DETECTION OF P53 and Bax genes ASSOCIATED WITH HELICOBACTER PYLORI INFECTION

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ABSTRACT

H. pylori is considered one of the most common human pathogen worldwide, with infection rates much higher in developing countries than in developed nations. The aim of this study is the investigation of some candidate genes include P53 and Bax genes mutation associated with H. pylori infection and study the role of H. pylori virulence genes on the induction of P53 and Bax genes mutation. The results show a total of 92 gastric biopsies gave positive results for the presence of H. pylori diagnosed by molecular technique. Only (49) cag a positive H. pylori strains obtain from gastric biopsy of patient suffering from H. pylori infection which also harboring cag E and vacA genes. Single Strand Conformation Polymorphism (PCR-SSCP) technique used to identify mutations in the p53 gene in several exons. Where 16 alterations in exon E5A, 19 alterations in exon E5B6A, 17 alterations in exon E7 and only two in exon E6B; but no alteration was appeared in exon E8 of P53 gene. In the same way, PCR-SSCP analysis detected alterations in Bax gene where only 14 positive samples gave alteration in the sequence of Bax gene in exons E1, E4 and E6. Regarding to exon 1 of Bax gene, it was appeared that only 10 samples gave positive gene locus mutation. This may attribute to H. pylori which stimulate Bax mutation through its ability to produce cag A protein which has the ability to cause changing in gene sequence and function. It is concluded that p53 and Bax genes mutation is important tool in the screening of genetic alteration associated with H. pylori infections.

Key words: H. pylori, Gastric biopsy, PCR-SSCP technique, P53 gene mutation, Bax gene mutation.

INTRODUCTION

Helicobacter pylori was definitively identified by Robin Warren and Barry Marshall through cultivation of bacteria from gastric biopsy samples which usually seen for nearly a century by pathologists. The WHO and the IRAC recognized this pathogen as a group one carcinogen for stomach cancer during 1994, also currently it is counted the most common etiologic agent of infection associated with cancer diseases, which account about 5% of the worldwide cancer problem. Moreover, it influences the prognosis of gastric cancer (Wroblewski and Peek, 2013; Yong et al., 2015).

Mostly, H. pylori colonization does not produce any symptoms. Conversely, long-term H. pylori infection significantly enhances the risk of developing gastro duodenal illness include gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma (Bauer and Meyer, 2011). Besides, some researches supplied clear evidence that H. pylori infection significantly enhances the risk of gastric cancer (Oda et al., 2002; Farzam et al., 2014; Graham, 2014). However, development of gastric cancer was reported in about 3% of H. pylori infected patients (Malnick et al., 2014).

The most important host factor associated with severe illness caused by H. pylori infection are P53 (tumor-suppressor gene), and Bax gene dysfunction. p53 which is responsible for regulation of cell proliferation and apoptosis and Bax is also involved in the regulation of apoptosis. However, p53 and Bax alteration is correlated with particular consequences of gastric carcinogenesis (Shadifar et al., 2015).

MATERIALS AND METHODS

Patients: A total of 92 biopsy specimens were taken from patients with H. pylori infection, who admitted to specialized center of digestive system and endoscopy unit of Medical Marjan City at Babylon governorate/ Iraq during a period from February to September 2016, Samples were collected according to ethical approval of Ministry of Iraqi health. Evaluation of H. pylori infection was tested by cultivation and PCR amplification of ureC and ureA using gastric biopsy specimens. H. pylori infection was considered when any one of these assays were positive. Besides, molecular investigation of H. pylori virulence markers include cagA, cag E and vacA was also done. The PCR primers design and conditions was done with some modification according to that reported in He et al., (2002) and Bessa et al., (2014).

Investigation of P53 and Bax Gene Mutations Related to H. Pylori Infection: Genomic DNA extraction from gastric biopsy is achieved according to the method recommended by the manufacturing company (Geneaid/ Korea) using (gSYNC™ DNA Mini Kit). The PCR-primer and their corresponding genes are shown in (table-1) PCR amplification of exons (5 – 8) of the p53 gene and Bax gene (Deguchi et al., 2001 and Oda et al., 2002). The PCR conditions were involved: after an initial denaturation, (35 – 40) cycles at 94°C for 1–1.5 min, (58°C - 62°C) for 1–2 min and (72°C) for 1–2 min was carried out in a thermal cycler (Clever, USA). The molecular markers used to amplify exons (1, 4, 5, 6) of the Bax gene amplification conditions were an initial denaturation, (35–40) cycles of the reaction mixture at...
(94°C) for 1 min, (52° - 62°C) for 1 min and (72°C) for 1 min were included. PCR amplicon were investigated by 1.6% agarose gel electrophoresis.

These genes were screened for detection novel mutations. And a group of samples was analyzed by SSCP technique. The amplicons were diluted 1/10 with sterile distilled water. Equal amounts of diluted sample and SSCP loading buffer were heated to (95°C) for 3 minutes to denature the samples, and directly put on ice to avoid re-annealing of DNA strands. Then electrophoresis of 3 μl of each sample was done. The SSCP gels consisted of 1× TBE buffer (Tris–borate–EDTA), (8-10)% polyacrylamide and 5% glycerol. Setting agents were 15μl (25%w/v) ammonium persulphate and 15μl N, N',N'-tetramethylethylenediamine (TEMED) for every 10ml of non-denaturing gel and SYBR green. Electrophoresis was done at room temperature (20–24°C), using 0.5× TBE buffer. The gels were electrophoresed for 2–3 hours at 170 Volt. Single strands of DNA were seen and the pattern of each mutant band was compared with that of normal pattern of healthy individual biopsy.

Table 1: Primers sequences and their corresponding genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers 5'→3'</th>
<th>Product size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53-E5A</td>
<td>Forward: TCCTCTTTCCCTGCAGTACTC</td>
<td>152 bp</td>
<td>Saxena et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCGTCATGTGCTGTGACTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53-E5B6A</td>
<td>Forward: GCCATCTCAACAGAGTCACA</td>
<td>167 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCAGACCTAAGAGCAATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53-E6B</td>
<td>Forward: TTAGGTCTGGCCCCTCCTCA</td>
<td>132 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: AGTTGAAACCCAGACCTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53-E7</td>
<td>Forward: TGTGCTCTAGGTGGGCTCT</td>
<td>136 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CAAGTGGCTCCTGACCTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53-E8</td>
<td>Forward: TGGTAATCTACTGGGACGGA</td>
<td>149 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CTGCTTGCTTACCTCGCTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax-E1</td>
<td>Forward: CGTTCAAGGGGGCTCTCA</td>
<td>207 bp</td>
<td>Deguchi et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAGGCCGGTAGAGGATGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax-E4</td>
<td>Forward: TCTCCTGCAGGATGATTGC</td>
<td>209 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCCCAGTCTACAGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax-E5</td>
<td>Forward: CAGGCGAATGAGGCAAGGTT</td>
<td>192 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GGGGTGGGTGGGCTGAGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax-E6</td>
<td>Forward: CCCCTGCGCGAGCTACTGAA</td>
<td>237 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: AATGCCCATGTCCCCCAATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS
Detection of P53 Gene Mutation: Among 92 H. pylori infected patients diagnosed by cultivation and PCR detection of ureC and ureA genes as a diagnosis biomarker, only patients were infected with H. pylori strain harboring full signal of virulence genes include cag A, cagE and vacA indicted that these strains were more virulent.

Beside mutations of the p53 gene in exons (5 to 8) were identified by PCR-SSCP technique. However, PCR was used to amplify exons 5,6,7 and 8 of p53 gene which are identified to be mutational hot spots. PCR-SSCP technique has detected mutations in the p53 gene in several exons. Where sixteen alterations in exon E5A, nineteen alterations in exon E5B6A, seventeen alterations in exon E7 and only two in exon E6B but no alteration was seen in exon E8 of P53 gene.

All samples were positive PCR amplification of P53 genes after detection by agarose gel electrophoresis. The second step in the mutational screening analysis were the denaturation prior to loading, which is the essential step for SSCP technique. The altered patterns of DNA bands observed on the SSCP gels indicated of the confirmational change occur in the single stand DNA when seperated in a polyacrylamide gel with electrophorasis(1&2).

Detection of Bax Gene Mutation: PCR-SSCP analysis detected alterations in Bax gene were only positive samples gave alteration in the pattern of Bax gene in exons E1, E4 and E6. As shown in the figures (figures numbers pleas). As for the exon 1
of Bax gene, it was seen that only 10 samples had positive gene locus mutation. This may attribute to H. pylori which stimulate Bax mutation through its ability to produce cag A protein which have ability to cause changing in gene sequence and function.

According to the results which obtained in this study, different DNA bands size were detected in Bax mutant (more than three bands) whereas the original gene gave only three bands. In case of exon 4 mutation in Bax gene, it was noticed in only two sample, and the other after digestion gave the same that bands which indicated of absence of alteration in Bax gene at this locus. At the same time, it was found that only two mutations were detected in exon 6, where the mutant gene gave four bands compared to the three bands presented in the normal genes. Whereas no alteration in exon 5 of Bax gene was observed in the current study.

Figure 1: A Polyacrylamide gel electrophoresis for variant detection of P53-E5B6A using SSCP technique, visualized under U.V. Light after staining with SYBR green. B for P53-E5B6A

Figure 2: A Polyacrylamide gel electrophoresis for variant detection of Bax-E1 using SSCP technique, visualized under U.V. light after staining with SYBR green. B Polyacrylamide gel electrophoresis for variant detection of Bax-E4 using SSCP technique, visualized under U.V. light after staining with SYBR green.

DISCUSSION

In this study, PCR-PCR-SSCP method is used for P53 mutation detection and analysis which is based on the phenomenon that a change of base sequence (mutations) has an effect on the folding conformation of single-stranded DNA and the observation of the effect of SSCP on the migration of the DNA during electrophoresis gel. Detection of mutations by this method has an approximately 90% sensitivity and specificity. There are five regions located within tumor suppressor gene (p53) that have a high degree of homology in vertebrates and that are also greatly conserved in evolution. Any alteration in the sequences of these regions have been correlated with cellular transformation. The most common of p53 mutations in human tumors appear within four exons originate in these conserved regions represented exons (5,6,7,8).

In the current study, all p53 mutations were detected in cagA positive H. pylori patients. Suggested that the oncogenic function of H. pylori cagA protein which was injected to host cell by type four secretion system may induced P53 mutation. This result was correlated with Szkardkiewicz et al., (2015) who indicated that the development of H. pylori (cagA+) positive gastric adenocarcinomas is associated with abnormalities in function of p53 protein and suggested that H. pylori (cagA+) may induce p53 mutations already at the early phase of carcinogenesis. These findings seems to be suppor-
ted by studies of Kountouras et al., (2008) who detected p53 mutations in 30% cases of intestinal metaplasia and in 58% patients with gastric dysplasia.

Many researchers study the relation between p53 gene mutation and H. pylori infection associated with more severe illness. Some studies indicated that H. pylori infection induces p53 gene alteration in gastric carcinogenesis are correlated with the results of this study (Masaaki et al., 2007; Rafiei and Hosseini, 2012; Shadifar et al., 2015).

Furthermore, CagA is the most important pathogenic element in the gastric tumorigenesis of H. pylori infection. Conspicuously, the p53 gene is highly expected to carry p53 mutations in gastric tumors with CagA-positive H. pylori strains. Many searches proposed that epithelial cells infected with CagA-positive H. pylori encourage the expression of cytidine deaminase, which may be a mechanism of p53 mutation accumulation during H. pylori-associated gastric carcinogenesis (Matsumoto et al., 2007; Shimizu et al., 2014). Deguchi et al., (2001) who proposed that CagA positive H. pylori strains may have a critical role in the prognosis of stomach cancer in an individual with p53 alterations. Also, they recommended that investigation of CagA positive H. pylori may be valuable in finding patients with p53 mutation.

Molecular markers that can find patients with a past of H. pylori infection and associate with significant risk of progress of gastric cancer remain an important aim of research, as are predictive markers for gastric cancer development and targeted treatments. Investigation of powerful combination biomarkers depending on the bacterial genotypes, inflammation, and host genetic and phenotypic profiles should supply much essential tools for screening, prevention, and treatment of severe illness associated with H. pylori infection.

One of the most important biomarkers linked to carcinogenesis caused by H. pylori infection are P53 (tumor-suppressor gene), and Bax gene mutation. P53 which is responsible of regulation of cell proliferation and program cell death and Bax is also implicated in the regulation of apoptosis. Moreover, p53 and Bax alteration is correlated with the particular events in the gastric tumorigenesis (Rath et al., 2015). The experimental study done by Ohnishi et al., (2008) was directly demonstrated the oncogenic potential of CagA through the detection that transgenic mice systemically expressing cagA spontaneously progressed gastrointestinal carcinomas and hematopoietic malignancies. Also, transgenic expression of CagA enhanced intestinal carcinogenesis caused by a mutation in the tumor-suppressor gene P53 (Neal et al., 2013), giving more in vivo evidence for the oncogenic ability of CagA. Even though the contribution of other H. pylori proteins to disease development, for example vacuolating cytotoxin A (VacA) has also been appeared, collecting evidence clearly points to a central role of CagA in gastric carcinogenesis (Yamaoka, 2010; Antonia and Sepulveda, 2013; Ibraheem and Al-Ardhi, 2017; Abbas et.al, 2017).

However, Bartchewsky et al. (2010) demonstrated that in H. pylori infection, manifested by chronic gastritis, an increased Bax expression initiates cell apoptosis, but in patients with gastric cancer the pathogen induces the anti-apoptotic gene bcl-2. In addition, as presented in vitro investigation demonstrate that the H. pylori-secreted vacuolating cytotoxin – VacA exerts a pro-apoptotic effect on epithelial cells, acting in an opposite manner to anti-apoptotic action of CagA (O’ldani et al., 2009; Matsumoto et al., 2011).

Moreover, only few studies have illustrated a relationship of cag E with gastric cancer (Chomvamrin, et al., 2008; Bessa, et al., 2014). Whereas, other researches indicated the existence of such gene in early stage of tumor and a correlation with other pathogenic markers, representing that there is a role for cag E in gastric carcinogenesis (Lime, et al., 2010; Lime, et al., 2011).

CONCLUSION
It is concluded that recognition of genetic mutation for P53 and Bax genes may accrue due to effect of pathogenic strains of H. pylori.

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