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Polymorphisms of Filaggrin gene as a new Marker to Detection of Gastric Cancer Patients in Iraq

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Abstract

Gastric cancer is the most common cancer in the world specifically in Iraq, compared to other cancers, that affects patients of old ages of 30 years and more. This study deals with the verification of cancerous gene, through the filaggrin (FLG) gene, as well as to explain the relationship between filaggrin gene and other clinical indicators [Alp-fetoprotein (AFP), and Protein53 (P53)]in patients with gastric cancer, under study. Ninety patients with gastric cancer were selected from AL-Hussein Teaching Hospital of Kerbala and 100 healthy subjects represented as the control group, their ages were identical with the ages of patients.The clinical characteristics of patients were documented, which included age, smoking, family history, obesity, and chemotherapy drug. Statistical analysis of the results showed that 60% of patients were aged (51-65) year and 40% were between the ages of (30-50) year.Single Nucleotide Polymorphism (SNP) to Filaggrin (FLG) gene was measured in the blood of same patients and healthy group.

Chi-square test was used to compare the differences in genotypeand allele frequencies of FLGrs2065955 in each group of genetic study to filaggrin (FLG) gene. Also student`s t-test was used to analyze the results. The statistical results were showed the cytosine-cytosine (CC) genotype was found in 47 of 90 (52.2%) (P=0.003) in gastric cancer cases, and 13 of 100 (13%) in normal control, while the guanine-cytosine (GC) genotype was found in 23 of 90 (25.6%) (P=0.016) in gastric cancer cases, and 69 of 100 (69%) in normal control.Also the statistical results were documented a highly significant (P<0.000) increase in the level of AFP. There also was a highly significant (P<0.01) increase in P53 level, compared with the healthy group.

Keywords: Chemotherapy, Radiotherapy, Calcitonin, CEA.

Introduction

Gastric cancer (GC) is globally the fifth most common cancer and third leading cause of cancer death after the breast, bladder, lung, and colon carcinoma [1]. The development of gastric cancer is a complex, multi-step process involving multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, and signaling molecules [2]. Recognition of viruses with diversification levels of the cytokine response and immune system as Epstein–Barr virus (EBV) is also closely related to polymorphisms in host inflammatory response genes in gastric carcinoma [3].

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Analysis of SNPs (single nucleotide polymorphisms) in genes related to the inflammatory response in the gastric mucosa, and the associated risk for gastric malignancy has been the central focus of many studies [4].

Filaggrin (FLG) gene is an intermediate-filament-associated protein that aggregates keratin intermediate filaments in the epidermis. Thisprotein is composed of 324 amino acids, has a molecularweight of 37 KDa and it corresponds to 6% of the epidermis protein content [5].

Profilaggrin, which contains10-12 tandemly repeated filaggrin units and is processedinto filaggrin by specific dephosphorylation and proteolysis during terminal differentiation of the epidermalcells, is encoded by the FLG gene [6]. This gene is located onthe long arm of chromosome 1 at position 1q21.3, andit is located in the so-called EDC (epidermal differentiation complex) region, which brings together a group of more than 30 structural and evolutionarily related genes,which encode proteins of the epidermis, such as filaggrin, loricrin or involucrin, among others [7]. Filaggrin gene plays an important role in the function of skin as a barrier against invasion of harmful factors from the external environmental risks [8], and then the mutations in the FLG gene are associated with gastric cancer and skin disorders [9].

Elevated serum alpha-fetoprotein (AFP) levels in adults are considered abnormal. This parameter is used mostly in the diagnosis and follow-up of hepatocellular carcinomas and yolk sac tumors. Among the other rare tumors accompanied with elevated serum AFP levels, gastric cancer is the most common [10].

Protein 53 (P53) was a tumor antigen to host cellular mutations, and one of the common alterations observed in human tumors used a tumor suppresor, through suggests mechanism lead to regulate the cell cycle [11].

Materials and methods

Patients and control

This study included ninety patients with gastric carcinoma; those patients were enrolled from AL-Hussein Teaching Hospital of Kerbala in the period from April 2020 to January 2021, whose age ranges between (30-65) years. Blood samples of those patientswere obtained from oncology unit, diagnosed as gastric cancer by the histopathological examination.

The Control group was consisted of 100 healthy subjects who were free from signs and symptoms of cancer, and whose ages were identical with the age of patients.

Specimen Collection

Trained nurses collected venous blood samples (10 ml) from each individualof both gastric cancer and healthy control. Each blood sample was divided intotwo tubes:

1. Plain plastic tubes (5ml) for tumor marker studies.
2. EDTA tubes (5ml) for Molecular study.

Disposable syringes and needles were used to collection the blood specimens. The blood samples of tumor marker studies were centrifuged at3000 xg for 15 minute. A serum of these blood samples and a whole blood samples to molecular study were taken with its tubes and put to freeze at -70 ^{0}C until the analysis.

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Determination of Alpha-fetoprotein (AFP)

Serum AFP was identified using the enzyme-linked Immunosorbent Assay (ELISA) (Humanalpha-fetoprotein ELISA package, CSB-E04770h, CUSABIO,China).

Determination of Protein 53 (P53)

Serum P53was identified using the enzyme-linked Immunosorbent Assay (ELISA) (Human protein53 ELISA package, MBS824754, Mybiosource, USA.).

Genomic DNA Extraction

Blood DNA was isolatedby usingWizard®, Promega, Genomic DNA Purification Kit, 9FB022, USA.

Filaggrin Gene (*FLG*) Polymorphism Detection

Single neuclutide polymorphism (SNP) to filaggrin gene was used by Restriction Fragment Length Polymorphism (RFLP) Teaching Kit, HiPer®,Mumbai - 400 086, India.

Agarose Gel Electrophoresis Analysis

After DNA isolation was used electrophoresis by BIO RAD; D-10 Electrophoresis,DC1E488903, Module, Singapore.Agarose Gel Electrophoresis was performed. And then the DNA bands were visualized by using UV transilluminator.

PCR Analysis

PCR reactions were performed with 2 μL of DNA extracts (100 ng/μL) in a 25 μL reaction mixture containing standard PCR buffer, 1.5 mmol/L $MgCl_2$, 200μmol/L dNTPs, 1.25 μmol/L of each primer, and 0.2 U. TaqDNA polymerase (TaKaRa Biotechnology Co, Kyoto, Japan).

Statistical analysis

The Chi-square test was used to compare the differences in genotype and allele frequencies of FLGrs2065955 in each group. Nonconditional logistic regression was used to estimate the odds ratio (OR) and P-values to indicate the correlation between genotype and the risk of gastric carcinoma. Results were considered to be statistically significant when $P< 0.05$. Also Student t-test was used to analyze the results. All of the data were expressed as mean ±standard error (Sd.E), P-value ≤ 0.05 was considered significant. Statistical analyses were conducted using SPSS 19.0 statistical software (SPSS).

Results and Discussion

The results revealeda highly significant ($P<0.01$) increase in the concentration of serum tumor protein 53 (P53), compared with healthy group. In addition, there was a highly significant ($P<0.000$) increase in the concentration of serum alpha-fetoprotein (AFP), compared with healthy group (Table1).

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Table 1: The levels of parameters under study in patients with gastric cancer and control group.

Parameter	Age(30-50) n=36 Mean±Sd.E	Age(51-65) n=54 Mean±Sd.E	P-value
AFP *(ng/ml)*	**226.50±34.58**	**354.11±103.54**	**0.251**
P_{53} *((µg/L)*	**9.47±0.16**	**9.37±0.11**	**0.629**

Present study, to Follow-up of AFP levels documented that the AFP in metastatic gastric cancer patients with elevated AFP levels may allow prediction of early treatment response and could be more useful than the carcinoembryonic antigen (CEA) marker for follow-up in response evaluation [12].

The tumor suppressor gene p53 is one of the most frequently mutated genes in human cancers, and p53 mutations occur in 0 to 77% of stomach cancers. Mutation of p53 has been observed starting at the early stages of gastric cancer and this frequency increases as the malignancy progresses [13].

Demographic study

Age factor

In this study, the patients with gastric cancer were categorized into two groups according to their age. Group 1 consists of 36 patients (40%) with ages between 30-50 years. Group 2 consist of 54 patients (60%) with ages between 51-65 years (Figure 1). The statistical analysis of results did not show any significant variation (P>0.05) in all parameters under study between the two age groups

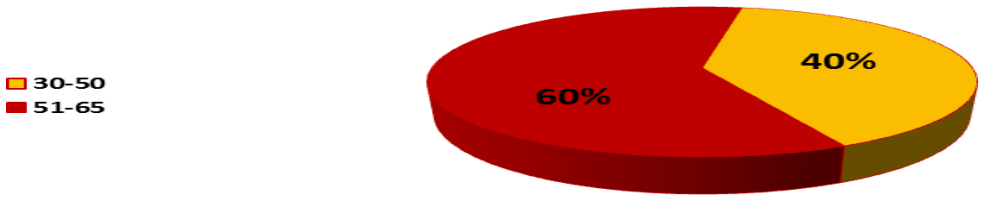

Figure 1: The percentage of patients according to their ages.

Table 2: The levels of parameters under study in patients with gastric cancer in two age groups.

Parameter	Patients n=90 Mean±Sd.E	Control n=100 Mean±Sd.E	P-value
AFP *(ng/ml)*	**9.41±0.09**	**8.46±0.05**	**0.000**

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P_{53} *(µg/L)*	**301.34±62.60**	**85.82±3.62**	**0.008**

The Patrick`s study (2018) revealed the older men are more likely to be diagnosed with gastric cancer. Although only 1 in 10,000 men under age 40 will be diagnosed, the rate shoots up to 1 in 38 for ages 40 to 59, and 1 in 14 for ages 60 to 69 [14].

In fact, more than 65% of all gastric cancers are diagnosed in persons over the age of 50 year. The average age at diagnosis of gastric cancer in the United States was 65 year. After that age, the chance of developing gastric cancer becomes more common than any other cancer in men or women [15].

Smoking factor

In this study, patients with gastric cancer were classified into two groups, smokers 30 case (33%), and non-smokers 60 case (67%) (Figure 2). The results did not show a significant (P>0.05) different in the concentration of all parameters under study between smoker and non-smoker patients, except there was significant (P<0.05) increase in concentration of serum CA72-4, in smoker patients compared with non-smoker patients (Table 3).

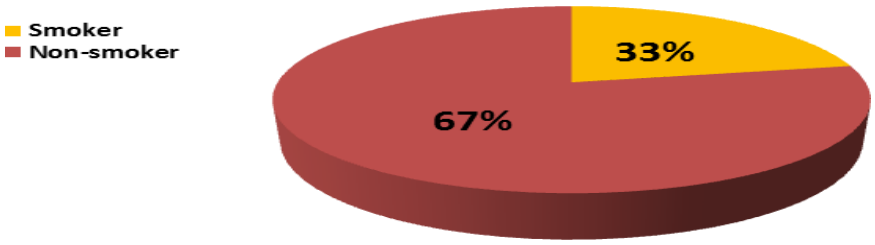

Figure 2: The percentage of smokers and non-smokers patients.

Table 3: The levels of parameters under study in smoker and non-smoker patients.

Parameters	Smoker n=33% Mean±Sd.E	Non Smoker n=67% Mean±Sd.E	P-value
AFP *ng/ml))*	**197.47±30.43**	**326.60±77.14**	**0.127**
P_{53} *(µg/L)*	**9.36±0.15**	**9.42±0.11**	**0.765**

Currently, there was no strong evidence that smoking, vasectomy, obesity or high alcohol intakes are risk factors in the development of gastric cancer. Results of the different epidemiological

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studies are controversial, probably because of differences in sampling and methods of analysis. In most cases only insufficient marginal differences can be established [16].

Molecular Analysis to Filaggrin *(FLG)* Gene

The 436 bp fragments of the FLG gene flanking the rs2065955 locus were successfully amplified in 90 gastric cancer patients, and 100 normal control samples (Figure 3). After digestion by ECORI, the homozygous wild-type genotype GG remained as only 'one 436 bp band. The homozygous mutant genotype CC showed two bands, 368bp and 68bp. The heterozygous genotype GC showed three bands, 368 bp, 68 bp, and 436 bp (Figure 4).

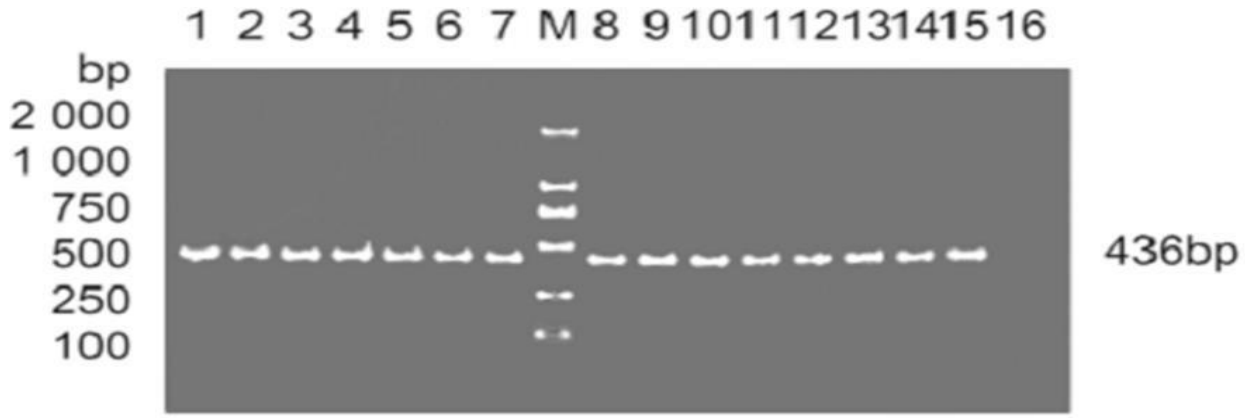

Figure 3:Electrophoresis results of FLG gene (rs2065955) PCR products. Lane M: DL 2000 DNA Marker; lane 16: negative control; lanes 1–15: 436 bp FLG (rs2065955) PCR products.

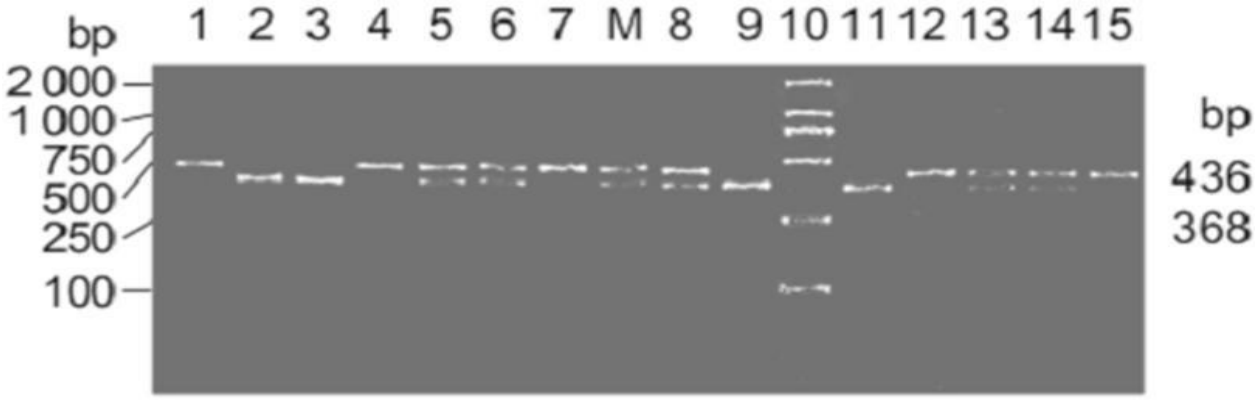

Figure 4: PCR-RFLP analysis of FLG gene (rs2065955) genotyping. Lane M: DL 2000 DNA Marker; lanes 1, 4, 7, 12. 15:GG genotype (homozygous wild-type); lanes 5, 6, 8, 9, 13, 14: GC genotype (heterozygous type); lanes 2, 3, 10, 11: CC genotype (homozygous mutated type).

The distributions of filaggrin (FLG)rs2065955 genotypes and alleles in gastric cancer patients and normal control are shown in Table 4. The CC genotype was found in 47 of 90 (52.2%) in gastric cancer cases, and 13 of 100 (13%) in normal control, while the GC genotype was found in 23 of 90 (25.6%) in gastric cancer cases, and 69 of 100 (69%) in normal control.

The distribution of the CC genotype was significantly different between the two groups (gastric cancer group vs normal control: $\chi^2 = 36.566$, $P< 0.01$. The CC genotype showed higher frequency in gastric cancer group than normal control group and may be a risk factor for gastric cancer patients vs normal control: OR = 8.081, 95% CI = 3.941–16.571.

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Allele C frequency was 70.6% and 48% in gastric cancer patients and normal control respectively. Allele C exhibited higher frequency in gastric cancer group than normal control and may be a risk factor for gastric cancer (gastric cancer vs normal control: χ^2 = 16.137, P< 0.01, OR = 2.687, 95% CI = 1.683–4.289).

Table 4: Genotypes and alleles distribution of *FLG* gene in gastric cancer patients and control samples.

***FLG* (rs2065955)**	**Gastric Cancer Patients n=90 47.4%**	**%**	**Control n=100 (52.6%)**	**%**	**Odd Ratio 95%CI***	***P* value**
Genotypic frequencies						
GG	20	22	18	18	0.380 – 1.862	0.669
CC	**47**	**52**	**13**	**13**	**0.103 – 0.636**	**0.003**
GC	23	26	69	69	1.198 – 7.410	**0.016**
Recessive Model						
Others	43	48	87	87	8.081	**< 0.01**
CC	47	52	13	13	(3.941–16.57)	
Dominant Model						
GG	20	22	18	18	0.989	0.978
Others	70	78	82	82	(0.450–2.073)	
Allelic frequencies						
C	127	71	96	48		
G	53	30	104	52	2.687 (1.683–4.289)	**< 0.01**

*Confidence interval

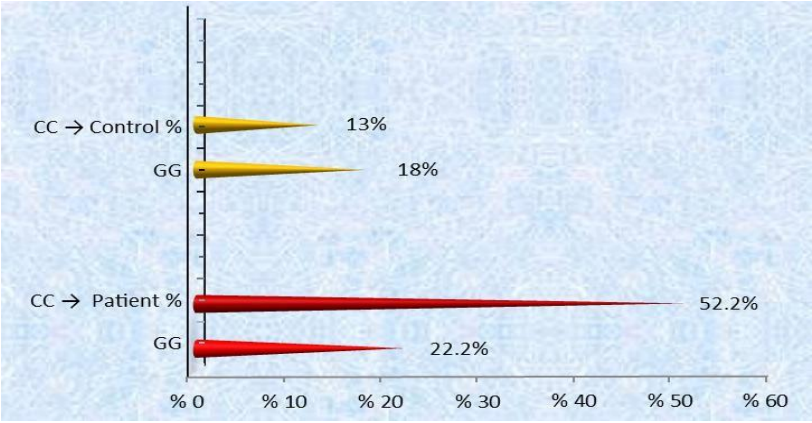

Figure 5: The percentage of genotypes distribution to *FLG* gene in gastric cancer patients and control.

In this study, we found that genotype CC of FLG showed higher frequency in gastric carcinoma, which may be a risk factor for gastric cancer development. To our knowledge, this is the first research in Iraq describing the association of FLG polymorphism with gastric cancer diseases.

Filaggrin proteins are crucial for the terminal differentiation of the epidermis by aggregating keratin filaments.They play important roles in the barrier function of skin, which may prevent the entry of harmful factors from environmental exposure including microorganisms, allergens, or chemicals (Catherine et al., 2019) [17]. Loss-of-function mutations in the FLG gene reduce epidermal filaggrin levels and may disrupt the skin barrier, and have been shown to be highly associated with ichthyosis vulgaris (Hai et al., 2020) [18], and atopic dermatitis (Ai-Young L 2020) [19]. In addition to skin, filaggrin proteins are also expressed in the oral cavity, cervix,

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endometrium, and vagina (Victoria et al., 2015) [20].

Conclusion

We can detect gastric cancer by measuring the Filaggrin (FLG) gene mutation. Filaggrin (FLG) gene can be clinically useful as a marker to treatment monitoring of gastric cancer patients.Gastric cancer is starts in middle ages in the most cases, that is about 50 years and over.

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