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Wnt1 and β-Catenin Expression in Lobular Capillary Hemangioma: Immunohistochemical Study

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ABSTRACT

Introduction: Lobular capillary hemangioma (LCH) is a vascular neoplasia with a pathogenesis that has not been fully clarified. The Wnt signaling pathway plays an essential role in vasculogenesis and angiogenesis. The increased activity in the Wnt signaling pathway may cause some neoplastic proliferations. This study revealed the role of the Wnt1/β-catenin signaling pathway in the development of LCH.

Methods: The study included 30 LCH tissue samples. Vascular structures in healthy tissue surrounding LCH lesions were used as controls. Wnt1 and β-catenin protein expressions in tumor tissue and surrounding tissue vascular structures were evaluated using the immunohistochemical method.

Results: Wnt1 expression was 1.10±0.16 and 0.33±0.10 in the LCH and control groups, respectively, indicating a statistically significant difference ($p<0.001$). β-catenin expression was 1.57±0.39 and 0.59±0.18 in the LCH and control groups, which was also statistically significant ($p<0.001$). Wnt1 and β-catenin expressions were similar in the mucosal and cutaneous LCH lesions ($p>0.05$).

Conclusion: In LCH, the Wnt1/β-catenin signaling pathway expression is increased. This increase may be due to the increased *Wnt1* gene expression, pathologies in different signaling pathways, or paracrine factors secreted from the tumor microenvironment. Treatment modalities targeting the Wnt1 signaling pathway may be promising for treating LCH.

Keywords: β-catenin, hemangioma, immunohistochemistry, pyogenic granuloma, Wnt β-catenin signaling pathway

Introduction

Lobular capillary hemangioma (LCH), a prevalent type of vascular lesion that affects the skin and mucous membranes, commonly emerges in young adults and children and affects both genders uniformly. Until recently, LCH was considered a vascular proliferation reactive to certain predisposing factors, including chronic trauma, inflammation, and hormonal factors. However, as knowledge of vascular proliferations and vascular tumor pathogenesis increases, the idea that LCH is a true neoplasia is becoming more commonly accepted (1).

During embryonic development, the Wnt signaling pathway is responsible for cell patterning, polarity and migration, cell fate determination, and regeneration of organs and tissues. Besides, there is increasing evidence that Wnt has a significant impact on angiogenesis and vasculogenesis (2). The Wnt family consists of 19 glycoproteins. These proteins use canonical or non-canonical pathways, depending on whether they use β-catenin as a downstream effector. In the canonical Wnt pathway, when Wnt is not present, a protein complex consisting of axin, adenomatous polyposis coli, and glycogen synthase kinase-3β (GSK3β) phosphorylates the cytosolic β-catenin. Upon Wnt proteins binding to Frizzled (Fz) proteins in the presence of coreceptor LDL-receptor-associated protein 5 or 6, the phosphorylation complex containing GSK3β is inhibited, and cytosolic β-catenin accumulates and then β-catenin translocates from the cytoplasm into the nucleus. Following this translocation, β-catenin interacts with transcription factors from the T-cell factor/lymphocyte enhancing factor family and modulates the expression of multiple target genes (2). The impairment in the Wnt signaling pathway can cause developmental defects, degenerative diseases, and some cancers. In the development of infantile hemangioma, one of the vascular tumors, the canonical Wnt pathway activity is increased. Similarly, an increase in β-catenin expression has been reported in pulmonary sclerosis hemangiomas (3,4).

Wnt1 in the chromosome 12q13 region is a member of the Wnt family that uses the canonical signaling pathway. *Wnt1* gene transfection into human umbilical vein endothelial cells has shown that it causes hyperproliferation in endothelial cells (5). In addition, the increased

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Wnt1 expression has been reported in some tumors, such as melanoma, breast cancer, and basal cell carcinoma (6).

In this study, we aimed to elucidate the involvement of the Wnt1/β-catenin signaling pathway in the pathogenesis of LCH.

Methods

In this study, we recruited thirty patients diagnosed with LCH based on histopathological analysis of a skin biopsy performed at Fırat University between March 2019 and May 2021. Patients who had previously undergone cryotherapy, electrocautery, surgical intervention for the lesions, and those who had received any topical treatment were not included in the study. Normal vascular structures in healthy tissues surrounding the lesions were used as the control group. The study was approved by the Fırat University Non-Interventional Research Ethics Committee (approval number: 3956, date: 28.09.2021). Because the samples were obtained from the archives of the Fırat University Faculty of Medicine, Department of Pathology, patient consent was not required.

Immunohistochemistry

Paraffin-embedded specimens were sectioned into 4-micrometer slices and mounted on glass slides coated with Poly-L-Lysine. To retrieve antigens, specimens were deparaffinized and then rehydrated through a series of graded ethanol solutions. Afterwards, the specimens were subjected to microwave boiling in citrate buffer for 12 minutes (7+5) at a pH of 6.0. After cooling the specimens to room temperature for about 20 min, they were subjected to three washes with phosphate-buffered saline (PBS) (P4417; Sigma-Aldrich, St. Louis, MO) for 5 min each. Afterwards, the specimens were incubated with hydrogen peroxide for 5 min to prevent endogenous peroxidase activity (Hydrogen Peroxide Block, TA-125-HP; Lab Vision Corp., New York, NY). Ultra V Block solution (TA-125-UB; Lab Vision Corp.) was applied to the specimens for 5 min. The primary antibodies WNT1 Rabbit pAb (WH1158730; Cat. no: A2475; AB Clonal) and β-catenin (E-5) sc-7963 [mouse monoclonal immunoglobulin G (IgG); Santa Cruz Biotechnology) were diluted at 1:200 and then incubated with the specimens in a humid environment at room temperature for 60 min. Following primary antibody incubation, the specimens were washed three times with PBS for 5 min and subsequently exposed to biotinylated goat anti-polyvalent secondary antibody (anti-mouse/rabbit IgG) (TP-125-BN; Lab Vision Corp.) for 30 min at room temperature. The sections were rinsed thrice with PBS, followed by incubation with streptavidin peroxidase (TS-125-HR; Lab Vision Corp.) for 30 min, and then placed in PBS. A solution of 3-amino-9-ethyl carbazole (AEC) substrate and AEC chromogen (AEC substrate, TA-015-HAS; AEC Chromogen, TA-002-HAC; Lab. Vision Corporation) was used to stain the specimens with AEC. When immunoreactivity became visible under light microscopy, the sections were washed with PBS. Counterstaining was performed using Mayer's hematoxylin. After rinsing the sections with PBS and distilled water, they were covered with a mounting medium (Large Volume Vision Mount, TA-125-UG; Lab Vision Corp.). The Zeiss Axio Scope A1 microscope (Zeiss, Gottingen, Germany) was used to examine and photograph the mounted sections. All samples were scored blindly by a pathologist and histologist. For immunohistochemical scoring, the histological score was calculated as follows: The staining distribution was evaluated using a scoring system where 0.1 represented less than 25%, 0.4 represented 26-50%, 0.6 represented 51-75%, and 0.9 represented 76-100%. Another scoring was used to assess the staining intensity, where 0 indicated no staining, 0.5 indicated very little staining, 1 indicated little staining, 2 indicated moderate staining, and 3 indicated very strong staining. Histoscores were obtained by multiplying the distribution score and intensity score according to the formula histoscore = distribution × intensity (7).

Statistical Analysis

Statistical Package for the Social Sciences (IBM Corp., Armonk, NY, version 25.0) was used for statistical analysis. Numerical variables are presented as mean ± standard deviation (SD) values. On the other hand, categorical variables were described using frequencies and percentages. Parametric distribution of the variables was assessed via the Kolmogorov-Smirnov test. For intergroup comparisons, numerical variables were analyzed using the Student's t-test and Mann-Whitney U test, while categorical variables were evaluated using the chi-square and Fisher's exact (two-sided) tests. Statistical significance was defined as a p-value of less than 0.05.

Results

General Characteristics of the Subjects

Among the 30 patients, an equal number of individuals (n=15) were male and female. The patients had a mean age of 33±21.1 years (mean ± SD). The means macroscopic size of the LCH lesions was 11±6.07 mm. Ten (33%) lesions showed mucosal localization. Of all the lesions, 17 (57%) were observed in the head and neck, seven (23%) in the upper extremity, five (17%) in the lower extremity, while only one (3%) was found on the trunk. Comparisons between genders revealed no statistically significant differences with respect to mean age, lesion size, and mucosal involvement (p>0.05). Table 1 displays a summary of the patients' demographic characteristics (Table 1) (Figure 1).

Table 1. Summary of the patients' clinical data

Characteristic	Female	Male	Total	p-value
Number	15 (50%)	15 (50%)	30 (100%)	
Age, mean (SD, min.-max.)	36 (20, 14-67)	32 (22, 1-67)	33 (21, 1-67)	0.568
Size (mm)	11.33±5.52	10.67±6.77	11.00±6.07	0.770
The mucosal involvement, n (%)				
Yes	7 (47%)	3 (20%)	10 (33%)	
No	8 (53%)	12 (80%)	20 (67%)	
Total	15 (100%)	15 (%)	30 (100%)	0.121

SD: Standard deviation, min.: Minimum, max.: Maximum

Wnt1 Immunoreactivity

Wnt1 expression was 1.10±0.16 in the LCH lesions and 0.33±0.10 in the healthy vascular structures in the tissue surrounding the lesions evaluated as controls, and a significant difference was found between these groups (p<0.001) (Table 2). Wnt1 immunoreactivity was nuclear and cytoplasmic in the vascular endothelial cells, and increased Wnt1 expression was observed in tumor stroma (Figure 2). The presence or absence of mucosal involvement did not yield a statistically significant difference in Wnt1 expression (p>0.05). Wnt1 expression was similar in both genders in the LCH and control groups. The analysis revealed no significant difference in Wnt1 expression with respect to lesion localization (p>0.05) (Table 3).

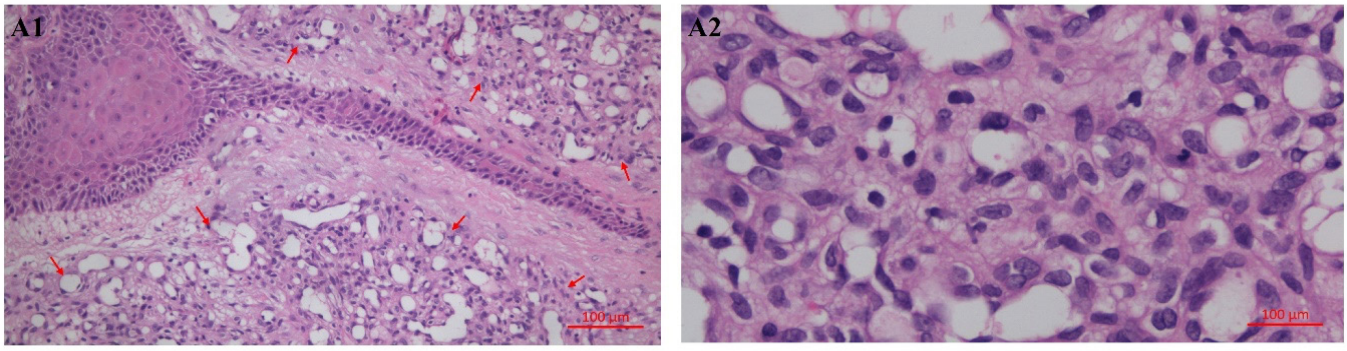

Figure 1. Histopathological examination of the lobular capillary hemangioma (hematoxylin and eosin). Lobular proliferation of capillary-sized vessels within the superficial dermis and mixed inflammatory infiltrates resembling the granulation tissue in the stroma. The infiltration consists of fibroblasts, lymphocytes, neutrophils, plasma cells, and mast cells (A1, A2)

Table 2. Wnt1 and β-catenin immunoreactivity of the study groups

Histoscores of the groups, mean (SD)	Control	LCH	p-value
Wnt1	0.33±0.10	1.10±0.16	**<0.001**
β-catenin	0.59±0.18	1.57±0.39	**<0.001**

SD: Standard deviation; LCH: Lobular capillary hemangioma

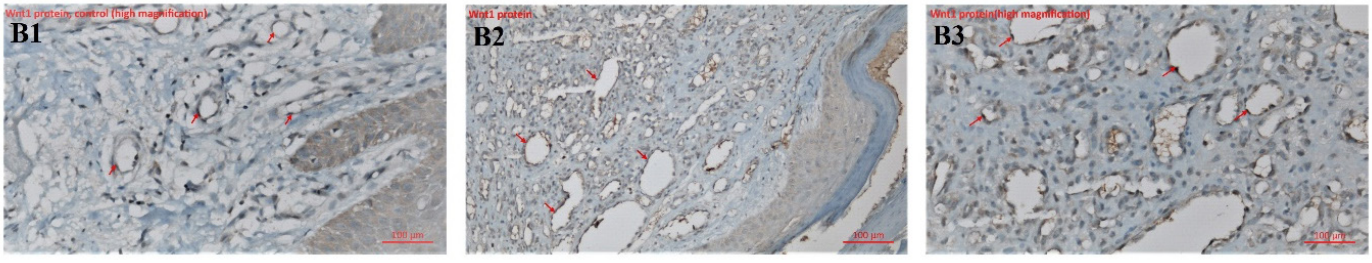

Figure 2. Immunohistochemical analysis of Wnt1 protein. Normal vascular structures in healthy skin adjacent to LCH (B1), vascular structures in an LCH lesion (B2, B3)

LCH: Lobular capillary hemangioma

Table 3. Histoscore of Wnt1 and β-catenin immunoreactivity of the study groups

Histoscore of groups, mean (SD)	Wnt1		β-catenin	
	Control	LCH	Control	LCH
Gender				
Female	0.35±0.11	1.10±0.15	0.57±0.72	1.56±0.36
Male	0.30±0.09	1.10±0.19	0.60±0.20	1.58±0.43
	p=0.124	p=0.795	p=0.741	p=0.802
Mucosal involvement				
Present	0.31±0.84	1.11±0.14	0.555±0.213	1.590±0.414
Absent	0.34±0.11	1.10±0.18	0.600±0.169	1.530±0.359
	p=0.0.547	p=0.818	p=0.533	p=0.700

SD: Standard deviation, LCH: Lobular capillary hemangioma

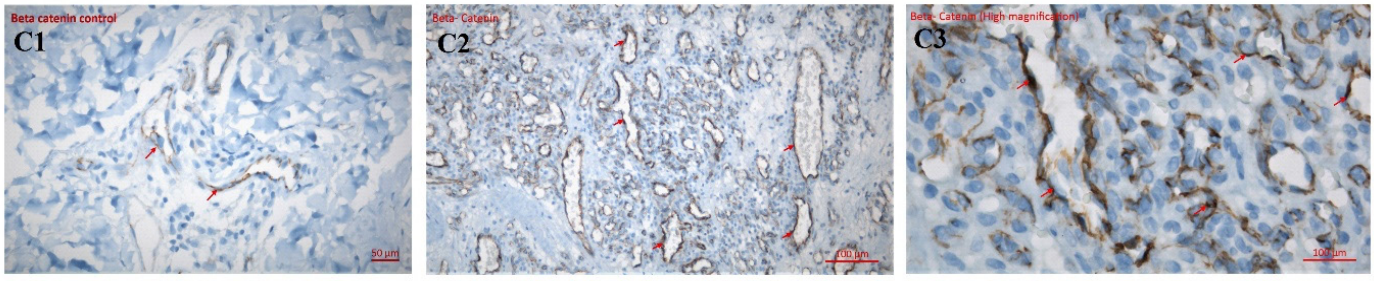

Figure 3. Immunohistochemical analysis of β-catenin protein. Normal vascular structures in healthy skin adjacent to LCH (C1), vascular structures in an LCH lesion (C2, C3)
LCH: Lobular capillary hemangioma

β-catenin Immunoreactivity

β-catenin expression was 1.57±0.39 in the LCH lesions and 0.59±0.18 in the controls, indicating a statistically significant difference ($p<0.001$) (Table 2). β-catenin immunoreactivity was nuclear and cytoplasmic in the vascular endothelial cells. There was no increase in β-catenin expression in tumor stroma (Figure 3). The expression of β-catenin showed no significant differences between lesions with and without mucosal involvement, between male and female patients in both the LCH and control groups, or among different lesion localizations ($p>0.05$) (Table 3).

Discussion

Until recently, LCH, namely pyogenic granuloma, was considered to be reactive vascular proliferation due to its emergence in areas of previous trauma or chronic irritation, increased frequency in some hormonal changes such as pregnancy, presence of Gram-positive (+) and Gram-negative (-) bacilli in microscopic examinations, and association with the use of some drugs (8). However, in recent comprehensive studies, trauma history was observed in only 4.5-15% of the patients, and the observed bacilli were elements of the flora and not found to be a predisposing factor in most patients (9,10). First, Pagliai and Cohen (10) proposed that pyogenic granuloma was a benign acquired vascular neoplasia and used the term "LCH" to describe this condition. In previous studies, hemangioblastic blood islands were observed within LCH lesions, which were observed to contain hematopoietic stem cells (11). Blackwell et al. (12) reported that in LCH, the embryonic stem cell markers OCT4, SOX2, pSTAT3 and NANOG revealed increased expression. They suggested that LCH originated from the primitive endothelium, similar to infantile hemangioma and developed because of *de novo* vasculogenesis (12).

The Wnt pathway has been recognized for its significant involvement in vessel remodeling, angiogenesis, and vasculogenesis. Wnt2, Wnt5a, Wnt7a, and Wnt10b were found to be expressed in endothelial cells, while Wnt-5a was found to be expressed in vascular smooth muscle cells (13). Transfection of the *Wnt1* gene into human umbilical vein endothelial cells increases endothelial cell proliferation (5).

Wnt signaling pathway activation is also known to increase cell proliferation and cause some cancers such as skin melanoma, basal cell carcinoma, colorectal carcinoma, squamous cell carcinoma, and hematological malignancies (2,6,14). It has been suggested that in addition to supporting an epithelial-mesenchymal transition, thereby promoting the maintenance of cancer stem cells, Wnt also increases immune tolerance and limits antitumor response by acting as a bridge between the tumor microenvironment and tumor cells (15). Stephenson et al. (4) showed that infantile hemangiomas, the most common vascular tumors, were derived from stem cells, and Wnt/β-catenin transcription activity was essential for this process, with the inhibition of this pathway also inhibiting the expression of stem cell factors (4). Another study that investigated the Axin, C-myc, and β-catenin expressions in pulmonary sclerosis hemangioma, another vascular tumor originating from the primitive respiratory epithelium, high cytoplasmic β-catenin and C-myc expressions were observed in polygonal cells, suggesting that polygonal cells have higher proliferation capacity (3). In this study, we showed that there are high levels of Wnt1 and β-catenin expressions in LCH. These findings suggest that LCH is a vascular neoplasia resulting from the increased canonical Wnt1 signaling pathway expression. This increase is observed in both mucosal and cutaneous LCH, revealing that this pathway is jointly used in developing mucosal and cutaneous LCH.

In LCH, we observed increased Wnt1 expression in vascular endothelial cells and tumor stroma. The nuclear and cytoplasmic β-catenin expression in vascular endothelial cells was remarkable. Wnt1 immunoreactivity in tumor stroma suggests that paracrine factors may have influenced this increase. Wnt ligands can be secreted from the tumor microenvironment (16). In addition, some growth factors secreted from stromal and inflammatory cells can activate the expression of Wnt through a secondary route. For example, it has been shown that there is an increase in the hepatocyte growth factor receptor in colorectal carcinoma, which further contributes to tumor progression by increasing the expression of β-catenin (16). In addition, the nuclear translocation of β-catenin and the promotion of epithelial-mesenchymal transition are increased by platelet-derived growth factor (PDGF) (17). Studies on inflammatory cells and tumor interaction in tumoral tissues reveal that macrophages also play a crucial role in tumor progression and angiogenesis (18). Producing Wnt7b, macrophages activate the canonical signaling pathway through paracrine mechanisms in vascular endothelial cells and contribute to tumoral angiogenesis (19). LCH presents a mixed inflammatory

infiltrate, containing lymphocytes, neutrophils, plasma cells, and macrophages. We consider that Wnt ligands secreted from the tumor microenvironment and some growth factors, such as fibroblast growth factor (FGF) and PDGF, may be involved in Wnt signaling upregulation in the pathogenesis of LCH on an inflammatory background.

Epulis gravidarum is a mucosal form of LCH that occurs during pregnancy or oral contraceptive use in women. Female sex hormones have been implicated in the pathogenesis of this condition, and it has been suggested that these hormones cause vascular proliferation by increasing the secretion of basic FGF, vascular endothelial growth factor, and interleukin 1β (20). Katoh reported that Wnt1 was overexpressed in human breast cancer, and β-estradiol upregulated Wnt1 in human breast cancer cell culture (MCF-7 cells) (6). This interaction between estradiol and the Wnt1 signaling pathway may contribute to the increased incidence of LCH during oral contraceptive use or pregnancy.

LCH, especially in the presence of mucosal and finger involvement, may be caused by chronic trauma (21). Mechanotransduction stimulates lymphatic vessel development through activation of the canonical Wnt pathway (22). It is also known that *de novo* vasculogenesis can secondarily develop from endothelial stem cells due to trauma in adults (2). Studies that will reveal the relationship of the Wnt1 signaling pathway with vasculogenesis secondary to trauma and mechanotransduction may illuminate the pathogenesis of LCH caused by trauma.

Myofibroblasts are cells with ultraslow contractility found in inflammatory processes, cancer tissues, and fibrotic tissues. It has been shown that myofibroblasts are also in LCH along with endothelial and pericyte cells (23). They are intensely observed in patients with atypical mucosal LCH and multiple LCHs (24). The importance of the signaling pathway of canonical WNT/ß-catenin has been shown in transforming mesenchymal stem cells into myofibroblasts (25). We think that Wnt1 upregulation is likely to increase the transformation of mesenchymal stem cells into myofibroblasts, which contributes to the fibrotic process in late lesions. Further studies in early and late LCH lesions can demonstrate the relationship between myofibroblasts and the Wnt1 signaling pathway.

Currently, therapeutic approaches that target aberrant canonical Wnt signaling pathway are gaining considerable attention in the field of cancer treatment. Blockade of Wnt1-mediated signaling with the monoclonal anti-Wnt1 antibody has been shown to inhibit survivin and rapidly and significantly induce apoptosis in lung, breast, mesothelioma, and sarcoma cancer cell cultures (26). Similarly, monoclonal antibodies designed to bind Wnt1 and Wnt2 have been shown to suppress tumors in several malignancies, such as colorectal cancer, melanoma, and non-small-cell lung carcinoma (27). Treatment modalities targeting Wnt1 may also be promising in the treatment of LCH.

Study Limitations

The most important limitation of this study is that Wnt1 and β-catenin expression in LCH lesions was evaluated only with the immunohistochemical method. Our second limitation is that we did not screen predisposing factors influential in the emergence of lesions. We consider that the evaluation of genetic mutations associated with the Wnt1/β-catenin signaling pathway and the demonstration of its relationship with predisposing factors will significantly contribute to the elucidation of the pathogenesis of LCH.

Conclusion

This study revealed that the Wnt1/β-catenin signaling pathway in LCH is activated. Further studies on this subject will help reveal the cause of this activation and elucidate the pathogenesis of LCH. Treatment methods targeting Wnt1 may be promising for treating LCH.

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Ethics Committee Approval: The study was approved by the Fırat University Non-Interventional Research Ethics Committee (approval number: 3956, date: 28.09.2021). Because the samples were obtained from the archives of the Fırat University Faculty of Medicine, Department of Pathology, patient consent was not required.

Informed Consent: Because the samples were obtained from the archives of the Fırat University Faculty of Medicine, Department of Pathology, patient consent was not required.

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