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Original Article

Real-Time Ultrasound Elastographic Features and Color Doppler Imaging of Mitral Valve Prolapse

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ABSTRACT

Background The aim of this study was to investigate the elasticity of ocular structures in patients with mitral valve prolapse (MVP).

Material and Methods This prospective study included a total of 35 patients with MVP (study group) and 35 healthy volunteers (control group). The elastography value of the ratio of orbital fat-sclera (ROF/S) was measured with real-time ultrasound elastography. For each eye, central retinal artery (CRA), posterior ciliary artery (PCA), and ophthalmic artery (OA) were evaluated, respectively.

Results The mean ages of the patients in the study and the control groups were 31.77±11.40 years, and 30.65±7.45 years, respectively (p=0.511). Mean ROF/S were 1.95±0.81 and 1.37±1.06 (p=0.001) in the study groups and control, respectively. The mean resistance index (RI) of the OA was 0.67±0.05 in the control group, 0.67±0.05 (0.55; 0.87) in study group. The mean RI of the PCA was 0.66±0.05 in the control group, 0.68±0.06 in study group. The mean RI of the CRA was 0.66±0.05 in the control group, 0.66±0.06 in study group. The RI value was not a significant difference between control and study group (p>0.05).

Conclusions Scleral elasticity was significantly increased in MVP patients. These could be related to ocular pathologies such as glacouma, kerataconus in MVP.

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Introduction

Mitral valve prolapse (MVP) is a common valvular disease, seen in 2-6% of cases, characterized by the bulging of one or both mitral leaves into the left atrium more than 2 mm above the plane of the mitral annulus during systole, due to progressive myxomatous degeneration of the mitral valve leaflets and chorda tendinea.[1] Kerataconus is associated with most of ocular manifestations described in MVP.[2,3] In some studies, retinal vascular occlusion, retinal emboli and glacuoma are also releated with MVP.[4-6]

Real-time elastography (RTE) performed in the differential diagnosis of tumour, inflammation, and normal tissue is a relatively new imaging technique that provides a noninvasive and painless assessment of tissue stiffness and elasticity.[7] Ultrasound elastography show changed elasticity of tissues resulting from from radiofrequency signals during externally applied compression–relaxation cycles.[8] Color doppler imaging (CDI) also is a non-invasive and painless technique that used to evaluted of blood flow velocity.[9] Assessment of blood flow and the retrobulbar vascular parameters (central retinal artery, posterior ciliary artery, and ophthalmic artery) have been researched with CDI in many studies.[10] In this study, we aimed to evaluate the ocular elasticity and retrobulbar vascular properties in patients with MVP by comparing them with the stiffness in the eyes of age and sex-matched healthy controls using ocular real-time ultrasound elastography and doppler ultrasonography.

Material and Methods

Ethical considerations

The study protocol was approved by the local ethics committee. The study followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study population and design

This prospective cross-sectional study was conducted at the departments of cardiology ophthalmology, and radiology of a tertiary referral hospital in Turkey. A total of 70 participants without any major comorbidities were enrolled to the study. Of them 35 patients with a diagnosis of MVP consisted of the study group while 35 healthy subjects consisted of the control group. Participants' basic demographic characteristics, elastography value of the ratio of orbital fat-sclera (ROF/S) as well as central retinal artery (CRA), posterior ciliary artery (PCA), and ophthalmic artery (OA) were assessed and then compared between the groups. Patients who had a history of significant ocular disease, intraocular pressure readings greater than 21 mmHg, glaucoma, ocular trauma, or tumor, ocular inflammatory disease, history of uveitis, retinal disease, diabetes, hypertension were excluded from the study.

Measurements of transthoracic echocardiography

All procedures of transthoracic echocardiography using a 1-5 MHz transducer (Vivid 7, GE Vingmed Sound Horten, Norway) were performed by two experienced cardiologist. All volunteers were investigated using parasternal long-axis and short-axis approaches and apical four- and two-chamber views in supine and left lateral positions. According to the suggestions of the American Society of Echocardiography primary MVP was diagnosed when at least 1 mitral leaflet was prolapsed into the left atrium and passed the mitral annulus by at least 2 mm along the parasternal long axis during the systolic phase.[11]

Ocular ultrasound elastography measurement

The image analyzes were performed by the same radiologist who carried out the ultrasound (USG) examinations. All measurements were acquired in the supine position. All images were obtained from both eyes of both groups. Freehand RTE measurement was carried out with a high frequency linear probe (13-15 MHz) on a Hitachi Arietta 65 ultrasound machine (Hitachi Aloka Medical, Ltd, Japan) by the same radiologist experienced on B-scan USG and elastography.

The all ocular measurements were assessed in the same direction as the blood flow with in an angle (<60°). The measurements have been carried out by the same experienced radiologist and the method was previously described.[12,13] The peak systolic velocity (PSV) and the end-diastolic velocity (EDV) values were recorded for each artery. Then, the vascular resistance index (RI) was calculated (RI= PSV–EDV/PSV). After assessment of orbit with conventional USG, the

probe was put onto the lids of the patient and elastography was carried out with the existing elasto software. Elasticity images were obtained by moving the probe continuously and obtaining compression and relaxation waveforms. After 8-10 compression and relaxation cycles, the elastographic examination was finalized, and strain rate measurements were acquired. We performed at a relatively slow speed, very gentle compression/release 3 to 5 times in intervals of 1 to 2 seconds and repeated measurements. At least 10 attempted elastography examinations were made for each eye until the color displayed in the ROI was completely stable to allow reliable measurement results. The strain images were put over gray-scale images in a color layout: red (largest strain, softest tissue), green (mean strain, intermediate tissue) and blue (lowest strain; hardest tissue). The ratio of orbital fat to sclera (S) was measured according to the semi-quantitative evaluation of the elastographic images. The region of interest (ROI) of the sclera was drawn, and the corresponding ROI in the adjacent normal fatty tissue was later drawn as a control for the measuring ROF/S (ratio of orbital fat-sclera) (*Figure 1*). The analyzed region in the sclera was the same dimension for all subjects and it was placed at a fixed distance from the surface. Higher elastography ratio values indicated stiffer tissue, while lower elastography ratio values showed increased elasticity. The image analyses were performed by the same radiologist who carried out the USG examinations. All measurements were acquired in the supine position. All images were obtained from both eyes of both groups.

Statistical analysis

All statistical analyses were applied using SPSS version 26 software (SPSS Inc., Chicago, IL, USA). The conformity to normal distribution of each continuous variable was assessed using the Kolmogorov-Smirnov test. The Chi-square test were used to analyze the categorical variables between the groups. An independent *t* test was used to compare continuous variables. Continuous variables were presented as mean±standard deviation while categorical variables were presented as number (percentage). A value of $p<0.05$ was accepted as statistically significant.

Results

Demographic characteristics

The mean age was 31.77±11.40 years in the study group and 30.65±7.45 years in the control group. There was no statistically significant difference between the two groups in respect of mean age ($p=0.511$). The female-to-male ratio was 20:15 in the study group and 20:15 in the control group, with no significant difference between the groups ($p>0.05$).

USG elastography results

The mean ROF/S was 1.95±0.81 in the study group, 1.37±1.06 in the control group ($p=0.001$). The mean ROF/S was significantly higher in the study group than the control group. The mean RI of the OA was 0.67±0.05 in the control group, 0.67±0.05 (0.55;0.87) in study group. The mean RI of the PCA was 0.66±0.05 in the control group, 0.68±0.06 in study group. The mean RI of the CRA was 0.66±0.05 in the control group, 0.66±0.06 in study group. The RI value was not a significant difference between control and study group ($p>0.05$). The RTE and CDI measurements of both control and study are summarized in Table 1.

Discussion

Our study evaluated the blood flow rates of retrobulbar vascular structures and elasticity of the eye in MVP patients. The results indicated that MVP patients had higher ROF/S values compared with the healthy participants. In the comparison of the MVP patients with a control group, statistically significant differences were found in the EDV, and PSV values of the PCA and CRA. A significant relationship was releated with ocular elasticity. MVP may occur as a result of various pathological mechanisms involving any of the functional components of the mitral valve apparatus. Primary MVP, which is seen as a result of valvular collagen disruption and myxomatous infiltration, is the prominent condition among them. MVP may be familial, sporadic, non-syndromic, or part of a well-defined syndrome of heritable connective tissue disorders, such as Marfan syndrome, Ehlers-Danlos syndrome, polycystic kidney disease among others.[14]

Table 1. Comparison of the ratio of sclera to orbital fat and retrobulbar hemodynamics measurements between the groups.

	Study Group	Control Group	P-value [a]
ROF/S	1.95±0.81	1.37±1.06	0.001
Ophthalmic artery			
RI	0.67±0.05	0.67±0.05	0.975
PSV (cm/s)	17.30±9.53	20.60±8.79	0.051
EDV (cm/s)	5.69±4.01	6.62±3.20	0.171
Posterior ciliary arteries			
RI	0.68±0.06	0.66±0.05	0.069
PSV (cm/s)	18.04±8.59	23.50±9.36	0.001
EDV (cm/s)	5.67±3.28	7.98±3.72	<0.001
Central retinal artery			
RI	0.66±0.06	0.66±0.05	0.978
PSV (cm/s)	19.66±8.60	26.17±9.13	0.004
EDV (cm/s)	5.80±2.52	8.53±3.41	<0.001

RI; resistance index, PSV; peak systolic velocity, EDV; end-diastolic velocity.
[a]Independent samples t-test.

Patients with primary MVP have excessive connective tissue, which causes thickening of the spongiosa layer due to the excess of dermatan sulfate, which is a glycosaminoglycan. This condition weakens the structure of the mitral leaflets and adjacent tissues, resulting in elongation of the chordae tendineae.[6]

Ocular involvement can potentially lead to vision threatening disease. MVP can be associated with ophthalmological diseases such as keratoconus (KC), glaucoma, chronic progressive external ophthalmoplegia, and retinal artery embolism.[15] According to the literature information some studies showing a relationship between MVP and ocular involvement. Duru et al.[2] reported thinning of Bowman layer in the inferior half of the cornea in patients with MVP. Lichter et al.[3] found 22.2% keratoconus patients with MVP. Akcay et al.[15] reported that KC prevalence is higher than control individuals in MVP patients and the biomechanical properties of the cornea are altered in patients with MVP.

Chiang et al.[6] reported that MVP is a significant predictor for the development of open angle

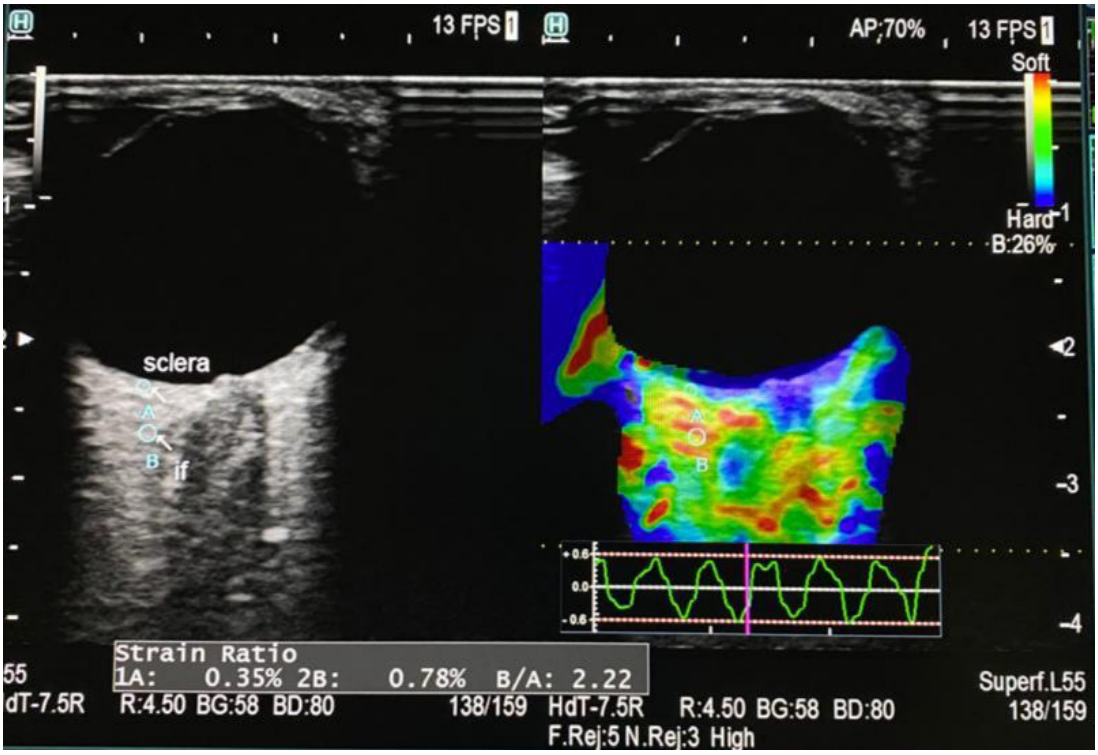

Figure 1. B-mode ultrasonography image (1) and the related elastography image (2) of scleral tissue (A) versus intraconal fat (B). The circles (A and B) indicate where the elastographic measurements were taken. B intraconal fat, A sclera tissue.

glaucoma, after adjusting for possible confounding factors. In MVP, the disease duration induces scleral tissue alterations which can lead to scleral destruction. Thus can be related with ocular involvement in MVP disease.

Previous studies have demonstrated the elastographic characteristics of ocular tissue. Unal et al.[16] investaged the optic nerve head (ONH) characteristics in patients with primary open angle glaucoma (POAG) using real-time elastography. They found that human scleral rigidity of POAG patients was greater than the control eyes. Agladioglu et al.[17] reported the correlation between primary open angle glaucoma and ocular elasticity in adults and demonstrated that anterior vitreous/posterior vitreous strain ratio increases in glaucoma patients. Kazemi et al.[18] found that ocular rigidity was significantly lower in glaucomatous eyes than control eyes.

In addition to these associated with ophthalmological diseases, involvement of retinal vasculer manifestations retinal embolizm and retinal vascular occlusive disorders. Previous studies have reported abnormal elastic properties of the vascular systems in MVP. Kardesoglu et al.[19] found that the aortic stiffness index was increased. Erolu et al.[1] reported that increased elasticty of the aorta in childhood.

Limitations of the study

The present study had several important limitations. The main limitations of the study were relatively small number of participants in the groups and its single-centered design. Another main limitation was that the evaluated medical data was limited.

Conclusions

To the best of our knowledge, our study is the first to compare the blood flow rates of retrobulbar vascular structures and elasticity of the eye at the same time in MVP patients. This study demonstrated that MVP patients has a statistically significant difference scleral elasticity. The mean ROF/S values were significantly increased in patients with MVP as compared to the control group. The results of this study showed a significant difference between the control, and MVP groups

in respect of the EDV and PSV values of PCA, and CRA. From these results, it can be said that vascular extracellular matrix changing in the vessels feeding the eye. This histological alteration can cause the feeding of tissues will be impaired with a reduced flow and consequently more ocular complications will be seen such as retinal vascular occlusive disorders. Nevertheless, there is a need for further studies to evaluate the importance of early determination of ocular vascular damage in MVP with CDI and RTE.

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Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

Bursa City Hospital Clinical Research Ethics Committee approved the study protocol (Decision number: 2020-8/5, date: 07.10.2020).

Authors' Contribution

Study Conception: DT, MEC, EKT; Study Design: DT, MEC, EKT, BU; Supervision: DT, MEC, EKT, BU, MFK, MEA; Literature Review: DT, BU, MFK, MEA; Critical Review: EKT, BU, MFK, MEA; Data Collection and/or Processing: DT, MEC, EKT, BU, MFK, MEA; Statistical Analysis and/or Data Interpretation: DT, MEC, BU; Manuscript preparing: DT, MEC. Echocardiography: DT, BU; Ocular ultrasound elastography: EKT; Ocular measurements: CRA, PCA, OA.

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Original Article

Do Hormonal Disorders Contribute to the Pathology of Hereditary Angioedema?

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ABSTRACT

Background Hereditary angioedema (HAE) is an autosomal dominant disorder characterized by recurrent episodes of angioedema without urticaria or pruritus. In this study, we compared the levels of anabolic hormones, such as insulin, insulin-like growth factor, growth hormone, and thyroid hormones (thyroid-stimulating hormone [TSH], triiodothyronine [T3], and thyroxine [T4]), and the levels of hormones that are considered catabolic, such as adrenocorticotrophic hormone (ACTH) and cortisol, between HAE patients and controls. We also discussed the contribution of these hormones to the pathophysiology of HAE.

Material and Methods The study included 18 patients (9 diagnosed with HAE type 1 and 9 with HAE type 2) who were followed in the immunology and allergy clinic between January 2013 and January 2020. The control group comprised 28 age- and gender-matched healthy control subjects. For determination of hormone levels enzyme standard radioimmunoassay, immunometric and immunoluminometric assays were used.

Results The HAE type 1, HAE type 2, and healthy control groups showed no significant differences in insulin, insulin-like growth factor, ACTH, cortisol, TSH, or T4 levels. The C-peptide and T3 levels were significantly different between the groups (p=0.011 and p=0.027, respectively). Post-hoc pairwise comparison revealed no significant difference in C-peptide level among the groups, but a significant difference in the T3 level was detected between HAE type 1 patients and controls (p=0.029).

Conclusions Although no significant differences were observed in other anabolic hormone levels between the controls and HAE patients, T3 levels were significantly lower in type 1 HAE patients. Close monitoring of low T3 levels is required, particularly in patients with type 1 HAE.

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Introduction

Hereditary angioedema (HAE) is an autosomal dominant disorder characterized by recurrent episodes of angioedema without urticaria or pruritus. Attacks often involve the skin, upper respiratory mucosa, and gastrointestinal tract. Angioedema attacks are self-limiting and the sufferer completely returns to normal within 2-5 days. However, fatal asphyxia can occur due to laryngeal involvement.[1,2] During an attack, plasma bradykinin levels rise well above normal, and the development of angioedema has been attributed to the increase in bradykinin.[3] Agents such as antifibrinolytics (tranexamic acid), partial androgen analogs (danazol), plasma-derived C1 inhibitor concentrates, and humanized plasma kallikrein monoclonal antibodies are used as prophylactic treatment for the disease.[4-7] Plasma-derived and recombinant C1 esterase inhibitor concentrates, bradykinin b2 receptor antagonists, and kallikrein inhibitors can be used to treat an acute attack.[8] Danazol is a partial androgen that has been used successfully for many years to treat patients with HAE.[9,10] In animal experiments, danazol prevents attacks by increasing liver production of the C1 esterase protein.[11,12] Other hormones increase the synthesis of serum proteins from the liver during the pathogenesis of HAE, and may be useful in the treatment of the disease. Although activation of factor XII and plasma prekallikrein on the endothelial cell surface is important in the initiation of attacks, information is limited as to why HAE patients show deficits in the synthesis of the C1 esterase protein, and whether they have adequate levels of anabolic hormones that aid in the synthesis of the C1 esterase protein by the liver. In this study, we compared the levels of anabolic hormones, such as insulin, insulin-like growth factor, growth hormone, and thyroid hormones (thyroid-stimulating hormone [TSH], triiodothyronine [T3], and thyroxine [T4]), and catabolic hormones, such as adrenocorticotrophic hormone (ACTH) and cortisol, between HAE patients and controls. We also discuss the contribution of these hormones to the pathophysiology of HAE.

Material and Methods

The study included 18 patients (9 diagnosed with HAE type 1 and 9 with HAE type 2) followed in the immunology and allergy clinic between January 2013 and January 2020. We also included 28 age- and gender-matched subjects as controls. Patients with known hypothyroidism or hyperthyroidism who took medication, had another chronic disease that may cause euthyroidism, had a diagnosis of widespread inflammatory disease or diabetes mellitus, or received other medical treatment were excluded from the study. The study protocol was approved by the local ethics committee (decision no: 2018/1576, date: 16.10.2018), and written informed consent was obtained from all patients.

All HAE patients were under on-demand treatment and none were receiving prophylactic treatment, such as tranexamic acid or danazol, for HAE. Blood samples were taken from all HAE patients when they were asymptomatic for HAE attacks.

Venous blood samples were drawn for biochemical analyses after at least 10 h of fasting early in the morning (7.00 am). All biochemical analyses were performed in the central biochemistry laboratory of the Meram Faculty of Medicine. Complete blood counts were performed with the Cell Dyn 3700 device (Abbott Laboratories, Abbott Park, IL, USA). Serum C4 levels were measured with the Advia 2400 Clinical Chemistry System (Siemens, Tarrytown, NY, USA) using a colorimetric method. Serum C1 esterase inhibitor levels were measured with the Siemens BN II/BN ProSpec system and a nephelometric assay. Serum C1 esterase inhibitor function was assessed by the chromogenic method using the Stago Compact Max device (Stago, Parsippany, NJ, USA).

Concentrations of insulin, c-peptide and ACTH were assessed using standard radioimmunoassay (RIA) (Pharmacia Insulin RIA 100, Pharmacia & Upjohn, Inc., Uppsala, Sweden; Lumitest ACTH, Brahms Diagnostica GmbH, Berlin, Germany). For determination of cortisol, TSH, free T3, and free T4, enzyme immunometric and immunoluminometric assays were used, respectively (Enzymun-Test Cortisol ES 300 and Elecsys, Roche Molecular Biochemicals, Mannheim, Germany). All samples were measured

in duplicate in the same assay. Serum insulin-like growth factor (IGF-1) level was determined using an immunofluorometric assay with acid-ethanol serum extracts.

Statistical analysis

The analysis was performed with IBM SPSS Statistics software (ver. 22.0; IBM Corp., Armonk, NY, USA). Normally distributed parameters are presented as mean±standard deviation, and skewed parameters as medians (interquartile range). Descriptive data are presented as frequencies and percentages, and were compared using the chi-square test. Baseline characteristics were compared using the independent Student's *t*-test, Mann-Whitney rank-sum test, Fisher's exact test, or chi-square test, as appropriate. We used one-way analysis of variance for analyzing normally distributed parameters with homogeneity of variances. We used the Kruskal-Wallis method if a parameter was not normally distributed or heterogeneity was detected. Tamhane's T2 test was used for the post-hoc analysis. A p-value >0.05 was considered significant.

Results

In total, 18 HAE patients (12 females and 6 males) and 28 age- and gender-matched healthy subjects (18 females and 10 males) were included in the study. In the HAE group, nine patients with a diagnosis of type 1 HAE, and nine with type 2 HAE, were followed up. The demographic and laboratory characteristics of the patients are summarized in Table 1.

The average ages of the HAE and control groups were 36.33±11.81 and 38.07±12.21 years, respectively. No differences in age or gender were observed between the patients and controls (p=0.634 and p=0.747, respectively). Although the insulin, IGF-1, growth hormone, ACTH, cortisol, TSH, and T4 levels tended to be lower in HAE patients than controls, the differences were not significant (*Figure 1*). However, a significant difference in C-peptide levels was observed between the HAE patients and controls (p=0.030). In addition, the T3 levels were significantly lower in the HAE patients (p=0.130) (*Table 2*) (*Figure 2*).

No significant differences in insulin, IGF-1, ACTH, cortisol, TSH, or T4 levels were detected among the HAE type 1, HAE type 2, and control groups. Significant differences in C-peptide and T3 levels were observed among the groups (p=0.011 and p=0.027, respectively) (*Table 3*). Post-hoc pairwise comparisons revealed no significant difference in C-peptide levels among the groups, but a significant difference in T3 levels was observed between HAE type 1 patients and controls (p=0.029) (*Table 4*).

Table 1. Demographic and laboratory parameters of the hereditary angioedema patients (n: 18).

Parameters	Values
Age (years)	36.33±11.81
Females n (%)	12 (66.7)
HAE type I n (%)	9 (50)
HAE type II n (%)	9 (50)
Consanguinity n (%)	6 (33.3)
C1 esterase inhibitor level (mg/dL)	11.00 (3-50)
C1 esterase inhibitor activity (%)	14 (3-171)
Age of onset of symptoms (years)	9 (2-56)
Age at diagnosis, years	20.50±12.71
Diagnostic delay, years	3.50 (0–34)
Neutrophil count at diagnosis (mm^3)	5,488.89±1,681.00
Lymphocyte count at diagnosis (mm^3)	2,007.78±667.92
Platelet count at diagnosis (mm^3)	281,388.89±92,430.67
ANA positivity n (%)	3 (16.67)
C4	0.06 (0.01-0.23)

HAE: hereditary angioedema, C1: complement factor 1, ANA: antinuclear antibody.

Discussion

HAE is a rare autosomal dominant disease characterized by a C1 esterase inhibitor deficiency and/or decreased activity. In our study, a significant difference in the T3 level was observed between HAE patients and healthy controls, due to the difference between type 1 HAE patients with a C1 esterase inhibitor deficiency and controls. The

Table 2. Comparison of clinical parameters of hereditary angioedema and control patients.

Parameters	HAE (n: 18)	Controls (n: 28)	P value
Age (years)	36.33±11.81	38.07±12.21	0.634
Females n (%)	12 (66.7)	18 (72)	0.747
Glucose (mg/dL)	87.50±11.03	98.12±9.97	0.934
Insulin (mU/L)	11.25 (4.92-48.22)	14.03 (2-81.80)	0.350
C-peptide (ng/mL)	2.95 (1.81-9.72)	2.51 (0.39-7.05)	0.030
IGF-1 (ng/mL)	163.31±68.01	180.01±57.58	0.260
Growth hormone (ng/mL)	0.24 (0.04-3.15)	0.35 (0.05-8.24)	0.866
ACTH (pg/mL)	17.64 (10.48-31.03)	17.73 (6.20-64.20)	0.620
Cortisol (mcg/dL)	11.33±4.26	14.47±6.03	0.045
TSH (mU/L)	1.62 (0.77-4.86)	1.65 (0.53-5.27)	0.311
Free T3 (ng/L)	3.04±0.34	3.30±0.31	0.013
Free T4 (ng/dL)	1.29 (0.023-0.151)	1.34 (1.10-14.0)	0.131

Data were given as mean±SD or median (min-max). HAE: hereditary angioedema, IGF-1: insulin-like growth factor 1, ACTH: adrenocorticotropic hormone, TSH: thyroid-stimulating hormone, T3: triiodothyronine, T4: thyroxine.

Table 3. Hereditary angioedema type I, type II, and control patients' demographic and laboratory parameters.

Parameters	HAE type 1 (n: 9)	HAE type 2 (n: 9)	Controls (n: 18)	P value
Age (years)	37.67±10.89	35.00±13.19	38.07±12.21	0.804
Females n (%)	6 (66.7)	6 (66.7)	6 (64.3)	0.986
Glucose (mg/dL)	85.07±12.28	90.63±9.50	88.12±10.27	0.525
Insulin (mU/L)	11.29 (4-36.68)	11.20 (5.35-48.22)	14.03 (2-81.80)	0.637
C-peptide (ng/mL)	2.95 (1.87-5.91)	2.94 (1.81-9.72)	2.51 (0.39-7.05)	0.011
IGF-1 (ng/mL)	159.53±45.63	167.09±87.84	180.01±57.58	0.657
Growth hormone (ng/mL)	0.16 (0.4-3.15)	0.28 (0.04-1.40)	0.35 (0.05-8.24)	0.523
ACTH (pg/mL)	17.10 (10.48-31.03)	18.17 (11.13-27.49)	17.73 (6.20-64.20)	0.884
Cortisol (mcg/dL)	11.92±4.80	10.74±3.85	14.47 ± 6.03	0.160
TSH (mU/L)	1.61 (0.82-2.52)	1.65 (0.77-4.86)	1.65 (0.53-5.27)	0.559
Free T3 (ng/L)	2.97±0.34	3.11±0.34	3.30±0.31	0.027
Free T4 (ng/dL)	1.19 (1.04-1.45)	1.33 (1.03-1.50)	1.34 (1.10-14.00)	0.184

Data were given as mean±SD or median (min-max). HAE: hereditary angioedema, IGF-1: insulin-like growth factor 1, ACTH: adrenocorticotropic hormone, TSH: thyroid-stimulating hormone, T3: triiodothyronine, T4: thyroxine.

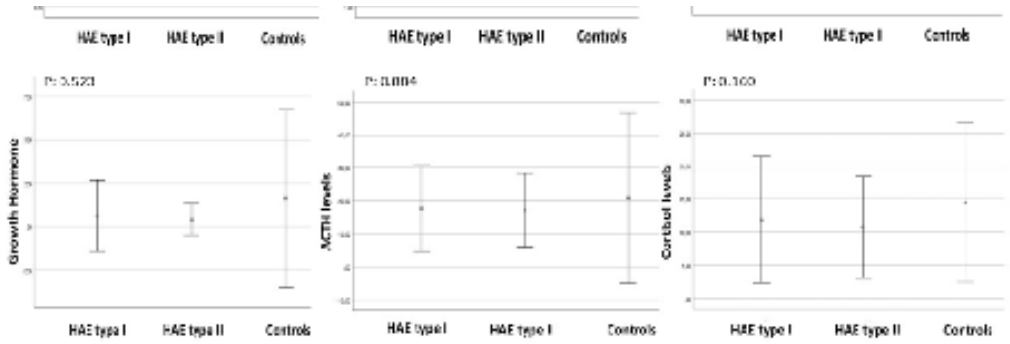

Figure 1. Comparison of hormone levels among the hereditary angioedema type 1, hereditary angioedema type 2 and control groups.

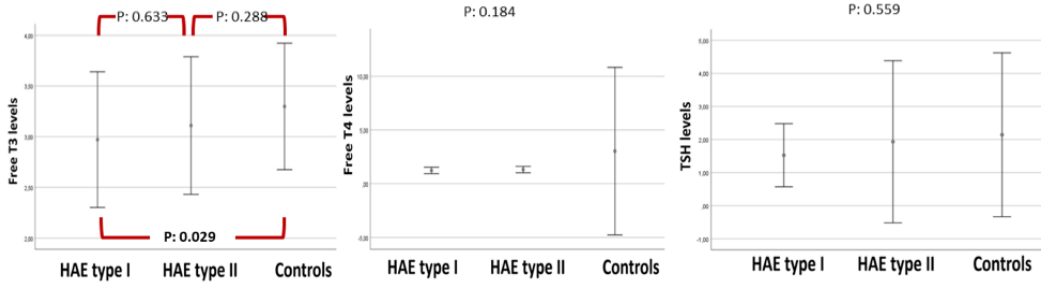

Figure 2. Comparison of thyroid hormone levels among the hereditary angioedema type 1, hereditary angioedema type 2 and control groups.

Table 4. Post-hoc analysis of HAE patients according to T3 and C-peptide levels.

Parameters			P value
T3	HAE type I	HAE type II	0.633
		Controls	0.029
	HAE type II	HAE type I	0.633
		Controls	0.288
C-peptide	HAE type I	HAE type II	0.907
		Controls	0.185
	HAE type II	HAE type I	0.907
		Controls	0.326

HAE: hereditary angioedema, T3: triiodothyronine.

levels of other anabolic hormones, such as insulin, IGF-1, growth hormone, TSH, and T4, tended to be lower in HAE patients than control groups, although the differences were not significant.

Thyroid hormones control and regulate the synthesis of many plasma proteins[13-15], including transferrin, prothrombin and angiotensinogen, as well as complement, fibrinogen, and lipoproteins. Zhang et al.[16] showed that thyroid hormone and complement levels were correlated in patients with multiple sclerosis. Karkhaneh et al.[17] reported that serum T3 and T4 levels were correlated with complement levels in normal-weight humans. Czaller et al.[18] reported lower free T3 and free T4 levels in patients with type 1 HAE compared to controls. In the same study, free T4 levels were lower in patients who had more versus less frequent

HAE attacks. These findings suggest that thyroid hormones have an effect on complement in many diseases, and may also play a role in the course of HAE, which is considered a complement system disease.

Thyroid hormones also have many effects on the coagulation and fibrinolytic systems.[19-21] Numerous studies have examined the relationship between histamine-mediated angioedema and thyroid hormone disorders[15,22-25], but few have investigated the relationship between HAE and thyroid hormones. Moreover, these studies mostly examined the relationships of hyperthyroidism, hypothyroidism, and thyroiditis with HAE attacks.[26,27] Thyroid disorders are among the most common autoimmune disorders in type 1 HAE patients.[28] Liu et al.[29] reported a case of coexisting Graves' disease and type 1 HAE. Farkas et al.[26] showed that the frequency of attacks is lower in HAE patients taking anti-thyroid drugs or thyroid hormone replacement therapy. The findings of our study and those of Farkas et al.[26] suggest that the metabolic effects of thyroid hormones are involved in the pathogenesis of HAE.

Type 1 HAE progression is associated with a low C1 esterase inhibitor level, and type 2 HAE progression with dysfunction in the C1 esterase inhibitor.[30] The finding that the T3 level was significantly different between our type 1 (but not type 2) HAE patients and controls supports our hypothesis that T3 may affect the course of the disease by synthesizing the C1 esterase inhibitor.

In the most recent primary immunodeficiency disease (PID) classification system, HAE was included in the complement deficiency subclass of primary immunodeficiencies, and in the immunodeficiency group with susceptibility to infections.[31] Although frequent infections are not the main clinical feature of HAE, recurrent angioedema attacks in these patients may cause low T3 by including euthyroid sick syndrome, which is seen during chronic and serious diseases.

The main limitations of this study were its single-center design and relatively small population. The absence of a patient group taking a normal C1 inhibitor, and of patients evaluated for mutations known to be involved in the etiology of HAE (such as SERPING, FXII, angiopoietin, and plasminogen mutations), are further limitations.

Conclusions

We hypothesized that the levels of anabolic hormones might be lower in the HAE patient group than controls, based on long-term prophylaxis with danazol, and the increased expression of complement proteins and many serum protein syntheses due to the anabolic effects of danazol. Although no significant difference was observed between the controls and HAE patients in of the levels of other anabolic hormones, the T3 levels were significantly lower in type 1 HAE patients than controls. Close monitoring of low T3 levels is required, particularly in patients with type 1 HAE. Larger studies are needed to assess the effects of T3 replacement on C1 esterase inhibitor levels and attack frequency in euthyroid HAE patients.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

Local ethics committee approved the study protocol.

Authors' Contribution

Study Conception, Design, Supervision, Critical Review, Manuscript preparing: GA, HO, IB, FC, EY, SA, AZC; Literature Review: HO; Statistical Analysis and/or Data Interpretation: IB.

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Original Article

Comparison of The Expression of GATA-3 Protein from The Transcription Factor Family and Pathological Prognostic Parameters in Invasive Ductal Carcinomas of The Breast

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ABSTRACT

Background GATA binding protein 3 (GATA-3) is one of the six transcription factor family members and is important for glandular development in the breast. Its expression becomes important in breast cancer. We aimed to compare GATA-3 immunoreactivity and pathological prognostic factors in patients with invasive ductal carcinoma.

Material and Methods Our study was conducted retrospectively with 300 breast invasive ductal carcinoma patients who were operated on in our hospital between May 2013 and June 2014. Patient reports, slides and blocks in the pathology archive were scanned. GATA-3 immunohistochemical (IHC) staining was evaluated according to the nuclear staining, intensity and percentage. The relationship between clinicopathological prognostic parameters and GATA-3 IHC staining results was investigated.

Results A positive staining was observed in 286 (95.3%) cases. According to the GATA-3 staining intensity and percentage, 210 (70%) cases stained strongly and 246 (82%) stained +4, respectively. There was a significant relationship between GATA-3 immunoreactivity with ER, PR, Cerb-B2, Ki-67, mitotic degree, mitotic count and histological grade.

Conclusions There was a correlation between the high expression of GATA-3 and good prognostic markers. Hormone receptors can be evaluated with Cerb-B2 and Ki-67 and used as prognosis determinants in breast cancers. They can be used to identify both primary and secondary breast tumors.

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Introduction

Breast cancers are the most common malignancies among women and constitute approximately 26% of all cancer cases, and 17.6% of these cases result in death.[1,2] The most important cause of mortality in breast cancers is metastatic progression. Metastasis development is associated with various risk factors such as primary tumour size, histological grade, lymph node involvement, tumour type, and biomarkers.[3] It has been reported that the 5-year survival rate in patients with metastatic breast cancer is below 30%.

One of the most important prognostic indicators in breast cancers is changes in GATA-3 (GATA binding protein 3) transcription factor expression. The decrease in GATA-3 expression has been reported to be associated with aggressive tumour development and low patient survival.[2] GATA-3, a member of the GATA transcription factor family, is a gene that has a regulatory role in the differentiation and specialization of many tissues such as mammary glands, skin, inner ear, central nervous system, and epithelial structures of the kidney.[4] In the mammary glands, it gains importance, especially in the differentiation of luminal cells.[5-7] However, while the loss of expression in the GATA-3 gene region is associated with the development of breast cancer, low expression of this gene region was determined to be related to estrogen receptor (ER) and progesterone receptor (PR) negativity. It is thought that overexpression of GATA-3 contributes to abnormal aromatase expression in breast tumours.[8] In the conducted studies, when the cases in which GATA-3 was expressed high and low were compared, the tumours with a low expression profile indicated poor prognosis. Expression of GATA-3 was observed in 97% of patients with ten years or more survival time.[8,9]

Material and Methods

The research was approved by hospital ethical committee (04 February 2015, No: 2015-02/153). In our study, all incisional biopsy, excisional biopsy, lumpectomy and mastectomy specimens diagnosed with invasive ductal carcinoma in our centre between May 2013 and June 2014 were retrospectively analyzed. The microarray study identified three hundred cases and was included in the study.

Our study used hematoxylin-eosin (HE) stained preparations and paraffin blocks of the 300 patients diagnosed with invasive duct carcinoma. The tumoral area was marked on selected HE-stained slides, and a piece with a 5 mm diameter was removed from the corresponding tumoral area on the paraffin block with a manual tissue microarray device. Subsequently, this area was transferred onto recipient paraffin with 20 holes. At the same time, GATA-3 (–) non-breast control tissue (endomyometrium, cervix, liver) was also embedded in these recipient holes to determine the negative control and starting point. Seventeen paraffin blocks were obtained for a total of 300 patient specimens (depending on tissue quality or other technical reasons), and 3 μm sections were taken on adhesive slides, and GATA-3 (mouse monoclonal antibody, clone/L50-823) immunohistochemical (IHC) staining was applied. GATA-3 IHC study slides were examined under a light microscope. Nuclear staining was considered to be significant. Membranous and cytoplasmic stainings were not evaluated. The positive staining was evaluated according to the intensity of staining as negative/weak/moderate/strong and the staining percentage (*Table 1*).

Table 1. Evaluation score according to the percentage of GATA-3 immunoreactivity.

0	Less than 5% tumor cell nuclei
+1	5-25% tumor cell nuclei
+2	26-50% tumor cell nuclei
+3	51-75% tumor cell nuclei
+4	More than 76% tumor cell nuclei

Clinicopathological prognostic parameters such as the results of GATA-3 immunoreactivity and previous studies of ER, PR, Cerb-B2, Ki-67 IHC, patient's age, tumour size, presence of in situ carcinoma accompanying the tumour, presence of lymphovascular invasion (LVI), axillary lymph node involvement, the histological grade of the tumour and mitotic degree were compared.

Statistical Analysis

Data analysis was performed with SPSS for Windows 11.5 package program. The significance of the difference between the groups in terms of means was evaluated with Student's t-test when the number of independent groups was two. The significance of the difference between groups in terms of median values was examined with the Mann-Whitney U test when the number of independent groups was two. In contrast, the Kruskal-Wallis test analysed the difference between more than two groups. If the results of the Kruskal-Wallis test statistics were significant, the situation(s) causing the difference was determined using Conover's non-parametric multiple comparison test. Pearson's Chi-Square and Fisher's exact or Likelihood ratio tests were used for categorical variables. The results were considered statistically significant with a p-value of <0.05.

Results

Our study was carried out on 300 patient samples diagnosed with breast invasive duct carcinoma. Of all our cases, 286 (95.3%) were GATA-3 positive, and 14 (4.7%) were negative. The related data of GATA-3 immunoreactivity, GATA-3 staining degree, and GATA-3 staining percentages of the cases are shown in Table 2 and Figure 1. All-female patients' ages ranged between 22 and 80, and the mean age was 53.7±12.8 years.

ER positivity was found in 78.3% of the cases, and secondly, PR positivity was found in 56%. The rate of patients with both ER and PR positivity was 54.7%. Cerb-B2 was found to be positive in 26% of all cases. When evaluated in histological grading, most patients (61.3%) had a high grade with Grade 3. In most of our cases (67.7%), the tumour size was 2-5 cm, and the T stage was T2. Distant metastasis (bone, lung, liver, and brain) was found in 4%. 55.7% of the cases had TNM result stage 2 (*Table 3*).

When the mean ages of the patients in both the GATA-3 positive and negative groups were examined, they were found to be similar. The relationship between mean age and GATA-3 staining intensity and the percentage was also not statistically significant. All cases that showed ER and PR positivity were correlated with GATA-3

Table 2. Distribution of cases according to GATA-3 immunoreactivity, staining intensity and percentage.

GATA-3 immunoreactivity	
Negative	14 (4.7%)
Positive	286 (95.3%)
GATA-3 staining intensity	
Negative	14 (4.7%)
Weak	25 (8.8%)
Moderate	51 (17.0%)
Strong	210 (70.0%)
GATA-3 staining intensity	
Negative	14 (4.7%)
1+	0 (0%)
2+	15 (5.0%)
3+	25 (8.3%)
4+	246 (82.0%)

Table 3. Demographic distribution and pathological characteristics of the cases.

Variables	n: 300	Variables	n: 300
Age	53.7±12.8	LN+	138 (46.0%)
ER+	235 (78.3%)	LN+ number	3 (1-31)
PR+	168 (56.0%)	T-stage	
Cerb-B2+	78 (26.0%)	1	67 (22.3%)
Ki-67	25 (1-95)	2	203 (67.7%)
Mitotic count	12 (1-81)	3	25 (8.3%)
DCIS+	195 (65.0%)	4	5 (1.7%)
LVI	67 (22.3%)		
Mitotic degree		N-stage	162 (54.0%)
1	97 (32.3%)	1	72 (24.0%)
2	109 (36.3%)	2	38 (12.7%)
3	94 (31.3%)	3	28 (9.3%)
Histological grade		M-stage	
1	18 (6.0%)	M0	286 (95.3%)
2	98 (32.7%)	M1	14 (4.7%)
3	184 (61.3%)	TNM-stage	
Tumour size		1	48 (16.0%)
≤2 cm	71 (23.7%)	2	167 (55.7%)
2-5 cm	203 (67.7%)	3	71 (23.7%)
>5 cm	26 (8.7%)	4	14 (4.7%)

ER: estrogen receptor, PR: progesterone receptor, DCIS: ductal carcinoma in situ, LVI: lymphovascular invasion, LN: lymph node metastasis.

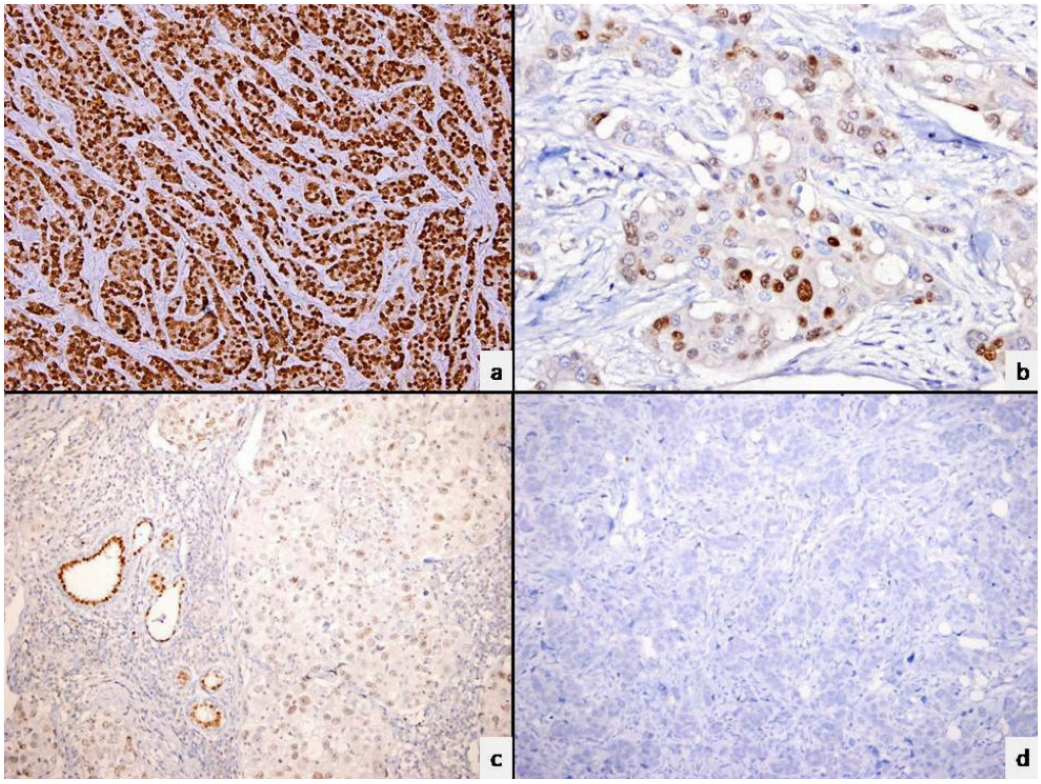

Figure 1. a: GATA-3 immunoreactivity is strong according to staining intensity and +4 according to percentage (GATA-3 IHC, 200x); b: Moderate and +3 GATA-3 immunoreactivity (GATA-3 IHC, 400x); c: The case that GATA-3 immunoreactivity was evaluated as weak and +2 per percentage. Benign glands with strongly stained luminal epithelium are seen on the left side of the figure (GATA-3 IHC, 200x); d: A case with no GATA-3 immunoreactivity (GATA-3 IHC, 200x).

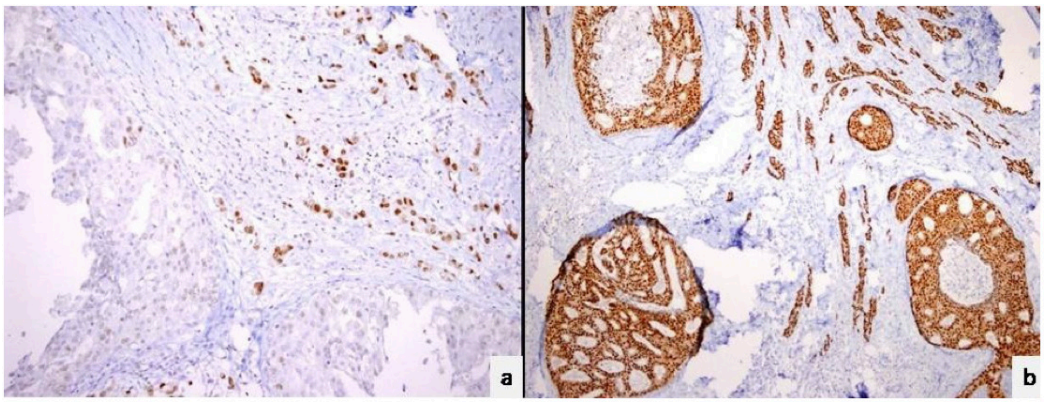

Figure 2. a: In situ carcinoma showing weak GATA-3 immunoreactivity in the sparsely is seen below left part, strong GATA-3 immunoreactivity in the invasive carcinoma area in the upper right part (GATA-3 IHC, 200x); b: Diffuse and strong GATA-3 immunoreactivity in in situ and invasive carcinoma areas (GATA-3 IHC, 20x).

Table 4. Distribution of GATA-3 immunoreactivity of the cases in positive and negative groups.

Variables	Negative (n: 14)	Positive (n: 286)	P-value	Variables	Negative (n: 14)	Positive (n: 286)	P-value
Age	55.5±15.2	53.6±12.7	0.598*	LN	7 (50%)	131 (45.8%)	0.758†
ER+	0 (0%)	235 (82.2%)	<0.001¶	LN– number	3 (1-15)	3 (1-31)	0.613ϕ
PR	0 (0%)	168 (58.7%)	<0.001¶	T stage			0.178ϕ
Cerb-B2+	1 (7.1%)	77 (26.9%)	0.125¶	1	1 (7.15)	66 (23.1%)	
Ki-67	55 (10-95)	25 (1-90)	<0.001¶	2	11 (78.6%)	192 (67.1%)	
Mitotic count	19 (8-81)	12 (1-48)	0.002ϕ	3	2 (14.3%)	23 (8.0%)	
DCIS+	9 (64.3%)	186 (65%)	1.000*	4	0 (0%)	5 (1.7%)	
LVİ	3 (21.4%)	64 (22.4%)	1.000*				
				N-stage			0.859ϕ
Mitotic degree			0.005ϕ	0	7 (50%)	155 54.2%)	
				1	4 (28.6%)	68 (23.8%)	
				2	2 (14.3%)	36 (12.6%)	
				3	1 (7.1%)	27 (9.4%)	
1	1 (7.1%)	96 (33.6%)					
2	4 (28.6%)	105 (36.7%)					
3	9 (64.3%)	85 (29.7%)					
Histological grade			0.003ϕ	M-stage			1.000¶
1	0 (0%)	18 (6.3%)		M0	14 (4.9%)	272 (95.1%)	
2	0 (0%)	98 (34.3%)		M1	0 (0%)	14 (4.9%)	
3	14 (100%)	170 (59.4%)		TNM stage			0.973ϕ
Tumour size			0.118ϕ	1	1 (7.1%)	47 (16.4%)	
<2 cm	1 (7.1%)	70 (24.5%)					
2-5 cm	11 (78.6%)	192 (67.1%)					
>5 cm	2 (14.3%)	24 (8.4%)					

*Student's t test, ¶ Fisher's exact test, † Pearson Chi-Square test, ϕ Mann-Whitney U test.
ER: estrogen receptor, PR: progesterone receptor, DCIS: ductal carcinoma in situ, LVI: lymphovascular invasion, LN: lymph node metastasis.

positivity (p<0.001). Ki-67 positivity was inversely correlated with GATA-3 positivity, which was statistically significantly lower (p<0.001). All the GATA-3 negative groups and 59.4% of the GATA-3 positive group had a histological grade of 3. The mitotic count was lower in the GATA-3 positive group compared to the negative group (p=0.002) (*Table 4*).

Although ductal carcinoma in situ (DCIS) was found in 195 cases, it was observed in only 55 of the IHC slides because the microarray study only consisted of invasive tumoral areas. Weak GATA-3 immunoreactivity was observed in 20% of these cases, and strong GATA-3 immunoreactivity in 80% (*Figure 2*).

Cerb-B2 positive cases were mostly in the weak and moderately stained groups. Ki-67 proliferation index decreased gradually from negative to strong staining (p<0.001). While all of the GATA-3 negative cases had a histological grade of 3, only 55.2% of those with strong and +4 staining had a histological grade of 3. While 64.3% of the GATA-3 negative group had a mitotic degree of 3, those with strong staining with GATA-3 were mostly in groups 1 and 2. The mitotic count decreased gradually from the GATA-3 negative group to the group with strong GATA-3 staining (p<0.001). The mitotic count decreased statistically significantly from the GATA-3 negative group to the 4+ group (mitotic count, 19, 20, 16, 12, respectively). The distribution of GATA-3 immunoreactivity according to the staining percentage and intensity in our cases is shown in Table 5.

GATA-3 staining was observed in all ER+, PR+, and ER+/PR+ groups. Cerb-B2 positive

Table 5. Distribution of the GATA-3 immunoreactivity of the cases in the groups by staining percentage and intensity.

Variables	Negative (n: 14)	Weak (n: 25)	Moderate (n: 51)	Strong (n: 210)	P-value
ER+	0 (0%)	4 (16%)	32 (62.7%)	199 (94.8%)	<0.001*
PR+	0 (0%)	4 (16%)	23 (45.1%)	141 (67.1%)	<0.001*
ER+ and PR+	0 (0%)	3 (12%)	20 (39.2%)	141 (67.1%)	<0.001*
Cerb-B2+	1 (7.1%)	7 (28%)	26 (51%)	44 (21%)	<0.001*
Ki-67	55 (10-95)	40 (10-90)	30 (5-90)	25 (1-90)	<0.001¶
Histological grade					0.002¶
1	0 (0%)	1 (4%)	2 (3.9%)	15 (7.1%)	
2	0 (0%)	6 (24%)	13 (25.5%)	79 (37.6%)	
3	14 (100%)	18 (72%)	36 (70.6%)	116 (55.2%)	
Mitotic count	19 (8-81)	19 (1-45)	14 (3-45)	12 (1-48)	<0.001¶
Mitotic degree					<0.001¶
1	1 (7.1%)	4 (16%)	10 (19.6%)	82 (39%)	
2	4 (28.6%)	7 (28%)	21 (41.2%)	77 (36.7%)	
3	9 (64.3%)	14 (56%)	20 (39.2%)	51 (24.3%)	
Variables	Negative (n: 14)	+2 (n: 15)	+3 (n: 25)	+4 (n: 246)	P-value
ER+	0 (0%)	4 (26.7%)	8 (32%)	223 (90.7%)	<0.001ϕ
PR+	0 (0%)	3 (20%)	6 (24%)	159 (64.6%)	<0.001*
ER+ and PR+	0 (0%)	3 (20%)	5 (20%)	156 (63.4%)	<0.001*
Cerb-B2+	1 (7.1%)	7 (46.7%)	13 (52%)	57 (23.2%)	0.002ϕ
Ki-67	55 (10-95)	60 (20-90)	30 (7-80)	25 (1-90)	<0.001¶
Histological grade					<0.001¶
1	0 (0%)	0 (0%)	1 (4%)	17 (6.9%)	
2	0 (0%)	2 (13.3%)	3 (12%)	93 (37.8%)	
3	14 (100%)	13 (86.7%)	21 (84%)	136 (55.3%)	
Mitotic count	19 (8-81)	20 (10-36)	36 (4-30)	12 (1-48)	<0.001¶
Mitotic degree					<0.001¶
1	1 (7.1%)	0 (0%)	2 (8%)	94 (38.2%)	
2	4 (28.6%)	5 (33.3%)	12 (48%)	88 (35.8%)	
3	9 (64.3%)	10 (66.7%)	11 (44%)	64 (26%)	

* Pearson Chi-Square test, ¶ Kruskal-Wallis test, ϕ Likelihood Ratio test.
ER: estrogen receptor, PR: progesterone receptor.

cases were mostly in the 2+ and 3+ groups. The Ki-67 proliferation index was gradually decreased from negative to +4 staining with respect to the percentage (p<0.001). Similar characteristics were observed when the GATA-3 immunoreactivity was evaluated according to the staining percentage and intensity. There was no significant relationship between age, LVI, metastatic lymph node number, tumour size, T stage, N stage, M stage, TNM final result stage, presence of DCIS, and GATA-3 immunoreactivity.

Discussion

Breast cancer is the most common cancer in women and the second most common cause of cancer-related deaths after lung cancer.[10] The prognosis of the disease is determined according to the patient's age, tumour size, presence of in situ carcinoma accompanying the tumour, LVI, axillary lymph node involvement, and histological grade of the tumour. Also, ER, PR, Cerb-b2, and

Ki-67 IHC studies of pathological specimens affect the treatment and prognosis. Nevertheless, different prognostic data may emerge from patient to patient with the same results. GATA-3, which has come to the fore in recent years as a new immune marker for breast carcinoma, has a high incidence and is one of the six transcription factor family members. It plays an important role in cell death. It is understood that it is a critical determinant of luminal cell differentiation in adult mammary glands.[11] GATA-3 IHC staining was performed on 300 cases in our study, and a positive result was obtained in 95.3% (286/300) of the cases. Wendroth et al.[12] found the rate of GATA-3 immunoreactivity of invasive ductal carcinoma as 92.7% (51/55), Miettinen et al.[13] as 92% (163/178), Clark et al.[14] as 91% (177/186) and Lui et al.[15] as 91% (90/99). These results are similar to our data.

One of the most important risk factors for breast cancer is age. In their study, Voduc et al.[16] found the mean ages as 60 and 58 years in the GATA-3 positive and negative groups, respectively, and reported a linear relationship between GATA-3 and age. However, they stated that the difference was little.[16] In our study, the mean age was 53.7 years, the youngest patient was 22 years old, and the oldest patient was 88 years old. No significant relationship was found with age when GATA-3 was evaluated according to positive/negative staining intensity and staining percentage.

When ER+ breast cancers and ER– breast cancers are compared, it is known that the ER– group is more aggressive and poorly differentiated. In ER+ and ER– breast cancer groups, loss of expression of GATA-3 was observed in the ER– group in many microarray studies and was associated with a poor prognosis.[17,18] In their research, Bong et al.[19] found a rate of 80% of GATA-3 positivity in the ER+ group in breast cancers in Malaysia, similar to our study. They emphasized the significant relationship between ER and GATA-3 positivity.[19] Graham et al.[20] analyzed the ER+ and ER– groups genetically and reported that GATA-3 was overexpressed in the ER+ group. In their study with a series of 305 cases, Mehra et al.[9] evaluated GATA-3 staining in 83 ER+ cases as high and low, and they observed high expression in 38 (45.8%) cases and low expression in 45 (54.2%) cases. They reported that the prognosis of the ER+ and high expression of the GATA-3 group was better, and the recurrence and/or metastasis rate of the ER+ and low expression of the GATA-3 group was high.[9] Fang et al.[21] found a strong relationship between ER and GATA-3 and suggested that GATA-3 can be a clinical marker in response to hormonal therapy. Hosodo et al.[22] reported higher expression of GATA-3 in ER+ premenopausal women compared to postmenopausal women and stated that disease-free survival was longer in these cases. Besides, they said there was a correlation between PR and GATA-3 in premenopausal women.[22] In our study, there were 235 (78.3%) ER+ cases. All of these cases were GATA-3 positive. According to the staining intensity of the cases, 199 (84.6%) were strongly stained, and according to the percentage of staining, 223 (94.9%) were +4. As the GATA-3 staining intensity and the percentage increased, the rate of ER positivity cumulatively increased. In our study, the relationship between ER positivity and GATA-3 expression was found to be significant ($p<0.001$). The results were consistent with the literature.

In our study, the number of ER– cases was 65. No GATA-3 positivity was found in 14 (21.5%) cases. Eleven patients were evaluated as strong, 19 cases as moderate, and 21 as weak staining. Liu et al.[23] found that the expression of GATA-3 was 69% (66/96) in 96 ER– cases and determined that GATA-3 was the most specific marker for the breast. In our study, the expression of GATA-3 was 78.5% (51/65) in the ER– group, which was higher than in Liu et al.'s study.[23] However, unlike our study, their study also included metaplastic carcinomas. Albergaria et al.[24] reported in their study that there was an inverse relationship between GATA-3 and histological grade and GATA-3 and Cerb-B2 in hormone receptor (HR) negative tumours.

PR positivity is important for hormone therapy, even though not as much as ER positivity. In our study, a positive/negative relationship was found between GATA-3 and PR in terms of the percentage and intensity of staining. Like our study, Yoon et al.[8] found a significant association with PR. Besides, they emphasized the relationship between low GATA-3 expression and high tumour grade, large tumour size, and ER negativity.[8]

Histological tumour grade is a prognostic

factor independent of staging. Grade 1 tumours are reported to have better survival.[25] Usary et al.[26] said that high GATA-3 expression correlated with low grade and slow proliferation rate. In our study, 18 (6%) cases were evaluated as histological grade 1, 98 (32.6%) cases as histological grade 2, and 184 (61.6%) cases as histological grade 3. Histological tumour grade was statistically lower in the GATA-3 positive group compared to the GATA-3 negative group (p=0.003). The grade distribution was statistically significant when evaluated according to the intensity and percentage of GATA-3 staining (p=0.002).

The mitotic count has been used alone to predict prognosis for many years. Another parameter used in the histological grading system is the mitotic degree.[27] In our study, the mitotic degree was lower in the GATA-3 positive group than in the negative group (p=0.005). According to the scoring system made according to the mitotic count, 97 (32.3%) cases were evaluated as score 1, 109 (36.4%) cases as score 2, and 94 (31.3%) cases as score 3. It was observed that the average number of mitoses counted at ten high power fields was 12 (min: 1, max: 81). In our study, the mitotic count was lower in the GATA-3 positive group compared to the negative group (p=0.002). The mean number of mitosis was 12 (1-48) in the GATA-3 positive group and 19 (8-81) in the GATA-3 negative group.

Cell proliferation markers (Ki-67) are used to determine the degree of malignancy in breast cancer, a follow-up response to treatment, and determine prognostic features.[28] Usary et al.[26] also found a significant relationship between high GATA-3 expression and low proliferation rate. In our study, the percentage of Ki-67 was lower in the GATA-3 positive group than in the GATA-3 negative group. The mean Ki-67 staining percentage was 25 in the GATA-3 positive group and 55 in the GATA-3 negative group. The relationship between GATA-3 staining percentage and intensity and Ki-67 was statistically significant.

Most studies indicate that HER-2/neu gene amplification and protein overexpression are associated with a poor prognosis, especially in breast cancer.[29] In our study, according to Cerb-B2 (Her2/neu) scoring system, 222 (74%) cases were evaluated as negative and 78 (26%) as positive. There was no significant difference when the GATA-3 positive and negative groups were compared, while a significant difference was observed when the percentage and intensity of staining were compared. Albergaria et al.[24] reported an inverse relationship between GATA-3 and Cerb-b2 in HR-negative tumours.

With the widespread use of cancer screening in recent years, the breast cancer detection rate in the early stages has increased.[30] 55.2% of our cases were TNM stage 2. No significant relationship was found between GATA-3 and positive/negative patients, the percentage and intensity of staining, T stage, lymph node involvement, and distant organ metastasis. In the animal experiment conducted by Yan et al.[31], they investigated GATA-3 in 36 rats with invasive ductal carcinoma. They found the expression of GATA-3 in 21 cases. In the study, the GATA-3 negative group contained 5-6 times more distant metastases than the GATA-3 positive group. In addition, they reported that the disease-free survival time of the GATA-3 positive group was longer.[31] Only 14 (4.7%) of our cases contained distant metastases. This explains that the relationship between GATA-3 and distant metastasis is not statistically significant due to the reduction of distant metastasis with early diagnosis and developing treatment methods. Gonzalez et al.[32] applied GATA-3 IHC to male and female breast cancer cases. Unlike female breast cancers, they did not find the relationship between GATA-3 and ER/PR and distant organ metastasis in male breast cancers statistically significant. Also, in their study, they reported that the GATA-3 immunoreactivity rate was found as 31.6% in male breast cancers and 82.3% in female breast cancers. They did not find a statistically significant relationship between lymph node metastasis and Cerb-B2 in both genders.[32] Mehra et al.[9] reported a significant relationship between low GATA-3 expression and large tumour size, positive lymph node, high grade, ovarian expression of Her-2, and recurrence and metastasis rate. Unlike this study, in our research, we found a significant relationship between GATA-3 and Her-2 when evaluated according to the histological grade and staining percentage. Voduc et al.[16] found an association between GATA-3 and grade 1/2 tumours and tumour size of >5 cm. However, the difference was small. They found no significant relationship with

lymph node metastasis.[16] Hosodo et al.[22] found an inverse relationship between GATA-3 and tumour size and lymph node metastasis in premenopausal women.

LVI is an important step in breast cancer metastasis and is one of the significant causes of mortality and morbidity. LVI detection in the primary tumour is an important marker for metastasis potential.[33] Jacquemier et al.[34] found a relationship between LVI and GATA-3 and reported that it could be used in determining prognosis. In our study, LVI was detected in 20.3% of the cases. However, the relationship with GATA-3 was not significant. This led us to think that more sampling should be done more carefully, especially in high-grade cases with aggressive progression potential. If necessary, IHC should be performed to detect LVI.

There is no publication in the literature investigating the relationship between the presence of in situ carcinoma and GATA-3. However, Asselin-Labat et al.[35] investigated GATA-3 expression in 11 cases with only in situ carcinoma. They reported that GATA-3 positivity was associated with recurrence-free survival in cases with in situ carcinoma.[35] In our study, the in situ component could be evaluated in 55 GATA-3 stained IHC slides. However, no statistically significant correlation was found between GATA-3 and positive/negative staining percentage, intensity, and in situ component.

Mammaglobin and GCDFP-15 positivities are widely used as descriptors in breast cancers. It has been reported that mammaglobin positivity is found in 23-74% of breast cancers, and GCDFP-15 positivity is found in 48-72%.[36] Although the specificity and sensitivity of both IHC markers were reported to be low, GATA-3 immunoreactivity was seen as high as 95.3% in our study. As a result of this study, it was understood that the GATA-3 immune marker is more reliable in identifying breast carcinomas. Having GATA-3 in the immune panel is important and guiding while determining the prognosis in primary breast cancers and investigating the primary metastatic cancers. In their study with 30 male breast cancer cases, Biserni et al.[37] compared GATA-3, NY-BR-1, mammaglobin, and GCDFP-15. They showed that GATA-3 is more sensitive in males breast cancers as well than other immune markers.[37] We could not evaluate this finding because there were no male cases in our study.

Conclusions

There was a correlation between the high expression of GATA-3 and good prognostic markers. In addition, with their high immunoreactivity in breast cancers, HRs, Cerb-B2, and Ki-67 can be evaluated together and used as prognosis determinants. The high incidence of GATA-3 immunoreactivity in most cases suggested that GATA-3 was the most specific and sensitive breast marker ever found. Therefore, it should be used to identify both primary and secondary breast tumours.

Conflict of interest

The authors declare that they have no conflict of interest.

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There are no funding sources to declare.

Ethical Approval

For this study, approval was obtained local ethics committee.

Authors' Contribution

Study Conception: LP, OK; Study Design: LP, OK; Supervision: LP, OK; Literature Review: LP; Critical Review: OK; Data Collection and/or Processing: LP; Statistical Analysis and/or Data Interpretation: LP, OK; Manuscript preparing: LP.

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Original Article

Paraoxonase Activity an Independent Contributor in SARS-CoV-2 Infection

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ABSTRACT

Background The aim of the present study was estimation of serum paraoxonase (PON1) activity in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Material and Methods In this cross sectional study we estimated serum paraoxonase activity in 73 patients with SARS-CoV-2 infection and 73 healthy controls.

Results The results showed that PON1 activity was significantly decreased in patients with SARS-CoV-2 (1.30±0.55 kU/L) than in healthy controls (1.913±0.48 kU/L, $p<0.05$). In addition we found that the level ALT/AST, bilirubin, creatinine and urea tests were significantly increased in patients with SARS-CoV-2 than normal subjects ($p<0.05$). Multivariate logistic regression reveals PON1 activity is independently associated with SARS-CoV-2 infection.

Conclusions SARS-CoV-2 may decrease the PON1 activity in patients which needs more clarification.

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Keywords: *Paraoxonase, severe acute respiratory syndrome coronavirus 2, high density lipoprotein.*

Introduction

Paraoxonase (PON) is an aryldialkylphosphatase (EC 3.1.8.1) located on the long arm of chromosome 7 (7q21-22). The PON gene family has three members (*PON1, PON2* and *PON3*); they share structural properties and enzymatic activities.[1] PON1 is shown to reside over high density lipoprotein (HDL) tightly bound to Apo A1 having organophosphate hydrolase, lactonase, arylesterase activities and also has both antioxidant and antiatherogenic functions.[2-5] It also shows various polymorphisms.[6,7] It was observed that patients with low levels of HDLs showed an increased risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and worse outcome.[8-10] Therefore, it was assumed that PON1 activity may be associated with SARS-CoV-2 infection.

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Coronaviruses are a large family of viruses that causes clinical conditions ranging from common colds to severe lung conditions, such as severe acute respiratory syndrome (SARS) caused by SARS-CoV and Middle East respiratory syndrome (MERS) caused by MERS-CoV. SARS-CoV-2 is a novel strain of coronavirus, and has been identified as the causal pathogen of an ongoing worldwide epidemic since 2019.[11]

As antioxidant enzyme, PON1 is inactivated under inflammation-induced oxidative stress and PON1 activity is found to be decreased in endothelial dysfunction, various inflammatory and infectious diseases.[3,4,6] In SARS-CoV-2 infected patients clinical deterioration often occurs 7-10 days after the onset of symptoms, in association with declining viral titres,[12] suggesting that pathology is driven by inflammatory cascade than direct viral injury. As suggested by increase in inflammatory markers in patients with severe SARS-CoV-2.[13,14] With this backdrop the present study was carried to evaluate activity of PON1 in SARS-CoV-2 patients.

Material and Methods

This is a cross sectional case-control study designed to assess the activity of PON1 in SARS-CoV-2 patients admitted to S.R.T.R. Govt. Medical College Ambajogai. The study has been undertaken with due approval from the Institutional Ethics Committee and consent were taken from study participants. Cases and controls were selected randomly. Inclusion criteria for cases were RT PCR-positive subjects admitted in hospital covid wards. Inclusion criteria for controls, they were subjects attending the outpatient department (OPD) for regular medical check-up and are RT PCR-negative. Exclusion criteria for cases (n: 73) and controls (n: 73) were a history of cardiovascular, renal or hepatic disease, diabetes mellitus, hypertension and endocrine disorders. With all aseptic precautions early morning fasting blood samples were collected by venepuncture from cases and control subjects, blood samples were collected in plain tube, centrifuged, serum was separated and stored at -80 ºC until analysis. Serum samples from RT PCR-positive patients were collected within 48 hours of admission.

Paraoxonase Activity Assay

The rate of formation of p-nitrophenol was measured on fully automated clinical chemistry analyzer XL-640 (Erba Mannheim/Transasia) using working reagent containing 25 mmol/L of triethanolamine-HCL buffer of pH 7.4 and 1 mmol/L $CaCl_2$ over 225 sec after 100 sec lag time in total volume of 600 µl using 2 µl of serum. The activity expressed in kU/L based on the molar absorptivity (14,000 M^{-1}/cm^{-1}) of p-nitrophenol at 405 nm. As p-nitrophenol liberated is being measured, its linearity was checked and if the activity was beyond the linearity, then the serum was diluted to linearity concentration of p-nitrophenyl acetate.[7,15] Intra assay CV 1.6% and inter assay CV was 6%.

Serum ALT and AST activity, bilirubin, creatinine, urea, $Na^+$and K^+ levels were estimated in XL-640 autoanalyzer using Erba Mannheim kits.

Statistical Analysis

The continuous variables were tested for normality by Shapiro-Wilk test. Results are presented as mean±standard deviation. Student's unpaired t-test was used for statistical analysis of continuous variables, Chi square test was used for categorical variables, Pearson's correlation was performed for correlation of paraoxonase with other variables under study. Univariate logistic regression was performed to assess contribution of variable under study towards presence of SARS-CoV-2 infection. Variables found significant in univariate logistic regression were modelled through multivariate logistic regression. $p<0.05$ was considered as statistically significant. Statistical analysis was performed using Microsoft excel and Mystat 12 software.

Results

PON1 activity in SARS-CoV-2 infected patients was significantly decreased than in control (*Figure 1*). All other baseline or biochemical parameters (age, sex, bilirubin, AST, ALT, creatinine, urea and electrolytes) shows significant difference ($p<0.05$) between cases and controls while Na^+ and K^+ were not significantly different ($p>0.05$) (*Table 1*). To assess the association of variables under study, univariate logistic regression was

Table 1. Baseline parameters.

Variables	Cases (n: 73) (Mean±SD)	Controls (n: 73) (Mean±SD)	P-value
Age (years)	53.93±18.02	45.17±16.62	0.002*
Sex (M/F)	48/25	42/31	NS
Total bilirubin (mg/dL) (Diazo method)	1.16±1.81	0.72±0.65	0.05
AST (IU/L) (IFCC method)	46.33±32.27	30.43±16.36	<0.001*
ALT (IU/L) (IFCC method)	41.06±29.84	26.3±13.79	<0.001*
Creatinine (mg/dL) (Creatinase enzymatic)	1.04±1.00	0.75±0.25	<0.001*
Urea (mg/dL) (GLDH method)	47.04±37.22	27.72±9.56	<0.001*
Sodium (mmol/L) (ISE method)	142.17±6.46	141.64±4.43	0.560
Potassium (mmol/L) (ISE method)	3.96±0.82	3.87±0.61	0.480

*p<0.05

Table 2. Univariate logistic regression.

Variables	Estimate	Odd's Ratio	95% Confidence interval		P-value
			Lower	Upper	
Age	-0.029	0.971	0.953	0.991	0.003**
Sex	0.349	1.417	0.725	2.770	0.308
NPON	2.277	9.746	4.253	22.331	0.000**
Bilirubin	-0.474	0.622	0.351	1.103	0.023*
AST	-0.030	0.970	0.953	0.988	0.000**
ALT	-0.038	0.963	0.942	0.984	0.000**
Creatinine	-1.528	0.217	0.067	0.705	0.001**
Urea	-0.062	0.940	0.912	0.968	0.000**
Sodium	-0.018	0.983	0.926	1.042	0.558
Potassium	-0.162	0.850	0.541	1.336	0.479

done which shows association of variables like age, paraoxonase, bilirubin, AST, ALT, creatinine towards the SARS-CoV-2 infection which was significant except Sex, Na^+ and K^+ (*Table 2*). Multivariate logistic regression (*Table 3*) showed low PON1 activity (odd's ratio 2.7-18.8) and urea were independently associated with SARS-CoV-2 infection. The inclusion of PON1 with these parameters in the diagnostic algorithm had high sensitivity and specificity (AUC=0.853) (*Figure 2*).

Table 3. Multivariate logistic regression

Variables	Estimate	Odd's Ratio	95% Confidence interval		P-value
			Lower	Upper	
Age	0.016	1.016	0.987	1.046	0.271
NPON	1.980	7.239	2.784	18.827	0.000**
Bilirubin	-0.256	0.775	0.459	1.307	0.33
AST	0.000	1.000	0.973	1.028	0.989
ALT	-0.018	0.982	0.950	1.016	0.302
Creatinine	0.316	1.371	0.247	7.613	0.718
Urea	-0.050	0.952	0.914	0.991	0.017*

Discussion

In present study, it was found that the PON1 activity was significantly decreased in SARS-CoV-2 patients (1.30±0.55 kU/L) compared to healthy individuals (1.913±0.48 kU/L). We also found that serum PON1 activity was decreased in cases, to levels near about half of that of the controls.

Defective high density lipoprotein and endothelial dysfunction leads to increase in oxidative stress which could be the possible mechanism behind low PON1 activity.[16,17] Vascular endothelium activation and damage occur as part of SARS-CoV-2 infection.[18-21] SARS-CoV-2 infected patients are known to often have low HDL levels[22] and recent studies reported that patients with severe SARS-CoV-2 had decreased HDL cholesterol and/or HDL functionality.[23-25] Begue et al.[26] found that the HDL cholesterol concentration of SARS-CoV-2 patients admitted to the Intensive Care Unit was about half that of healthy individuals and that their HDL particles were enriched in various inflammatory proteins and depleted in PON1. All these studies suggest that there is change in HDL molecules and its contents hence it becomes more inflammatory. This could be the reason behind dramatic decrease in PON1 activities.

This study found that liver (bilirubin, AST, ALT) and kidney function (urea, creatinine) values were impaired. While these parameters increased significantly when univariate logistic regression was performed, multivariate logistic regression showed that urea was independently associated with SARS-CoV-2 infection. These findings are in agreement with previous studies stating that liver injury occurs during highly pathogenic human coronavirus infections, moreover abnormalities in laboratory indexes of blood biochemical parameters, may be associated with the severity of multiple organ dysfunction.[14,27-29]

Out of 73 patients 13 patients died from SARS-CoV-2 infection having decreased PON1 activity (1.19±0.46 kU/L) as compared to survivors (1.31±0.56 kU/L) ($p>0.05$, data not shown). Among all variables examined, PON1 and urea were found to be associated with SARS-CoV-2 infection. Variables related to SARS-CoV-2 infection in univariate logistic regression were modelled by multivariate logistic regression, showing that PON1 and urea are independently related to SARS-CoV-2 infection. Low PON1 levels appeared to be associated with increased SARS-CoV-2 infections (Odd's ratio 7 with 95% confidence interval 2.7-18.8).

The PON1 enzyme could be a valuable biomarker for understanding SARS-CoV-2 viral

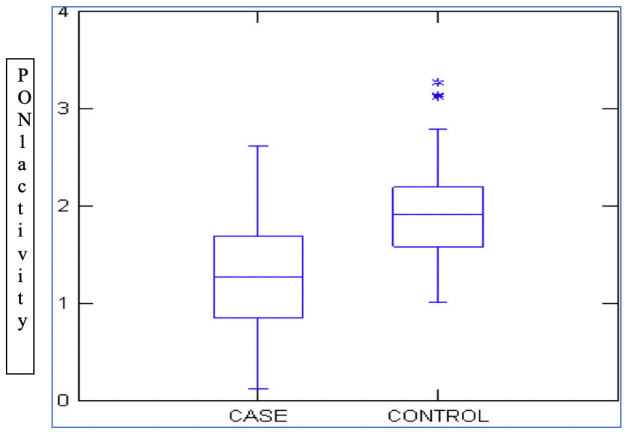

Figure 1. Boxplot for PON1 activity.

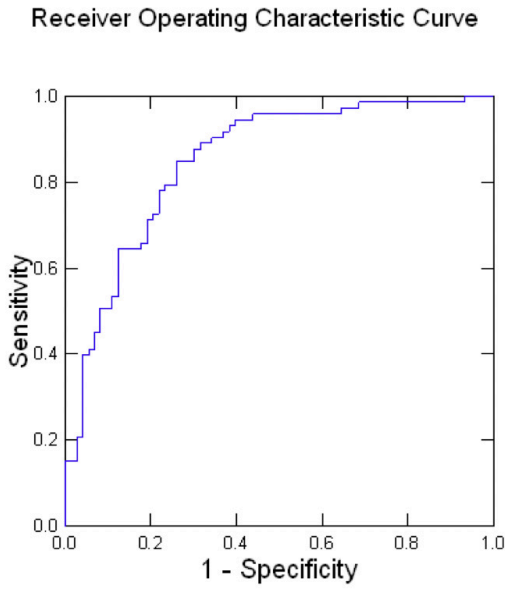

Figure 2. ROC Curve (Area under ROC=0.853).

infection. PON1 estimation requires inexpensive tools such as colourimeters available in remote healthcare facilities in developing and low-income countries. Determination of serum PON1 arylesterase activity is simple and economical, requires p-nitrophenyl acetate as substrate, and can be used as a primary screening test in developing and low-income countries where an ELISA reader is available. The detailed elucidation of inflammatory pathways, identification of inflammation triggers and the role of PON1 could eventually lead to the discovery of a new therapeutic target.

Conclusions

PON1 activity is significantly reduced in SARS-CoV-2 patients. In addition, as in univariate logistic regression, ALT/AST activity, bilirubin, creatinine and urea test levels increased significantly in patients with SARS-CoV-2 ($p<0.05$). Also, multivariate logistic regression reveals that PON1 activity and urea are associated with SARS-CoV-2 infection. In the future, including PON1 activity as a biomarker in SARS-CoV-2 infection may aid diagnostic algorithms with increased sensitivity and specificity. However, extensive multicenter studies may be required to determine its role in SARS-CoV-2 infection.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical Approval

This study has been duly approved by IEC (Institutional Ethical committee).

Authors' Contribution

MRM and PSR researched literature and conceived the study. MRM, MGD & RMZ was involved in protocol development, gaining ethical approval, patient recruitment and data analysis. PSR wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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Original Article

Comparison of Hemophagocytic Lymphohistiocytosis Diagnostic Criterias in Malignancy-associated Hemophagocytic Lymphohistiocytosis Patients

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ABSTRACT

Background Fulfilling diagnostic criterias of hemophagocytic lymphohistiocytosis (HLH) is challenging due to unavailable laboratory tests. Hence, we aimed to reveal malignancy-associated-HLH (M-HLH) patients in our center, which can not be reached in all tests.

Material and Methods Nine patients with M-HLH were analyzed retrospectively.

Results The median age was 59 years. The distribution of the underlying diseases were like diffuse large B cell lymphoma in 3 patients, acute myeloid leukemia in 2 patients, Hodgkin lymphoma in 2 patients, T cell non-Hodgkin lymphoma in 1 patient and small cell lung cancer in 1 patient. According to HLH-2004 diagnostic criteria except soluble CD25 and natural killer activity tests; one patient had 3/6, six patients had 5/6, two patients had 6/6 criteria while the median H-score was 258 at diagnosis. According to Tamamyan et al's criteria; at the diagnosis all patients had ≥7 (between 7-12) of 18 parameters. Patients fulfilled ≥5 parameters a median 15 days (3-52 days) before the diagnosis and on that time six patients had 3/6 criteria of HLH-2004. 88.8% of the patients died. The median duration of survival was 8.5 days (1-18 days).

Conclusions Unavailability of the tests in some countries and centers as in ours results in complicating to fulfill 5 of 8 criteria and being delayed to diagnose and treatment. We need to develop more specific and accessible criteria, and grading systems for M-HLH diagnose.

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Introduction

Breast hemophagocytic lymphohistiocytosis (HLH) is a rare and life-threatening disorder which is difficult to diagnose and to manage especially in adults. The pathogenesis of HLH consists of increased activation and stimulation of macrophages, cytotoxic T-lymphocytes and natural killer (NK) cells resulted in cytokine storm and hyperinflammatory syndrome due to a genetic mutation or a trigger mechanism.[1] Increased cytokines such as interferon γ, tumor necrosis factor-α, interleukin (IL)-6, IL-8, IL-10, IL-12, IL-18, and macrophage colony-stimulating factor and infiltration of tissues by overactivated cells induce tissue injury and multiorgan dysfunction and can prompt to death rapidly.[2] Histiocyte Society Steering Committee classified HLH into 3 categories as primary (familial) HLH, macrophage activation syndrome (MAS)-HLH and secondary HLH. If there is a known genetic defect associated with lymphocyte cytotoxicity or a family member who has HLH history is defined as primary (familial) HLH. When there is an auto-immune condition which triggers hyper and uncontrolled inflammation is called MAS-HLH. Ultimately, if HLH is originated from some sort of underlying medical status (infection, malignancy, metabolic diseases, inherited or acquired immunodeficiencies) it is named as secondary HLH.[3] Familial HLH is encountered in children commonly while MAS and secondary HLH are seen in adults generally.[4] In adults, the accurate incidence of HLH is not known due to the fact that mis or underdiagnose in critically ill. In a Japan nation-wide study, HLH rate in adults was 40% of all HLH cases.[5] Malignancy-associated HLH (M-HLH) is common in adults especially in older age (1% of all malignancies and nearly 50% of adult HLH) and mostly originate from lymphoma (80%) especially NK/T cell lymphoma (35%) and lesser in multiple myeloma, acute leukemia, chronic leukemia and solid cancers.[6-8] M-HLH may begin during malignancy treatment or in remission probably due to immunosuppression and/or infection or before treatment even as a presenting symptom of malignancy at diagnosis or relapse.[4,7,9] Therapy-induced HLH is associated with immune activating therapies for malignancies lead to activation of $CD8^+$ T cells and drug induced hypersensitivity syndrome (DIHS).[10]

Clinical findings such as fever (>38.5 °C), cytopenia, splenomegaly, hepatomegaly, liver failure, increased aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin levels, ascites, elevated lactate dehydrogenase (LDH), D-dimer levels (even while international normalized ratio, partial thromboplastin time and fibrinogen levels are normal), encephalopathy, respiratory distress syndrome, renal failure, edema, purpura, bleeding, arthralgia, diarrhea, rash and neurological symptoms (loss of consciousness, convulsions, cranial nerve abnormalities and ataxia) can occur in HLH and these abnormalities can be seen together or progressively within a few days or weeks. These clinical and laboratory findings also help to differential diagnosis and to assess the response to treatment with supporting diagnose.[4,8] The HLH-2004 diagnostic criteria (*Table 1*) of the Histiocyte Society which was developed for children is in use also for adult HLH although there is no validation data for adult patients. According to HLH-2004 diagnostic criteria patients should have ≥5 of 8 criteria for diagnosis.[11] Fardet et al.[12] developed another diagnostic scoring model which is called H-score and was validated in adult and children regarding reactive and immune hemophagocytic syndrome in adults (*Table 2*). Tamamyan et al.[13] announced extended 18 parameters to help early diagnosis in M-HLH in 2016 (*Table 3*). The patients who fulfilled 5 parameters of 18 were considered as high likelihood of M-HLH.[13]

During the treatment the main aim to restrict the hyperinflation besides that to provide the balance between HLH specific and malignancy specific treatment and to success this aim without any guideline. Infection is also a known trigger of HLH in immunocompromised patients. Especially, HLH specific treatment can be devastating in such a group of patients instead of anti-inflammatory approach.[14] First line treatment is generally corticosteroids however dose-adjusted etoposide (50-100 mg/m^2) can be chosen if the multiorgan failure is in the foreground before the malignancy specific treatment according to disease adapted HLH-94 treatment protocol.[7] Etoposide can also be used with CHOP (cyclophosphamide, doxorubicin, vincristine, etoposide, prednisone as

CHOEP) or CHOP-like (dose-adapted etoposide and CHOP as DA-EPOCH) chemotherapy protocols.[15]

Diagnosis of HLH is increasing in recent years depending on raising awareness with this disorder.[16] Nevertheless, soluble CD25 (soluble IL-2 receptor), NK activity according to HLH-2004 diagnostic criteria are not always accessible and obtained criterias in every country and center. Due to impossibilities and lack of standard suggestion in these circumstances we thought to share our experience of diagnosis and treatment in M-HLH patients in our tertiary university hospital in the last three years.

Material and Methods

This retrospective study was conducted between October 2018-October 2021 at our hospital and approved by the Institutional Ethical Committee (BAEK 2021/448). We determined nine patients who were approved and treated as M-HLH. Patients' all data were collected from electronic medical files. Patients' age, gender, underlying disease, date of diagnosis, status of the underlying disease, last date of chemotherapy, presenting symptoms of HLH, physical examination findings, laboratory tests, microbiological tests, radiological images (such as X-ray, computerized tomography, magnetic resonance), positron emission tomography (PET) images, pathology of biopsies, presence or suspicion of infection, exposure to antibiotics, treatment of HLH and/or specific malignancy, response to treatment, outcomes and survival period of the patients were evaluated. HLH-2004 diagnostic criteria was used for diagnosis.[11] However, the diagnosis of HLH was done based on 6 criteria instead of 8 due to the absence of soluble CD25 (soluble IL-2 receptor) and NK activity laboratory tests in our center. Therefore, if the patient did not fulfill five of six criteria the diagnosis of HLH was supported with other suspicious findings and tests such as hepatomegaly, elevated hepatic transaminase and bilirubin levels, LDH, hypoalbuminemia, coagulopathy and strong consideration of HLH as a clinician. Besides that, H-score was calculated for every patient.[12] We also reassessed the patient group in terms of Tamamyan et al.'s extended criteria.[13] Response to treatment was determined as improving clinical findings and abnormal laboratory tests. Survival period of the patients was defined as the days after diagnosed HLH. According to pathology evaluation and PET findings hemophagocytosis or remission/non-remission status of the malignancy were revealed. We decided to infection status according to microbiology-culture and scanning tests and response to specific antimicrobial treatment. HLH-94 protocol or corticosteroids were used as etoposide 150 mg/m^2 first and second days in the first two weeks and subsequent once weekly and dexamethasone 10 mg/m^2 per day in first week and 5 mg/m^2 per day in the second week, 2.5 mg/m^2 per day in the third week, 1.25 mg/m^2 per day in the fourth week or with only dexamethasone for HLH specific treatment.[17]

Statistical analysis was not applied.

Table 1. HLH-2004 diagnostic criteria.[11]

Fever
Splenomegaly
Cytopenia (≥2 lineages in the peripheral blood)
Hemoglobin <9 g/L or platelets <100,000/mm^3 or neutrophils <1,000/mm^3
Hypertriglyceridemia and/or hypofibrinogenemia
Fasting triglycerides ≥3.0 mmol/L (i.e., ≥265 mg/dL) and/or fibrinogen ≤1.5 g/L
Hemophagocytosis in bone marrow or spleen or lymph nodes
Low or no natural killer cell activity
Ferritin ≥500 mg/L
sCD25 (i.e., soluble IL-2 receptor) ≥2,400 U/mL

Table 2. H-score system.[12]

Findings	Points
Known underlying immunosuppression	No:0
	Yes:18
Temperature (°C)	<38.4: 0
	38.4–39.4: 33
	>39.4: 49
Organomegaly	No: 0
	Hepatomegaly or splenomegaly: 23
	Hepatomegaly and splenomegaly: 38
Number of cytopenia (hemoglobin 9.2 g/L and/or leukocyte ≤5x10 ^{9} /L and/or platelet ≤110x10 ^{9}/L)	1 lineage: 0
	2 lineages: 24
	3 lineages: 34
Ferritin (mg/L)	<2,000: 0
	2,000-6,000: 35
	>6,000: 50
Triglyceride (mmol/L)	<1.5: 0
	1.5-4: 44
	>4: 64
Fibrinogen (g/L)	>2.5: 0
	≤2.5: 30
Aspartate aminotransferase (U/L)	<30: 0
	≥30: 19
Hemophagocytosis on bone marrow aspirate	no: 0
	yes: 35

Results

In our tertiary hospital which serves generally Marmara region of Turkey on an average 190 patients are diagnosed newly with hematologic malignancy per year. Lymphomas which are the most accused hematologic etiology of HLH represent nearly 40% of this number of patients. In our patient group, the mean age of patients was 54 (24-78 years) and the median age was 59. Six patients (66.6%) were male while three patients (33.4%) were female. The distribution of the underlying diseases in patients was like three patients with diffuse large B cell lymphoma (DLBCL), two patients with acute myeloid leukemia (AML), two patients with classical Hodgkin lymphoma (HL) and the others with small cell lung cancer and T cell non-Hodgkin lymphoma (T-NHL). One of these patients who was counted as AML had diagnosed and treated for DLBCL two and half years before. One of the patient was diagnosed with HLH and DLBCL simultaneously. In only one patient who had T-NHL developed HLH during malignancy specific therapy. Rest of patients had

Table 3. Tamamyan et al.'s extended criteria[13]

Bone marrow/ lymph node/spleen hemophagocytosis per pathology evaluation	Renal failure (≥50% increase in creatinine over baseline)
Fever	Elevation of liver enzymes (≥2.5 times the upper limit of normal)
Splenomegaly (clinically palpable spleen)	Hypofibrinogenemia (fibrinogen ≤150 mg/dL)
Hepatomegaly (clinically palpable liver)	Hyperferritinemia (ferritin ≥500 µg/L)
Anemia (hemoglobin <9.0 g/L)	Coagulopathy (prothrombin time ≥1.5 times the upper limit of normal and/or partial thromboplastin time ≥1.5 times the upper limit of normal, and/or D-dimer ≥10.0 µg/mL)
Thrombocytopenia (platelets <100x10^{9}/L)	Hypoalbuminemia (<3.5 g/dL)
Neutropenia (absolute neutrophil count <1.0x10^{9}/L)	Elevated β2-microglobulin (≥2 mg/L)
Monocytosis (absolute monocyte count >1.0x10^{9}/L)	Elevated lactate dehydrogenase (≥2.5 times the upper limit of normal)
Hypertriglyceridemia (≥265 mg/dL)	Elevated soluble IL-2 receptor

Table 4. Characteristics of malignancy-associated hemophagocytic lymphohistiocytosis patient group.

Age (median-years)	59
Male (%)	66%
Underlying malignancy (number of patients)	Diffuse large B cell lymphoma (3) Acute myeloid leukemia (2) Hodgkin lymphoma (2) T cell non-Hodgkin lymphoma (1) Small cell lung cancer (1)
Clinical and laboratory findings at diagnosis (%) (HLH-2004 diagnostic criteria) [11]	Fever (100%) Splenomegaly (88.8%) Hypertriglyceridemia (88.8%) Hyperferritinemia (88.8%) Anemia (77.7%) Thrombocytopenia (66.6%)
Clinical and laboratory findings at diagnosis (Tamamyan et al.'s extended criteria) [13]	Fever (100%) Splenomegaly (88.8%) Thrombocytopenia (88.8%) Hepatomegaly (77.7%) Anemia (66.6%)
Median H-score at diagnosis [12]	258
Duration of application to hospital to HLH diagnosis (days)	16 (4-53)
Number of criteria at diagnosis (HLH-2004 diagnostic criteria) [11] (number of patients)	3/8 (1) 5/8 (6) 6/8 (2)
Mortality rate (%)	88.8
Median duration of survival (days)	8.5 (1-18)

active malignancy such as relapse, uncompleted therapy or newly diagnosed when diagnosed with HLH. The most common application symptom was fever (6 patients) and followed by dyspnea (4 patients) and weakness equally. Somnolence was the concomitant symptom to fever and weakness in one patient. The mean duration of application to hospital to HLH diagnosis 23.4 days (4-53 days), median time was 16 days. According to HLH-2004 diagnostic criteria in 8 criteria apart from soluble CD25 (soluble IL-2 receptor) and NK activity laboratory tests; one patient had 3/6, six patients had 5/6, two patients had 6/6 criteria when they were approved as HLH at the time of diagnosis. Six patients of nine had hemophagocytosis in bone marrow biopsy. The mean H-score was 256 while median score was 258 at diagnosis. One patient's whom had 3/6 HLH-2004 diagnostic criteria H-score was 219. The patients' whom had 5/6 HLH-2004 diagnostic criteria H-score were between 173 and 317. Two patients' H-score were 213 and 307 with 6/6 HLH-2004 diagnostic criteria. When medical records were reviewed due to Tamamyan et al.'s study[13], we could reach data in all patients mostly except soluble IL-2 receptor level and β2-microglobulin level. According to this extended criteria; at the diagnosis of HLH, all patients had ≥7 (between 7-12) of 18 parameters. When we analyzed the time which patients fulfilled ≥5 parameters it was a mean 17.7 days and median 15 days (3-52 days) before the diagnosis of HLH. On that time six patients had 3/6 criteria of HLH diagnostic criteria while the other patients had 2-4-5/6.

Regarding ferritin which was found one of the most related laboratory finding to HLH due to level, eight of nine patients had elevated ferritin (≥500 μg/mL) level while six of them had ≥1500 μg/mL. Defect of coagulation parameters and bleeding diathesis were not remarkable however

Table 5. Features of malignancy-associated hemophagocytic lymphohistiocytosis patient group.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Age (years)	68	40	30	67	24	54	99	66	78
Gender	Male	Male	Male	Female	Female	Male	Male	Female	Male
Underlying malignancy	T NHL	HL	AML	DLBCL	AML	SCLC	HL	DLBCL	DLBCL
HLH-2004 diagnostic criteria[11]									
Fever	+	+	+	+	+	+	+	+	+
Splenomegaly		+	+	+	+	+	+	+	+
Hemophagocytosis	+	+	+	+	+	+		+	
Hemoglobin <9 g/L					–	+	+	–	+
Neutrophils <1,000/mm³			–				+		
Platelets <100,000/mm³			–		–	+	+	–	+
Hypertriglyceridemia	+	+	+	+		+	+	+	+
Hypofibrinogenemia	+	+							
Ferritin ≥500 ng/L		+	+	+	+	+	+	+	+
sCD25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Low or no NK cell activity	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H-score (at the time of diagnosis)[7]	219	317	307	272	268	307	238	213	173
Tamegnon et al.'s extended criteria (at the time of diagnosis)[7]									
Hemophagocytosis	+	+	+	+	+	+		+	
Fever	+	+	+	+	+	+	+	+	+
Splenomegaly		–	–	+	–	+	+	–	+
Hepatomegaly		–	–	+	–	+	+	–	
Anemia (hemoglobin <9.0 g/L)	+				+	+	+	+	+
Thrombocytopenia (platelets <100×10⁹/L)		+	+	+	+	+	+	+	+
Neutropenia (absolute neutrophil count <1.0×10⁹/L)			+				+		
Monocytosis (absolute monocyte count >1.0×10⁹/L)			+	+	+		+	+	
Hypertriglyceridemia	+	–	–	+		+	+	–	+
Renal failure		–				+	+		
Elevation of liver enzymes		+				+	+		
Hypofibrinogenemia	+	+							
Hyperferritinemia		+	+	+	+	+	+	+	+
Coagulopathy	+	+	+		+				
Hypoalbuminemia	+			+	–	+		–	+
Elevated β2-microglobulin								–	
Elevated lactate dehydrogenase		+		+	+			+	+
Elevated soluble IL-2 receptor	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

T NHL: T cell non-Hodgkin lymphoma, HL: Hodgkin lymphoma, AML: acute myeloid leukemia, DLBCL: diffuse large B cell lymphoma, SCLC: small cell lung cancer, LDH: lactate dehydrogenase, N/A: not available

D-dimer levels were ≥10.0 μg/mL whom was performed.

Seven patients were receiving broad-spectrum antibiotics at HLH diagnosis and two of them were also using antifungal treatment. In only one patient's concurrent two blood culture were resulted as "*Acinetobacter baumannii*" on the same day she deceased. Regarding antifungal treatment, it was given as "possible" fungal infection according to host and clinical features with imaging findings without mycological evidence.

Two of the patients could not have treatment while two of seven patients had dexamethasone and five of them had etoposide and dexamethasone protocol as mentioned before. Four of these five patients had also chemotherapy during HLH treatment. Chemotherapies were performed as CHOP protocol, 7+3 protocol (100/200 mg/m^2 /day cytarabine, 7 days and 60 mg/m^2 /day daunorubicin, 3 days), brentuximab or rituximab and bendamustine. Plasmapheresis for 2 days (one volume with fresh frozen plasma) and IVIG (0.5 g/kg) were performed to one patient but he did not respond to these interventions.

Outcomes of nine patients; only one patient recovered from HLH clinic status however he

deceased related to progression of the primary disease. Remained of the patients whether treated or untreated died related to uncontrolled HLH status and multiorgan failure in the intensive care unit. The mean survival time of the treated patients was 12.6 days (7-21 days). Characteristics and features of M-HLH patient group are summarized in Table 4 and Table 5.

Discussion

Infections or malignancies can initiate HLH pathogenesis if they are not managed properly nevertheless infections can seem a trigger of HLH deceptively in patients with underlying autoinflammatory or malignant disorders.[18] Mortality rate is extremely high along with bleeding, infections, loss of consciousness and refractory hypotension due to multiorgan involvement and failure.[4,8] Machazka et al.[6] announced the incidence of M-HLH as 1% in hematological malignancies. Cumulative incidence rate was reported as 2.8% in malignant lymphoma patients while 9% in acute myeloid leukemia patients after remission treatment.[19,20] Hematological malignancies are the most common cause of M-HLH in a rate of 40-70% and up to 93.7% in a review of M-HLH. Distribution of them is like 35-40% T/NK cell lymphoma-leukemia, 30-40% B-cell lymphoma, 5-8% Hodgkin lymphoma, 8% acute myeloid leukemia, 8% myelodysplastic syndrome.[21,22] In our study, the distribution was like DLBCL in 3 patients, AML in two patients, HL in two patients. Median age of our patient group was 59 and it was similar to the other studies.[13,18,22,23] In terms of clinical and laboratory findings, fever (100%), splenomegaly (88.8%), hypertriglyceridemia (88.8%), hyperferritinemia (88.8%), anaemia (77.7%), thrombocytopenia (66.6%) were seen mostly and the least met criteria was neutropenia (11.1%) according to HLH-2004 diagnostic criteria. This was supported by German registry study and a multicenter study from USA regarding fever, splenomegaly, anemia, thrombocytopenia, hyperferritinemia but neutropenia was determined 43% and 76% in these studies, respectively.[18,23] Fever (100%), splenomegaly (88.8%), thrombocytopenia (88.8%), hepatomegaly (77.7%), anemia (66.6%) were determined frequently regarding Tamamyan et al.'s[13] extended criteria. The distribution of the criterias was different from the original study as fever (42%), splenomegaly (35%), thrombocytopenia (48%), anaemia (39%).[13] This diversity could be associated with the underlying disease, ethnicity or small patient group that we examined. Besides that, monocytosis was seen in four patients (44.4%) and two of them were diagnosed with AML while it was 17% (6 patients) in the original study and three of them were AML. Therefore, we think monocytosis could be confusing in terms of HLH probability when the underlying disorder is AML in M-HLH.[13] Hyperferritinemia was not found as a distinctive indicator for adult HLH patients nevertheless it was found as an important marker for diagnostic work-up in Schram et al.'s study.[24] Tamamyan et al.'s[13] and our study correlate in terms of hyperferritinemia which was found nearly 90% in the patient groups. In our patient group, 75% of patients (6 patients) with hyperferritinemia had >1,500 μg/mL ferritin level. The other criteria of extended criteria that are defect of coagulation parameters and bleeding diathesis were not remarkable however D-dimer levels were ≥10.0 μg/mL whom was performed. Any patient could not fulfilled these criteria with only high D-dimer level. In Schram et al.'s study[23], 85% of M-HLH patients represented coagulopathy. This parameter may be separated for assessment HLH because of D-dimer could be find high due to underlying malign disorder not only with bleeding diathesis or disseminated intravascular coagulation.

Fever, bicytopenia with increased risk of bleeding and splenomegaly are triad and suspicious findings for adult HLH and in patient who has these findings it is recommended that to initiate diagnostic examinations regarding HLH rapidly.[4,8] If the clinical status does not respond and even gets worse with accurate antimicrobial therapy, this condition could be approved as a possible signal of HLH. Re-assessment of clinical findings, physical examination and laboratory tests closely are the fundamental for diagnosis.[4] However, treatment is recommended in patients who are suspected strongly for HLH despite cannot provide 5 of 8 HLH-2004 diagnostic criteria and we also initiated treatment in a patient with 3/6 criteria due to significant clinical support.[4] Besides that,

H-score can be used for diagnosis however H-score could not be adequate to determine M-HLH due to population of validation groups. Tamamyan et al.[13] also reported extended diagnostic criteria to this patient group who has poor prognosis for providing earlier intervention. Therefore, they suggest to workup for HLH and to pretend as HLH in patients who has hepatic or renal failure of unknown etiology, sudden-onset multiorgan failure, culture negative sepsis or encephalopathy of unknown etiology.[13]

We think that reaching to all HLH-2004 diagnostic criteria which as mentioned before soluble CD25 (soluble IL-2 receptor) and NK activity laboratory tests is difficult in every country and center as, in our country and in a tertiary hospital. Unavailability of these tests results in complicating to fulfill 5 of 8 criteria and being delayed to diagnose and treatment. Therefore, we aimed to reveal the process of diagnose and outcomes in limited patient group. Hemophagocytosis in bone marrow or lymph node or spleen is another criteria which is difficult to interpret in status of malignancy and inflammation.

The mean duration of HLH diagnosis 23.4 days according to HLH-2004 diagnostic criteria in 6 criteria apart from soluble CD25 (soluble IL-2 receptor) and NK activity laboratory tests. In terms of the differentiation efficiency of HLH patients with H-score, the number of patients are limited to conclude however the score was not correlate with HLH-2004 diagnostic criteria due to co-occurrence of lower HLH 2004 criteria and high-level H-score. According to Tamamyan et al.'s criteria all patients had HLH clinic status probably on average 17 days before the day of HLH diagnosis and on that time most patients had 3 or 2/6 criteria of HLH-2004 diagnostic criteria.[13] We cannot reveal any benefits to determine patients with HLH earlier however we think that to change our approaching to this patient group and to be on alert regarding unfulfilled HLH-2004 diagnostic criteria to do not miss the cases and be late for diagnosis.

Regarding diagnosis and treatment, HLH patients need multidisciplinary approach along with hematology, oncology, pathology, infectious diseases and intensive care. M-HLH is the poorest prognostic HLH subtype. There is still no specific diagnostic tool or treatment or guideline for M-HLH due to lack of prospective, randomized and controlled studies.[25] HLH which occurs during treatment and is with prolonged neutropenia, fever despite of antibiotic treatment benefit from especially corticosteroids due to anti-inflammatory feature and IVIG nevertheless excluding of non-remission malignancy should be done.[4]

Mortality rate was 88.8% in our patient group and the median duration of survival was 8.5 days (1-18 days) in all patients while it was 12.6 days in treated patients. Mortality rate was reported 80% and the median survival time 1.5 months in Tamamyan et al.'s study[13] and mortality rate were 100% and the median survival time was 22 days in another study from Sweden in M-HLH.[26] These mortality rates were not so different from our results however the median survival time is shorter in our patient group probably due to being late for diagnosis.

In the latest study which has the largest M-HLH patient group, sCD25 >3,900 U/mL and ferritin> 1,000 ng/mL were reported as "Optimized HLH Inflammatory" (OHI) index. And this index suggests to provide more accurate pathological state of HLH.[22] However, diagnosing and managing of M-HLH is still challenging especially unavailability of tests which are associated to reveal hyperinflammation.

Conclusions

Mortality rate of HLH in adults especially in M-HLH still high. Adult HLH especially M-HLH are candidates for improvements and some studies are going on regarding treatment currently. Considering HLH in differential diagnosis and awareness of HLH are still essential for diagnosis and treatment. We need to develop more specific and accessible criteria and grading systems for diagnosis and more targeted therapy options for M-HLH.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical Approval

For this study, approval was obtained local ethics committee.

Authors' Contribution

Study Conception: HOK, TAK, UD, SKG, VB, EGU, AMD; Study Design: HOK, TAK, UD, SKG, VB, EGU, AMD; Supervision: HOK, TAK, UD, SKG, VB, EGU, AMD; Literature Review: HOK; Critical Review: HOK; Data Collection and/or Processing: HAK, TAK, UD, SKG, VB; Analysis and/or Data Interpretation: HAK, TAK, UD; Manuscript preparing: HAK, TAK, UD, SKG, VB, EGU, AMD.

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Case Report

Multiple Lymph Node and Bone Metastases From An Occult Breast Cancer: A Case Report

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ABSTRACT

Cancer of unknown primary is defined as cancer with an unknown primary origin. Occult breast cancer (OBC) is a rare diagnosis in which physical examination, imaging methods, and even surgical procedures are insufficient to put on a primary tumoral site in the breasts. This definition leads to only 0.3-1% of all breast cancer cases. The diagnosis of OBC is usually obtained with an axillary lymph node biopsy. A 52-year-old female presented with right arm weakness and a neck lump. On the physical examination, multiple masses were palpated at the right axillary, right supraclavicular, and anterior cervical areas. Mammography, breast ultrasonography, breast magnetic resonance imaging, and 18-fluorodeoxyglucose positron emission tomography (18-FDG PET) scan could not show a suspicious tumoral area as the primary origin. 18-FDG PET scan put on the massive tumoral burden at multiple bones and lymph nodes, but there were no lesions to suspect as the tumoral origin. Finally, the supraclavicular lymph node biopsy result has revealed the diagnosis; hormone receptor-positive and C-erbB2 positive occult breast cancer. Cervical lymph node metastasis is also a scarce condition for breast cancer. The lymphatic drainage pathway is not clear in explaining the breast cancer involvement of the cervical.

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Keywords: *Cancer of unknown primary, breast cancer, occult cancer, metastases.*

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Introduction

Cancer of unknown primary (CUP) syndrome is referred to as a state whose primary site is unclear, but malignant cells have metastasized in a widespread. Occult breast cancer (OBC) is clinically recognizable axillary metastatic carcinoma from an undetectable primary breast tumour. With negative preliminary imaging and a biopsy-confirmed diagnosis of metastatic site, the patient is now classified as having OBC with an unknown primary site. The incidence of OBC is less than 1% of all patients who present with breast cancer (BC). OBC denotes the clinical status of axillary lymph node metastases consistent with a breast carcinoma arising in the absence of any identifiable primary breast tumour (pT0N+) in the staging system.[1]

Mammography, breast ultrasonography, and chest x-ray are the first screening methods after detecting pathological axillary lymph nodes. Magnetic resonance imaging (MRI) of the breast, likely to reveal a suspicious lesion in 76% of patients, can identify the primary occult tumour in 62% of this sub-group, with a false positive rate (FPR) of 29%. Although criticized for its high false-positive results in routine BC diagnosis, the role of MRI is crucial in the diagnostic dilemma of OBC.[1]

The incidence of ipsilateral supraclavicular lymph node metastasis in BC is reported to be as low as 1-4.3% even though breast cancer is known as the most common primary cancer to metastasize to neck lymph nodes when primary head and neck cancers are excluded.[2,3] Even though there is not a primary tumoral origin, in this case, extended tumoral spread with multiple metastases at lymph nodes and bones makes this case precious.

Case Report

A 52-year-old woman presented with persistent and progressive neck swelling and weakness in her right arm. On her physical examination, multiple masses were palpable on the right anterior cervical chain, right supraclavicular, and axillar areas. The largest one was at the right supraclavicular area, 4 cm in diameter. There was minimal restriction in

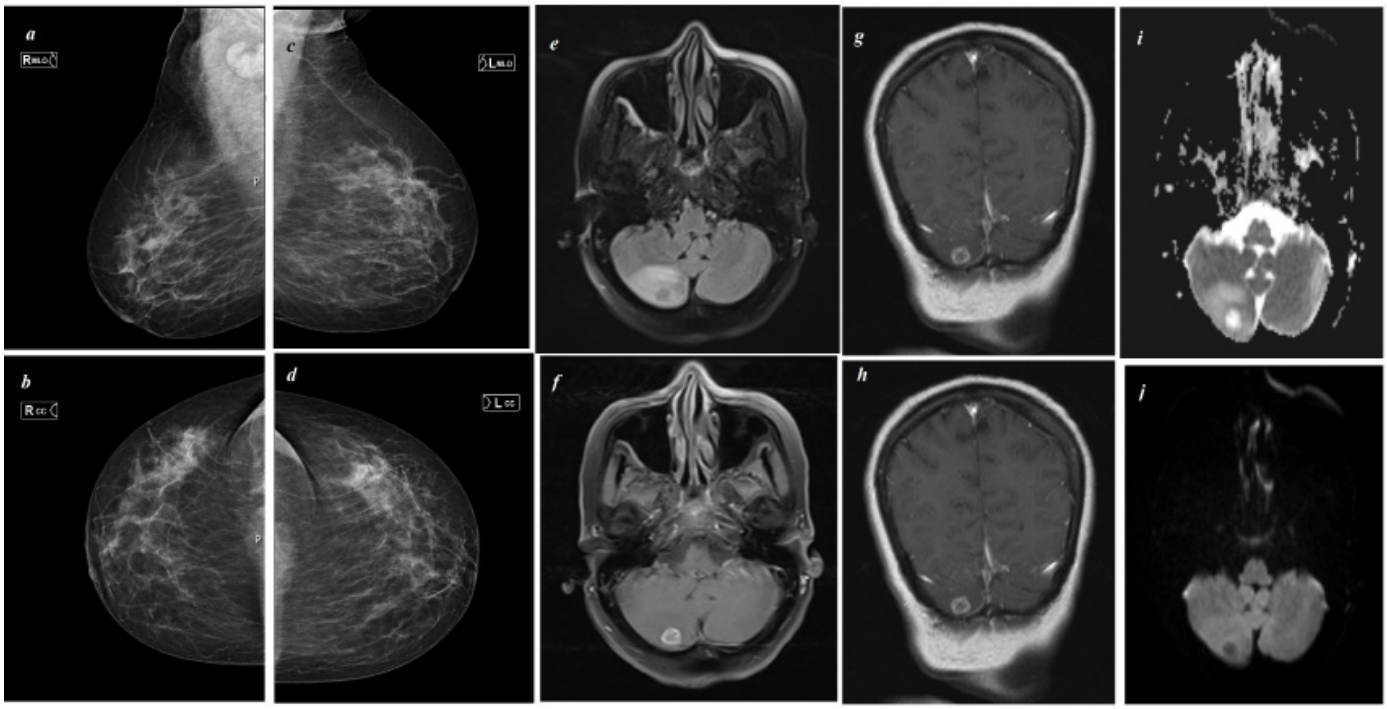

Figure 1. BIRADS-3 findings were observed in the mammography imaging (a,b,c,d). In cranial MR imaging; right cerebellar hemisphere posteromedial section has T2-Flair A (e) peripheral hyperintense edema area measuring approximately 10x12 mm in its widest dimension, showing intense contrast enhancement in T1 hypointense postcontrast T1 series (f,g,h) and causing mild limitation in diffusion examination (i,j) mass lesion observed.

right shoulder abduction and flexion movements, tenderness, and weakness in the right arm. Each of the largest ones in axillary and cervical masses was approximately 3 cm in diameter. There was no palpable mass in both of the breasts. Her right arm muscle strength examination was 3/5. She had only a beta-thalassemia minor in her medical history. She was not under any medications except the nonsteroidal anti-inflammatory drugs she used for her right arm pain.

In the laboratory tests, haemoglobin was 10.4 g/dL, MCV 65 fL, and LDH 321 IU/L. There were no other abnormal results at routine biochemistry tests. With the suspicion of breast cancer, as she had an axillary mass, we performed mammography and breast ultrasonography. Both of them could not find a tumoral site in the breasts with the Breast Imaging Report and Data System (BIRADS) 3 (*Figure 1*). Electroneuromyographic electrophysiological findings were consistent with the lesion where the medial and lateral cord of the brachial plexus was severely affected, and the posterior cord was moderately affected. In cranial magnetic resonance imaging (MRI), the right cerebellar hemisphere posteromedial section has T2-Flair a peripheral hyperintense oedema area measuring approximately 10x12 mm in its broadest dimension, showing intense contrast

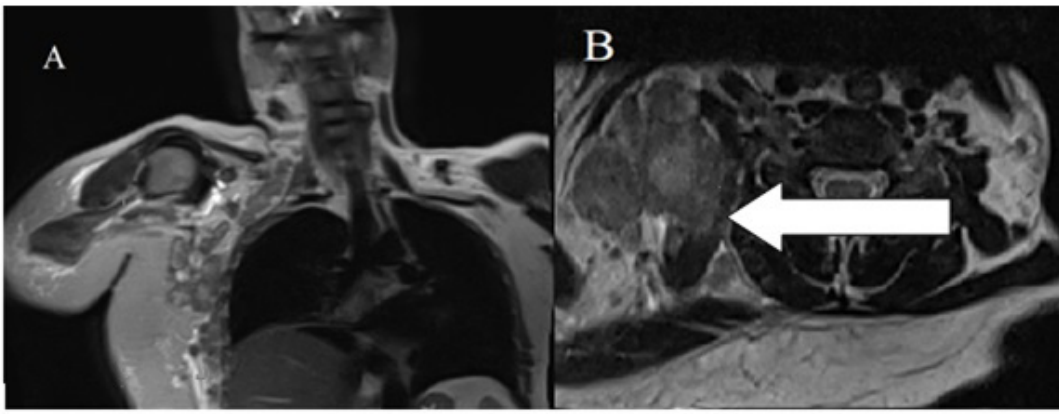

Figure 2. MRI images showing extensive involvement of the right brachial plexus. In A (coronal) and B (axial) T1-weighted sequences showing an infiltrating mass encasing the right brachial plexus.

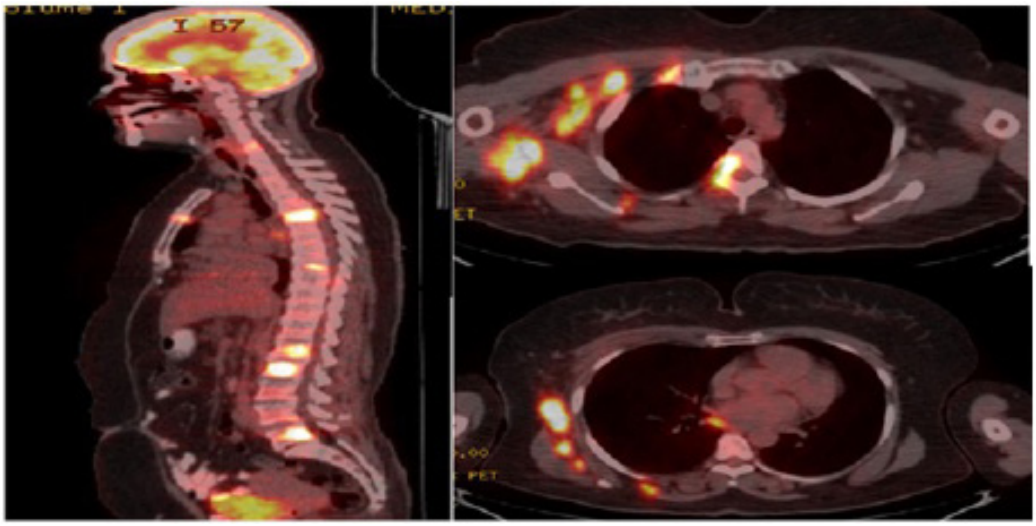

Figure 3. 18 FDG whole body positron emission tomography (PET) findings.

enhancement in T1 hypointense postcontrast T1 series and causing mild limitation in diffusion examination mass lesion observed (*Figure 1*).

With the ongoing suspicion of breast cancer, we performed a breast MRI. There was no focus to maintain our suspects at both breasts, but the right supraclavicular, retro pectoral, and right deep axillary lymph nodes were seen in the MRI scan. Besides, T2 hyperintense, T1 hypointense, and T1-T2 hypointense irregular-shaped heterogeneous multiple lesions (*Figure 2*) were observed in the vertebral column and sacral bone. In the 18-fluorodeoxyglucose positron emission tomography (18-FDG PET) scan, which was performed to choose the best site for taking the biopsy sample and for staging the disease, multiple hypermetabolic lymph nodes were observed at the right supraclavicular fossa with the largest one 41x38 mm in dimensions, the interpectoral area with the most prominent one 35x35 mm in sizes, right retro pectoral area with the largest one 40x36 mm in dimensions, right axillary fossa, right parasternal and subcarinal area, left axillary fossa and right jugular area. Also, multiple metastatic lesions were seen in all the bones of the pelvic arch, both femurs, sternum, 3rd cervical-, 3rd, 4th, 8th, and 9th thoracic, 1st, 4th, and 5th lumbar vertebral corpus and 2nd, 3rd lumbar vertebral pedicles. However, there was no pathological FDG uptake in both breasts or any other solid organ (*Figure 3*).

Excisional biopsy was performed from the supraclavicular Lymph node because it was easy to reach and one of the highest SUV max and most prominent lymph nodes detected with an 18-FDG PET scan. With positive estrogen receptor (ER) and progesterone receptor (PR) results of enzyme immunoassay and positive C-erbB2 gene mutation proven by PCR analysis, lymph node biopsy was reported as breast cancer metastasis. The follow-up and treatment of the patient are proceeding at the clinical oncology department with the diagnosis of OBC.

Discussion

Here, we described an occult BC patient with progressive enlargement of axillary, supraclavicular, and retro pectoral lymph nodes that have involved the brachial plexus in a 52-year-old woman. Advanced neuropathic findings and aggressive enlargement of the involved lymph nodes made us suspicious of malignancy, especially breast cancer. Mammography, breast ultrasonography, and chest x-ray were not enough to give a clue about the primary site of the tumour. MRI of the breasts also failed to expose any lesions to the suspect as the primary origin. 18-FDG PET scan was also unable to put on a primary focus on the breasts or any solid organ, but multiple metastatic lesions at the bones and the lymph nodes in a widespread pattern. After all, an excisional supraclavicular lymph node biopsy was taken. The histopathological assessment of the biopsy sample was reported as breast cancer metastasis with positive ER, PR, and CerbB2.

Breast cancer may be specifically suspected when axillary nodes are involved in women. Even if the ultrasonography and mammography are negative, a breast MRI is recommended if the findings are like CUP syndrome.[4,5] The use of MRI identifies a lesion which may be the primary focus in 72% of patients whose mammography and ultrasound results are negative. Over 85% of the suspected lesions shown by MRI are proven as the primary breast carcinoma upon biopsy.[6] In our case, MRI was also helpless to determine a malignant focus in breasts. MRI is also the distinguished image modality for evaluating the brachial plexus and its pathological involvement. Among 104 cases with non-traumatic brachial plexopathy, radiation fibrosis (31% of cases), metastatic BC (24%), and primary or metastatic lung cancer (19%) were the most common causes.[7,8]

It is well known that OBC includes various subtypes, from hormone receptor-positive tumours to human epidermal growth factor receptor 2 (HER2) positive and triple-negative tumours. Negative ER/PR immunohistochemistry results do not exclude breast cancer diagnosis. Still, they are not enough for the diagnosis as different malignancies can synthesise hormone receptors, such as colon, ovarian, and endometrium. Blood tumour marker studies such as CEA and CA 15-3 can provide a more reliable diagnosis. Immunohistochemical stains for lactalbumin, CEA, ER, and PR are recommended for diagnosing breast cancer when in doubt.[9-11]

Conflict of Interests

The authors declare that they have no conflict of interest.

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Informed Consent

Written consent was obtained from the patient.

Authors' Contribution

Literature Review, Critical Review, Manuscript preparing held by all authors.

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Case Report

Persistent Stomach Pain in the Young Age Patient: A Case of Primary Gastric Burkitt's Lymphoma

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ABSTRACT

Lymphoma is the second most common gastric cancer, following gastric adenocarcinoma. Most gastric lymphomas are mucosa-associated lymphoid tissue lymphomas or diffuse large B-cell lymphomas. Primary gastric Burkitt's lymphoma is a subtype of non-Hodgkin lymphoma and represents an aggressive and rare malignity with the fewest cases reported globally. Primary gastrointestinal non-Hodgkin lymphoma is a rare condition. Burkitt's lymphoma is an aggressive form of B-cell lymphoma endemic to Africa while it is not endemic to the rest of the world. Here we presented a young immunocompetent male patient who had weight loss and was admitted with a stomachache. Upper gastrointestinal endoscopy and biopsy detected a large primary gastric Burkitt's lymphoma. While long-term survival rates are possible with early diagnosis and timely appropriate treatment, delay in treatment can be fatal for such patients.

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Keywords: *Burkitt's lymphoma, stomach ache.*

Introduction

Highly malignant Burkitt's lymphoma derived from B-cells was first described by Dennis Burkitt in 1958 in a Ugandan child's jaw. Burkitt's lymphoma is a B-cell non-Hodgkin lymphoma (NHL) with a high proliferation rate. After six years, Epstein-Barr virus (EBV), a gamma herpes virus, was isolated from cultured Burkitt's lymphoma cells.[1] This tumour is mainly observed in patients in Sub-Saharan Africa.[1] It is associated with the t (8;14) (q24;q32) translocation of C-myc and IgH genes; IgH-myc fusion is characteristic.[2] Sporadic forms are rarely diagnosed in medical centres in Europe and Asia, with 4 to 5 cases annually.[3] Although Burkitt's lymphoma is regarded as a nodal lymphoma, extranodal involvement is present in 80% of the cases.[3]

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Extranodal Burkitt's lymphoma is especially common in gastrointestinal, head, and neck areas. Also, though rare, bone marrow, genitourinary system, bone, central nervous system and liver involvement have been reported.[3,4] It is a rare condition in adults. Overall survival is less than five months in more than 60% of the cases; among the negative prognostic factors are bone marrow and central nervous system involvement and late diagnosis.[3] Regarding primary sporadic Burkitt's lymphoma of the stomach, few cases with the secondary spread from retroperitoneal Burkitt's lymphoma are diagnosed due to admittance with symptoms related to gastric cancer. However, to this day, less than 200 papers have been published on gastric Burkitt's lymphoma. In this paper, we are presenting an extraordinary case of aggressive Burkitt's lymphoma.

Case Report

A 19-year-old male patient presented with a stomachache, involuntary weight loss, and persistent stomach pain history in the last three months. The patient had no associated comorbidity, family history, drug use, smoking, alcohol or drug habit. In the physical examination, the epigastric region was stiff and tender during the abdominal examination. There was not any lymphadenopathy, hepatosplenomegaly or palpable mass. The biochemistry and complete blood count of the patient were normal. He had no anemia. EBV DNA or HIV RNA tests were not detected in the patient. The patient was initiated on proton pump inhibitor and anti-acid treatments. However, the patient's complaints were not subdued. In endoscopy of the upper

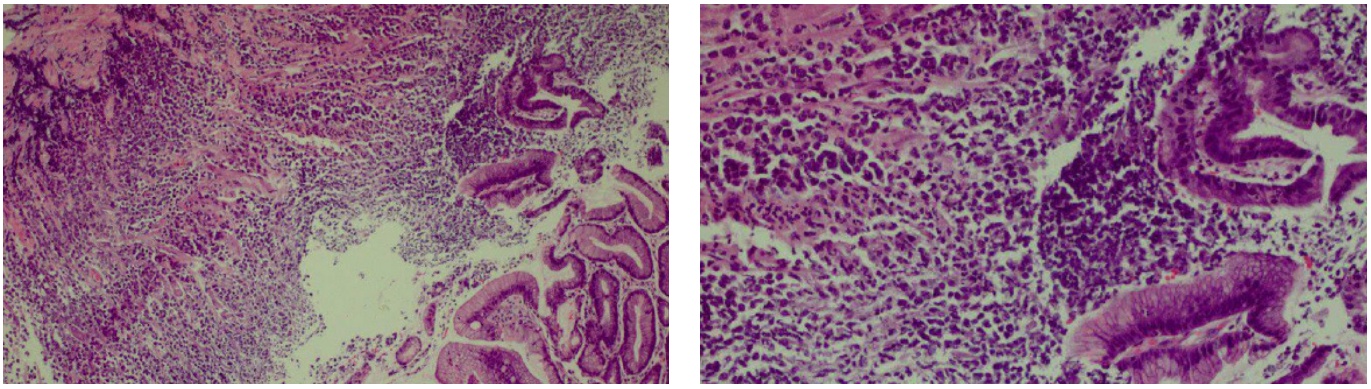

Figure 1. Microscopic findings of gastric Burkitt lymphoma. Proliferation of monomorphic medium-sized atypical lymphocytes with multiple mitotic figures and apoptotic figures in Hematoxylin-Eosin (x10).

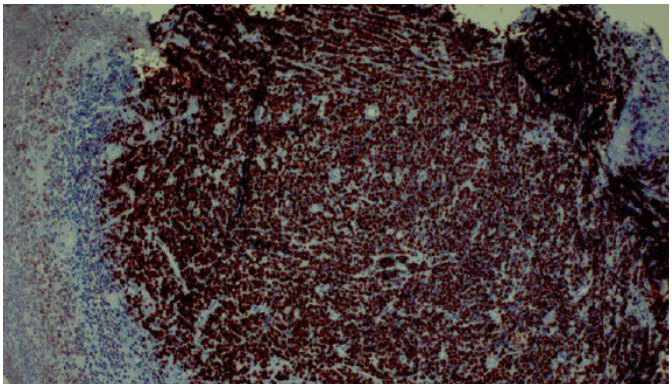

Figure 2. Ki67 staining was found close to 100% (x10).

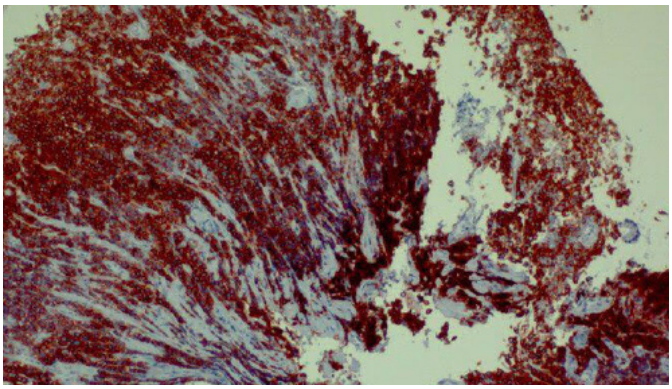

Figure 3. Immunohistochemical study revealed strong and diffusely positive neoplastic cells for LCA, c-myc, CD20 (x10).

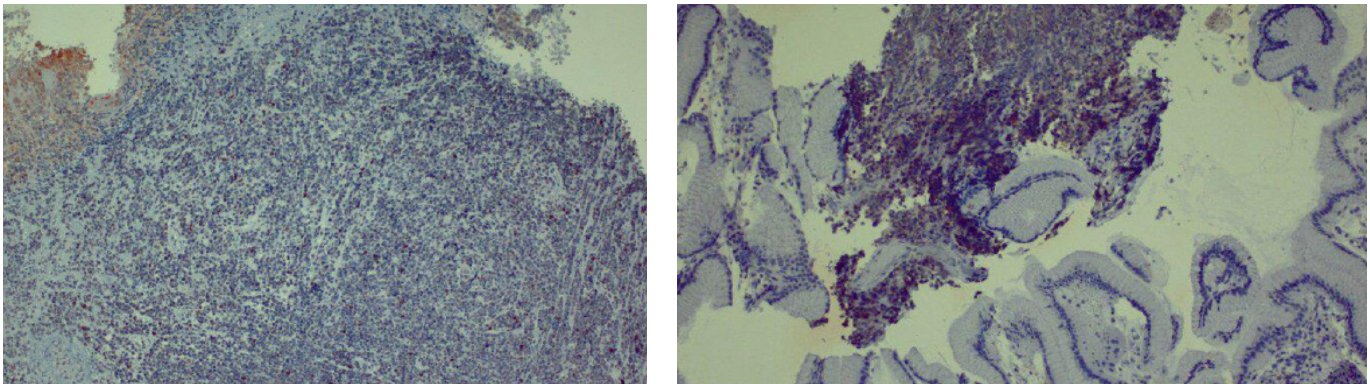

Figure 4. CD10 and bcl-2 were negative for tat, CD5, CD3 and cyclin D1.

gastrointestinal system, a giant malignant ulcer was detected in the antrum. Histological examination showed the proliferation of mid-sized monomorphic atypical lymphocytes containing many mitotic and apoptotic figures (*Figure 1*). The pathology was compatible with high-grade B-cell lymphoma. Ki67 staining was detected as near 100% (*Figure 2*). The immune histochemical study detected strong and diffuse positive neoplastic cells for LCA, c-myc, and CD20 (*Figure 3*). It was negative for CD10 and bcl-2, TdT, CD5, CD3, and cyclin D1 (*Figure 4*). MIB1 proliferation index was almost 100%. In the whole abdominal computed tomography, a filling defect with a wide opening and 42 mm diameter was observed in the gastric antrum level towards the lesser curvature. In PET-CT, in the stomach at the lesser curvature-antrum localization, increased F-18 FDG uptake (SUV max: 12.49) with approximately 4 cm diameter and soft tissue density concentrated in the region spanning towards the lumen was detected. No uptake was seen in the other areas of the body in PET-CT (*Figure 5*). The patient was referred to the hematology department for further tests and prompt treatment initiation.

Discussion

Burkitt's lymphoma is one of the most aggressive forms of B-cell NHL with a replication approaching 100% and has three clinical forms; endemic, sporadic and immunodeficiency-associated forms. An endemic variant is prevalent in Africa, while a sporadic variant is present in the USA and Western Europe, and the immunodeficiency variant is observed primarily in HIV patients. Sporadic variant includes 30% of pediatric lymphomas and less than 1% of adult NHLs.[5] The most commonly affected region, aside from lymph node involvement, is the gastrointestinal system (30-50%).[6] Primary gastrointestinal lymphoma is rare. Secondary involvement of the gastrointestinal system is common in lymphoma. Primary gastrointestinal lymphoma manifests with localized or mainly dominant symptoms in the gastrointestinal tract. Though gastric lymphomas are more common than intestinal lymphomas, primary gastric involvement is rare in Burkitt's lymphoma. For non-endemic Burkitt's lymphoma, the gastrointestinal system is the most common region, followed by retroperitoneal, kidney, ovary and testicular involvement.[7] Incidence of Burkitt's gastric lymphoma in adults is exceptionally rare. Burkitt's lymphoma is a highly aggressive malignancy and is one of the fastest-growing malignancies among human malignancies.[5] It requires immediate and aggressive treatment. Fortunately, regardless of being a rapidly growing malignancy, it responds to aggressive chemotherapy.[8]

In the literature, few cases related to this issue were found in the childhood age group. Our case is in the young age group. No new cases were found in the literature in the last ten years. Published patients present with mass effect, vomiting and abdominal pain. In our case, however, obstruction due to the mass did not occur yet, and only a persistent stomach was present. The presence of alarm symptoms is a priority in upper gastrointestinal endoscopy patients.[9] Our patient did not have any alarm symptoms. Duodenal ulcer, gastritis, and gastroesophageal reflux are most common in younger patients.[9]

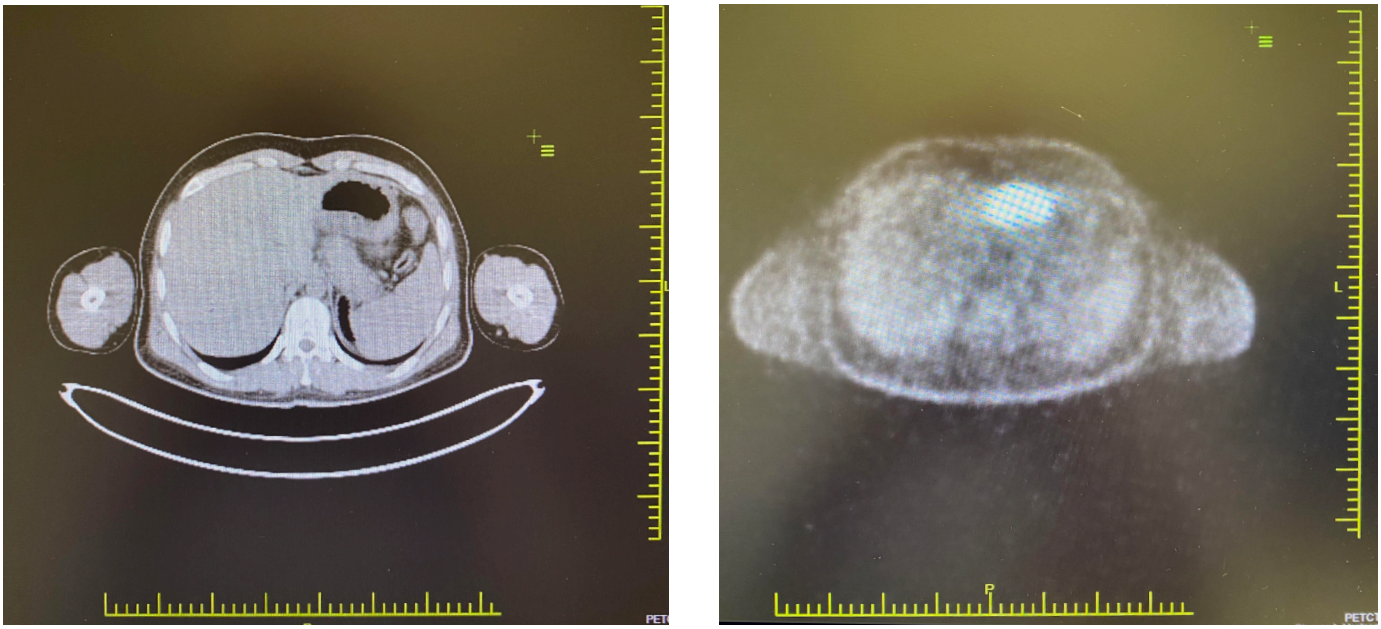

Figure 5. In PET-CT, intensely increased F-18 FDG uptake (SUV max: 12.49) was detected in the area extending to the lumen with a soft tissue density of approximately 4 cm in the small curvature-antrum localization of the stomach.

Conclusions

Gastric Burkitt's lymphoma is a rare form of non-Hodgkin lymphoma with a high rate of proliferation, aggressive nature, and poor prognosis in adult patients. Optimum treatment for this disease is still unknown. Aggressive chemotherapy should be a part of all treatment regimens. The advanced stage is associated with poorer outcomes in older age groups. While long-term survival rates of 70-80% may be possible with early diagnosis and timely appropriate treatment, delay in treatment might be fatal for these patients. Although it is not an alarming symptom, it should be kept in mind that gastric lymphoma can be seen in the young age group, and upper gastrointestinal endoscopy should be performed without delay. Early diagnosis of gastric lymphoma prolongs the life of the patient.

Ethical Approval

Our institution does not require ethical approval to publish an anonymous case report. Informed consent was obtained from the patient for the use and publication of data and images in the case report.

Conflict of Interests

The authors declare that they have no competing interests. The funders had no role in the design, conduct, analysis, or interpretation of data or in writing the manuscript.

Authors' Contribution

HH wrote the first draft of this paper. All authors approved the final version.

The case report has written in an anonymous characteristic. Thus secret and detailed data about the patient has been removed. Editor and reviewers can know and see these detailed data. These data are backed up by editors and by reviewers.

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Case Report

Isolated Unconjugated Hyperbilirubinemia in Adults: The Gilbert's Versus Criggler Najar Syndrome Type 2 Conundrum.

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ABSTRACT

Gilbert's syndrome is a genetic disorder characterised by non-hemolytic unconjugated hyperbilirubinemia. It is caused by mutations in the UGT1A1 gene which codes for the enzyme uridine diphosphate glucoronosyl transferase-1, which conjugates bilirubin for excretion. Affected individuals are usually asymptomatic apart from a mild jaundice and investigations reveal a mild isolated indirect hyperbilirubinemia. This may be exacerbated in the face of environmental and physical stressors. It is very similar in presentation to Criggler-Najjar syndrome (CNS) type 2. There is a small risk of kernicterus in patients with CNS type 2 needing daily phenobarbitone therapy. This risk is miniscule in Gilbert's syndrome. Genetic testing for polymorphisms of the UGT1A1 gene is the diagnostic clincher for Gilbert's syndrome, but it can also be picked up by evaluating the response to phenobarbitone and fasting, particularly in resource poor settings. Due to limited availability, case reports documenting the genetic mutational analysis are sparse. We reported one such rare case with an unusually high indirect hyperbilirubinemia in Gilbert's syndrome confirmed by both phenobarbitone response and genetic analysis.

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Introduction

Unconjugated hyperbilirubinemia is a cause for evaluation in adults. In the absence of hemolysis and liver disease, genetic disorders should be sought. Our case report shows that rarely, Gilbert's may present with a very high unconjugated hyperbilirubinemia in adults. Although genetic analysis clinches the diagnosis, in resource poor settings, the phenobarbitone test becomes particularly useful to differentiate it from Criggler-Najjar syndrome (CNS) type 2.

In CNS type 2 also there is unconjugated hyperbilirubinemia. However, unlike Gilbert's, the hyperbilirubinemia is much more marked and jaundice persists as serum bilirubin never touches normal. Intercurrent illnesses, fasting and stressors can raise bilirubin enough to even cause kernicterus in CNS type 2. Therefore a daily single bedtime dose of phenobarbitone is recommended in CNS type 2 whereas Gilbert's needs no specific therapy in daily life. Hence, a good clinical suspicion can distinguish the two and the right advice and diagnosis can help abate or manage further episodes of jaundice in patients of Gilbert's syndrome.

Case Report

A 40-year-old labourer presented to the emergency with complaints of yellowish discolouration of skin and eyes and passage of highly coloured urine for seven days. This was not associated with any pain abdomen, nausea, vomiting, the passage of clay-coloured stools or any pruritus. There was a history of heat exhaustion, loose stools and fever lasting 2-3 days, about a week back, after which the patient noticed deepening jaundice. On probing further, the patient gave a history of recurrent jaundice at least three to four times over the past five years. It was insidious and resolved on its own over a month each time. There was no history of addictions, blood transfusions, chronic ailments, weight loss, or intemperate habits. He was the fourth sibling out of six, born from a non-consanguineous marriage and father of four children. There was no history of similar complaints in the family. On general physical examination, he was icteric but with no other signs of liver cell failure. He was hemodynamically stable, and systemic examination was also unremarkable.

Investigations showed haemoglobin of 13.1 g/dL, a total leukocyte count of 10,000/mm^3 and a platelet count of 1.6 lac/mm^3. A liver function test (LFT) showed total bilirubin of 12.9 mg/dL, of which the indirect component was 10.1 mg/dL and the direct fraction was 2.8 mg/dL. Liver enzymes were normal, and there was no coagulopathy. The renal function test (RFT) had urea of 97 mg/dL and a creatinine of 2.8 mg/dL. Serum electrolytes and serum protein were within normal limits. Blood tests for hepatitis B, C and HIV were negative. No evidence of ongoing hemolysis, as confirmed by normal serum lactate dehydrogenase (LDH) and no hemoglobinuria. Direct and indirect Coomb's tests were both negative. Leptospira IgM and IgG were also both Negative. Ultrasonography of the abdomen was normal.

The patient seemed dehydrated, and the RFT also responded to hydration, bringing the creatinine to 1.2 mg/dL within two days, with adequate urine output. Since there was a history of recurrent jaundice and an isolated unconjugated hyperbilirubinemia, we suspected a genetic disorder of conjugation, our differentials being CNS Type 2 and Gilbert's syndrome. As the immediate genetic analysis was unavailable, oral phenobarbitone was empirically started in a dose of 60mg thrice a day. The patient was discharged with advice to follow up with serial LFTs. The fall in bilirubin was quite dramatic (as seen in *Figure 1*). As the patient had developed slight transaminitis, the dose of phenobarbitone was reduced to twice daily, yet the bilirubin continued to fall and turned normally. This proved the diagnosis of Gilbert's syndrome, with jaundice possibly ensuing the dehydration episode. Further, we withheld the phenobarbitone, and the patient's bilirubin remained normal.

The genetic analysis done in follow-up showed heterozygosity for UGT1A1*6 G71R and, mainly, a homozygous polymorphism for UGT1A1*28/28 genotype, A(TA)7TAA. This results in two extra (TA) bases in the promoter region of UGT1A1, which was suggestive of minimal enzyme activity and, thus, Gilbert's syndrome.

Discussion

The Gilbert syndrome, also known as Gilbert-Meulengracht syndrome after its discoverers in France and Germany, is a hereditary condition heralded by intermittent isolated unconjugated hyperbilirubinemia in the absence of any other cause, such as hepatocellular disease or hemolysis.[1] It is the most common congenital hyperbilirubinemia syndrome, occurring in 3-13% of the population and varying with ethnicity.[2,3] There is at least a 50% decrease in the hepatic bilirubin UGT activity.[4]

The patients are mostly unaware of their diagnosis as they have characteristic asymptomatic jaundice, usually noticed by the onlookers.[5] The degree of hyperbilirubinemia is typically less than 5 mg/dL, and conjugated bilirubin is less than 20% of the total bilirubin fraction.6 Our case is perhaps the only case of Gilbert's syndrome documented in the literature, with a high degree of hyperbilirubinemia.[7]

What is also noteworthy in Gilbert's syndrome is the response to phenobarbitone. This becomes particularly useful to differentiate it from its close friend, another cause of unconjugated hyperbilirubinemia in adults, CNS type 2. CNS type 2 is also a hereditary disorder of bilirubin metabolism characterized by marked unconjugated hyperbilirubinemia due to a much reduced (<10%) activity of hepatic bilirubin glucuronosyltransferase coded by UGT1A1. There is persistent jaundice as the bilirubin levels never reach normal and intercurrent illnesses, fasting, and other stressors may elevate bilirubin enough to cause kernicterus. Hence a single bedtime dose of phenobarbitone is recommended. CNS Type 2 is less severe than CNS type 1 where this hepatic enzyme is absent, and kernicterus, progressive bilirubin encephalopathy, ensues, causing mortality in most cases. The chances of kernicterus are very minimal in Gilbert's syndrome.

The phenobarbitone test is a simple test in which the fall in bilirubin levels in response to phenobarbitone administration has to be evaluated. In Gilbert's syndrome, following phenobarbitone administration, the bilirubin levels normalise entirely. In CNS type 2, the fall in bilirubin levels is usually more than 30 percent, but the levels never normalise. The dose of phenobarbitone used is 1-5 mg/kg/day, titrated to 60-180 mg/ day in single or divided doses.[8] Other tests that can be used as alternatives include the caloric deprivation test, in which an increase in jaundice can be seen after 48 hours of a 300 kcal/ day caloric deprivation. Patients with Gilbert's syndrome had a 3 to 5-fold exaggeration of the baseline bilirubin level.[9] Alternatively, phenytoin and phenazone have also been used in diagnosis. In genetic analysis, we found a homozygous polymorphism for UGT1A1*28/28 genotype, A(TA)7TAA, which is a commonly reported variant in the Indian ethnic group.[10]

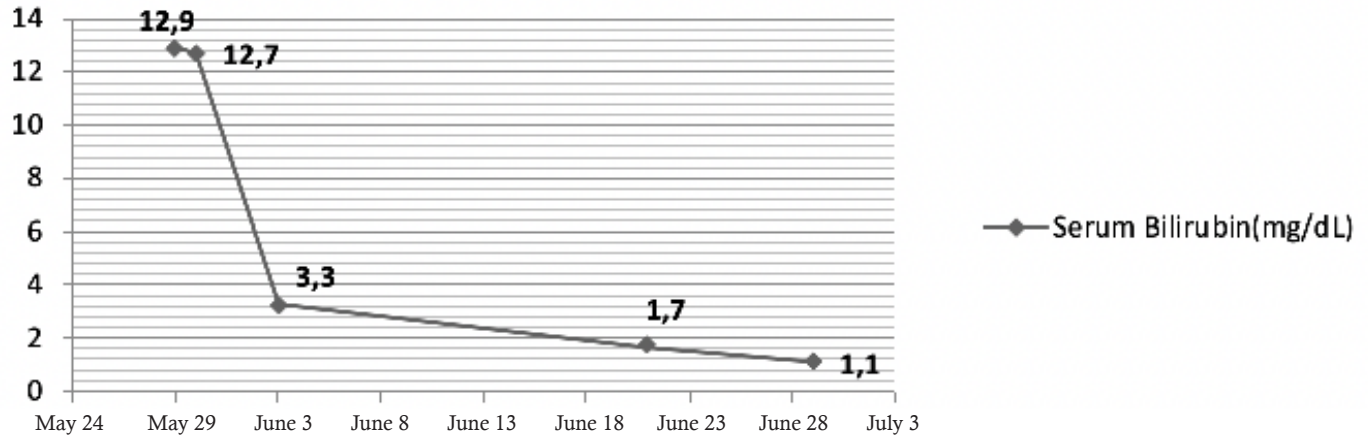

Figure 1. Showing fall of serum bilirubin after adding phenobarbitone on June 3.

In resource-poor settings where genetic confirmation is unavailable or delayed, the response to phenobarbitone can conveniently differentiate between CNS type 2 and Gilbert's syndrome. While CNS type 2 therapy is lifelong, Gilbert's syndrome may need only awareness and no continuous treatment. Patients must be sounded about triggers such as dehydration, intercurrent illnesses, exhaustive exercise, menstruation and hepatotoxic drugs, which can precipitate disproportionate jaundice.[5]

Concluisons

When evaluating a case of chronic megaloblastic anaemia, we should not forget this rare association with G-NETs. They can perpetuate the anaemia through ulceration and bleeding. There is also the risk, even in sporadic cases, for them to evolve into a malignant lesion, thus changing the patient's prognosis. The most commonly known is the association of gastric neuroendocrine tumour type I with macrocytic anaemia due to vitamin B12 deficiency. To our knowledge, there are no reports in the literature about an association of this type of tumour with folate deficiency-induced anaemia. Therefore we consider the publication of this case to help our colleagues.

Conflict of Interests

The authors declare that they have no conflict of interest.

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Informed Consent

Written consent was obtained from the patient.

Authors' Contribution

Literature Review, Critical Review, Manuscript preparing held by all authors.

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