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 is identified as an oncogene. It contributes to many processes involved in cell survival, proliferation and apoptosis which may 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). It may be that EGFR tyrosine kinase gene polymorphisms differ between populations and screening could be useful in gastric cancer patients who might benefit from tyrosine kinase inhibitor therapy.

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 were diagnosed as symptomatic cases of gastric neoplasm, and informed consent was obtained from all participants. The ethical committee approved the research protocol and methods. Patients were divided in two groups: controls (40 volunteers) and cases (83 gastric cancer patients). All patients had undergone surgical resection with a minimum follow up of 24 months. The clinical records of all patients were reviewed to extract demographic and clinical information. DNA was isolated from peripheral blood lymphocytes using the DNA isolation kit (Qiagen, Hilden, Germany) and then stored at -80°C. EGFR genotyping analysis was performed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method. Genomic DNA (2 μg) was amplified by PCR (25 cycles) using forward primer 5′-TCA CAG TCA GGT CAA ACC ATT-3′ and reverse primer 5′-GCT GCT CGA TGT CAG TCA AGA-3′ (Moutinho et al., 2008) in total volume of 50 μl containing 1×PCR buffer, 2.5 mM MgCl₂, 200 μM of each dNTP, 2 units of Taq polymerase (Qiagen, Hilden, Germany), and 2 μg of DNA. Then, 10 μl of PCR product was denatured (5min at 95°C) and then loaded into 10% non-denaturing polyacrylamide gel and electrophoresis was performed under cooling condition at 20°C. DNA sequencing was conducted for different mobility shift bands. Finally, the data were statistically analyzed using the chi-2 test and the SPSSver.16 program.
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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 58\textsuperscript{th} and 78\textsuperscript{th} nucleotide of mRNA respectively. Between two mutations, T>A mutation changed codon and caused Cys 781 Ser amino acid

Figure 1. PCR-SSCP of Exons 18-21. A&B) SSCP Pattern Observed in Exon 18 of EGFR Gene. A) Lane1 shows wild type genotype but lane 2 shows a heterozygous genotype with mutation G>A (Table 2), it was present in 7 subjects of gastric cancer and 3 subjects of control groups, B) lane3 shows a homozygous genotype with mutation G>A that just observed in 2 patients. C) exon 19 didn’t demonstrate mobility shift bands. D) exon 20 showed different aberrant migrate bands, lane 1: this pattern was prevalent between patients and controls. Lane2 and 3 shows mobility shift bands due to mutations. E) SSCP pattern of exon 21 was same in all patients and controls

Figure 2. DNA Sequencing of Exon 20 of EGFR Gene. A) sample of gastric cancer patient showed a mutation T>A that cause changing codon TGC to AGC that alter an amino acid on EGFR tyrosine kinas pocket, B) a sample of normal that is lack of this mutation . Also Figure A&B Show a mutation G>A that presented in all study population with homozygous and heterozygous genotype and cause no amino acid change in protein

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.

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 94ºC, the PCR reaction mixture was set at 35 cycle including 30s at 94ºC for denaturing, 45s annealing at 62ºC for exon 18-20 and 58ºC for exon 21, 45s extension at 72ºC, final extension was done 7 min at 72ºC. 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%, Zylen syanol 1%) and was denatured at 95ºC 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 (χ\textsuperscript{2}) test and fisher exact test was used for comparison between variants. P≤0.05 was considered statistically significant.
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 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) 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 Mimmoni 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 spliced 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-2database (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-sarfati 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 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

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Table 1. Primer Sequence, Annealing Temperature & PCR Product Length of EGFR Gene (9)

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Annealing Temperature</th>
<th>Primer sequence</th>
<th>EXON</th>
</tr>
</thead>
<tbody>
<tr>
<td>283 bp</td>
<td>62</td>
<td>F: TGGGCAATCCCTGCGACTGC</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACAGCTTGAAGACGCTGG</td>
<td></td>
</tr>
<tr>
<td>241 bp</td>
<td>62</td>
<td>F: TCACTGGGACGATGTTGCA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGCTTGAGCAGACATGAAAAA</td>
<td></td>
</tr>
<tr>
<td>295 bp</td>
<td>62</td>
<td>F: CCTGTCGGACGACTGCAAA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CGCTATGAGAGACCTGCT</td>
<td></td>
</tr>
<tr>
<td>241 bp</td>
<td>62</td>
<td>F: TCACTGGGACGATGTTGCA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGCTTGAGCAGACATGAAAAA</td>
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</table>

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

<table>
<thead>
<tr>
<th>Exon</th>
<th>Genotype</th>
<th>EGFR Mutation status</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male/Female</td>
<td>Male/Female</td>
</tr>
<tr>
<td>exon18</td>
<td>GG</td>
<td>Without mutation</td>
<td>52/22</td>
<td>20/17</td>
</tr>
<tr>
<td>GA</td>
<td>rs17337107</td>
<td>Intrinsic variant</td>
<td>2/5</td>
<td>0/3</td>
</tr>
<tr>
<td>AA</td>
<td>rs17337107</td>
<td>Intrinsic variant</td>
<td>2/0</td>
<td>0/0</td>
</tr>
<tr>
<td>exon20</td>
<td>GA</td>
<td>rs1050171</td>
<td>silent mutation</td>
<td>20/5</td>
</tr>
<tr>
<td>AA</td>
<td>rs1050171</td>
<td>silent mutation</td>
<td>24/12</td>
<td>3/12</td>
</tr>
<tr>
<td>TA</td>
<td>T2347A/Cys 781 Ser</td>
<td>15/7</td>
<td>7/9</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>rs1050171</td>
<td>silent mutation</td>
<td></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>Score</th>
<th>Prediction data</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIFT</td>
<td>0.09</td>
<td>Tolerated</td>
</tr>
<tr>
<td>PolyPhen-2</td>
<td>0.65</td>
<td>Seq rep: 781 C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitivity: 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Specificity: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prediction: Damaging</td>
</tr>
</tbody>
</table>

change (Figure 1). In addition, G>A (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

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.
References


