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Investigation of P16 gene hypermethylation in serum of gastric cancer patients in Ardabil province

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ABSTRACT

Gastric cancer is the second leading cause of cancer death in the world. There is evidence that shows genetic and epigenetic change in stomach cancer is as a result of a variety of oncogenes, tumor suppressor genes, genes that repair the DNA, genes regulating cell cycle and cell adhesion molecules. P16 is an Inhibitor of protein kinase that is dependent on Cyclin D. Inactivation of this gene as a tumor suppressor gene, cause to cancer. This study was conducted to Investigation the P16 hyper-metylation status in serum of gastric cancer patients in Ardabil province as the prognostic factor for early diagnosis of gastric cancer. 82 Blood samples of patients with gastric cancer was prepared from Aras clinic of Imam Khomeini hospital. After DNA extraction from serum, using methylation-specific PCR assay it was examined the existence of hypermethylation in the P16 gene promoter. Incidence of free DNA in the serum of patients was 59.8%. P16 gene promoter was hyper methylated in 20.4% of subjects. Data analysis showed a statistically significant association between P16 methylation status and types of the adenocarcinoma. But there was no statistically significant association between methylation status of P16 with other features (age, sex, tumor location). This study showed P16 gene methylation of occur in some of gastric cancer patients. This result suggests that p16 methylation may be a prognostic marker for early diagnosis of gastric cancer.

Keywords: gastric cancer, P16 gene hypermethylation, MS-PCR.

INTRODUCTION

Gastric cancer is the most common and second cause of cancer death in the world with very different geographical prevalence and this occurred more than 70 percent of new cases and deaths in developing countries. The highest incidence of gastric cancer is in Japan, South America, East Europe and East Asia. In many countries, there is a decrease in the overall incidence and mortality of gastric cancer among men and women over the past 75 years. However, gastric cancer remains as one of the major killer among cancers [1-6].

Aberrant DNA methylation in the promoter region of the gene is leading to inactivation of tumor suppressor genes and other genes related to the cancer cells. There is evidence that gastric cancer is a multistep process of genetic and epigenetic alterations of several proto-oncogene encodes the protein, tumor suppressor genes, DNA repair genes , adhesion molecules and cell cycle regulators.

P16 is inhibitor gene protein kinase dependent on cyclin-D and acts as a tumor suppressor gene. P16 gene is cell cycle regulators, which inhibit the function of protein kinase 4 and 6 depending on cyclin-D, causes cell cycle arrest at G1 and in this way it is involved in the phosphorylation of retinoblastoma protein [6,10]. Several mechanisms are involved in the inactivation of P16 including homozygous deletion, point mutation and the development of CPG methylation of the promoter region of the gene to exon 1. In GC, 32-67% of cases inactivation of P16 occurs by methylation, whereas homozygous deletions and point mutations have been reported both less than 10%. Therefore, methylation of CG is a major mechanism for inactivation of P16. Inactivation of p16 as tumor suppressor genes disrupts the cell cycle regulation mechanisms, and causes facilitation carcinogenesis. It has been reported deletion or mutation of p16 in various tumors such as pancreatic cancer [57%], Glicoblastoma [40-70%], esophageal cancer [50%] and lung cancer [20%]. The relationship between inactivation of P16 has shown in gastric cancer. 40% of gastric cancer is associated with inactivation of P16.

Studies show that gastric adenocarcinoma is the most lethal cancer in Iran that has very difference in death rate in various provinces. Based on these studies, Ardabil Province is the highest prevalence of gastric cancer in Iran that is the most common cancer. So that 49.1% of men and 25.4% of women are effected with gastric cancer in Ardabil province [7, 8]. Therefore this study was designed to assess p16 promoter methylation in serum samples of gastric cancer patients in Ardabil province

MATERIALS AND METHODS

Blood samples from 82 patients with gastric cancer were taken from the Aras clinic of Imam Khomeini Hospital. Serum

DNA was extracted using Traizol, chloroform, ethanol 100% and 10% sodium citrate and sodium hydroxideand and the Bi-sulfate treatment of DNA was performed using distilled water, soda, hydroquinone, sodium bisulfate, mineral oil, isopropanol 80%, glycogen, NH4Ac, ethanol 10 and 70%. Then the using methylation-specific PCR assay it was examined the existence of hypermethylation in the P16 gene promoter. For this purpose, it was used in each reaction from 5 Mmkcl, Taq DNA polymerase, 20 mM Tris-HCL (pH 8.4), 1.2-2.5 mM MgCl2, 200 mM dNTPS, and Mineral oil Destilled deioniz water. Sequencing of Primers used for methylated DNA was F=5′ TTATTAGAGGGTGGGGGGGATCGC3′ and R=5′ GACCCCGAACCGCGACCGTAA3′ and for non-methylated DNA was F=5′TTATTAGAGGGTGGGGTGGATTGT 3' and R=5′ CAACCCCAAACCACAACCATAA3′.

Samples that were put through PCR processed in the beginning at 95˚C for 5 min, then 35 cycles of 95˚C for 40 sec, 35 cycles of 64˚ C for 40 sec, 35 cycles of 72˚C for 40 sec, and finally at 72˚C for 10 min. PCR products were electrophoresed at 1% agarose gel.

Statistical analysis was performed using SPSS 16 software. The relationship between variables was assessed by $\chi 2$ and Fisher test and significant relationship between the variables was considered $P < 0.05$.

RESULTS

Age of gastric cancer patients was 37-86 years and the mean age was 65.5 years with a standard deviation of 1.09. 72% of patients were male and 28% female; 34.1% of tumors were diffuse type and 65.9% intestinal type.

The tumors that located on small curvature of the trunk had a higher frequency of the other tumors position and frequency of multifocal site with adjacent centers were higher than frequency of multifocal sites with distant center. The frequency of serum free DNA in studied patients was 59.8 %. p16 promoter was hyper methylated in 20.4% of subjects . Data analysis indicated a significant association between methylation status of P16 and type of adenocarcinoma that shown in Table 1. However, no significant relationship was found between gender and type of adenocarcinoma ($df = 1$, $X^2 = 0.923$, $P = 0.337$), gender and location of the tumor ($p = 0.262$), age group and type of adenocarcinoma ($p = 0.639$), tumor location and type carcinoma ($P = 0.345$), gender and the presence of free DNA ($df = 1$, $X^2 = 1.892$, $P = 0.169$), age group and the presence of free DNA ($P = 0.399$), type of adenocarcinoma and presence of free DNA ($df = 1$, $X^2 = 0.363$, $P = 0.547$), tumor location and presence of free DNA ($P = 0.135$),

gender and methylation status (df = 1, X2 = 0.043, p = 0.835), age group and methylation status (P = 0.418) P16, methylation status and tumor location (P = 0.419) P16.

Table1. Relationship between methylation statuses of P16 and adenocarcinoma

Total	Non-methylated	Methylated	Methylation status Adenocarcinoma
18	17	1	Diffuse
31	22	9	Intestinal
49	39	10	Total
X^2=3.864		df =1	P=0.049

DISCUSSION

There are several methods to suppress the expression of genes in eukaryotes which hyper methylation promoter of these genes especially in nucleotide C is one of the most important procedure. Although silencing of these genes cause to the cell development and differentiation But in some cases, aberrant methylation could be disrupted cell regulatory systems and sometimes lead to formation of the tumor, association of between hyper methylation in some genes and some cancer cell lines and primary tumors had been observed (11, 12, 13, 14, 15). But it is important to examine this issue in cell-free DNA released from tumor cells in to the blood stream in some ways. Noninvasive assessment of serum or plasma can be detected easily and minimal risk, the risk of cancer and or reveal existence of tumors at the early stage and undetectable by the other methods.

Some studies have done related to PI6 promoter gene hypermethylation in the serum of patients with gastric cancer. For example, a study conducted in 2002 by Wai Leung et al who it was investigated in samples of tumor tissue and serum samples from 54 patients with gastric cancer, the 5 abnormal promoter genes Hypermethylation such as DAP-Kinase, E-cadherin, GSTP1, P15, P16 MS-PCR method. Promoter methylation of genes) DAP-Kinase70.3%), P16 (% 66.7), P15 (68.5%), GSTP1 (78.5%), E-cadherin (75.9%) existed in the sample patients and it has been gene methylation in serum samples of DAP-Kinase (48.1%), P16 (51.9%), P15 (55.6%), GSTP1 (14.8%), E-cadherin (57.4%) and there was no methylation in any of the 30 cases in the control group.

Japanese researchers in 2003 studied, Promoter Hyper methylation of the P16 gene using MS-PCR technique at Tumor and serum samples of 60 patients, There is aberrant methylation of P16 in tumor samples of 23 patients (38%) of 60 patients, 6 patients out of 23 patients (26%) indicated that changes in serum DNA. In this study, no methylation was found in the control group. The findings suggest that DNA methylation of P16 is as a marker for tumor detection in serum of patients with gastric cancer.

Another study conducted in 2005 by Livia Huang and colleagues, which has been paid to the study of P16 gene promoter hypermethylation in plasma of patients with gastric adenocarcinoma. In this study, tumor tissue and serum samples were collected from 84 patients with adenocarcinoma of the stomach before and after surgery. Tumor tissue of 26 patients (31%) and two cases of tumor adjacent tissue (0.02%) and plasma of 12 patients (14.3%) before surgery show broke hyper methylation of P16, but not in serum, plasma 15 controls hypermethylation of P16.

In 2008, according to a study in Iran on 52 patients with gastric cancer, promoter methylation of P16 is shown in 44.2% of tumor cells, and 60.9% DNA in the serum of these patients was methylation. But the control group showed no methylation in serum and tissues. P16 protein expression in 61.5% of patients has decreased considerably, which is associated with hyper methylation of P16. This study has pointed to the possibility of using DNA methylation as a biological marker for the early detection of gastric cancer.

According to a study in 2009, the P16 genetic mutation was associated with hereditary melanoma. Removal and inactivation of P16 has been reported in 75% of pancreatic cancers, 40 Glico-blastoma 40-70%, esophageal cancer, lung cancer 20%. The relationship between the deactivation of P16 has been shown in gastric cancer. 49% of gastric adenocarcinoma is associated with inactivation of the P16.

In 2013, South Korean researchers studied the DNA methylation of P16 gene using methylation-specific PCR in 53 patients with gastric cancer. The P16 gene methylation was detected in 79.2% of preoperative serum DNA and in 54.7% of postoperative serum DNA. P16 methylation patterns did not change after surgery has been reported in 37.7%, and the appearance of methylation of 13.2%, so that no methylation of P16 after surgery was associated with prolonged survival.

In the present study, serum markers in patients with gastric cancer were investigated in order to identify markers of cancer specialists. Of course, this study exist by the other investigations of genetic markers and epigenetic and most importantly, the comparison between the serum of gastric cancer patients and controls for presence of free DNA hyper methylation of certain genes, and finally a little comparison of serum free. Due to late diagnosis of the tumor, resulting in low patient survival after diagnosis is to find a marker to predict the value of prevention and early detection is crucial tumor formation.

Although previous studies have found that significant association between P16 promoter hyper methylation in the tumor and tumor samples, however, due to the invasiveness of sampling, is not useful in practice. This causes epigenetic issues were addressed in serum as a marker for non-invasive technique helped to detect the disease earlier.

This study, like most previous studies, which showed methylation of P16 gene in the percentage of patients with gastric cancer and in some cases, a significant correlation was found between hypermethylation and pathologic data such as tumor grade. Data analysis revealed a significant association between methylation status and adenocarcinomas of the P16. However, the methylation status of P16 with other data (age, sex, tumor location) found a statistically significant association. Thus, according to findings in the percentage of patients can be helped physicians in the early detection of gastric cancer. According to accompanied studies we can suggest that the investigation of quantity of methylation has accompanied in patient serum and the comparison of methylation in patient's tumor and serum.

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