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REGULATION OF P53 ACTIVITY BY METHIONINE SULFOXIDE REDUCTASES

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ABSTRACT

Aim: p53 is one of the important tumor suppressor proteins in humans and plays a role in many biological functions such as cell cycle arrest, DNA repair, and apoptosis. The aim of this research was to examine the activity of methionine and the methionine sulfoxide reductase (Msr) enzyme on oxidized p53.

Methods: Shuttle vectors that can replicate in both yeast cells and bacterial cells were used in the research. A high copy of the human p53 protein was created by Gateway cloning technology. Escherichia coli bacteria were used for the plasmid DNA transformation. The isolation experiment was then completed. The accuracy of the process was checked by a run-on experiment in gel electrophoresis. In addition, the β-Galactosidase assay was performed in the study.

Results: Mutants lacking the Methionine Sulfoxide Reductase genes had higher p53-dependent Lac-Z activity compared to the control cells. However, overexpression of Msr genes did not cause a change in p53 activity. Differences in p53 activity were observed upon treatment of mutants with 1mM hydrogen peroxide. Specifically, cells lacking both the Msra and Msrb genes were found to have less p53 activity.

Conclusions: The loss of activity in p53 in oxidative stress was concluded to be protected against and controlled by the Msr enzymes.

Key words: p53, cancer, methionine sulfoxide reductase enzymes, oxidative stress

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INTRODUCTION

p53 is a tumor suppressor protein [1]. The p53 protein is described as a "genome guardian" that regulates cell division by preventing the grow than division of cells, that is preventing the development of tumors [2]. Studies have shown that various events such as DNA damage, heat shock, hypoxia, and oncogene overexpression result in the activation of p53, and that p53 plays an important role as a regulatoryprotein that regulates various biological responsesand is responsiblefor genetic stabilityby preventing genome mutation [3,4]. A meta-analysis study has shown that abnormal p53 status was associated withworse survival in every subgroup of small cell lung cancer [5]. In another meta-analysis study, the p53 gene was found to be generally associated with an increased risk of invasive cervical cancer; however, the country of residence, cellular structure, and HPV type have been considered to be effective factors in these relationships [6].

Tumor cells are known to continue to divide despite having damaged DNA by inactivating the control molecules in the division process seen in normal cells [7]. For this reason, the role of the p53 gene in cancer has been a scribed great importance. The p53 gene is the tumor suppressor gene that is most mutated in bladder cancer [8]. p53 gene mutations have been commonly detected in people diagnosed with cancer [6, 8]. On the other hand, it is known that interventions that can affect p53 in humans require costly and lengthy efforts. Research and cancer gene therapy development studies continue on the effectiveness of treatments that specifically target the p53 gene [9, 10]. In addition, one of the areas where the change of the p53 gene can be examined more quickly and with lower cost is bacteria [11]. The p53 gene plays an important role in cancer treatment. Therefore, obtaining information about enzyme activities related to the p53 gene will contribute more to the literature.

Many posttranslational mechanisms controlling p53 activity are known, but its control byoxidative stress is not clearly known. Methionine amino acids in the structure of p53 form methionine sulfoxide groups (metO) in an oxidation state, which disrupts the function of p53. Methionine SulfoxideReductases, which are antioxidant group enzymes, reduce methionine sulfoxides back to methionine. This study aimed to determine whether there is an activity control mechanism involving methionine oxidation/reduction on p53 and Msr group enzymes.

METHODS

MATERIALS

To carry out the necessary procedures for the study, a deep freezer, refrigerator, ice maker, Dislyte water device, digital water bath, precision scale, incubator, shaking incubator, micropipette, autoclave, pH meter, centrifuge, vortex mini mixer, electrophoresis system, spectrophotometer, gel imaging device, automatic pipette, fume hood, 96-well plate, microwave, and heater were used.

Among the chemical and biological materials used in the research were LBClonase enzyme, Proteinase-K, RNA-ase-A suspension, Elution buffer, Tango buffer, X honey, Evor-V, Lysis solution, Neutralization solution, Wash solution, Agar, Ethidium bromide, LIOA-C (Lithium acetate), SSDNA, PEG-mix, ONPC (O-nitrophenyl β-D-galactopyrronoside),

H_2O_2 (Hydrogen Peroxide), Z buffer, $NaCO_3$ (Sodium Carbonate), Methionine amino acid, Histidine amino acid, and Uracil amino acid.

EXPERIMENTAL PROCEDURES

The pRS315 RE-Z and pRS316PGKp53 plasmids were used in the research. These vectors are shuttle type vectors that can replicate in both yeast cells and bacterial cells. These plasmids have low copy properties. The pRS315 RE-Z plasmid contains the Lac-Z gene, which can be expressed under the control of a specific DNA sequence to which the p53 protein can bind as a transcription factor. The LEU2 gene is the auxotrophic marker gene of the plasmid. The plasmids used in the research were provided by Dr. Gray Merrill.

The PAG413p53 vector, which is an empty vector, was used to create a high copy of the human p53 protein. PAG413p53was also the vector we would use for Msrs. The LR reaction was established using Gateway Cloning Technology.

To propagate the PAG413P53 plasmid, it was transformed into Escherichia coli bacteria. This plasmid we used in the transformation contains the Ampicillin resistance gene. E.coli is also sensitive to this antibiotic. Therefore, we used an Ampicillin medium as a medium for the PAG413p53 plasmid to grow in bacteria.

After the plasmid DNA was propagated in E. coli, an isolation experiment was performed with a DNA purification kit obtain this plasmid in pure form.These kits contained special solutions and spin columns to hold DNA. These special solutions used enable the physical dissolution of DNA; columns were used to capture DNA. There is a matrix layer in the columns. Since substances other than DNA cannot adhere to the matrix, they are removed from the environment. At the end of the process, there is an elution stage to remove the DNA from the matrix, and with this stage, the DNA is separated from the matrix and takes its final form.

After cloning the plasmid DNA, it was tested whether the p53 transcription factor was cloned correctly. Run-on gel electrophoresis was used for this verification. Gel electrophoresis is a method that separates proteins and nucleic acids according to properties such as size and electrical charge. Molecules move in the presence of electricity and move towards the opposite side according to the size of the molecule. To ensure that the p53 gene has been cloned correctly, the decision is made by running it on the gel and looking at its molecule size.The mutant yeast cells we used in yeast transformation are the BY4741 strain of Saccharomyces cerevisiae and their genetics are MATahis3leu2ura3met15. The Msra single Msrb single and Msra and Msrb double mutants which we used to measure human p53 activity in mutant yeast cells and oxidative stress and are also used as antioxidant enzymes, were available in the laboratory because they were used in previous studies.

As a result of the yeast transformation experiment, at least three colonies were taken from BY4741 cells growing in yeast nitrogen base solid medium for each medium and after adding the appropriate amino acids to the medium, they were re-planted in YNB medium. Our goal was to keep the medium fresh for a certain period of time. And now, in the last case, we had the transformantsBY4741REZp53, BY4741REZ-p53Msra, BY4741REZ-p53Msrb, and BY4741REZ-p53Msrab. We used Kippert's method to quantify β-galactosidase activity in yeast cells [12].

After testing the p53 activity in the yeast system with Msra single Msrb single and Msrab double mutants in case of oxidative stress, we wanted to test it with the Msrc enzyme, which we did not test. In the yeast system, in the new case, there were the pRS315 RE-Z and pRS316PGKp53 plasmids and Msra single Msrb single and Msrc single mutants. In order to study this in the yeast system, the p423-GPD vector was used as an empty vector. All of the plasmids and Msrs we were to use were propagated in bacterial cells by bacterial transformation and plasmid DNA isolation, the methods of which we have previously explained, and obtained in pure form, and their concentrations were measured. The subsequent yeast transformation and β-galactosidase assay procedure was the same as the procedure we followed before.

ETHICS

This research does not require ethics committee approval.

STATISTICAL ANALYSIS

The effect of methionine sulfoxide reductase (Msr) enzymes on p53 activity was examined in yeast cells under normal conditions and oxidative stress conditions. In the study, the increase and decrease in p53 activation were evaluated with descriptive statistics such as median and percentage. The test results of all groups were analyzed with the Kruskal-Wallis H test since the groups did not show a normal distribution. Normal distribution of the data was checked with the Kolmogorov–Smirnov test and the Shapiro-Wilk Test. Additionally, analysis was performed using the Kruskal-Wallis H Post-Hoc Test for pairwise comparisons between groups. In the study, the significance level was accepted as α=5. The IBM SPSS 22.0 software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) program was used to perform the analyses.

RESULTS

Whetherp53 was cloned correctly was tested by the result of the gel electrophoresis experiment. In the gel electrophoresis experiment, the expected molecular size of p53 in the gel was approximately (base pair) (1200)+(656). Since the molecular weight of p53 was within the expected range, it was concluded to have been cloned correctly.

According to the Kruskal-Wallis H test, the change between the groups was found to be statistically significant, and the difference between the binary measurements was statistically significant ($p<0.05$). Below are the comparison results between the groups (Table 1).

Table 1 Comparison of the results obtained from the research

	WT (I)	Msra (II)	Msrb (III)	Msrab (IV)	X^2	I-II	I-III	I-IV	II-III	II-IV	III-IV
Lac-Z enzyme activity	0.37 (0.26-0.45)	0.61 (0.53-0.78)	0.64 (0.60-0.73)	0.47 (0.32-0.54)	19.84**	<0,05	<0,05	-	-	-	<0.05
p53 activation status	1.44 (1.06-1.75)	1.37 (1.03-1.63)	1.43 (1.22-1.69)	1.56 (1.10-1.86)	0.52	-	-	-	-	-	-
Mutants under oxidative stress	0.28 (0.25-0.29)	0.32 (0.28-0.38)	0.25 (0.23-0.29)	0.23 (0.20-0.25)	16.83*	-	-	-	<0.05	<0.05	-
Msr enzymes and oxidative stress	0.18 (0.15-0.21)	0.13 (0.11-0.15)	0.15 (0.12-0.18)	0.17 (0.15-0.23)	11.03*	-	-	-	-	<0.05	-

Median (Q1-Q3), X^2=Kruskal-Wallis H Test, *<0.05, **<0.01

In cells lacking the Msra enzyme, a 40% increase in p53 activity was observed compared to the control group. A 52% increase in p53 activity was detected in cells lacking the Msrb enzyme compared to the control group. A 20% increase in p53 activity was detected in cells lacking Msra Msrb double mutants compared to the control group (Figure. 1)

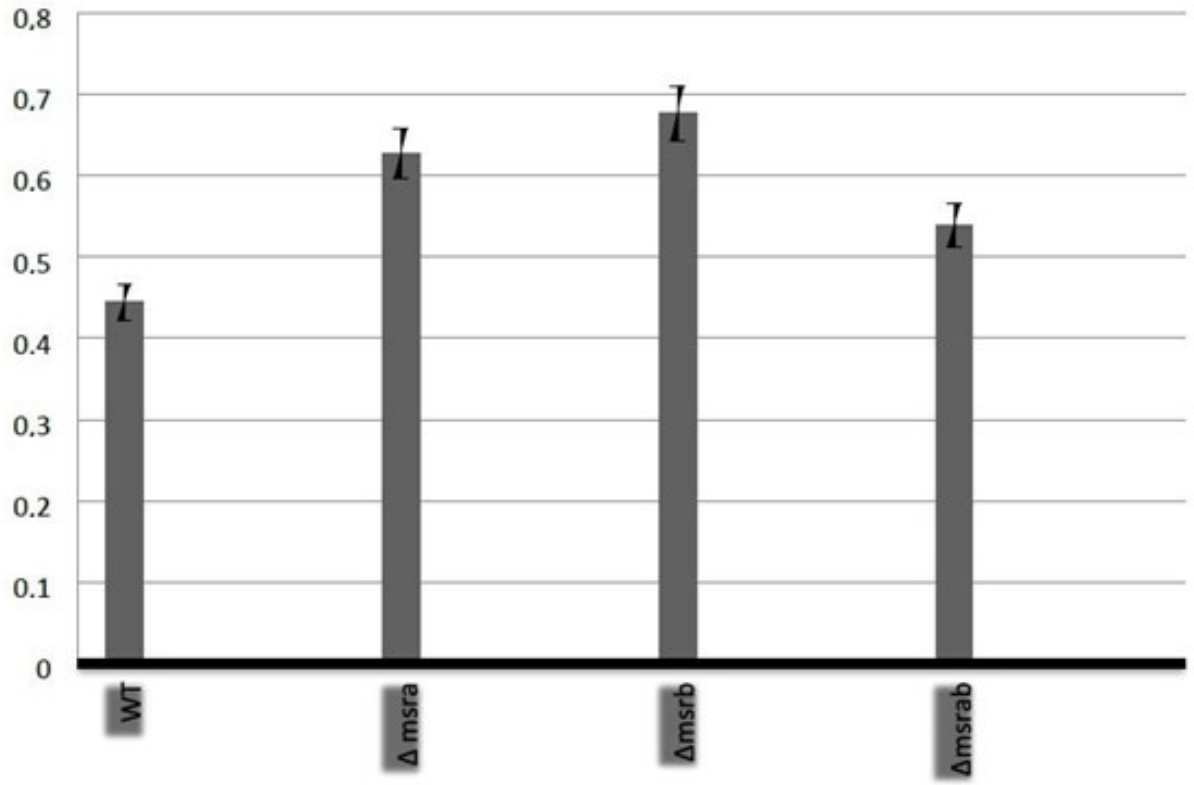

Figure 1. p53 activity was found by measuring p53-dependent Lac-Z enzyme activity
These are the average values obtained from measurements.

It was determined that p53 activity decreased by 3% in cells with the Msra enzyme compared to the control plasmid in cells overexpressing Msr enzymes. In overexpression of the msrb enzyme, a 3% increase in p53 activity was observed compared to the control plasmid, and in overexpression of the msrc enzyme, a 5% increase in p53 activity was detected compared to the control (Figure 2).

Compared to the control group, a 14% increase in p53 activity was observed in cells lacking the Msra enzyme in case of oxidative stress. Compared to the control group, an 11% decrease in p53 activity was detected in the absence of the Msrb enzyme in oxidative stress. Finally, a 22% decrease in p53 activity was detected in the case of oxidative stress in the absence of MsraMsrb double mutants (Figure 3.)

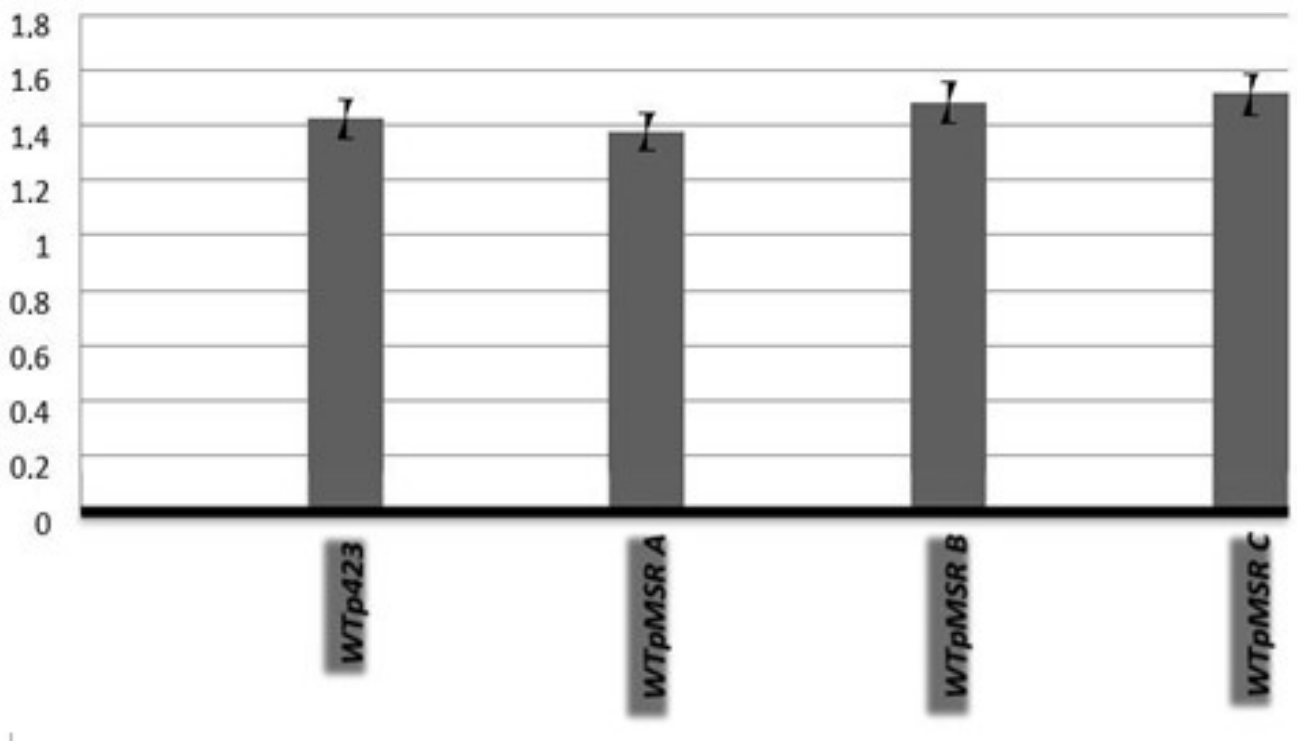

Figure 2. p53 activation status of wild-type cells in which MSR genes are overexpressed

These are the average values obtained from measurements.

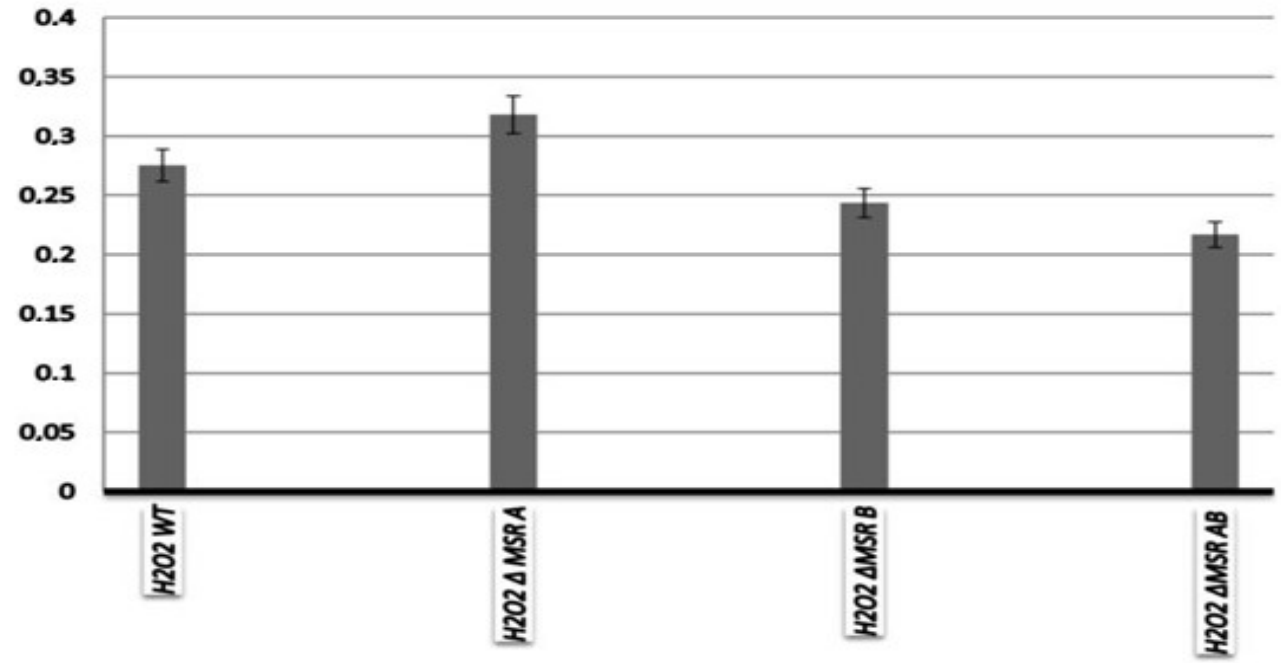

Figure 3. p53 activation states of wild type (BY4741) and MSR mutants under oxidative stress

These are the average values obtained from measurements.

In the study, a 29% decrease in p53 was detected under oxidative stress in cells where the Msra enzyme was overexpressed compared to the control plasmid. A 17% decrease in p53 activity was detected in overexpression of the Msrb enzyme. Finally, a 5% decrease in p53 activity under oxidative stress was detected in Msrc overexpression (Figure 4.)

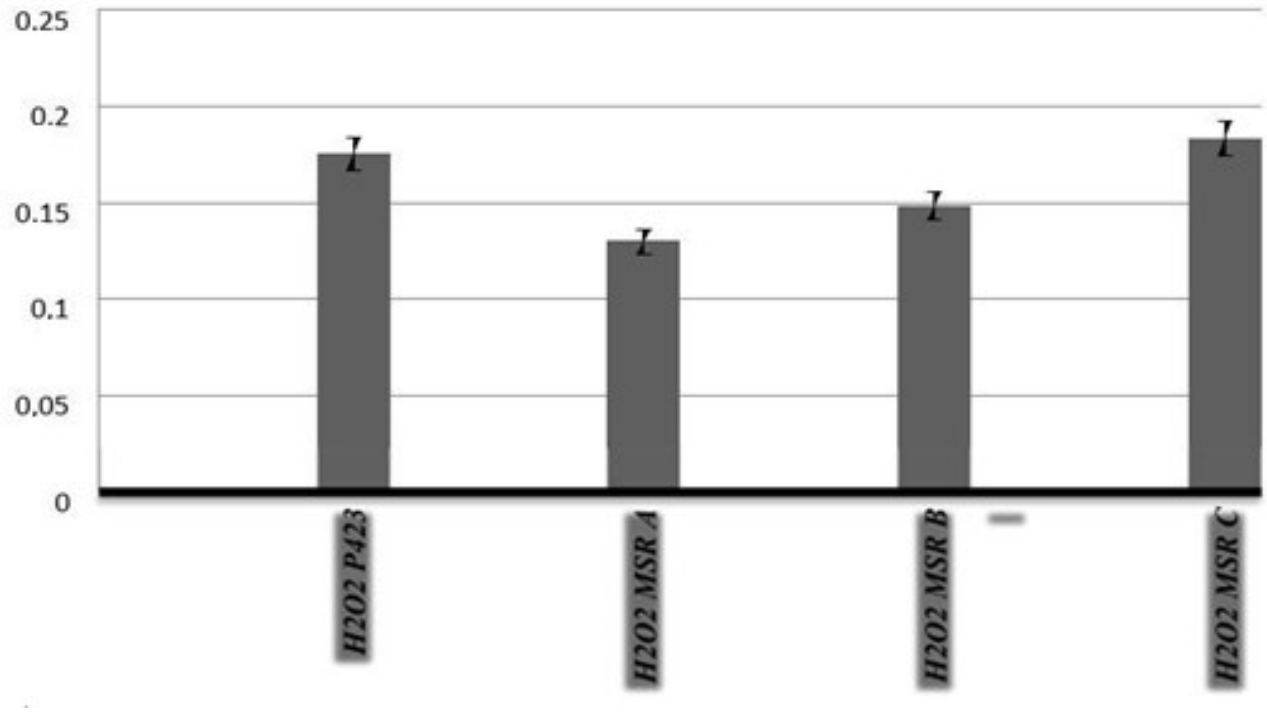

Figure 4. p53 activation conditions under overexpression of Msr enzymes and oxidative stress
These are the average values obtained from measurements.

DISCUSSION

This study showed that the activity of p53 in oxidative stress could be controlled by methionine sulfoxide reductases. Methionine sulfoxide reductases are antioxidant enzymes found in all eukaryotes. Methionine sulfoxides are formed as a result of the oxidation of methionine amino acids, and methionine sulfoxides are reduced back to methionine by methionine sulfoxide reductase enzymes. Methionine sulfoxide reductases have been studied in many organisms and their positive effects on life extension have been determined in these organisms [13]. Methionine sulfoxide reductases, which are anti-oxidant enzymes, also play an important role in protecting against oxidative stress.

p53 is a tumor suppressor protein and is a tetramer protein that stimulates the cell cycle, development, and differentiation by regulating the expression of other genes. The fact that p53 has a tetramer structure is important for its tumor suppressor function [14]. While p53 is kept at low levels in healthy cells, its level increases significantly in situations such as DNA damage,

dNTP deficiency, situations that create cellular stress, and telomerase absence [15]. Proteins have very important biological effects in the body but are highly sensitive to oxidative stress. The methionine amino acids are especially quite open to oxidation. Since there are 12 methionine amino acids in the structure of p53, these methionine amino acids in the structure of p53 will be easily oxidized in case of oxidative stressand cause the function of p53 to deteriorate.

When methionine amino acids undergo oxidation, methionine sulfoxide (metO) is formed and this causes loss of function in proteins. In a previous study, methionine (met340) in the tetramerization region of p53 was oxidized by H_2O_2, methionines were transformed into methionine sulfoxide, and it was determined that the tetramer structure of p53 was disrupted and there was a loss of activity in the protein [16]. This study suggested that the p53 protein may be inhibited if the methionine in the structure of p53 is oxidized, but a detailed study has not been conducted by including other methionines in the structure of p53 and methionine sulfoxide reductases.

As a result of the research, Themethionines in the structure of p53 are shown to be opened to oxidation in oxidative stress. In our experiment, we wanted to test whether human p53 protein activity can be controlled by methionine sulfoxide reductases, which are antioxidant enzymes, under oxidative conditions in the yeast system. When the experimental results were examined, the p53-dependent Lac-Z activity of mutants lacking msr genes was higher than control cells. However, overexpression of msr genes did not cause a change in p53 activity.

LIMITATIONS

The results obtained from this study are limited to those related to the human p53 gene transferred into bacterial cells. Therefore, it should be kept in mind that different results may be obtained in human populations.

CONCLUSIONS

Mutants that lack methionine sulfoxide reductase genes had slightly higher p53-dependent LacZ activity than control cells. However, overexpression of Msr genes did not change p53 activity. The mutants had a different p53 activity pattern under oxidative stress when the cells were treated with 1 mM of hydrogen peroxide. Specifically, the cells that lack both MSRA and Msrb genes showed less p53 activity. The loss of activity occurring in p53 in oxidative stress has resulted from the protection and control of Msr enzymes.

Conflicts of interest: The authors have nothing to disclose.

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