RESEARCH ARTICLE

Epidermal Growth Factor Receptor Gene Polymorphisms and Gastric Cancer in Iran

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Abstract

Background: Epidermal growth factor receptor (EGFR) is a transmembrane receptor which contributes to many processes involved in cell survival, proliferation and inhibits apoptosis, that may lead to cancer development. Gastric cancer is one of the most common diseases of digestive system that has low 5-year-survival. The aim of this research was to determine the significance of EGFR tyrosine kinase domain gene polymorphisms in gastric cancer in Iran. Materials and Methods: In the present study, 83 patients with gastric cancer and 40 normal subjects were investigated for EGFR gene polymorphisms in exons 18-21 by PCR-SSCP. Then, 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. Results: Exon 18 of EGFR gene showed three different bands in SSCP pattern and DNA sequencing displayed one mutation. SSCP pattern of Exons 19 and 21 did not show different migration bands. Exon 20 of EGFR gene revealed multiple migrate bands in SSCP pattern. DNA sequencing displayed 2 mutations in this exon: one mutation was caused amino acid change and another mutation was silent. Conclusion: 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.

Keywords: Cancer - EGFR - DNA sequencing - mutations - therapeutic implications

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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; Krasinskas 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

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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 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 reveres 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^2) test and fisher exact test was used for comparison between variants. P≤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

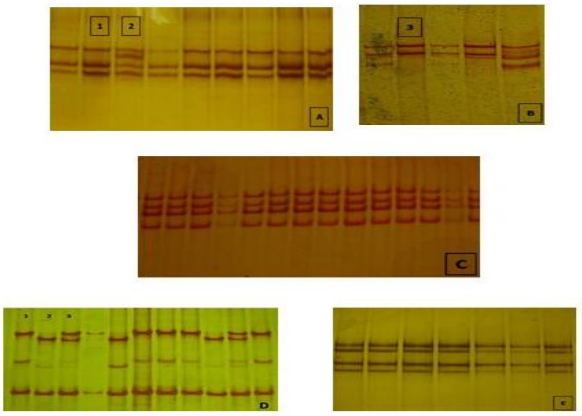

Figure 1. PCR-SSCP of Exons 18-21. A&B) SSCP Pattern Observed in Exon 18 of EGFR Gene. A) Lane1 shows wild type genotype but lane 2 shows a heterozygous genotype with mutation G>A (Table 2), it was present in 7 subjects of gastric cancer and 3 subjects of control groups, B) lane3 shows a homozygous genotype 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

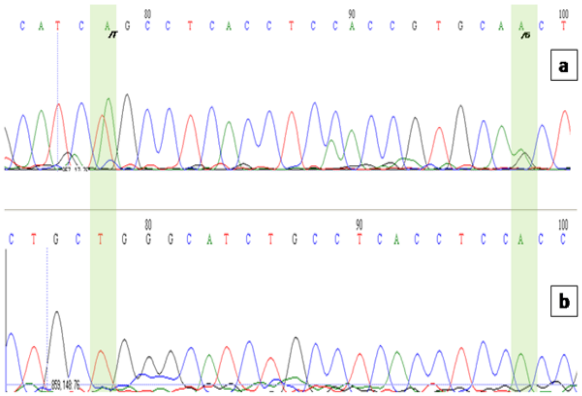

Figure 2. DNA Sequencing of Exon 20 of EGFR Gene. A) sample of gastric cancer patient showed a mutation T>A that cause changing codon TGC to AGC that alter an amino acid on EGFR tyrosine kinas pocket, B) a sample of normal that is lack of this mutation . Also Figure A&B Show a mutation G>A that presented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

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

PCR product	Annealing Temperature	Primer sequence	EXON
283 bp	62	F : TGGGCCATGTCTGGCACTGC R : ACAGCTTGCAAGGACTCTGG	18
241 bp	62	F : TCACTGGGCAGCATGTGGCA R: CAGCTGCCAGACATGAGAAA	19
295 bp	62	F : CCTTCTGGCCACCATGCGAA R : CGCATGTGAGGATCCTGGCT	20
265 bp	58	F : ATTCGGATGCAGAGCTTCTT R : CCTGGTGTCAGGAAAATGCT	21

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

Exone	Genotype	EGFR Mutation status	Patients Male/Female	Controls Male/Female
exon18	GG	Without mutation	52/22	20/17
	GA	rs17337107 /Intronic variant	2/5	0/3
	AA	rs17337107/Intronic variant	2/0	0/0
exon20	GA	rs1050171/silent mutation	20/5	1/8
	AA	rs1050171/silent mutation	24/12	3/12
	TA	T2347A/ Cys 781 Ser	15/7	7/9
	AA	rs1050171/ silent mutation		

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

Tools	Prediction data	
SIFT	Score	0.09
	Prediction	Tolerated
	Seq rep*	0.65
	Sequences at position	781 C
PolyPhen-2	Score	1
	Sensitivity	0
	Specificity	1
	Prediction	Damaging

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

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 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 kinas 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-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.

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References

Alanazi M, Abduljaleel Z, Khan W, et al (2011). Silico analysis of single nucleotide polymorphism (SNPs) in human b-globin gene. *PLoS ONE*, **6**, 25876.

Aly RM, El-Sharnobyand MR, Hagag AA (2011). Prognostic significance of NRAS gene mutations in children with acute myelogenous leukemia. *Mediterr J Hematol Infect Dis*, **3**, 2011059.

Capon F, Allen MH, Ameen M, et al (2004). A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. *Human Mol Genet*, **13**, 2361-68.

Crew KD, Neugut AI (2006). Epidemiology of gastric cancer. *World J Gastroenterol*, **12**, 354-62.

Dahabreh IJ, Linardou H, Kosmidis P, Bafaloukos D, Murray S (2011). EGFR gene copy number as a predictive biomarker for patients receiving tyrosine kinase inhibitor treatment: a systematic review and meta-analysis in non-small-cell lung cancer. *Annals Oncology*, **22**, 545-52.

Dicken BJ, Bigam DL, Cass C, et al (2005). Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg*, **241**, 27-39.

Farrokhi E, Shayesteh F, Asadi Mobarakeh S, et al (2011). Molecular Characterization of Iranian Patients With Possible Familial Hypercholesterolemia. *Ind J Clin Biochem*, **26**, 244-8.

Gravalos C, Jimeno A (2008). HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Annals Oncology*, **19**, 1523-9.

Galizia G, Lieto E, Orditura M, et al (2007). Epidermal Growth Factor Receptor (EGFR) Expression is Associated With a Worse Prognosis in Gastric Cancer Patients Undergoing Curative Surgery. *World J Surg*, **31**, 1458-68.

George P, Doss C (2012). In Silico profiling of deleterious amino acid substitutions of potential pathological importance in haemophlia A and haemophlia B. *J Biomed Sci*, **16**, 19-30.

Gharahkhani PA, O'Leary C, Kyaw-Tanner MA, et al (2011). A non-synonymous mutation in the canine PKD1 gene is associated with autosomal dominant polycystic kidney disease in bull terriers. *PLoS One*. **6**, 22455.

Gonçalves A, Esteyries S, Taylor-Smedra B, et al (2008). A polymorphism of EGFR extracellular domain is associated with progression free-survival in metastatic colorectal cancer patients receiving cetuximab-based treatment. *BMC Cancer*, **8**, 169.

Johnson MM, Houck J, Chen C (2005). Screening for deleterious nonsynonymous single-nucleotide polymorphisms in genes involved in steroid hormone metabolism and response. *Cancer Epidemiol Biomarkers Prev*, **14**, 1326-9.

Kimchi-sarfaty C, Oh JM, Kim IW, et al (2007). A silent polymorphism in the MDR1 gene changes substrate specificity. *Sciences*, **315**, 525-8.

Krasinskas AM (2011). EGFR signaling in colorectal carcinoma. *Patholog Res Int*, **2011**, 932932

Lee JW, Soung YH, Kim SY, et al (2005). Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clin Cancer Res*, **11**, 2879-82.

Lee JW, Soung YH, Kim SY, et al (2005). Absence of EGFR mutation in the kinas domain in common human cancers besides non-small cell lung cancer. *Int J Cancer,* **113**, 510-1.

Liakakos T, Xeropotamos N, Ziogas D, Roukos D (2008). EGFR as a prognostic marker for gastric cancer. *World J Surg*, **32**, 1225-26.

Liang Z, Zeng X, Gao J, et al (2008). Analysis of EGFR, HER2, and TOP2A gene status and chromosomal polysomy in gastric adenocarcinoma from Chinese patients. *BMC Cancer*, **8**, 363

Liu Y, Lu X, Luo YR, et al (2009). Effect of single nucleotide polymorphism of IRF1 gene on cytokine traits in three pig breeds. *J Anim Vet Adv*, **18**, 2346-50.

Malekzadeh R, Derakhshan MH, Malekzadeh Z, et al (2009). Gastric cancer in Iran: epidemiology and risk factors. *Arch Iran Med,* **12**, 576-83.

Mammano E, Belluco C, Sciro M, et al (2006). Epidermal growth factor receptor (EGFR): mutational and protein expression analysis in gastric cancer. *Anticancer Res*, **26**, 3547-50.

Marchetti A, Martella C, Felicioni L, et al (2005). EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol*, **23**, 857-65.

Masoodi TA, Al Shammari SA, Al-Muammar MN, Almubrad TM, Alhamdan AA (2012). Screening and structural evaluation of deleterious Non-Synonymous SNPs of ePHA2 gene involved in susceptibility to cataract formation. *Bioinformation*, **8**, 562-7.

Mimori K, Nagahara H, Sudo T, et al (2006). The epidermal growth factor receptor gene sequence is highly conserved in primary gastric cancers. *J Surg Oncol*, **93**, 44-46.

Moutinho C, Mateus AR, Milanezi F, et al (2008). Epidermal growth factor receptor structural alterations in gastric cancer. *BMC Cancer*, **8**, 8-10

Mu XL, Li LY, Zhang XT, et al (2005). Gefitinib-sensitive mutations of the epidermal growth factor receptor tyrosine kinase domain in chinese patients with non-small cell lung cancer. *Clin Cancer Res*, **11**, 4289-94.

Nishio K, Arao T, Kato T, Yokote H (2006). EGFR mutation in various tissues. *Cancer Chemother Pharmacol*, **58**, 39-41.

Paez JG, Jänne PA, Lee JC, et al (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*, **304**, 1497-500.

Pao W, Miller V, Zakowski M, et al (2004). EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA*, **101**, 13306-11.

TJ Pugh, G Bebb, L Barclay, et al (2007). Correlations of EGFR mutations and increases in EGFR and HER2 copy number to gefitinib response in a retrospective analysis of lung cancer patients. *BMC Cancer*, **7**, 128.

Varadhachary G, Ajani JA (2005). Gastric cancer. *Clin Adv Hematol Oncol*, **3**, 118-24.

Yang SY, Yang TY, Li YJ, Chen K-C and et al. EGFR exon 19 in-frame deletion and polymorphisms of DNA repair genes in never-smoking female lung adenocarcinoma patients. *Int J Cancer*, **132**, 449-58.

Zhang X, Chang A (2008). Molecular predictors of EGFR-TKI sensitivity in advanced non–small cell lung cancer. *Int J Med Sci*, **5**, 209-17ß