Int J Clin Exp Pathol 2015;8(7):8038-8047
www.ijcep.com /ISSN:1936-2625/IJCEP0010508

Original Article

Effects of hypoxia and its relationship with apoptosis, stem cells, and angiogenesis on the thymus of children with congenital heart defects: a morphological and immunohistochemical study

A Bahar Ceyran[1], Serkan Şenol[1], Füsun Güzelmeriç[2], Eylem Tunçer[3], Aybala Tongut[3], Babürhan Özbek[3], Ömer Şavluk[2], Abdullah Aydın[1], Hakan Ceyran[3]

[1]Department of Pathology, Medeniyet University, Göztepe Training and Research Hospital, İstanbul, Turkey; [2]Department of Anesthesiology, Koşuyolu Training and Research Hospital, İstanbul, Turkey; [3]Department of Pediatric Cardiac Surgery, Koşuyolu Training and Research Hospital, İstanbul, Turkey

Received May 21, 2015; Accepted June 28, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Introduction: The thymus slowly involutes with age after puberty. Various stress conditions accelerate the involution of the thymus and cause changes in the histologic structure of the gland. Objective: The present study performed histomorphological and immunohistochemical (IHC) evaluations of the thymus glands removed during surgical repair in patients with cyanotic or acyanotic congenital heart disease (CHD). The thymus glands in the hypoxic group were compared to those in the non-hypoxic group. This study suggested that the activation of HIF-1 alpha promotes tumor progression and impair prognosis due to the inhibition of apoptosis, increased population of stem cells, and induction of angiogenesis also suggested that inactivation of HIF-1 alpha in tumor-infiltrated tissues could halt tumor progression and improve prognosis. Materials and methods: The study included 76 thymus glands removed from patients who underwent an operation due to CHD. Of these cases, 38 had cyanotic CHD, and constituted the hypoxic group. The remaining 38 patients had acyanotic CHD, and constituted the non-hypoxic group. IHC procedures were performed for HIF-1 alpha, FoxP3, CD44, Bcl-2, and CD34. Results: There were statistically significant differences between the hypoxic and non-hypoxic groups only in terms of medullary enlargement toward the cortex and effacement of the corticomedullary junction. In the immunohistochemical examination for five markers, staining intensity and staining rates increased with decreasing oxygen saturation. Conclusion: It can be concluded that the activation of HIF-1 alpha promotes tumor progression and impair prognosis due to the inhibition of apoptosis, increased population of stem cells, and induction of angiogenesis.

Keywords: Hypoxia, thymus, congenital heart defects, apoptosis, stem cells, angiogenesis, HIF-1 alpha, FoxP3, CD44, Bcl-2, CD34

Introduction

The thymus is a lymphoepithelial organ that is essential for the maturation of T lymphocytes, and it is located in the anterior mediastinum. Thymopoiesis involves reciprocal tissue interactions between the epithelial cells derived from the endoderm of the embryonic pharynx (branchial region) and neural crest-derived mesenchyme [1]. The thymus is a pyramid-shaped, bilobed organ covered by a capsule and divided into cortical and medullary segments. It attains its greatest relative weight toward the end of the fetal period, but its absolute weight increases to a maximum of 30 to 40 g at puberty. Thereafter, the gland slowly involutes with age [2].

The two major cell types are endodermal-derived epithelial cells and bone marrow-derived lymphocytes [3]. The cortex appears dark because it is densely populated by immature lymphocytes (cortical thymocytes). In the medulla, the lymphocytes are less densely packed than in the cortex. In addition to the thymic epithelial meshwork that forms the scaffolding, characteristic Hassall's corpuscles with concentric keratinization or central cavitation are

Table 1. The number, proportion, intensity and localization of staining of IHC markers

IHC Marker	The number and proportion of staining			The intensity and localization of staining
	+1	+2	+3	
HIF-1 α	≤5/1HPV	5-10/1HPV	>10/1HPV	Strong nuclear staining (average 10 HPV)
Fox P3	≤5/1HPV	5-10/1HPV	>10/1HPV	Medium/strong nuclear staining (average 10 HPV)
CD 44	≤40%	40-70%	>70%	Strong cytoplasmic membranous staining (average 10 HPV)
Bcl2	≤40%	40-70%	>70%	Medium/strong cytoplasmic membranous staining (average 10 HPV)
CD 34	≤10/1HPV	10-25/HPV	>25/1HPV	Strong cytoplasmic staining containing lumen (average 5 HPV)

HPV: high power view.

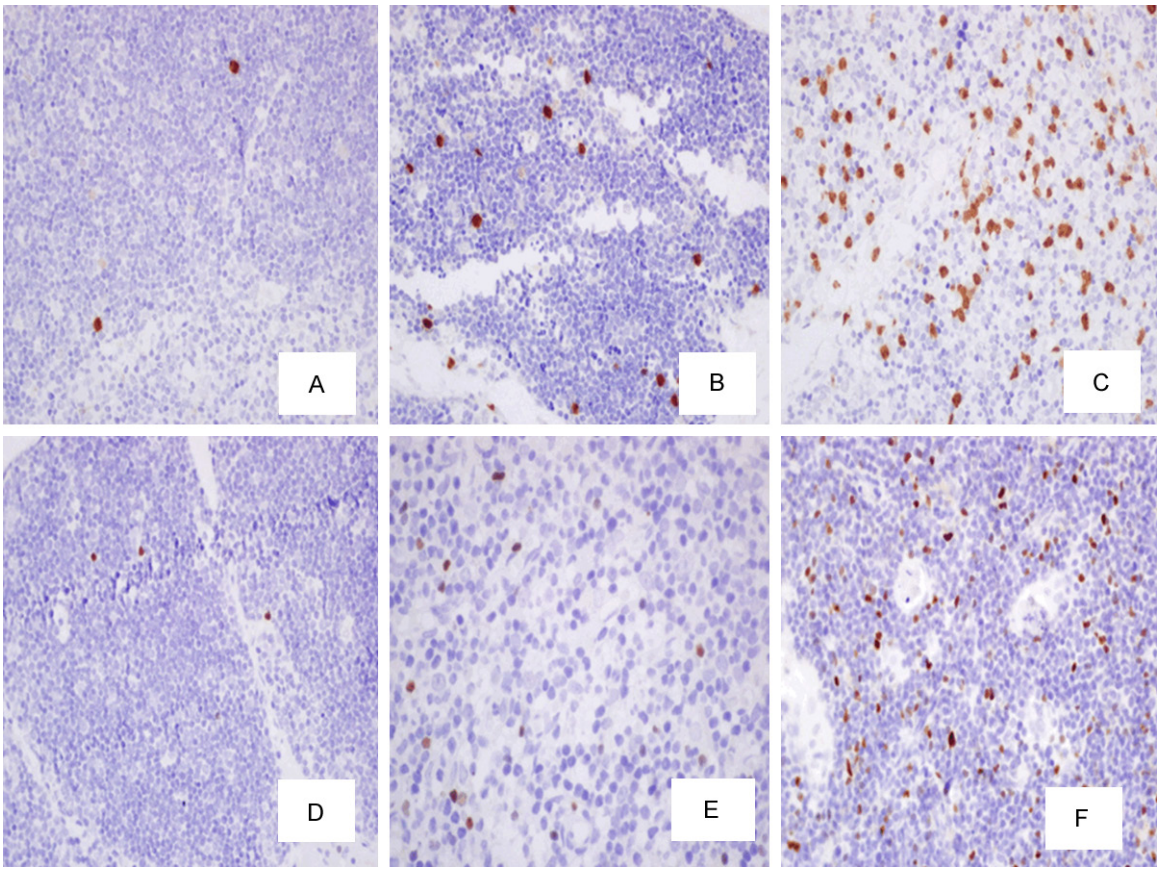

Figure 1. IHC staining for HIF-1 alpha and FoxP3. A. 1+ staining for HIF-1 alpha in nonhypoxic thymus, ×400, B. 2+ staining for HIF-1 alpha in hypoxic thymus, ×400, C. 3+ staining for HIF-1 alpha in hypoxic thymus, D. 1+ staining for FoxP3 in nonhypoxic thymus, ×400, E. 2+ staining for FoxP3 in hypoxic thymus, ×400, F. 3+ staining for FoxP3 in hypoxic thymus, ×400.

present [2]. Other cells normally present in the thymus include B cells, interdigitating reticulum cells, Langerhans cells, mast cells, eosinophils, and the usual non-specific types of stromal cells [3]. Between the cortex and medulla, the cortico-medullary junction is recognized as an area rich in blood vessels and a site where connective-tissue septa reach the medulla region [1].

Approximately 0.4% to 1.2% of infants are born with moderate or severe congenital heart defects (CHD) [1, 4]. These CHD are etiologically heterogeneous, and genetic and environmental causes have been proposed for many specific defects [1, 5].

The most common acyanotic birth defect is ventricular septal defect (VSD), and the most

Table 2. The distribution of histomorphological findings

Histomorphological findings	All cases		Hypoxia Group		Non-hypoxia Group		P
	N=76	%	N=38	%	N=38	%	
*Cortex:Medulla ratio (≤1/2) and effacement of the corticomedullary junction	48	63.2	36	94.7	12	31.6	*P<0,05
Cortical "starry sky" appearance	45	59.2	24	63.2	21	55.3	
Changes in Hassall's corpuscles (increase number, cystic enlargement, calcification)	72	94.7	38	100	34	89.5	

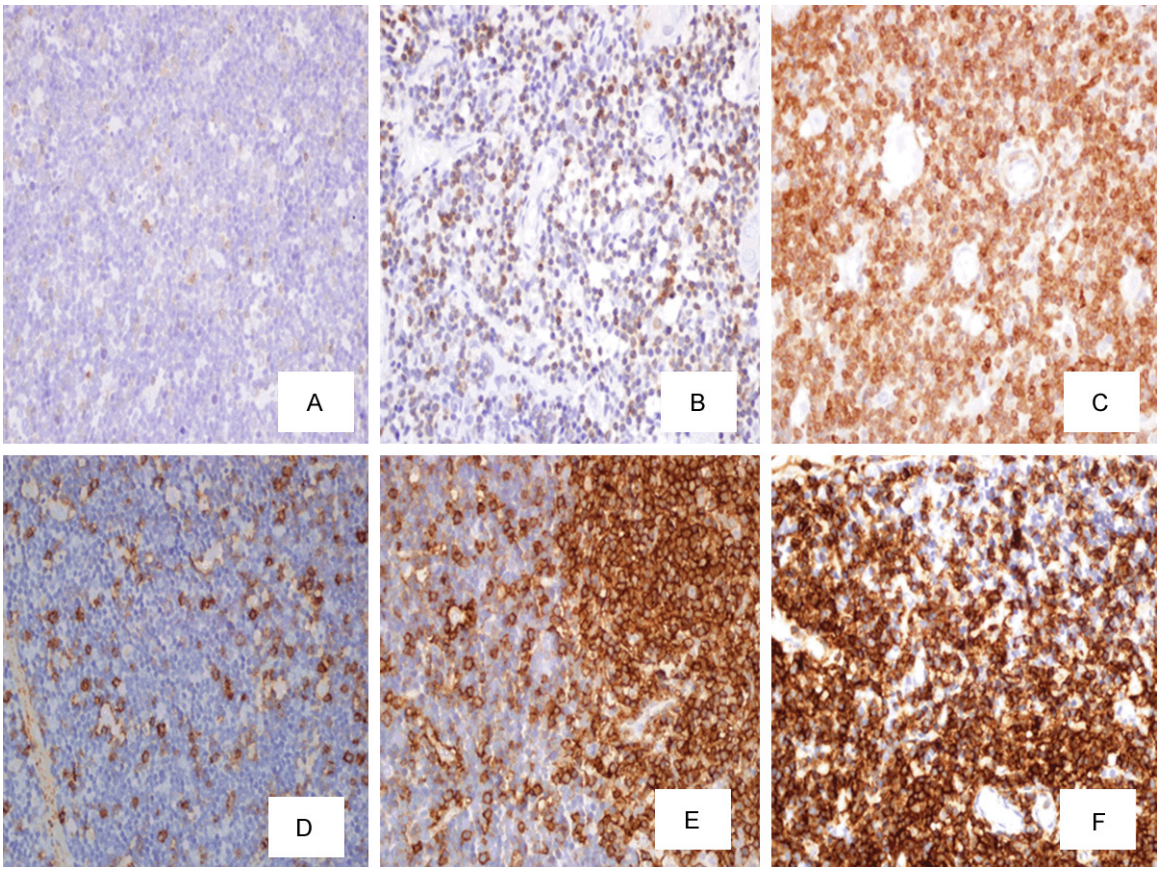

Figure 2. IHC staining for Bcl-2 and CD 44. A. 1+ staining for Bcl-2 in nonhypoxic thymus, ×400, B. 2+ staining for Bcl-2 in hypoxic thymus, ×400, C. 3+ staining for Bcl-2 in hypoxic thymus, D. 1+ staining for CD44 in nonhypoxic thymus, ×400, 2E) 2+ staining for CD44 in hypoxic thymus, ×400, F. 3+ staining for CD44 in hypoxic thymus, ×400.

common cyanotic birth defect is tetralogy of Fallot (TOF) [4, 6]. Congenital heart diseases result in hypoxia, pulmonary hypertension, recurrent pulmonary infections, malnutrition, and growth retardation in the childhood period. Various stress conditions accelerate the involution of the thymus and cause changes in the histologic structure of the gland [7].

A partial or total thymectomy is frequently performed to improve access to the heart and great vessels in pediatric cardiac surgery [8].

The present study performed histomorphological and immunohistochemical (IHC) evaluations of the thymus glands removed during surgical repair in patients with cyanotic or acyanotic CHD. The thymus glands in the hypoxic group were compared to those in the non-hypoxic group. Histomorphological changes in the thymus gland are characterized by apoptosis of cortical thymocytes occurring in the early stages of hypoxic stress and phagocytosis by the macrophages. The changes in Hassall's corpuscles and effacement of the cortico-medullary junction are striking findings in the later stages. In the IHC examination, HIF-1 alpha, FoxP3, CD44, Bcl-2, and CD34 expressions were assayed.

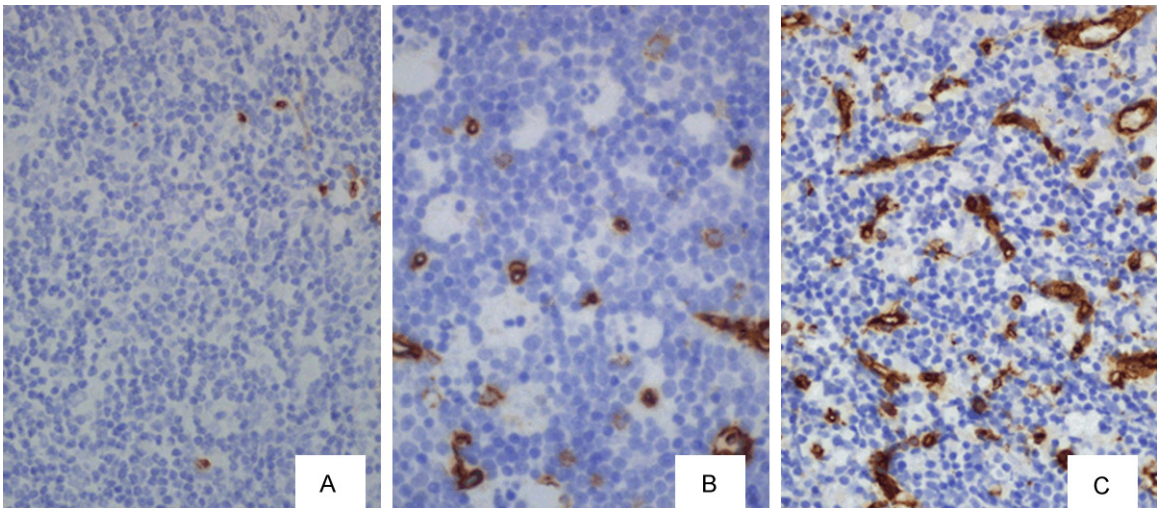

Figure 3. İHC staining for CD34. A. 1+ staining for CD34 in nonhypoxic thymus, ×400, B. 2+ staining for CD34 in hypoxic thymus, ×400, C. 3+ staining for CD34 in hypoxic thymus, ×400.

HIF-1 alpha is a basic helix-loop-helix heterodimeric complex composed of a novel HIF-1 alpha subunit and HIF-1 beta [9]. HIF-1 alpha has been shown to up-regulate several genes to promote survival in hypoxic environments. The HIF-1 alpha expression also plays a role in cancer. There is evidence that tumor hypoxia promotes metastasis through the induction of MET overexpression by HIF-1 alpha [10].

FoxP3 is a member of the forkhead/winged-helix family of transcriptional regulators and is highly conserved across mammals. It is essential for normal immune homeostasis; FoxP3 is the key transcription factor for the differentiation of regulatory T cells. It is stably and constitutively expressed at a high level in CD25+ CD4 positive regulatory T cells. Hypoxia promotes FoxP3 and regulatory T-cell function [11].

CD44 is a cell surface glycoprotein expressed in lymphocytes, monocytes, and granulocytes [12]. CD44 is a multistructural and multifunctional cell surface molecule in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, and growth factors [13]. The HCELL glycoform was originally discovered on human hematopoietic stem cells and leukemic blasts and subsequently identified on cancer cells [14].

Bcl-2 is an integral inner mitochondrial membrane protein and is frequently overexpressed in many lymphoid malignancies. This protein also interferes with programmed cell death independent of promoting cell division.

CD34 is expressed in the capillary endothelial cells and also in immature hematopoietic stem/progenitor cells, embryonic fibroblasts, and rare glial cells in nervous tissue. The CD34 protein is a member of a family of single-pass transmembrane sialomucin proteins [15].

The aim of the present study was to investigate the relationship between hypoxia and apoptosis, regulator T cells, stem cells, and angiogenesis. The following phase of the study planned to investigate the association of HIF-1 alpha activation related to hypoxic stress in tumor-infiltrated tissues in various organs with poor prognosis. The authors hypothesize that inactivation of HIF-1 alpha in tumor-infiltrated tissues could halt tumor progression and improve prognosis.

Materials and methods

The study included 76 thymus glands removed from patients who underwent an operation due to CHD at İstanbul Kartal Koşuyolu Education and Research Hospital between 2013 and 2015. Of 76 patients, 37 were females and 39 were males. All patients had CHD. Of these cases, 38 had blood oxygen saturation below 85% and constituted the hypoxic group. The remaining 38 patients had acyanotic CHD, and blood oxygen saturation was above 95% (**Table 1**). Of 38 patients in the hypoxic group, 17 were

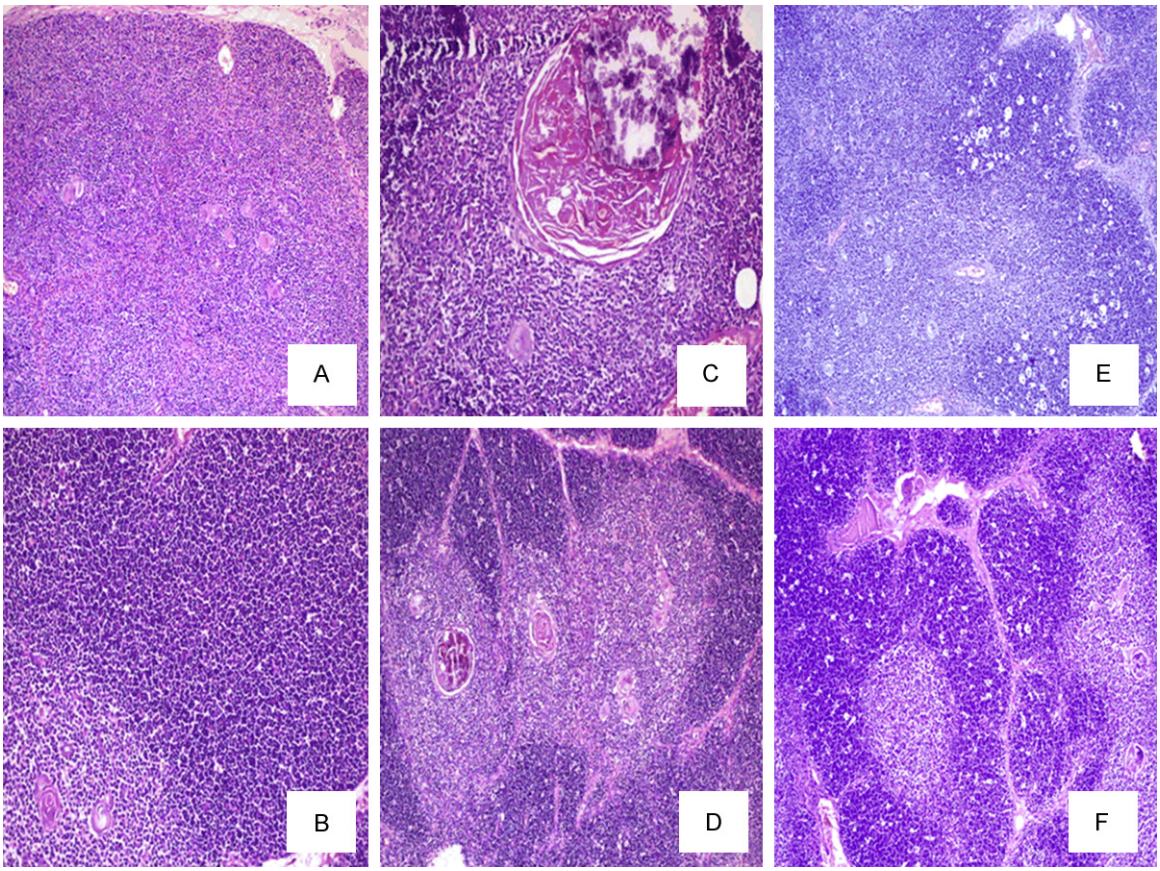

Figure 4. Histomorphological findings in hypoxic and nonhypoxic thymus. A. Cortical thinning and effacement of the corticomedullary junction in hypoxic thymus, ×200, H&E, B. Thick cortex and distinctive corticomedullary junction in nonhypoxic thymus, ×200, H&E, C. Hassall's corpuscle, cystic enlargement in the hypoxic thymus, ×200, H&E, D. Increase in the number or size of the Hassall's corpuscles in nonhypoxic thymus,×100, H&E, E. Starry sky appearance in hypoxic thymus, ×100, H&E, F. Starry sky appearance in nonhypoxic thymus, ×100, H&E.

males and 21 were females. Of 38 patients in the non-hypoxic group, 22 were males and 16 were females. The mean age was 1.65±4.32 years (range: 0 months-2.5 years) in the hypoxic group and 3.25±9.29 years (range: 1 month-12 years) in the non-hypoxic group.

After subtotal or total resection of the thymus glands during surgical repair of cardiac defects, the specimens were fixated in 10% formalin solution for 24 hours. After fixation, two pieces were taken from each thymus gland. Thymus pieces were embedded in paraffin blocks after routine visualization using an automated imaging device (Leica) and 4-5 micron thick sections were stained with H.E.

Histomorphological assessment

H.E.-stained sections were assessed in terms of the ratio of thymic cortex to medulla, clarity of corticomedullary junction, "starry sky" appearance in the cortex, and changes in Hassall's corpuscles (number, size, cystic enlargement, and calcification). Histomorphological features were assessed using qualitative methods.

Immunohistochemical methods

IHC staining was performed with a Leica Bondmax automated staining device. In all cases, IHC procedures were performed for HIF-1 alpha, FoxP3, CD44, Bcl-2, and CD34. For the formalin-fixed, paraffin-embedded tissue sections (thickness 4-5 micron), as well as the suitable positive and negative controls, the standard technique (avidin-biotin-peroxidase) was applied. The primary antibodies HIF-1 alpha (rabbit monoclonal, clone EP1215Y, dilution 1:100-1:300, Biocare Medical, Concord, CA, USA), FoxP3 (rabbit monoclonal, clone SP97, dilution

Table 3. The number and percentage of cases according to the staining intensity and proportion of IHC markers

IHC Marker	Hypoxic Group						Non-hypoxic Group					
	1+		2+		3+		1+		2+		3+	
	n	%	n	%	n	%	n	%	n	%	n	%
HIF-1α	38	0	10	26.3	28	73.7	36	94.7	2	5.3	0	0
Fox P3	38	0	12	31.6	26	68.4	35	92.1	3	7.9	0	0
CD 44	38	0	4	10.5	34	89.5	31	81.6	7	18.4	0	0
Bcl2	38	0	7	18.4	31	81.6	32	84.2	6	15.8	0	0
CD 34	38	0	10	26.3	28	73.7	35	92.1	3	7.9	0	0

1:100, Spring Bioscience, Pleasanton, CA, USA), CD44 (mouse, clone 156-3C11, dilution 1:100-1:200, Thermo Scientific, CA, USA), Bcl-2 (mouse, clone 100, Ready-to-Use, BioGenex, San Ramon, CA, USA), CD34 (mouse monoclonal, clone QBend 10, Ready-to-Use, Genemed, South San Francisco, CA, USA) were used.

As a positive control, normal breast tissue was used for HIF-1 alpha, tonsils were used for FoxP3, Bcl-2, and CD34, and benign urothelium was used for CD44.

Immunohistochemical assessment

In IHC assessment, a semi-quantitative assessment was performed based on nuclear or cytoplasmic membranous staining for the markers. For each marker, the intensity and proportion of staining were scored from 1 positive (+) to 3 positive (+++) using a semi-quantitative scoring system.

For HIF-1 alpha, strong nuclear staining: ≤5/1 HPF: 1+; 5-10/1 HPF: 2+; >10/1 HPF: 3+.

For FoxP3, moderate-strong nuclear staining: ≤5/1 HPF: 1+; 5-10/1 HPF: 2+; >10/1 HPF: 3+.

For CD44, strong cytoplasmic membranous staining: ≤40%: 1+; 40-70%: 2+; >70%: 3+.

For Bcl-2, moderate- strong cytoplasmic membranous staining: ≤40%: 1+; 40-70%: 2+; >70%: 3+.

For CD34, luminal and cytoplasmic strong staining: ≤10/1 HPF: 1+; 10-25/1 HPF: 2+; >25/1 HPF: 3+.

The number and percentage of cases according to the staining intensity and proportion of IHC markers are shown in **Table 1**.

A total of ten high power fields were taken into consideration for HIF-1 alpha, FoxP3, CD44, and Bcl-2, and the average of the densest five HPFs was taken for CD34.

Statistical methods

The statistical analysis was conducted using IBM SPSS Statistics version 19.0 (IBM Corporation, New York, USA) software package. The numeric variables were expressed as mean and standard deviation (mean ± SD), and categorical variables were expressed as frequency and percentage (n, %). The numeric data were compared using the Mann-Whitney U-test, and the categorical data were compared using the chi-square (χ^2) test. The Spearman's rank correlation test was used to examine the relationship between the results of immunohistochemical staining and arterial blood oxygen saturation. Two-sided *P* values less than 0.05 were considered statistically significant.

Results

Histomorphological findings of the patients in the two groups were assessed using a qualitative method.

Cortical thinning, medullary enlargement, and decrease in the cortex: medulla ratio (cortex: medulla <1/2): This finding was observed in 48 out of 76 cases (63.2%). There was effacement of the corticomedullary junction in 48 cases that had also more than twofold medullary enlargement compared to the cortical area. Of 48 cases, 36 were in the hypoxic group and 12 were in the non-hypoxic group. Finding #1 was observed in 36 out of 38 cases (94.7%) in the hypoxic group and 12 out of 38 cases (31.6%) in the non-hypoxic group.

There was a significant difference between the hypoxic group and non-hypoxic group in terms of Finding #1. In the hypoxic group, the medulla was enlarged compared to the cortex; there was remarkable cortical thinning and effacement of the corticomedullary junction (**Figure 1A**, **1B**).

Cortical "starry sky" appearance: This finding was observed in 45 out of 76 cases (59.2%). This finding was substantially remarkable in only one case. Of these 45 cases, 24 (53.3%) were in the hypoxic group and 21 (46.7%) were in the non-hypoxic group. Finding #2 was observed in 24 out of 38 cases (64.4%) in the hypoxic group and 21 out of 38 cases (55.3%) in the non-hypoxic group.

No significant difference was observed between the hypoxic group and non-hypoxic group in terms of Finding #2 (**Figure 1C**, **1D**).

Changes in Hassall's corpuscles: There was a change in the number or size of the Hassall's corpuscles, cystic enlargement in the enlarged corpuscles, and rare calcifications in 72 out of 76 cases (94.7%). The changes in Hassall's corpuscles were observed in all except four cases, which were in the non-hypoxic group.

No significant difference was observed between the hypoxic group and non-hypoxic group in terms of Finding #3 (**Figure 1E**, **1F**).

The distribution of histomorphological findings is presented in **Table 2**.

The results of immunohistochemical examination on thymus glands of the patients were grouped as the following:

Results of HIF-1 alpha assay: Of 38 patients in the hypoxic group, 28 (73.7%) showed 3+ staining, and 10 (26.3%) showed 2+ staining for HIF-1 alpha. No patients showed 1+ staining. Of 38 patients in the non-hypoxic group, 36 (94.7%) showed 1+ staining, 2 (5.3%) showed 2+ staining, and none of the patients showed 3+ staining. The rate of staining for HIF-1 alpha was significantly higher in the hypoxic group than in the non-hypoxic group ($P<0.005$) (**Figure 2A-C**).

Results of FoxP3 assay: Of 38 patients in the hypoxic group, 26 (68.4%) showed 3+ staining, 12 (3.16%) showed 2+ staining, and none of the patients showed 1+ staining. Of 38 patients in the non-hypoxic group, 35 (92.1%) showed 1+ staining, 3 (7.9%) showed 2+ staining, and none of the patients showed 3+ staining. The rate of staining for FoxP3 was significantly higher in the hypoxic group than in the non-hypoxic group ($P<0.05$) (**Figure 2D**, **2E**, **2F**).

Results of Bcl-2 assay: Of 38 patients in the hypoxic group, 31 (81.6%) showed 3+ staining, 7 (18.4%) showed 2+ staining, and none of the patients showed 1+ staining. Of 38 patients in the non-hypoxic group, 32 (84.2%) showed 1+ staining, 6 (15.8%) showed 2+ staining, and none of the patients showed 3+ staining. The rate of staining for Bcl-2 was significantly higher in the hypoxic group than in the non-hypoxic group ($P<0.05$) (**Figure 3A-C**).

Results of CD44 assay: Of 38 patients in the hypoxic group, 34 (89.5%) showed 3+ staining, 4 (10.5%) showed 2+ staining, and none of the patients showed 1+ staining. Of 38 patients in the non-hypoxic group, 31 (81.6%) showed 1+ staining, 7 (18.4%) showed 2+ staining, and none of the patients showed 3+ staining. The rate of staining for CD44 was significantly higher in the hypoxic group than in the non-hypoxic group ($P<0.05$) (**Figure 3D-F**).

Results of CD34 assay: Of 38 patients in the hypoxic group, 28 (73.7%) showed 3+ staining, 10 (6.3%) showed 2+ staining, and none of the patients showed 1+ staining. Of 38 patients in the non-hypoxic group, 35 (92.1%) showed 1+ staining, 3 (7.9%) showed 2+ staining, and none of the patients showed 3+ staining. The intensity of microvasculature observed with CD34 staining was significantly higher in the hypoxic group than in the non-hypoxic group ($P<0.05$) (**Figure 4A-C**).

The number and percentage of cases according to the staining rates in the assay are presented in **Table 3**.

When staining rates were compared to blood oxygen saturation of the patients, the staining rates for five markers were significantly higher in the hypoxic group than in the non-hypoxic group. The rate of staining increased as blood oxygen saturation decreased (Figure S1).

Discussion

Owing to the close relationship between embryonic development of the thymus gland and the

heart, similar changes have been reported to occur in thymus glands of children with CHD [1, 16]. Congenital heart disease is one of the most common phenotypic manifestations of 22q11 deletion syndrome, which has been reported to be involved in 14-18% of patients with ventricular septal defect (VSD) and 10% of infants with tetralogy of Fallot [17]. This chromosomal disorder is commonly associated with other extracardiac anomalies involving the head-neck region. These anomalies are associated with a group of syndromes known collectively as "CATCH 22", which also includes thymus aplasia or hypoplasia. Chaoui et al. suggested that ultrasonographic absence or hypoplasia of the fetal thymus could be used as the marker of cardiac defects and chromosome 22q11 deletions [18].

The review of the literature shows that histomorphological changes in Hassall's corpuscles are the most common conditions observed in the thymus glands of children with CHD [1, 7, 19]. The increases in the number and size of the corpuscles, cystic central enlargement, and degenerative changes as calcifications are common findings. In 76 patients in the present study, all but four patients showed changes in Hassall's corpuscles. These changes did not significantly differ between the hypoxic and the non-hypoxic group. Similarly, there was no significant difference between the two groups in terms of cortical starry sky appearance. Although these patients did not develop hypoxia, it is suggested that this finding is associated with morphological and functional disorders of the thymus gland commonly accompanying CHDs and early occurrence of thymic involution changes.

There were statistically significant differences between the hypoxic and non-hypoxic groups only in terms of medullary enlargement toward the cortex and effacement of the corticomedullary junction. The cortical thinning and enlargement of the medullary region compared to the cortical region are considered to be caused by the loss of thymocytes and increased cortical and medullar vasculature associated with hypoxia. Owing to the dense vascular network, particularly in the corticomedullary junction, effacement in the junction occurs as a result of increasing vascularization from this region toward the cortex. The findings of IHC support this notion. In the immunohistochemical examination for five markers, staining intensity and staining rates increased with decreasing oxygen saturation.

Oxygen deprivation reduces the ability of cellular growth and survival. The exposure to hypoxia activates HIF-1 alpha and thereby causes changes in the expression of various genes in order to increase oxygen supply to the tissues and cellular survival. Thus, HIF-1 alpha contributes to the maintenance of oxygen homeostasis by inducing erythropoiesis, glycolysis, and angiogenesis [9, 20]. The higher rate of staining for HIF-1 alpha parallel to the decreases in oxygen saturation in the present study was associated with the activation of HIF-1 alpha in order to maintain oxygen homeostasis.

HIF-1 alpha-dependent Bcl-2 expression was reported in order to prevent hypoxia-induced apoptosis [9, 20]. In the present study, the intensity and rate of staining for Bcl-2 in cortical thymocytes significantly increased parallel to the increase in HIF-1 alpha that was associated with decreasing oxygen saturation. Increased staining for Bcl-2 indicates the presence of hypoxia-induced apoptosis and a loss of cortical thymocytes. The loss of cortical thymocytes results in cortical thinning, as evidenced by histomorphological examination (**Figure 1A**).

Previous studies suggested that hypoxia and HIF-1 alpha could have a regulatory role in the development, survival of, and cytokine production by T cells [11, 21-23].

FoxP3 is a key transcription factor strongly induced by hypoxia for the differentiation of regulator T cells. Further studies showed that oxygen availability promotes Tregs through a T cell intrinsic HIF-1 alpha pathway [11]. The increase in the intensity and rate of staining for FoxP3 parallel to the decrease in oxygen saturation and increase in HIF-alpha is consistent with the current literature (**Figure 2**).

CD44 is a marker of stem cells and cancer stem cells that has many essential functions in both cell types. CD44 is required for the initial interactions of hematopoietic progenitor cells with thymic stroma that promote the initiation of thymocyte development [24]. In the present study, substantial increases in the intensity

and rate of staining for CD44 in parallel to the decrease in oxygen saturation in the hypoxic group indicate stimulator effects of hypoxia and HIF-1 alpha expression on the stem cells (hematopoietic progenitor).

CD34 is a capillary endothelial cell marker, and it was used to identify the intensity of the microvasculature in the present study. The intensity of microvasculature was significantly higher in the hypoxic group than in the non-hypoxic group. The intensity of microvasculature increases parallel to the increase in HIF-1 alpha expression, which is induced by the decrease in oxygen saturation. The increase in the intensity of microvasculature causes enlargement in the medulla, in which neovascularization is more prominent, and effacement of the corticomedullary junction due to vessel proliferation extending into the cortex.

Previous studies also reported the induction of angiogenesis by overexpression of HIF-1 alpha that mediates VEGF secretion and c-MET protein expression in mesenchymal cells under hypoxic conditions [25].

Conclusion

The authors of the present study consider that HIF-1 alpha activation under hypoxic conditions induces apoptosis, regulator T cell differentiation, and the stimulation of stem cells and angiogenesis. Tumor development is known to produce a hypoxic state in the tissue. It can be concluded that the activation of HIF-1 alpha promotes tumor progression and impair prognosis due to the inhibition of apoptosis, increased population of stem cells, and induction of angiogenesis. It is suggested that inactivation of HIF-1 alpha in tumor-infiltrated tissues could halt tumor progression and improve prognosis. In the second phase of the study, the authors plan to investigate the relationship between HIF-1 alpha activation in tumor-infiltrated tissues in various organs and poor prognosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. A Bahar Ceyran, Department of Pathology, Medeniyet University, Göztepe Training and Research Hospital, İstanbul, Turkey. E-mail: baharceyran@gmail.com

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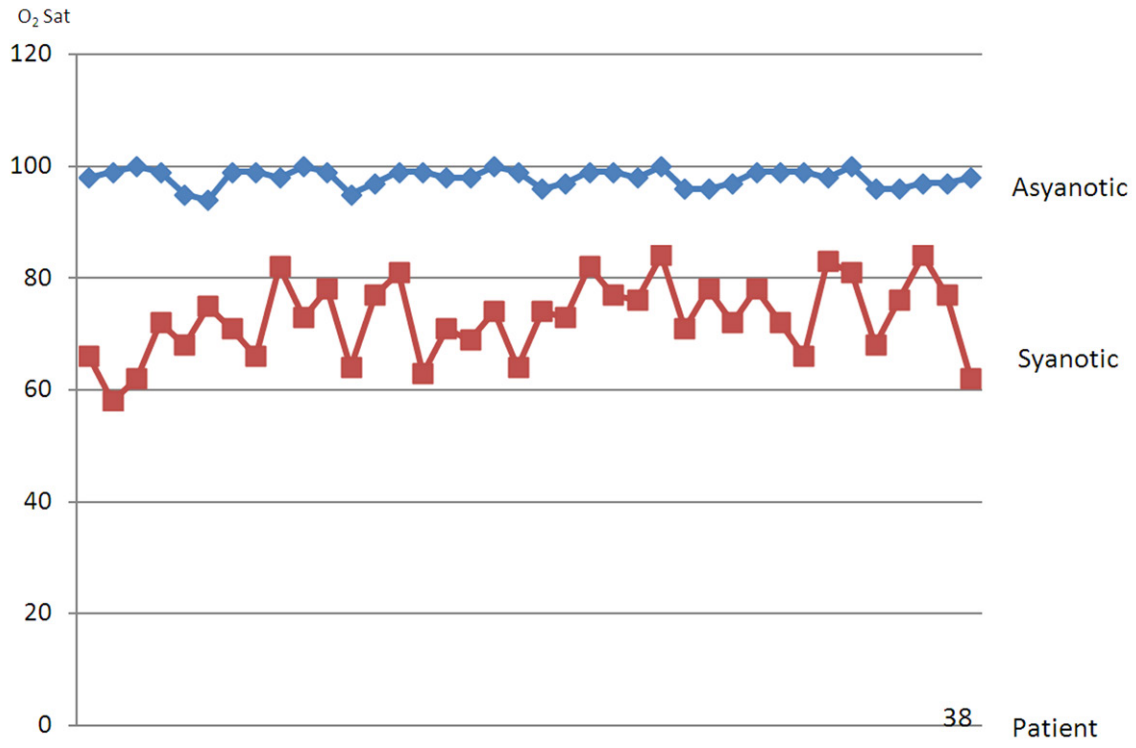

Figure S1. Patient's O_2 Saturations.