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Relation of Heme oxygenase-1 Enzyme Gen Polymorphism and Levels of Last Products with Chronic Obstructive Pulmonary Disease and Exacerbations

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ABSTRACT

Genetic predisposition is one of the causes of Chronic Obstructive Pulmonary Disease (COPD). This study is designed to investigate effect of the activity of an important antioxidant enzyme heme oxygenase-1 (HOX-1) by (GT)n dinucleotide repeat (GT)n ≤ 26 S allel, 27≤(GT)n≤ 31 M allel, (GT)n ≥ 32 L (long) allel), levels of serum iron and bilirubin, the last products of enzyme, on severity of COPD and exacerbations of disease.

Three different staged 90 COPD patients and 93 controls were included. By increasing the severity of disease, bilirubin levels were coming closer to the lower limit within normal range. Iron levels of Group 2 (moderate) and 3 (severe) were significantly lower than Group 1 (mild), *(p=0,025, p=0,035)*. There was an inversely related statistically significant correlation between exacerbation numbers and iron *(p=0,016)*. Allel frequency and carrier of L ((GT)n ≥ 32) allel were similar between patient and control group, and patient group in its own *(p=0,458, p=0,445)*. There was no significant relation between individuals with L allel (SL, ML, LL) and without L allel (SS, SM, MM) according to levels of iron and bilirubin, number of exacerbations and FEV1 *(p=0,631, p=0,065, p=0,356)*.

In conclusion; by the progression of COPD, levels of bilirubin and iron are liable to decrease while the number of exacerbations is increasing. (GT)n polymorphism of COPD patients in the study group is not a risk factor for development and stage of disease, frequent exacerbation, low iron and bilirubin levels.

Key Words: COPD, heme oxygenase-1, (GT)n polymorphism, FEV_1, iron, bilirubin, exacerbation

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterised with partially reversing airway obstruction, chronic bronchitis and emphysema; which is related with abnormal inflamatory response of lungs to environmental and personal risc factors at genetically responsive people. COPD is a systemic disease with effects out of lungs and seriousness of disease is effected by additional diseases. The fact that; COPD development at non-smokers, or airway obstruction at childhood conversion to COPD at elderly are supporting the idea of familiar genetic component of disease.

It is absolutely known that *alpha-1 antityripsin (AAT)* deficiency is a cause of COPD. Except this, there are lots of genetic factors that are suggested for disease development. *Microsomal epocsid hydrolase, Glutation-S- transferase, Citocrom p450 1A1, Extracelluler superoxid dismutase* and *Hem oxygenase-1 (HOX-1)* are designated as cause of reduced antioxidan effect.

Heme oxygenase (HO) is the rate limiting enzyme of reaction which heme catalyzed in to biliverdin by exposing carbon monoxide (CO) and free iron. Polymorphism at the enzimatic code region effects the enzymes activity; so anti-inflamatory, anti-oxidant and antiapoptotic properties of last products cannot occur. There are 3 types of enzyme defined so far. HOX-1 is an inducible form of HO. While HOX-2 is the founding enzyme, the function of HOX-3 has not been confirmed yet (1). Two potential polymorphisms in the 5 'promoter region of the HOX-1 gene can affect the endogenous protective property of this

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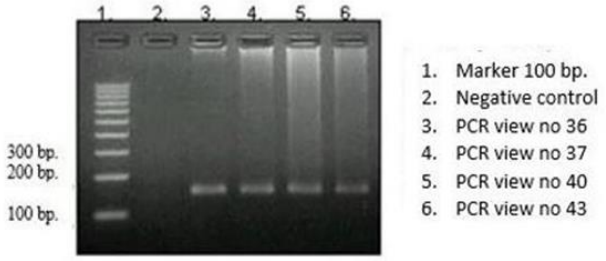

Fig. 1. PCR amplification agarose gel electrophoresis view

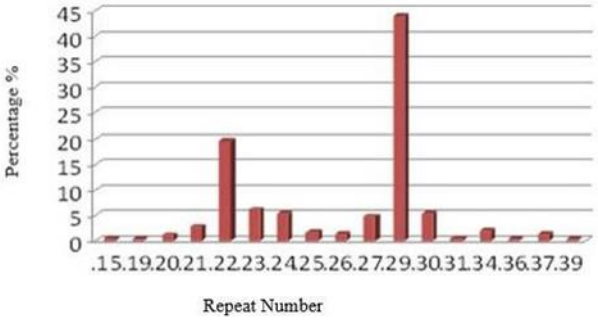

Fig. 2. Allel frequency distribution of all participants [(GT) n ≤ 26=S, 27≤(GT)n≤ 31=M, (GT)n ≥ 32=L]

enzyme These are; G (-1135) A and T (-413) A and (GT) n dinucleotide length polymorphism with two separate single nucleotide polymorphisms (SNP). In in vivo studies it was found that no transcriptional regulator protein could bind to the region where (GT) n repeats occur (2). Therefore, the enzyme cannot activate as the number of dinucleotide repeats increases.

The number of (GT) n repeats in polymorphism varies between 10-40. It was found that HOX-1 basal promoter activity or increased transcription stimulation against different stimuli was significantly higher in the group in which the dinucleotide repeat length was ≤25 compared to the group in which the number of repeats was> 25 (3). It has been reported that HOX-1 protects the lung against oxidative stress and perhaps is one of the important elements that provide balance between oxidant-antioxidants (4). The reason for this thought is the biological effects of the end products of the reaction catalyzed by the enzyme.

This study is planned to study the activity of HOX-1, which is an important antioxidant enzyme of body, and levels of enzymes last products, on severity and exacerbations of COPD.

Materials and Methods

Appropriate to Helsinki Declaration Princibles, acknowledgement instruct was taken from all paticipants. In order to investigate effect of the end products of the enzyme on the exacerbation

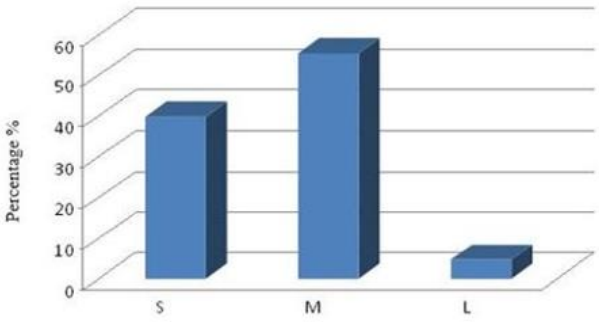

Fig. 3. Allel distribution

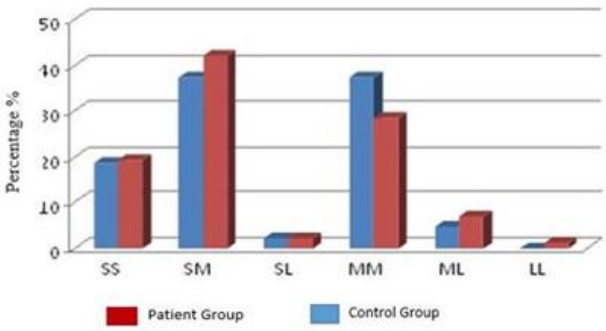

Fig. 4. Genotype frequency distrubution of all cases

frequency of the patients; instead of the GOLD 2017 ABCD classification which evaluates the risk of symptoms and exacerbation, patient classification designed with only respiratory function test values. 93 volunteer as control group (group 4) with normal respiratoy function test and older than 40 age; 90 COPD patient, who applied to chest disease polyclinic, classified as mild (group 1), average (group 2), heavy-very heavy (group 3) according to GOLD criterias. Totally 183 participants were included to study. Excluding criterias are; having any hematological, hepatic, gall bladder disease that effects bilirubin level; having any hematologic disease that effects Fe level; having any multisystemic disease like chronic renal failure, chronic hepatic failure, malignancy.

For molecular analysis, 10 ml venous blood was taken from all participants in to standart tubes with 0.072 ml %7.5 K3-etilendiamintetraasetic acid (EDTA). DNA was isolated from blood specimens by fenol chloroform extraction method to determine HOX-1 gene (GT) repeat. Samples were concealed at -80ºC.

Following to genomic DNA isolation, in order to determine (GT)n repeat number promotor region polymerase chain reaction was performed by using FAM marked F: 5'-AGAGCCTGCAGCTTCTCAGA-3'; R: 5'-ACAAAGTCTGGCCATAGGAC-3' primers. Fragment analyse was performed by conducting of

acquired polymerase chain reaction (PCR) products on ABI310 gel electrophoresis (Figure 1)

Allels were seperated in to three groups according to (GT)n repeat. (GT)n repeat number distrubution of all cases were analysed (Figure 2). Peak distributions were on three allel which have 22, 29, 34 repeats. According to distribution graphics; deadline value for S (short) allel is the lowest repeat after 1. peak, for M (medium) allel is the lowest repeat after 2. peak. As a result groups are classified as; (GT)n ≤ 26 S allel, 27≤(GT)n≤ 31 M allel, (GT)n ≥ 32 L (long) allel.

Genomic distribution according to (GT) n repeat number designed as SS, SM, SL, MM, ML, LL. We discussed the relation of genomic dispersion and iron, bilirubin levels and number of exacerbation between L allel carrier and not carrier group.

300μL periferic venous blood of each participant used for biochemical analyses of total bilirubin and Fe levels. *Roche Moduler P BiochemistryAutoanalyzer* was used for spectrofotometric method. Reference values of serum iron for male 60-170μg/dl, for female 50-140 μg/dl; 0,1-1,2 mg/dl for total bilirubin.

COPD patients were followed for 1 year in order to developing exacerbation. According to Anthonisen criterias; having any of sputum volume increase, sputum purulance increase and deterioration of dispne was accepted as exacerbation (4).

Statistical Analysis: Datas were analysed in *SPSS for Windows 11.5* packet program. Shapiro Wilk test was used to investigate if the distribution of constant variables were near normal. Descriptive statics for constant variables has shown as mean ± standard deviation or median (minimum-maximum), case number and (%) as for nominal variables. When the significance of the difference between the groups in terms of averages was two, the number of independent groups was evaluated with Student's t test and the significance of the difference between more than two groups was evaluated by *One-Way ANOVA*. The significance of the difference between the groups in terms of median values was investigated with the Mann Whitney U test when the number of independent groups was two, and the significance of the difference between more than two groups was investigated with the *Kruskal Wallis test*. In order to determine the situations that cause the difference if the *One Way Variance analysis* or *Kruskal Wallis test* statistics results are found to be important; post hoc Tukey or nonparametric multiple comparison test was used. Nominal variables were evaluated with *Pearson's Chi-Square test*. Results were considered statistically significant for p <0.05. *Bonferroni Correction* was made to control Type I error in all possible multiple comparisons.

Results

According to GOLD pulmonary function criterias 29 mild (Group 1), 31 medium (Group 2), 30 heavy-very heavy (Group 3) stage COPD patient and 93 volunteer as control group (Group 4) with normal lung function test and older than 40, totally 183 cases were included study.

There was no statistically significant difference according to bilirubin levels between patient and control group (p=0,235). Average bilirubin levels of Group 1-2-3 were 0,72 mg/dl, 0,59 mg/dl, 0,56 mg/dl.

In order to exclude patients with iron defficiency anemia (IDA), hemograms of male patients whose iron levels < 60 μg/dl and female patients whose iron levels < 50 μg/dl were analysed. If one of the values of average erythrocyte volume (MCV) <80 fL, erythrocyte dispersion width (RDW) >%16, average erythrocyte hemoglobine (MCH) <26 pg were corrected, patient classified as iron defficiency anemia (IDA). 3 cases of control group, 11 cases of patient group (1 from Group 1, 5 from Grouıp 2, 5 from Group 3) were excluded from multivariant analyses. There was no relation between control and patient group in point of iron levels. In consequence of iron levels of Group 2 and 3 were lower than Group 1 *(p=0,025* and *p=0,035),* there was significant different between patient groups (p=0,015). Mean iron levels of Group 1-2-3 were 92,4 μg/dl, 67,2 μg/dl and 68,1 μg/dl.

In patient group, there was statistically significant difference between stages according to number of exacerbation *(p=0,006*). The cause of this result is because number of exacerbation in Group 3 was higher than Group 1 *(p<0,001)* (Grup 1 avrg= 0,45, Grup 3 avrg=1,43 exacerbation). There was no statistically difference between Group 1 and 2, Group 2 and 3 *(p=0,062 ve p=0,129).* While number of exacerbation having a reversly related statistically relation with iron level *(p=0,016)*, it had no significant relation with bilirubin level*(p=0,691)* (Table 1).

While M was the most frequently seen allel with percentage of %55,5 (n=136), in terms of allel frequency distribution there was no significant difference between patient and control group

Table 1. Exacerbation Number, Iron and Bilirubin Levels in Patient Group According to Stages

Variables	Stage I	Stage II	Stage III	p
Exacerbation number [mean±sd]	0.5±0.7a	0.9±1.2ab	1.4±1.4b	0.006*
Iron(µg/dl)[mean±sd]	92.4±32.34a	67.3±32.17b	68.1±28.62b	0.003*
Bilirubin(mg/dl)[median(min-max)]	0.7(0.1-1.6)	0.5(0.3-1.4)	0.5(0.2-1.2)	0.130**

*One Way ANOVA, **Kruskal Wallis, a-b: There is no difference between groups with the same letter for each line (PostHoc: Tukey HSD and Bonferroni)

Table 2. Exacerbation Number, Iron and Bilirubin Levels According to Allel Frequency in All Cases

Variables	Not L carrier	L carrier	p
Exacerbation number [mean±sd]	1.0±1.2	0.6±0.7	0.356*
Iron [mean±sd]	75.5±35.14	85.0±31.81	0.631*
Bilirubin [median (min-max)]	0.5(0.1-2.2)	0.7(0.3-1.4)	0.065**

*(SS, SM, MM are not L carrier; SL, ML, LL are L allel carrier genotypes)
*Independent Samples t test, **Man Whitney U

(p=0,458) (Figure 3). Percentage of S and M allel carriers were %39,9 (n=106) and %4,6 (n=15). Genotypic distribution was similar in patient and control group; SM (n=69) with percentage of %39,7 was the most frequent, LL (n=1) with percentage of %0,6 was the most rarely seen genotype (Figure 4). Frequency of L allel carrier genoypes (SL, ML, LL) was similar in patient and control group *(p=0,445).*

There was no significant difference according to median bilirubin levels between L allel carrier and not carrier (SM, MM, SS) genotypes in all participants *(p=0,065)* (Table 2)

There was no statistical difference between L allel carrier and not carrier group according to iron level *(p=0,631)* (Table 2). In genotypic groups, between control and patient groups mean iron levels were statistically similar *(p=0,295).* There was no relation between L allel porter and exacerbation number in patient group *(p=0,356)* (Table 2).

When genotypic distribution according to disease stage was analysed; MM with percentage of %42,9 in Group 1, SM with percentage of %35,5 and %62,1 in Group 2 and 3 were the most frequently seen genoypes (Table 3).

According to stages, there was no statistically difference in terms of L carrier genotypes *(p=0,275)* (Table 4). Allel frequency distribution of stages were similar *(p=0,053).* M for Group 1 and 2 (%62,5 and %53,2), S for Group 3 (%55,2) were the most frequent allels.

Discussion

This study was designed in order to investigate the activity of HOX-1 enzyme, which is one of the antioxidant enzymes of body, in Turkish population and effects of last produts of enzyme on COPD exacerbations. To assess the adequacy of enzyme activity, the presence of polymorphism in the genetic coding region of the enzyme was investigated.

Polymorphisms do not directly cause illness like mutations, they can only be a risk factor for disease development. The frequency of polymorphisms in each society is different and this also affects the frequency of the disease associated with polymorphism in that society. First, Yamada et al. (3) examined the number of repetitions of (GT) n in Japan smoker patients, and found that patients with the L allele are susceptible to the development of emphysema. Fu et al. (5) reported that the number of (GT) > 32 repetitions, and that patients carrying the L allele are more likely to have advanced COPD in China. Guenegou et al. (6) in France, followed 749 patients aged 22-40, 40% of whom never smoked, for 8 years, and observed the fastest FEV1 and FEV1 / FVC reduction in the smoker and carrying L allele group. In contrast to all these results, He et al. (7) in Canada stated that; the genetic distribution in the white and black races was incompatible, therefore, a small number of blacks were not evaluated and they could not find a relationship between the number of repetitions of smokers (GT) and FEV1 decline.

To the best of our knowledge, there is no data from the Turkish community's (GT) n repeat polymorphisms. In our study, peak distributions were observed at 22, 29 and 34 repeats in terms of allele frequency. It is determined that the M allele, which is located between the boundaries of 27≤ (GT) n≤ 31 in Turkish society, is the dominant (55.5% in all cases, 52.8% in the patient group),

Table 3. Genotypic Distribution According to Disease Stages

Group (n)	SS (n/%)	SM (n/%)	SL (n/%)	MM (n/%)	ML (n/%)	LL (n/%)
1 (28)	4 (%14.3)	8 (%23.6)	1 (%3.6)	12 (%42.9)	3 (%10.7)	0 (%.0)
2 (31)	6 (%19.4)	11 (%35.5)	1 (%3.2)	16 (%32.3)	2 (%6.5)	1 (%3.2)
3 (29)	7 (%24.1)	18 (%62.1)	0 (%.0)	3 (%10.3)	1 (%3.4)	0 (%.0)

Table 4. Allel Frequency Distribution According to Stages

Group (n)	Not L carrier (n/%)	L carrier (n/%)
1 (28)	24 (%85.7)	4 (%14.3)
2 (31)	27 (%87.1)	4 (%12.9)
3 (29)	28 (%96.6)	1 (%3.4)

and L (n) 32 (allele) is the rarest allele. Genotypic distribution in patient groups did not show statistical significance between the patient and control groups. In group 3, which has the lowest FEV1 values, had the highest enzyme activity with S allele. There was no relation between pulmonary function test (PFT) values and enzyme activity. According to these results; different mechanisms shoud be responsible for lung function loss excluding the insufficient antioxidants.

Bilirubin, which is one of the final products of HOX-1 enzyme, is a natural antioxidant. It perform this effect by; leukocyte infiltration in tissues through different mechanisms such as reducing T cell proliferation (8), decreasing proinflammatory cytokines and increasing anti-inflammatory cytokines (9), and inactivating NFIB responsible for proinflammatory gene transcription (10). Bilirubin levels near the lower limit of normal predisposes to development of coronary artery disease (CAD) in patients with risk factors for vascular disease (11), diabetic patients (12), and men (13). Also there is an inverse relationship between the development of atherosclerosis in human and normal or slightly high bilirubin levels at the upper limit (13). In the joint evaluation of two large-scale studies investigating the location of azithromycin and simvastatin in preventing COPD exacerbations, higher bilirubin values were associated with less exacerbation (14). Again in a study examining serum bilirubin and disease course in early COPD patients; bilirubin was inversely related to the severity and progression of COPD (15).

In our study, there was no statistically significant difference between patient groups and control groups according to bilirubin levels. However, when the average of bilirubin values among the patient groups are examined; it has been determined that there is an increase from severe to very severe level to mild level (0.56 mg / dl, 0.59 mg / dl, 0.72 mg / dl). This result suggests that the levels of antioxidants, including bilirubin, are one of the factors that will explain the different course of the severity of the disease among groups with similar smoking history, average age and gender distribution. We also think that the disease may be milder in COPD patients with bilirubin values close to the upper limit of normal.

Free Fe released during heme destruction; has both oxidant/anti-oxidant properties. In the study of Karadag et al (16)., examination of elements involved in oxidative stress such as copper (Cu), zinc (Zn), Fe on COPD patients ; while low Zn, high Cu levels were related to the severity of the disease, Fe level was unrelated. In the study by McKeever et al. (17), high serum antioxidant vitamin and Fe levels were independently associated with high FEV1 values in patients with COPD.

In our study, iron levels of Group 2 and 3 were significantly lower than Group 1. Chronic disease anemia (CDA); which is frequently seen in COPD and progresses with chronic inflammation and increasing severity, may be the reason of this result. In addition, some of the patients were included in the study when they arrived during the exacerbation period. More frequent attacks on advanced stages and negative acute phase reactant property of Fe may be the reason of low iron levels of Group 2 and 3. We have the opinion that Fe level monitoring can be performed in order to make predictions about the progression of the disease, especially for patients who have more than one risk factor in terms of disease development and whose respiratory functions are close to normal.

HOX-1 gene polymorphism is just one of the genotypic differences that are thought to be responsible for the development of COPD. The

diverse distribution of polymorphism among communities, has made the deficiency of enzyme activity, in some countries, one of the responsible mechanisms for the development of COPD. In our study, we determined that the number of (GT) repeat polymorphisms was not a risk factor for the development of COPD and did not make significant changes in the level of their end products. Genetic factors play an important role in the development of COPD; however we believe that factors such as environmental exposure, compliance to treatment, and anatomical differences are more critical in the course of the disease.

Oxidant-antioxidant balance is important in terms of exacerbation development and disease seriousness. Despite the normal levels of heme, which is the substrat of HOX, level of enzyme's last products may be low because of decreased activity of enzyme. So we think that Fe may be used to get an idea about COPD progress. But different studies should be planned in order to support this foresight.

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