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ORIGINAL ARTICLE

Comparison of the tissue expressions of glutathione S transferase isoenzymes among patients with morphea and healthy controls: A preliminary study

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Abstract

Morphea is an inflammatory connective tissue disorder, which is characterized by sclerosis in skin and subcutaneous tissues with a chronic progress. The oxidative stress in pathogenesis of sclerosing diseases was proposed in several studies with conflicting results. To explore the tissue expressions of Glutathione S transferase (GST) isoenzymes in patients with morphea and compare these expressions with healthy controls. Twenty-two morphea patients and 20 sex and age matched healthy controls were enrolled in this study. Four millimeter punch biopsies were performed from the active sclerotic plaques of morphea patients. Tissue samples of control group were obtained from nonlesional normal skin biopsy specimens. The protein expressions of GST isoenzymes were analyzed immunohistochemically. Tissue expressions of GSTP1, GSTT1, and GSTA1 isoenzymes in morphea patients were found to be significantly higher than in control tissues. There was no significant difference in GSTM1 isoenzyme expression between the two groups. The increased tissue expressions of GSTA1, GSTP1, and GSTT1 isoenzymes in morphea may represent the activated GST enzymes in response to excessive free radical formation and may also support the hypothesis of increased oxidative stress in morphea etiopathogenesis.

KEYWORDS

dermatopathology, glutathione S transferase, immunohistochemistry, localized scleroderma, morphea, oxidative stress

1 | INTRODUCTION

Morphea, also known as localized scleroderma, is a rare, chronic, inflammatory disease of the connective tissue which presents with overactivation of fibroblasts leading to fibrosis of the skin, subcutaneous tissue, vessels and involvement of extracutaneous tissues.[1] The etiology of morphea is still obscure and it is suggested that there is a genetic predisposition with an autoimmune basis in addition to environmental triggering factors.[2] After it was reported that oxidative stress may cause scleroderma-like features, this mechanism has been also implicated in the pathogenesis of morphea.[3] Afterwards, an abnormal formation of reactive oxygen species (ROS) was shown in most of the pathologic features in systemic scleroderma patients.[4]

Oxidative stress occurs as a result of the imbalance between pro-oxidant and antioxidant systems and causes cellular damage. To avoid from this harm, various oxidative stress neutralizing enzymes and

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systems are present in cells. The glutathione S-transferase (GST) superfamily is one of the main member of cellular defense mechanisms, fighting against to harmful chemicals.[5] The increased tissue expressions of these enzymes may reflect the pronounced role of oxidative stress that role in the disease pathogenesis. In this study, we aimed to determine whether there is an alteration in the tissue expressions of GST isoenzymes in morphea patients and to compare these with healthy controls.

2 | MATERIALS AND METHODS

2.1 | Study design

The study enrolled consecutive patients with morphea who attended the outpatient clinic of Dermatology Department of Medeniyet University Medical Faculty, Department of Dermatology. All patients declared a written consent form before enrollment. The research included 22 morphea patients who have shiny, whitish sclerotic plaques and 20 sex and age matched healthy controls. Four millimeter punch biopsies were performed from skin lesions of patients. The control group was composed of 20 pathology specimens with normal skin histology. Exclusion criteria were a smoking history or any medical treatment history (including with drugs such as anti-inflammatory agents, immunosuppressant, sunscreens, and vitamin supplements) 6 months prior to tissue sampling.

2.2 | Immunohistochemical staining

The punch biopsies sections (5 μm) were stained with hematoxylin and eosin. Immunostaining for GSTT1, GSTM1, GSTA1, and GSTP1 was performed to evaluate the expression of enzymes, sequentially, in formalin-fixed paraffin-embedded tissues in line with the producer's instructions. In short, after the deparaffinization and dehydration of the slides, they were incubated in antigen retrieval solution (0.01 M citrate buffer (pH 6.0) in a domestic pressure cooker for 3 min. Following the blockage of endogenous peroxidases with methanol including 3% hydrogen peroxide for 10 min, the incubation of tissue sections was performed with primary antibody for anti-GSTT (GSTT1/glutathione S-transferase theta 1; [ab96592] Abcam, Inc. Cambridge, Massachusetts; 1:250 dilution), anti-GSTM (glutathione S-transferase mu 1/GSTM1 antibody; [ab113432] Abcam, Inc. Cambridge, Massachusetts; 1:250 dilution), anti-GSTA (GSTA1/glutathione S-transferase alpha; [ab180650], Abcam, Inc. Cambridge, Massachusetts; 1:250 dilution), anti-GSTP (glutathione-S-transferase P 1 (GSTP1; [ab53943] Abcam, Inc. Cambridge, Massachusetts; 1:250 dilution) for an hour at room temperature. The incubation of sections with secondary antibodies at room temperature lasted for an hour (Please mention that a horseraddish peroxidase system was used for detection. Otherwise the bron color could not be understood). Then, the sections were counterstained with hematoxylin for 30 s and analyzed via microscope Nikon Eclipse TE-200-U.

2.3 | Histopathological evaluation

After immunohistochemical staining, all sections were evaluated by two investigators—a biologist and a pathologist—who were not aware of the clinical diagnosis of the patient. The intensity, localization, and distribution of the immunostaining was recorded. The sections were evaluated according to brown color in the cytoplasm and/or nuclei of epithelial cells in the basal layer of epidermis. Same observers scored the immunoreaction and differences between two observers in scoring were resolved by consensus. The positivity of the response was evaluated according to intensity of the immunoreaction and classified into four groups as negative [−], weakly positive [1+], moderately positive [2+], and strongly positive [3+].

2.4 | Statistical analysis

NCSS (Number Cruncher Statistical System, 2007) and PASS (Power Analysis and Sample Size, 2008; NCSS, LLC, Kaysville, Utah) were used for all statistical calculations. In the evaluations of data descriptive statistical methods including mean, SD, median, frequency, ratio, minimum, maximum, the Mann-Whitney *U* tests, for unequally distributed data in group comparisons the Wilcoxon signed rank test were used. In evaluation of the comparisons of data between groups, Spearman correlations were used. $P < .05$ was considered as statistically meaningful.

3 | RESULTS

3.1 | Study group

Our study enrolled 22 morphea patients and 20 healthy controls. The age and sex distribution of both groups are shown in Table 1.

3.2 | Immunohistochemical staining

Intensity of expression of GSTP1, GSTM1, GSTT1, and GSTA1 between morphea patients and control group is shown in Table 2 (Figures 1-4). Statistically significant difference was detected in the expression of GSTT1, GSTA1, and GSTP1 in patients with morphea as compared to healthy controls ($P = .003$; $P < .01$, $P = .001$; $P < .01$, $P = .001$; $P < .01$; Figures 1-4). No significant difference was detected in GSTM isoenzyme expression between the two groups ($P > .05$).

4 | DISCUSSION

Murrell et al[7] first mentioned that free radicals and oxidative stress might play a role in scleroderma pathogenesis. Thereafter, various pathways, enzymes and nonenzymatic compounds have been

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TABLE 1 Age and sex distribution of morphea patients and controls

	Total (n = 42)	Morphea (*n* = 22; 52.4%)	Control (*n* = 20; 47.6%)	*P*
Age (year)				
Min-Max (Median)	13-85 (40)	13-85 (44.5)	19-68 (38)	0.075[a]
Med ± Ss	42.95 ± 16.42	47.36 ± 18.51	38.10 ± 12.48	
Sex; *n* (%)				
Female	25 (59.5)	15 (68.2)	10 (50.0)	0.231[b]
Male	17 (40.5)	7 (31.8)	10 (50.0)	

Note: $^{**}P < .01$.
[a]Mann-Whitney *U* Test.
[b]Pearson Chi-square Test.

TABLE 2 GSTP1, GSTM1, GSTT1, and GSTA1 expressions in morphea patients and controls

		Total (n = 42)	Morphea (n = 22)	Control (n = 20)	P[a]
GS TP1; *n* (%)	0	3 (7.1)	0 (0)	3 (15.0)	.003**
	1	25 (59.5)	10 (45.5)	15 (75.0)	
	2	14 (33.3)	12 (54.5)	2 (10.0)	
GS TM1; *n* (%)	0	3 (7.1)	2 (9.1)	1 (5.0)	.457
	1	11 (26.2)	5 (22.7)	6 (30.0)	
	2	21 (50.0)	13 (59.1)	8 (40.0)	
	3	7 (16.7)	2 (9.1)	5 (25.0)	
GS TT1; *n* (%)	0	9 (21.4)	0 (0)	9 (45.0)	.001**
	1	15 (35.7)	12 (54.5)	3 (15.0)	
	2	10 (23.8)	6 (27.3)	4 (20.0)	
	3	8 (19.0)	4 (18.2)	4 (20.0)	
GS TA1; *n* (%)	0	30 (71.4)	10 (45.5)	20 (100)	.001**
	1	7 (16.7)	7 (31.8)	0 (0)	
	2	5 (11.9)	5 (22.7)	0 (0)	

Note: $^{**}P < .01$.
[a]Fisher-Freeman-Halton Test.

FIGURE 1 Immunohistochemical staining with gsta1 in morphea and control lesions. A, negative immunreactivity (0); B, weak immunreactivity (1+); and C, moderate immunoreactivity (2+); (×10)

investigated to reveal their roles in disease pathogenesis, and some have even been used in the treatment of scleroderma for this purpose.[6]

Decreased antioxidant capacity such as lower levels of serum alpha-tocopherol, selenium, carotene and ascorbic acid, and higher values of oxidant molecules contribute to the oxidative stress mechanism in scleroderma pathogenesis.[3] In in-vitro studies it has been shown that fibroblasts cultured from scleroderma lesions, release increased amounts of reactive oxidizing species which on the other hand induce proliferation and activation of fibroblasts. This leads to increase in type I collagen synthesis, overproduction of pro-inflammatory and pro-fibrotic cytokines, and worsens vascular

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FIGURE 2 Immunohistochemical staining with gstm1 in morphea and control lesions. A, negative immunreactivity (0); B weak immunreactivity (1+); C, moderate immunoreactivity (2+); and D, strong immunreactivity (3+); (×10)

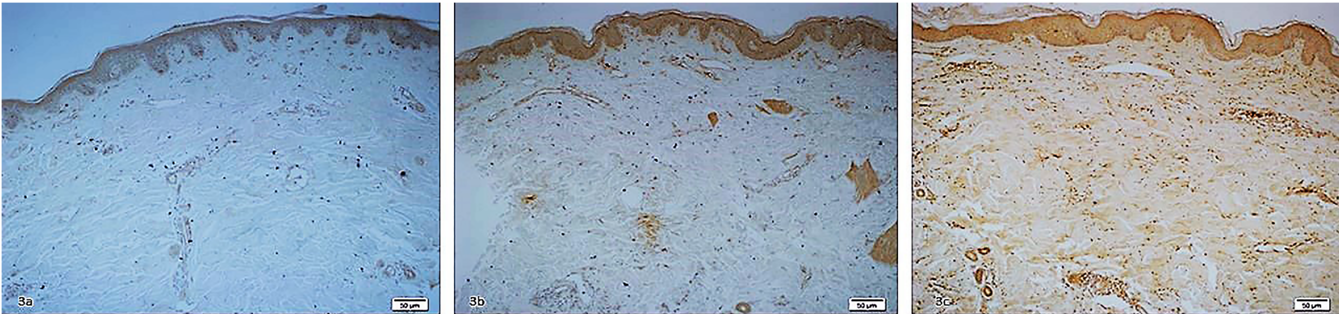

FIGURE 3 Immunohistochemical staining with gstp1 in morphea and control lesions. A, negative immunreactivity (0); B, weak immunreactivity (1+); and C, moderate immunoreactivity (2+); (×10)

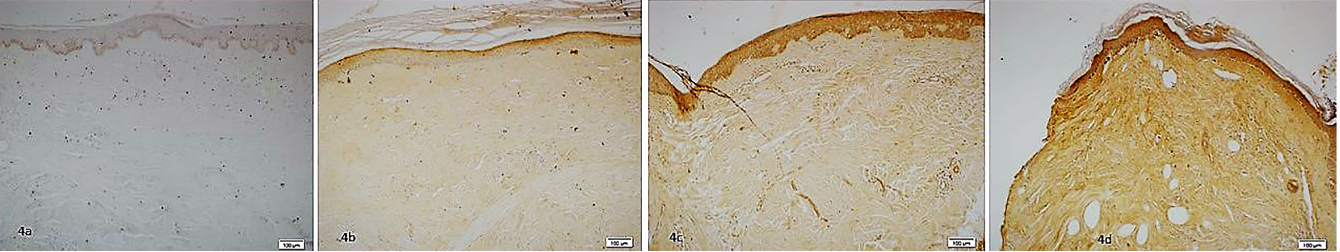

FIGURE 4 Immunohistochemical staining with gstt1 in morphea and control lesions. A, Negative immunreactivity (0); B, weak immunreactivity (1+); C, moderate immunoreactivity (2+); and D, strong immunreactivity (3+); (×10)

dysfunction.[7,8] It could be demonstrated that inhibition of ROS-generating NADPH oxidase in vitro and in vivo, is capable to suppress the activated fibroblasts and experimental skin fibrosis.[9,10] Some of the oxidative stress neutralizing compounds such as anti-oxidative enzymes including catalase, glutathione peroxidase, nonenzymatic compounds with antioxidant capacity such as vitamin C and E, selenium, glutathione, and oxidative stress markers such as lipid peroxidation products seem to contribute to fibrosis in morphea.[3]

Glutathione S transferases are a large superfamily that metabolize the detoxification of reactive-oxygen species of S-thiolated proteins that damage cells. They were classified into eight subgroups: alpha, theta, pi, omega, mu, kappa, sigma, and zeta that comprises various microsomal, cytosolic, and mitochondrial proteins in humans.[11,12] While GSTM1 and GSTP1 usually detoxify polycyclic aromatic hydrocarbons, GSTT1 detoxifies smaller hydrocarbons in human cells.[12] The genetic polymorphisms encoding these enzymes may result in functional impairment, which have been linked to different human disorders.

GST gene polymorphisms were detected in a wider spectrum of skin diseases.[13-19] In some of these studies, GST genotypes could modify a disease phenotype reflecting an association between cytogenetic damage and specific mutations in target genes.[11] GSTM1, GSTT1, and GSTP1 are the best-characterized polymorphisms in GST supergene family.[12]

The role of GST genotypes in scleroderma was first reported by Tew et al in 2001.[19] In this study, the authors reported that there was no significantly increase in M1 and T1 null genotypes in systemic scleroderma (SSc) patients as compared to ethnically matched controls. However, the frequency of GSTM1 and GSTT1 was significantly higher in patients with SSc who suffered from hypertension and

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pulmonary involvement.[19] Afterwards, Palmer et al[20] reported that alterations of GST genes, especially deletions in GSTM1 and GSTT1 genes, were associated with a moderate risk of early development of cardiovascular disease in SSc. The genetic polymorphisms of GST isoenzymes and manganese superoxide dismutase were studied by Tikly et al[21] among black South African patients who have SSc. They reported a significant decrease in the frequency of the GSTM1*B phenotype in SSc patients vs controls.[21] Fabrini et al[22] suggested that erythrocyte GST levels may be a clinical biomarker of disease activity in SSc patients, even not correlates to the impairment of a specific organ. Recently, Barańska et al[12] reported an increased frequency of null genotypes of GSTM1 and GSTT1 enzymes in SSc in the Polish population. Although these differences were not statistically relevant, all available published studies and genetic data show that the GSTM1 or GSTT1 null polymorphisms may be associated with scleroderma. In most of these studies, gene analysis was performed in serum samples and mucosal cells and defined by polymerase chain reaction.

Current research was designed to explore the tissue expressions of GST isoenzymes in patients with morphea and compare these with healthy controls. The tissue expressions of GSTM1, GSTA1, GSTP1, and GSTT1 isoenzymes were investigated by immunohistochemistry in skin biopsies of morphea patients and compared to control subjects. The expressions of GSTT1, GSTA1, and GSTP1 in tissue samples of morphea patients were significantly higher than control tissues. This significant increase of GSTA1, GSTP1, and GSTT1 expressions in morphea is probably associated with increased activation of GST enzymes and this finding may support the hypothesis of excessive free radical formation in morphea.

The GST isoenzymes are expressed in many tissues from various cells, in the normal skin GST stainings were predominantly found in the upper layer of keratinocytes and the outer sheath of the hair follicles in the epidermis, likewise in sebaceous and sweat glands and vessels in the dermis.[23,24] In this study, the tissue expressions of GST isoenzymes were mainly evaluated in keratinocytes, but also a mild staining can be seen in fibroblasts and vessels of dermal component.

To our knowledge, in morphea patients the tissue expressions of these enzymes has not been reported before in the literature. As we compare our results with the previous studies that focus on the GST gene polymorphisms in different diseases, we thought that the alterations in tissue expressions of these enzymes may be a better alternative way to show the oxidative damage.

5 | LIMITATIONS

The limited sample number is the main restriction of this research.

6 | CONCLUSION

The relationship between morphea, oxidative stress, and genetics is a trendy topic including the association with the GST enzymes and morphea. The significantly increased expressions of GSTA1, GSTP1, and GSTT1 may support the hypothesis of excessive free radical formation in morphea and the role of oxidative stress in disease pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design of study: Tugba Kevser Uzuncakmak, Seyma Ozkanli, Ayse Serap Karadag; acquisition of data: Tugba Kevser Uzuncakmak, Mahmut Can Koska, Seyma Ozkanli, Serpil Oguztuzun, Ayse Serap Karadag, Necmettin Akdeniz, Arzu Kaya Kocdogan; analysis and/or interpretation of data: Tugba Kevser Uzuncakmak, ayse Serap Karadag, Seyma Ozkanli, Serpil Oguztuzun, Uwe Wollina.

ETHICS STATEMENT

We conducted our research according to the World Medical Association Declaration of Helsinki and obtained the approval of local ethics committee.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

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