Diagnostic Value of Wilms Tumor 1 and CD44 in Langerhans Cell Sarcoma

Case Series of 4 Patients

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Abstract: Langerhans cell sarcoma (LCS) is a rare tumor with markedly malignant cytological features originating from Langerhans cells. LCS diagnosis is difficult and requires differentiation from other malignant tumors and Langerhans cell histiocytosis (LCH). Immunochemical antibodies, such as langerin, S-100 protein, and CD1a, have been used to diagnose LCS, but the results are crossed with LCH. To determine more significant biomarkers of LCS, we studied the expression and distribution pattern of Wilms tumor 1 (WT1) and cluster of differentiation 44 (CD44) in LCS.

A broad panel of antibodies was used for immunohistochemical technology. Simultaneously, dual immunofluorescence staining examination and fluorescence in situ hybridization staining methods were used to study the location of WT1 and CD44 in LCS tumor cells.

The results showed that tumor cells expressed WT1, CD44, and other special Langerhans cell markers (langerin, CD1a, and S-100 protein). LCS cells in all the cases showed normal cytogenetic findings without overexpression of WT1 and CD44. The expression of WT1 and CD44 was observed on langerin^{+} tumor cells by dual immunofluorescence staining examination in LCS.

Our results suggest that WT1 and CD44 are potential biomarkers for LCS diagnosis. Clear understanding of their functional roles may further explain the pathogenesis of this highly malignant tumor and develop some novel immunotherapy strategies.

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Abbreviations: CD44 = cluster of differentiation 44, FISH = fluorescence in situ hybridization, LCH = Langerhans cell histiocytosis, LCS = Langerhans cell sarcoma, WT1 = Wilms tumor 1.

INTRODUCTION

Langerhans cell tumors include Langerhans cell histiocytosis (LCH) and Langerhans cell sarcoma (LCS). LCS is a rare malignant dendritic cell tumor that displays overtly malignant cytology that includes the Langerhans cell phenotype. The overall survival of LCS is ~50% within 1.5 years even after being administered with a combination of radiotherapy and chemotherapy or with surgery and local radiotherapy.[1]

LCS diagnosis is difficult and requires differentiation from other tumors such as metastatic cancer, malignant melanoma, anaplastic large-cell lymphoma, myeloid sarcoma, and malignant fibrous histiocytoma, as well as from LCH. Various immunochemical antibodies, such as langerin, S-100 protein, and CD1a, have been introduced for LCS diagnosis, but the results were not ideal. Thus, more specific biomarkers should be determined for the diagnosis and differential diagnosis of LCS.

Wilms tumor 1 (*WT1*) gene is originally isolated as a tumor suppressor gene inactivated in the childhood kidney neoplasm Wilms tumor.[2] *WT1* gene plays a biological role by regulating the expression of different genes, such as transforming growth factor β, Bcl-2, and human telomerase reverse transcriptase. *WT1* is also a transcriptional regulator that plays a central role in the development of several organs and tissues, such as kidneys, gonads, and spleen.[2] Further research revealed that *WT1* gene is overexpressed in various cancer cells, including leukemia,[3] lung cancer,[4] and breast cancer.[5] These studies showed that WT1 expression is strongly associated with cancer progression and tumor-related patient prognosis. Additional studies demonstrated *WT1* as an oncogene in several tumors.

Cluster of differentiation 44 (CD44) is a cell-surface transmembrane glycoprotein involved in cell–cell interactions, lymphocyte activation, cell adhesion, recirculation and homing, adhesion of extracellular matrix, and angiogenesis, as well as cell proliferation, differentiation, and migration.[6] CD44 can also activate various receptor tyrosine kinases in many cancers. Moreover, CD44 plays an important role in invasion and metastasis of various tumor cells. These properties are associated with the pathologic activities of cancer cells.

However, WT1 and CD44 may serve as potential diagnostic and prognostic biomarkers, but no reports exist about the expression of WT1 and CD44 in LCS. In the present study, we detected the expression of the WT1 protein and CD44 in LCS using immunohistochemical and fluorescence detection to assay the expression of WT1 and CD44 on neoplastic cells and to estimate whether they serve as useful diagnostic and prognostic markers for LCS.

MATERIALS AND METHODS

Case Selection

Four cases of LCS were collected in this study. Three cases were diagnosed from the Department of Pathology, 150th Hospital of PLA, and the other was collected from Xinqiao

TABLE 1. Clinical Characteristics of Patients

Case No.	Sex/Age, y	Site	Therapy	Outcome
1	M/41	Soft tissue at anterior iliac spine and bilateral groin	Surgery, local radiotherapy, chemotherapy	Died of disease <1 y
2	M/37	Soft tissue at waist	Surgery, chemotherapy	Died after being diagnosed 5 mo
3	M/57	Right lower extremity	Surgery, chemotherapy	Died, month 8
4	FM/34	Lymph node	Local radiotherapy, chemotherapy	Died, month 2

Hospital, Third Military Medical University. The tissues were fixed in 10% neutral buffered formalin and paraffin embedded. A case of LCH was assayed in parallel as the control. Ethical approval for the study was obtained from the ethics committees of the 150th Hospital of PLA and the Xinqiao Hospital, Third Military Medical University. The clinical characteristics of patients were provided in Table 1.

Immunohistochemical Staining

The formalin-fixed and paraffin-embedded tumor blocks were cut into 3-μm-thick sections and prepared for hematoxylin and eosin and immunohistochemical staining.

For immunohistochemistry, sections were mounted on polylysine-treated glass slides. Endogenous peroxidase activity was blocked with 3.0% H_2O_2 for 15 minutes. Sections were incubated at 4°C overnight with primary antibody. The clones, dilutions, pretreatment, and sources of primary antibodies used in this study were listed in Table 2. On the second day, sections were washed and incubated with the corresponding secondary antibodies for 1 hour at room temperature. Peroxidase activity was visualized with diaminobenzidine (DAKO, Carpinteria, CA, USA) and brown coloration of tissues represented positive staining. The sections were lightly counterstained with hematoxylin, dehydrated through an ethanol series to xylene, and mounted. Sections incubated with concentration-matched immunoglobulin without primary antibodies were used as negative controls.

Fluorescence In Situ Hybridization Staining

To examine the expression of WT1 and CD44 better, fluorescence in situ hybridization (FISH) detection was employed. Briefly, 8 μL of hybridization buffer and 2 μL of the WT1 or CD44 probe (Empire Genomics, NY, USA) were added to the microcentrifuge tube, which was incubated at room temperature and then centrifuged for 3 seconds. The probe was denatured before being used. Slides were pretreated by preheating to 45°C to 50°C for 5 minutes, then 10 μL of the probe hybridization mixture was applied, sealed, and denatured at 73°C for 5 minutes. The slides were then incubated overnight in a humidified chamber at 37°C. The following day, the slides were washed with 0.4 × SSC/0.3% NP40 at 65°C for 2 minutes, rinsed twice in 2 × SSC/0.1% NP40 for 2 minutes, placed in 70% ethanol for 3 minutes, and, finally, air-dried. Ten microliters of 4′,6-diamidino-2-phenylindole was added immediately, and the slides were covered with cover slides. However, sections incubated with the related isotype control probes were used as isotype controls. The signal was observed using the fluorescence microscope (Zeiss Axioplan 2, Jena, Germany).

Dual Immunofluorescence Staining

For immunofluorescence staining, the formalin-fixed paraffin-embedded tumor samples were sliced in 3 to 5-μm-thick

sections, and then sections were incubated with primary antibodies (mouse monoclonal anti-langerin, anti-WT1, and anti-CD44 Abs) for 24 hours at 4°C. Alexa 568-conjugated goat antimouse/rat/rabbit immunoglobulin G (IgG) antibodies or fluorescein isothiocyanate-conjugated mouse antigoat IgG antibodies (Jackson Immuno Research, West Grove, PA) were added and treated for 1 hour. Isotype controls were conducted by using appropriate isotype control primary antibodies and fluorescently labeled secondary antibodies. The fluorescence

TABLE 2. Primary Antibodies Used in This Study and the Results in Tumor Cells

Antibody	Source	Clone	Dilution	Results
WT1	DAKO	Mouse	1:50	++
ALK	Abcam	Rabbit	1:100	−
Langerin	Abcam	Mouse	1:200	++
bcl-2	DAKO	Mouse	1:50	−
CD56	Millipore	Mouse	150	−
MC	Abcam	Mouse	1:50	−
MPO	Abcam	Rabbit	1:50	−
CD15	DAKO	Mouse	150	−
CD44	DAKO	Mouse	1:25	++
CD1a	DAKO	Mouse	1:50	++
CD34	DAKO	Mouse	1:25	−
S-100	Millipore	Mouse	1:400	++
CD35	DAKO	Mouse	1:25	−
LCA	DAKO	Mouse	1:50	−
CD68	DAKO	Mouse	1:50	Local +
OCT-2	Millipore	Mouse	1:75	−
P53	DAKO	Mouse	1:25	−
Vim	DAKO	Mouse	1:50	−
CD20	DAKO	Mouse	1:200	−
CD30	DAKO	Mouse	1:20	−
Lysozyme	Abcam	Mouse	1:200	−
PAX-5	Abcam	Rabbit	1:50	−
CD99	DAKO	Mouse	1:50	−
EBV	Abcam	Mouse	1:100	−
CD138	DAKO	Mouse	1:25	−
CD14	Abcam	Mouse	1:50	−
CD23	DAKO	Mouse	1:5	−
CD21	DAKO	Mouse	1:25	−
CD3	DAKO	Mouse	1:25	−
CD5	DAKO	Mouse	1:25	−
CD45RO	DAKO	Mouse	1:50	−

ALK = anaplastic lymphoma kinase, CD44 = cluster of differentiation 44, CD56 = cluster of differentiation 56, EBV = Epstein–Barr virus, LCA = leukemia cell antigen, MC = mesothelial cell, MPO = myeloperoxidase, Vim = vimentin, WT1 = Wilms tumor 1.

microscopy (Zeiss Axioplan 2, Jena, Germany) was used to analyze the results.

RESULTS

Histopathological Findings

Microscopically, tumor cells exhibited cytological features of malignancy and showed large cell sizes with irregular shapes and abundant cytoplasm with eosinophilic red staining, irregular, large, and variable prominent nuclei, and numerous neoplastic cells with longitudinal nuclear groove were observed. More than 30 mitoses per 10 high-power fields were detected. Large amounts of infiltrated inflammatory cells, such as plasma cells, eosinophils, and lymphocytes, were also observed (Figure 1A and B). Simultaneously, immunohistochemical staining showed that neoplastic cells were positive for langerin, CD1a, CD68, and S-100 protein (Figure 1 C−F). These cases were diagnosed as LCS based on histopathological characteristics and immunophenotypical findings. No tumor cells were expressed other than the antibodies listed in Table 2.

WT1 and CD44 Immunohistochemical Staining

Immunohistochemical staining showed that all 4 cases of LCS tumor cells were positive for WT1 (Figure 2A) and CD44 (Figure 2C). WT1 was observed in the cytoplasm of neoplastic cells, whereas CD44 was found on cell membranes and in the cytoplasm. Ki-67 proliferation index ranged from 70% to 90%. By contrast, the LCH tumor cells were negative for WT1 (Figure 2B) and CD44 (Figure 2D) but positive for CD1a, S-100 protein, and langerin.

Molecular and Cytogenetic Findings

FISH analysis was performed on paraffin-embedded tissue sections in all the cases using a fluorescence-label probe for WT1 and CD44. The LCS cells in all the cases showed normal cytogenetic findings without overexpression of WT1 (Figure 3A) and CD44 (Figure 3B).

Phenotypes of the WT1- and CD44-positive cells were further examined by fluorescence dual staining. Interestingly, the expression of WT1 and CD44 was observed on langerin$^+$ tumor cells, which indicate that WT1 and CD44 were
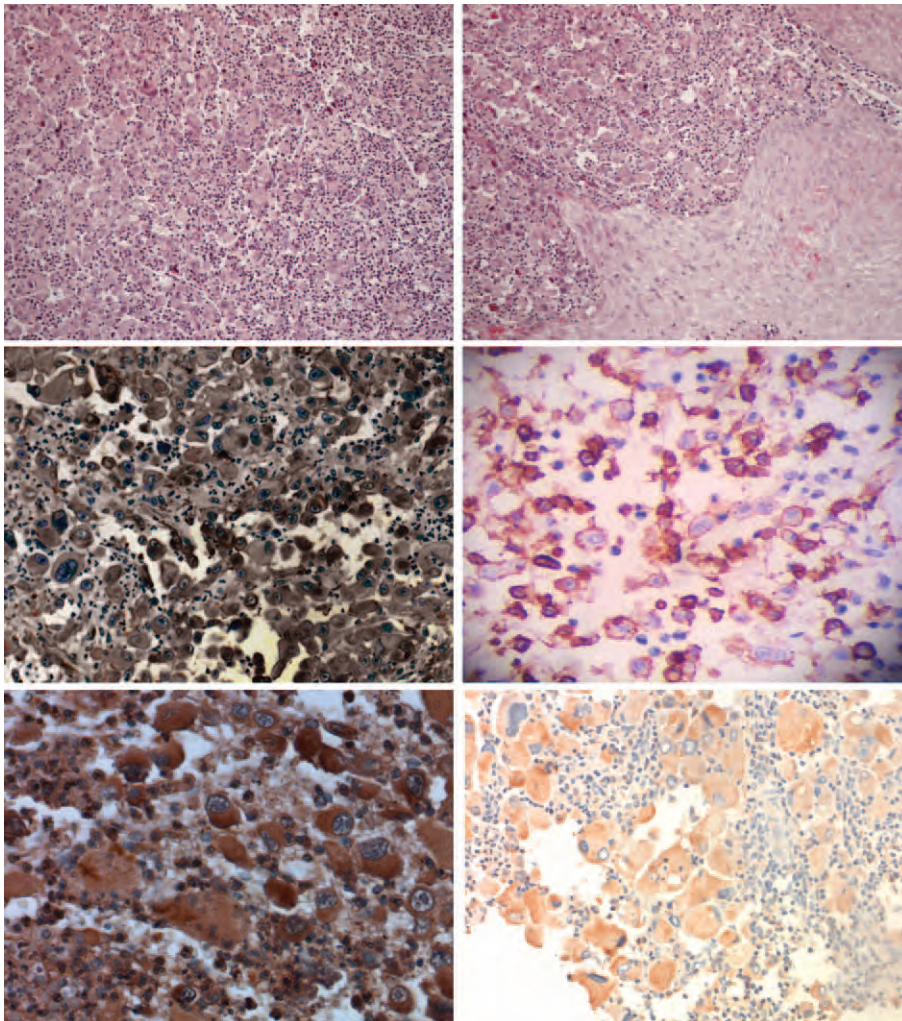
FIGURE 1. Morphological characteristics of LCS. Hematoxylin and eosin staining evidenced that the tumor cell with longitudinal nuclear groove and high mitotic rate was observed (A and B), (hematoxylin−eosin staining, magnification ×100); the tumor cells were positive for langerin (C), CD1a (D), CD68 (E), and S-100 protein (F). LCS = Langerhans cell sarcoma.
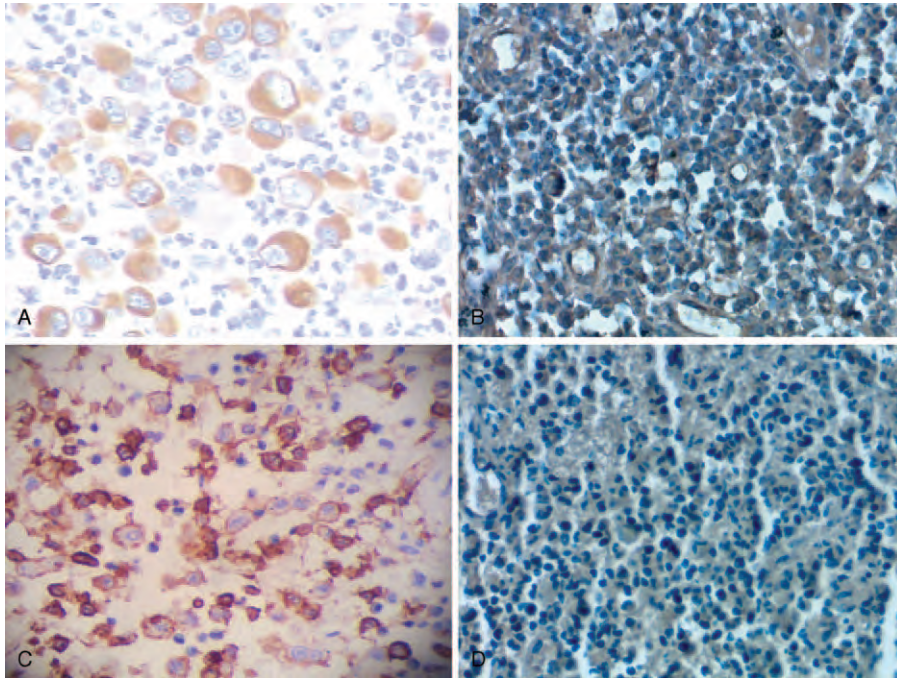
FIGURE 2. WT1 and CD44 expressed in LCS and LCH. The LCS tumor cells were positive for WT1 (A) and CD44 (C); but LCH tumor cells were negative for WT1 (B) and CD44 (D). CD44 = cluster of differentiation 44, LCH = Langerhans cell histiocytosis, LCS = Langerhans cell sarcoma, WT1 = Wilms tumor 1.

biomarkers for LCS diagnosis (Figure 4). However, the expression of WT1 and CD44 showed that they were found in infiltrated lymphocytes in LCH (Figure 5).

DISCUSSION

LCS is a malignant tumor that initially results from hematopoietic or mesenchymal stem cells. Thus, LCS is defined as a tumor of Langerhans cell hyperplasia with markedly malignant cytological features. Up to 35 cases of LCS have been reported in international journals from 1984 to 2012. LCS primarily involves the skin, lymph nodes, spleen, liver, bone marrow, thymus, lungs, and kidneys.[7–10] A recent report showed that LCS also involves the gallbladder and peritoneal lymph nodes.[11] LCS is a rare malignant dendritic cell tumor. The World Health Organization defined LCS as a high-grade neoplasm with overtly malignant cytology and Langerhans cell phenotype. The overall survival of LCS is ∼50% within 1.5 years even after being administrated in combination with radiotherapy and chemotherapy or with surgery and local
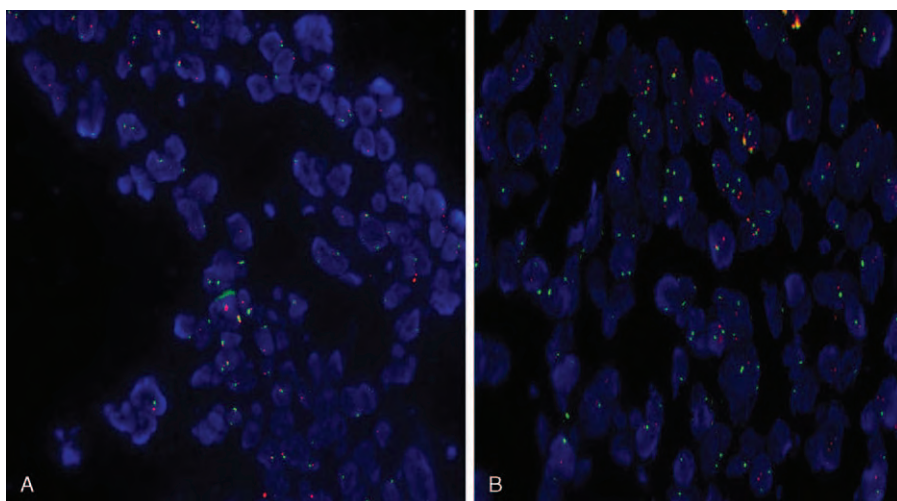

FIGURE 3. *WT1* and *CD44* gene amplification status of LCS; WT1 (A); CD44 (B) (FISH detection ×1000). CD44 = cluster of differentiation 44, FISH = fluorescence in situ hybridization, LCS = Langerhans cell sarcoma, WT1 = Wilms tumor 1.
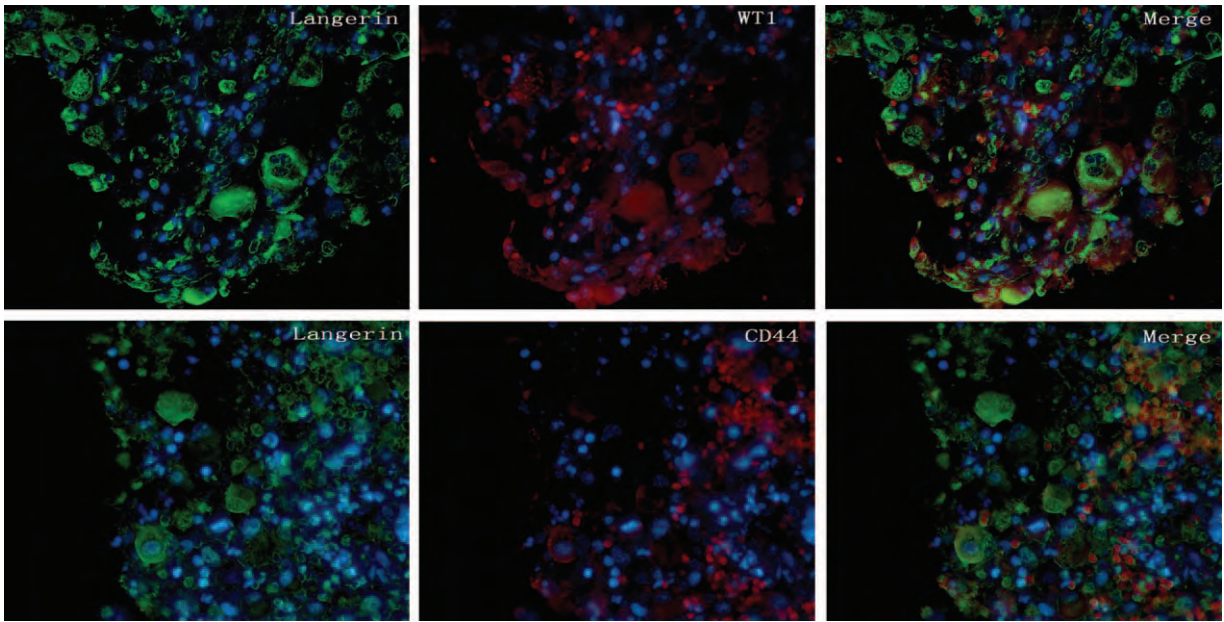
FIGURE 4. Characteristic expression of WT1 and CD44 on langerin cells in LCS was evidenced by dual fluorescence staining. The expression of WT1 and CD44 was found on langerin^{+} tumor cells. Blue color indicates nuclear DAPI staining. Scale bar = 20 µm. CD44 = cluster of differentiation 44, DAPI = 4',6-diamidino-2-phenylindole, LCS = Langerhans cell sarcoma, WT1 = Wilms tumor 1.

radiotherapy.[12] Thus, LCS is a highly malignant tumor with low survival rate and poor prognosis. LCS diagnosis is commonly based on histopathological characteristics, immunophenotype, and ultrastructure. In the current study, tumor cells with characteristics of Langerhans cells were observed in the sample sections and were strongly positive for CD1a, S-100 protein, CD68, and langerin. This finding indicates that these tumor cells were LCS, but ultrastructure data were unavailable.

WT1 and CD44 have been reportedly expressed in cancer tissues. The expression and function of CD44 and WT1 in various carcinomas have been well reported, but their distribution in LCS has not been illustrated. An understanding of their expression and anatomical distribution in LCS tissues is essential for the diagnosis and development of some novel treatment approaches for this highly malignant tumor.
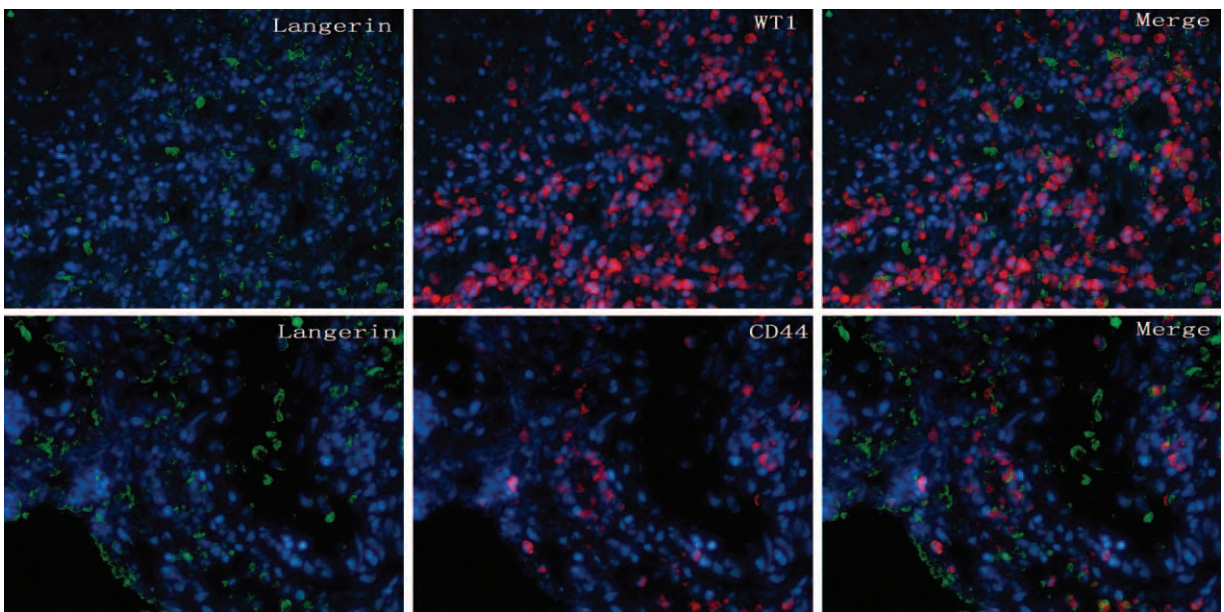
FIGURE 5. Characteristic expression of WT1 and CD44 on infiltrated lymphocytes in LCH was evidenced by dual fluorescence staining. The expression of WT1 and CD44 was found on different cells. Blue color indicates nuclear DAPI staining. Scale bar = 20 µm. CD44 = cluster of differentiation 44, DAPI = 4',6-diamidino-2-phenylindole, LCH = Langerhans cell histiocytosis, WT1 = Wilms tumor 1.

WT1, which induces cell apoptosis by upregulating the *Bcl-2* gene expression, functions as a tumor suppressor.[13,14] However, further research showed that *WT1* acted as an oncogene by inhibiting the Bcl-2 promoter.[15,16] Overexpression of WT1 in human prostate cancer cells inhibits proliferation, whereas WT1 expression in leukemic cells enhances proliferation.[17,18] One study showed WT1 downregulation by either micro RNA-15a/16-1 overexpression or specific small interfering RNA expression, which significantly inhibits the proliferation of leukemic cells.[19] WT1 plays different roles according to cell type. WT1 may act as a biomarker for LCS diagnosis and as a target against LCS tumor cells, which could also provide a novel therapeutic option for LCS. Immunohistochemistry analyses were conducted using WT1-specific mono-clone antibody (clone 24F.7G12) and goat IgG polyclonal antibodies. The results showed that WT1 was mainly expressed in neoplastic cells. Fluorescence dual staining found that WT1 was primarily expressed in langerin^{+} tumor cells. Our results indicated that WT1 expression was observed in tumor cells, which suggests that this protein might also be involved in regulating LCS development and can be used for LCS diagnosis.

CD44, which is located in chromosome 11p12-13 and occupies 20 exons, belongs to a widely expressed family of adhesion receptors. CD44 is mainly expressed in normal tissues. CD44 plays an important role in tissue integrity and is involved in multiple functions associated with cancer progression, such as cell migration,[20] resistance to apoptosis,[21] and presentation of growth factors and proteases.[22,23] CD44 has also been identified as a specific marker of cancer stem cells[24,25] and plays an important role in maintaining the stemness of breast cancer stem cells.[26] The current results showed that some malignant tumors could be treated by interfering with CD44 or reducing CD44 expression with a designed genetic therapy.[27] We also found that CD44 was expressed in langerin^{+} tumor cells. This expression pattern suggests that CD44 is also involved in regulating LCS development.

In summary, we described the expression pattern of CD44 and WT1 in LCS tissue samples. These 2 proteins were observed in langerin^{+} tumor cells, which indicate that they may function as useful diagnostic biomarkers for LCS. Considering the very low incidence of LCS, this highly malignant tumor should be differentiated from other neoplasms, and the valuable proteins found should be administrated in individualized therapy or immunotherapy for prognosis.

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