer or chronic gastritis were studied. Age, sex, *H. pylori* serology and presence of *p53* and *Ki-67* mutations were determined for all subjects. There was no significant difference between the groups for age ($p = 0.17$) but the groups were significantly different regarding sex ($p = 0.004$). The frequency of *H. pylori* seropositivity and presence of *p53* mutation were more common in case group ($p<0.001$ and $p<0.001$) while the difference between groups was not statistically significant for presence of *Ki-67* mutation ($p = 0.61$). After multivariate analysis the effect of *p53* mutation ($p<0.001$, adjusted OR = 126.56, 95% CI: 28.58-560.40) and *Ki-67* mutation ($p = 0.02$, adjusted OR = 0.21, 95% CI: 0.05-0.84) remained significant. *H. pylori* seropositivity lost its role in the presence of gastric cancer after multivariate analysis and the presence of *p53* and *Ki-67* were independent affecting factors for gastric cancer even in an endemic region for *H. pylori* such as Iran.

Key words: Gastric carcinoma, *p53*, *Ki-67*, *Helicobacter pylori*

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INTRODUCTION

Gastric cancer is the second most frequent malignant tumor in the world and contributes to significant cancer mortality particularly in Asia (Landis *et al*., 1998). *Helicobacter pylori* is a major cause of chronic active gastritis, gastric ulcer, duodenal ulcer, and gastric cancer (Eidt and Stolte, 1995; Kuipers and Meuwissen, 1996; Graham, 2000; Sepulveda and Graham, 2002; El-Omar *et al*., 2000; Uemura *et al*., 2001; Rezaii *et al*., 2008). It is also demonstrated that *p53* alteration is related to certain events in the multistep process in gastric carcinogenesis (Deguchi *et al*., 2001) but the reports concerning *Ki-67* in gastric cancer are few and controversial (Setälä *et al*., 1998). However, several studies have reported that there is no clear relationship between *H. pylori* infection and *p53* mutation in gastric cancer (Hongyo *et al*., 1995; Solcia *et al*., 1996; Palli *et al*., 1997; Blok *et al*., 1999).

Carcinogenesis is a multistep process in which regulation of both cell proliferation and apoptosis is involved. The role of affecting factors in the process of gastric carcinogenesis is not well determined. Only a few studies, with conflicting results, have addressed the potential role of *H. pylori* infection in relation with *p53* mutation in gastric cancer (Deguchi *et al*., 2001; Hongyo *et al*., 1995; Solcia *et al*., 1996; Palli *et al*., 1997; Blok *et al*., 1999). The purpose of this study was to evaluate the role of *H. pylori* infection in relation with *p53* and *Ki-67* mutations in a case-control study in Iran, an endemic region for *H. pylori* infection (Malekzadeh *et al*., 2004).

MATERIALS AND METHODS

Patients with histologically confirmed, earlier untreated adenocarcinoma of the stomach were recruited at the institution from March, 2003 to Dec., 2007. Cancer-free control subjects were selected from individuals presenting with either gastric ulcer or chronic gastritis in the gastroenterology clinic during the same time period. All subjects were nationals of Iran and were living in Tehran. Subjects were excluded if they had a known history of immune compromise (HIV positive, used steroids in the previous month, or received blood transfusion within the prior 6 months) or anti-*H. pylori* antibiotic therapy. After informed consent, each participant provided a blood sample.

The amount of immunoglobulin G (IgG) antibody to the high-molecular-weight cell-associated proteins of *H. pylori* was determined by using a well-characterized enzyme-linked immunosorbent assay (ELISA) (Captia™ *H. pylori* IgG ELISA; Trinity Biotech, Jamestown, NY) according to the manufacturer's instructions. The manufacturer-recommended cutoff points are values ≤0.9 ELISA units (EU) for negative, 0.9 to 1.1 EU for indeterminate, and ≥1.1 EU for positive. A pilot study (unpublished data) using the same ELISA kit on serum samples from 75 biopsy-positive cases and 48 biopsy-negative controls achieved a sensitivity of 100% and a specificity of 75%.

For the assays of *p53* protein, the sections were mounted on organosilane-coated glass slides, dewaxed and rehydrated. One slide of each case was transferred into citrate buffer and boiled twice for 5 min in a microwave oven. The sections were then incubated overnight at +4°C with monoclonal anti-*p53* antibody D07 (Dako, Glistrup, Denmark) diluted to 1:1000, washed and treated for 30 min with biotinylated secondary antibody (ABC Vectastain Elite Kit, Vector). The slides were incubated in preformed avidin-biotin-peroxidase complex, and peroxidase was made visible by immersing the slides in diaminobenzine tetrahydrochloride containing hydrogen peroxide (Sigma, Paisley, UK). Finally, the sections were counterstained with Mayer's haematoxylin. A sample of gastric cancer, previously shown to express *p53*, was used as a positive control.

For *Ki-67*, the sections were boiled in a household microwave oven six times, 5 min each, in citrate buffer. The *Ki-67* antigen was stained by applying a polyclonal antibody (Dako, Glistrup, Denmark) overnight as a 1:300 dilution and visualized as described above. Tonsil tissue was used as a positive control.

The analysis was performed in 2 steps. First, Chi-square and Student's t-test were used to compare the groups in univariate analysis. Statistically and clinically significant variables were extracted and included in multivariate binary logistic regression, in which Odds Ratios (OR) and 95% confidence intervals (95% CI) were determined and the effect of potential confounders was assessed. All analysis were done using SPSS 15.0 and the level of significance was always set at 0.05.

RESULTS

One case subject and one control subject had indeterminate results when tested for presence of *H. pylori*-specific IgG and were excluded from the analysis. Sixty-nine cases and 96 controls were eventually included. There was no significant difference between the groups for age ($p = 0.17$). Although male sex was more common in both case and control subjects but the groups were significantly different regarding sex ($p = 0.004$) (Table 1). The frequency of *H. pylori* seropositivity and

Table 1: Demographic data, *p53*, *Ki-67*, and *H. pylori* status for the groups

Studied variables	Case group (n = 69)	Control group (n = 96)	p-value
Age (years; Mean±SD)	62.5±7.0	64.7±12.3	0.17
Sex (M:F)	56:13	57:39	0.004*
p53 status (%)	66 (95.7)	18 (18.8)	<0.001*
Ki-67 status (%)	47 (68.1)	69 (71.9)	0.61
H. pylori seropositivity (%)	23 (33.3)	9 (9.4)	<0.001**

*p<0.05

Table 2: Multivariate regression on variables extracted from univariate analysis (Table 1)

Selected variables	p-value	Odds ratio (OR)	95% CI
Sex (Male)	0.842	0.89	0.28-2.83
p53 status	<0.001*	126.56	28.58-560.40
Ki-67 status	0.027*	0.21	0.05-0.84
H. pylori seropositivity	0.082	3.11	0.86-11.15*

*p<0.05

presence of *p53* mutation were more common in case group (p<0.001 and p<0.001) while the difference between groups was not statistically significant for presence of *Ki-67* mutation (p = 0.61).

Sex, *H. pylori* status, and presence of *p53* and *Ki-67* were selected for further analysis (Table 2). In multivariate binary logistic regression, sex, *H. pylori* status, and presence of *p53* were selected as covariates because of their statistically significance while presence of *Ki-67* was selected as covariate because of its clinically significance. The effect of *p53* mutation (p<0.001, adjusted OR = 126.56, 95% CI: 28.58-560.40) and *Ki-67* mutation (p = 0.02, adjusted OR = 0.21, 95% CI: 0.05-0.84) remained significant.

DISCUSSION

By multivariate analysis, which is the appropriate method when potentially confounding factors exist, we adjusted for sex and *H. pylori* seropositivity between cases and controls. They were already matched in age. The results showed that *p53* and *Ki-67* are independent significant affecting factors for gastric cancer. Mutations of the *p53* gene are the most common genetic changes found in human cancers thus for, occurring in over 60% of all cancers (Sipponen *et al*., 1998; Ranzani *et al*., 1995). According to the earlier studies, the expression rate of *p53* protein in gastric cancer varies from 25 to 61% (Joypaul *et al*., 1993). The *p53* database International Agency for Research on Cancer (IARC), listed 378 gastric cancer cases with point mutation in the *p53* gene (Hainaut *et al*., 1998). These mutation leads to error in the expression of cell cycle inhibitory and the final process lead to dysregulation of the cell growth.

H. pylori infects approximately half the world population (Malaty, 2007). The prevalence of infection is particularly high in developing countries, with nearly all adults being infected (Malaty, 2007). The prevalence of infection in adult Iranians has been estimated to be approximately 90% (Massarrat *et al*., 1995; Zendehdel *et al*., 2005). The prevalence, however, largely varies one region to another. A recent study on 300 randomly selected blood donors in Tehran revealed a prevalence of 51.7% (Khodarahmi *et al*., 2008), which is rather close to the one obtained in this study and might reflect a higher socioeconomic status of the participants compared with those in the study by Massarrat *et al*. (1995). In Japan, it has been reported that each year gastric cancer develops in 300,000 (0.5%) of 60 million people who are *H. pylori* positive, which means gastric cancer develops in 5% of *H. pylori* positive persons over 10 years (Uemura *et al*., 2001). Uemura *et al*. (2001) reported that gastric cancers developed in 2.6% *H. pylori*-positive Japanese patients and none of *H. pylori*-negative patients during a mean follow up period of 7.8 years.

The expression rates of *Ki-67* antigen in present study are a little higher than those previously reported (27-45%) and few reports concerning *Ki-67* and its association to stage and survival in gastric cancer are controversial (Kakeji *et al*., 1991; Kimura *et al*., 1992; Victorzon *et al*., 1996). In the present study, *Ki-67* expression had no impact on developing gastric cancer in univariate analysis but it found its protective role in multivariate analysis. A number of limitations accompanied present study. First, the groups were not originally matched in sex and *H. pylori* seropositivity status. Therefore, we had to adjust the variables by use of logistic regression. Second, we used only serology to diagnose *H. pylori* infection. Using direct methods to diagnose infection can significantly add to the accuracy of our results. Finally, the study was cross-sectional, over a period of 5 years. Carefully designed cohort studies with larger sample size can better demonstrate if *p53* and *Ki-67* expressions in relation with *H. pylori* seropositivity are associated with gastric cancer.

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