Introduction

Today, molecular pathways are recognized as an important point to disease understanding. It could provide a favorable success in disease control (Gravalos et al., 2008; Liakakos et al., 2008). Many molecules affect cancer process, in this way, epidermal growth factor receptor (EGFR) is a transmembrane receptor that identified as an oncogene. It contributes to many processes involved in cell survival, proliferation and apoptosis which lead to cancer development (Mammano et al., 2006; Goncalves et al., 2008; Zhang et al., 2008; Dahabreh et al., 2011). Mutations in EGFR gene is known in many tumor cells such as lung cancer, ovarian brain and what else (Nishio et al., 2006) and it is significantly correlated with poorly differentiated tumors (Lee et al., 2005). The studies suggested that EGFR gene polymorphism in exon 18-21 changes receptor functions in tyrosine kinas domain and causes ligand independent activation of receptor (Moutinho et al., 2008). Also, it was demonstrated that high level expression of EGFR protein lead to influence of cancer metastasis and poor prognosis. Hence, these mutations can be effect on responses to tyrosine kinase inhibitor drugs (Lee et al., 2005; Nishio et al., 2006; Gravalos et al., 2008; Moutinho et al., 2008; Krassinsk et al., 2011; Yang et al., 2013). Because of, there are ethnic differences in EGFR gene polymorphism, researchers try to screen of these mutations in different races and cancers (Paez et al., 2004; Mammano et al., 2006).

Gastric cancer is the most prevalent gastrointestinal malignancy (Malekzadeh et al., 2009). It is established that this cancer has low- five survivals and often is diagnosed in progressive stages of disease. It has high socio economic burden for treatment which affects on patient’s quality of life (Dicken et al., 2005; Varadhachary et al., 2005; Crew et al., 2006).

Objectives

The aim of this study is to analyze EGFR tyrosine kinas domain gene polymorphism for more knowledge about it in different population.

Materials and Methods

Study population

Study participants were consisted of 83 gastric cancer patients and 40 normal subjects. Informed subjects (patients and controls) were randomly collected from Mazandaran university medical science hospitals, Sari, Iran, from September 2010 to August 2011. The cancer...
patients verified with biopsy and pathology report and controls were free of any symptoms. Due to the presence of different ethnic in Iran, all samples selected from the same area and ethnic. All patients and controls were well informed about study and satisfied for participating in this study. This study was approved in local ethical committee.

DNA extraction & polymerase chain reaction
Genomic DNA was extracted from whole blood by DNA extraction kit (Roche, Germany) according to manufactures protocol. Polymerase chain reaction was performed for exon 18-21 of EGFR gene by primers explained in Table 1. PCR process for initial denaturing was 3 min at 94°C, the PCR reaction mixture was set at 35 cycle including 30s at 94°C for denaturing, 45s annealing at 62°C for exon 18-20 and 58°C for exon 21, 45s extension at 72°C, final extension was done 7 min at 72°C. After that PCR product observed on agarose 1% and Sibergreen staining.

Single strand conformation polymorphism (SSCP) analysis
For PCR- SSCP (Pugh et al., 2007; Aly et al., 2011; Farrokhi et al., 2011), PCR product was mixed 1:1 with formamaid loading dye (formamid 98%, EDTA 0.5 M, Bromophenolblue 1%, Zylene syanol 1%) and was denatured at 95°C for 5 min, immediately cooled on ice, electrophoresis was done 16H at 200V at room temperature on non-denaturing poly acryl amid gel 12%. Silver staining was carried out for observing SSCP pattern.

DNA sequencing
Different aberrant migrate bands in SSCP analysis were amplified in 500μg/μl concentration. Forward and revere primers were used for direct sequencing with ABI analyzer system. The sequences were aligned in public database NCBI, after that Sequence analysis was performed by Bioedit, ver. 7.0.5.3.

Bioinformatics analysis
SIFT, Sorting Intolerant from Tolerant, and PolyPhen-2 (Phenotyping Polymorphism) allow us to find some knowledge about the protein structure and its physiochemical properties based on non synonym SNP on the genome and mRNA. SIFT provide data related to protein function when there is an amino acid substitution in protein followed by a SNP. PolyPhen-2 predicts function of protein. All in all SIFT and PolyPhen-2 explores association between mutation and phenotype-2 (Alanazi et al., 2011; Gharahkhani et al., 2011).

Statistical analysis
Data was analyzed by SPSS ver.18 software. The Pearson’s chi-square ($\chi^2$) test and fisher exact test was used for comparison between variants. $P \leq 0.05$ was considered statistically significant.

Results
In this study, 83 informed gastric cancer patients and 40 normal subjects was recruited for polymorphisms analysis of exon 18-21 of EGFR gene.

Exon-18 of EGFR gene manifested 3 different mobility shift patterns in SSCP analysis. In addition, DNA sequencing showed one G>A mutation that presented on intronic variant that could not cause predicted changes in amino acid. It is summarized in Table 2. Exons 19 and 21 showed no mutation in SSCP analysis and DNA sequencing. There were different migrate bands in SSCP pattern of exon 20. Frequency of mutations and SSCP pattern of 4 exons are demonstrated in Table 2 and Figure 1 respectively. DNA sequencing indicated two mutations (NCBI Accession Number: NM_005228.3). including T>A and G>A in 58th and 78th nucleotide of mRNA respectively. Between two mutations, T>A mutation changed codon and caused Cys 781 Ser amino acid change.
Table 1. Primer Sequence, Annealing Temperature & PCR Product Length of EGFR Gene (9)

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Annealing Temperature °C</th>
<th>Primer sequence</th>
<th>EXON</th>
</tr>
</thead>
</table>
| 283 bp      | 62                       | F: TGGGCATGTCGTGGCAGCTGC  
R: ACGATCCTGAGACTGCTTG | 18 |
| 241 bp      | 62                       | F: TCACTGGCCAGCATGTCGGCA  
R: CAGCTGGCAGACATGAGAAAA | 19 |
| 295 bp      | 62                       | F: CCTTGCGCCACCTGCGCAA  
R: CGCATGGAGGATTCTGGCT | 20 |
| 265 bp      | 58                       | F: ATTCGAGATGCAGAGCTTCT  
R: CCTCTGTCAGAAAGATGCT | 21 |

Table 2. The Frequency of Genotype and EGFR Mutation Presented in Gastric Cancer Patients and Normal Subjects

<table>
<thead>
<tr>
<th>Exone Genotype</th>
<th>EGFR Mutation status</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>exon18 GG</td>
<td>Without mutation</td>
<td>52/22</td>
<td>20/17</td>
</tr>
<tr>
<td>GA rs17337107</td>
<td>Intronic variant</td>
<td>2/5</td>
<td>0/3</td>
</tr>
<tr>
<td>AA rs17337107</td>
<td>Intronic variant</td>
<td>2/0</td>
<td>0/0</td>
</tr>
<tr>
<td>exon20 GA rs1050171</td>
<td>silent mutation</td>
<td>20/5</td>
<td>1/8</td>
</tr>
<tr>
<td>AA rs1050171</td>
<td>silent mutation</td>
<td>24/12</td>
<td>3/12</td>
</tr>
<tr>
<td>TA T2347A/Cys781Ser</td>
<td>15/7</td>
<td>7/9</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Data was Provided by SIFT and PolyPhen-2 that Predict Function of Altered Protein via T>A Mutation

<table>
<thead>
<tr>
<th>Tools</th>
<th>Prediction data</th>
</tr>
</thead>
</table>
| SIFT    | Score: 0.09  
Prediction: Tolerated  
Seq rep*: 0.65  
Sequences at position: 781C |
| PolyPhen-2 | Score: 1  
Sensitivity: 0  
Specificity: 1  
Prediction: Damaging |

change (Figure 1). In addition, G>T (G2367A) mutation was present in all cases and controls. Mutation analyses of T2347A in SIFT-2 and PolyPhen-2 database explained in Table 3.

Discussion

Epidermal growth factor receptor is an ideal target for prognosis and treatment of cancers (Doss., 2012). Cancer development and response to receptor inhibitor drugs related to EGFR gene mutation. On the other hand, there are high expressions of these receptors on cancerous cells (Pao et al., 2004; Lee et al., 2005; Nishio et al., 2006). Thus, EGFR is as a target for cancer treatment. Many studies were done to find the relationship between cancers and EGFR condition (Mammano et al., 2006; Gravalos et al., 2008; Liakakos et al., 2008; Zhang et al., 2008) gene polymorphism and diseases development in various types of cancers (Marchetti et al., 2005; Mu et al., 2005; Liang et al., 2008). In gastric cancer, the Controversial role of EGFR mutation was reported by studies (Lee et al., 2005; Mimori et al., 2006; Liang et al., 2008). Lee et al. (2005) and Mammano et al. (2006) found no mutation in EGFR tyrosine kinase domain gene but Mimmori et al. (2006) found silent mutation in exon 20 of EGFR gene and the mutations in 18, 19 & 21 exons in intronic variant. These studies concluded that EGFR gene alteration in gastric cancer is rare or absent.

In the present study, the mutation found in exon 18 of EGFR gene in intronic variant that is spilled in EGFR mRNA and it seems that couldn’t effect on EGFR protein and its function. It is suggested that mutation in exon 18 in intronic variant may effect on EGFR gene transcription and high expression of receptor. Confirm to Lee et al. (2005) and Mammano et al. (2006), there was no mutation in exons 19 and 21 of EGFR gene.

In our study, there are two mutation in exon 20 of EGFR gene that one of them was silent mutation in 78th nucleotide of mRNA and another was a mutation in 58th nucleotide of mRNA (2347 T>A) with predicted amino acid change from Cys to Ser. This mutation was prevalent in study population either case or control groups. It seems that, this mutation could influence EGFR function because of different functional group of Cys and Ser. Ser substitution could change in EGFR tyrosine kinases domain pocket and lead to different function of receptor that seems a risk factor for activation of EGFR cascade, cancerous cell proliferation and cancer development. In the present study, the none synonym SNP was investigated in two database SIFT and PolyPhen-2. On the basis of its database, Serine and Cysteine amino acid are uncharged polar which could be Predict tolerated change for receptor (Johnson et al., 2005). Therefore, this change could lead to damaging mutation with different function of receptor which confirmed by PolyPhen-2 database (Masoodi et al., 2012).

High expression and silent mutation of EGFR gene was reported by studies (Lee et al., 2005; Galizia et al., 2007). Silent mutation didn’t change amino acid in protein. However, silent mutations may affect either splicing or mRNA stability (Capon et al., 2004; Kimchi-sarfaty et al., 2007) and be a factor of high level of EGFR mRNA. Our study showed a Silent mutation in exon 20 of EGFR gene that presented in all our study population which did not produce altered coding sequences and therefore, it isn’t expected to change the function of the protein and maybe influence on mRNA stability (Liu et al., 2009).

In this study, genomic DNA was extracted from whole blood that has less exposure to environmental factors. Therefore, our result suggested that there are different EGFR gene polymorphisms in different ethnics. It is concluded that EGFR gene mutation isn’t rare in gastric cancer patients and could be variant in different ethnics. Furthermore, it suggested that EGFR gene mutation is common in our study population and maybe a cause of high prevalence of gastric cancer in this area. Besides, study of EGFR gene can be basis of research on cancer cells in the others population.

Acknowledgements

This study was supported by Mazandaran University of medical sciences (grant No.89-41). Th authors would also like to thank of Mrs Samira Ramazany from Tooba Clinical Laboratory for technical assistance.
References


