Original Article

Squash smear cytology of Langerhans cell histiocytosis

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Abstract: Squash smear cytology of Langerhans cell histiocytosis (LCH) has rarely been reported. We described squash cytological findings of cranial LCH. Additionally, based on recent data that suggests an association of LCH with either viral infection or genetic alteration, we investigated the presence of several viruses or mutation of TP53 and BRAF in LCH tissue samples. Intraoperative squash smears of a small tissue fragment excised from the lesion demonstrated a mixed population of eosinophils, neutrophils, small lymphocytes and a high content of histiocytes. The histiocytes possessed abundant dense cytoplasm with round cell shape and eccentrically located nuclei with fine chromatin, delicate nuclear membranes and prominent nuclear grooves, indentations and pseudoinclusions. The cytologic features were consistent with Langerhans cells (LCs). Subsequent histopathologic examination confirmed the diagnosis of LCH. Immunohistochemically, the LCs were positive for S-100, CD1a and langerin, but negative for adenovirus, CMV, EBV, HHV-8, HPV, HSV, SV 40 and p53. BRAF V600E mutation was absent. Our findings did not support the role of viruses and genetic abnormalities in the pathogenesis of LCH. In summary, the presence of a mixed population of inflammatory cells and a high content of histiocytes with characteristic cytomorphology, along with radiologic evidence and appropriate clinical findings, is highly suggestive of LCH on the intraoperative squash smears. Awareness of characteristic cytological features of LCH is necessary for rapid and accurate diagnosis. Squash smear cytology is a potentially useful tool in the intraoperative diagnosis of LCH.

Keywords: Langerhans cell histiocytosis, squash cytological preparation, virus, Epstein-Barr virus, TP53, BRAF

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease that accounts for less than 1% of all tumor-like lesions of bone mainly affecting the skull, femur, mandible and ribs [1]. In the past, the disease was referred to as histiocytosis X with 3 variants: eosinophilic granuloma, Hand-Schuller-Christian disease and Letterer-Siwe syndrome that represent different expressions of the same disease, now known as LCH [2]. There is a wide spectrum of clinical manifestations of LCH that depends on the location and extent of the proliferation of Langerhans cell (LCs). LC is a major type of nonlymphoid mononuclear cell involved in both inflammatory responses and neoplastic processes. There is an ongoing debate on whether the pathogenesis of LCH includes a reactive or neoplastic process. Inflammatory reaction to viral infection is reportedly the potential etiology at disease onset [3-7]. However, no convincing evidence has emerged for an infectious cause of LCH. There are several reports on immune dysregulation and aberrant cytokine expression in patients with LCH, indicating that the nature of LCH is predominantly immune or inflammatory [8-10]. However, the role of cytokines and immune system regulators in the pathogenesis of LCH remains controversial. Meanwhile, the discovery of frequently recurring oncogenic BRAF mutations in LCH, along with TP53 mutation, supports the classification of this disease as a neoplasm [11]. However, there is also a debate about the source of pathologic LCs in LCH lesions. Furthermore, the presence of BRAF for TP53 mutation alone may not be sufficient for tumorigenesis. Even though the identification of genetic alterations in LCH strongly supports the hypothesis of its neoplastic nature, several questions remain unanswered.

Squash smear cytology is of great value in intraoperative pathologic consultations. It provides
a rapid and reliable intraoperative diagnosis and guidance to the surgeon during surgical resection and lesion targeting. It also helps the surgeon to monitor and modify the surgical approach. Using a very small amount of material, this technique makes it possible to detect cellular details and establish a provisional pathologic diagnosis [12]. In published series and case reports of LCH, its diagnosis has been established using routine histopathologic methods [4, 12-14]; the morphology of LCH is well recognized in processed biopsy or excised material. In contrast, the squash cytology description of LCH is limited. Knowledge of its cytomorphology, nevertheless, is of diagnostic importance [12, 15-17]. The appearance on squash smears has certain characteristic and diagnostic features. We described squash smear cytological findings for rapid and correct intraoperative diagnosis of cranial LCH in children. The findings of this study will increase the accuracy of the pathologists’ cytological diagnosis and guide appropriate management. Additionally, we investigated a possible relationship between LCH and viral infection or TP53 and BRAF mutation. The presence and cellular localization of viruses, as well as p53 expression status, was examined using immunohistochemical staining and in situ hybridization. The presence of BRAF V600E mutation was also examined using polymerase chain reaction (PCR).

Case presentation

Case 1

A 12-year-old boy presented to the neurosurgery outpatient department with a painless swelling of the frontal part of the head of 6 weeks duration. The lesion was not accompanied by fever or tenderness. There was no his-
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Table 1. Antibodies used for immunohistochemical staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Millipore Corporation, Bedford, MA, USA</td>
<td>20/11 &amp; 2/6</td>
<td>1:500</td>
</tr>
<tr>
<td>CD1a</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>10</td>
<td>1:50</td>
</tr>
<tr>
<td>CD3</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>CD20</td>
<td>Novocastra Laboratories, Newcastle upon Tyne, UK</td>
<td>L26</td>
<td>1:400</td>
</tr>
<tr>
<td>CMV</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>CCH2/DDG9</td>
<td>1:40</td>
</tr>
<tr>
<td>HHV-8</td>
<td>Novocastra Laboratories, Newcastle upon Tyne, UK</td>
<td>13B10</td>
<td>1:50</td>
</tr>
<tr>
<td>HPV</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>K1H8</td>
<td>1:100</td>
</tr>
<tr>
<td>HSV</td>
<td>Novocastra Laboratories, Newcastle upon Tyne, UK</td>
<td>20.7.1</td>
<td>1:3,000</td>
</tr>
<tr>
<td>p53</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
<td>BP53.12</td>
<td>1:5,000</td>
</tr>
<tr>
<td>S-100</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Polyclonal</td>
<td>1:5,000</td>
</tr>
<tr>
<td>SV 40</td>
<td>Calbiochem, Merck KGaA, Darmstadt, Germany</td>
<td>PAb416</td>
<td>1:100</td>
</tr>
</tbody>
</table>

History of trauma, headache or vomiting. Local examination revealed a firm and slightly mobile swelling of approximately 1.5 cm in diameter over the upper forehead. The overlying skin appeared normal. Systemic examination was otherwise unremarkable, and the patient was neurologically normal. He had no lymphadenopathy, organomegaly, skin rash or any other abnormality. Complete routine laboratory tests including hematological and biochemical investigations revealed no abnormality. A plain skull roentgenogram revealed a sharply demarcated, punched-out osteolytic lesion in the most anterior midline point on the frontal bone, approximately 3 centimeters above the glabella (Figure 1A). Sagittal (Figure 1B) and axial (Figure 1C) view of magnetic resonance imaging (MRI) scan demonstrated an osteolytic lesion in the frontal bone. Surgical excision with intraoperative frozen section and squash cytological preparations was performed. Subsequent permanent section diagnosis, followed by immunohistochemical staining, Epstein-Barr virus (EBV)-encoded RNA in situ hybridization (EBER-ISH) and analysis for BRAF V600E mutation detection, was also performed. The primary cytological diagnosis of LCH was made using squash preparations, and confirmed by the frozen and permanent sections. The postoperative course was uneventful, and the patient left the hospital 5 days later. Fourteen months after surgery, MRI scan showed no evidence of recurrence. Whole body positron emission tomographic scan showed no abnormal hypermetabolic lesion.

Case 2

A 6-year-old girl was referred to our hospital presenting with painful swelling over the left side of forehead for a month. There was no history of trauma, headache or vomiting. On examination, a fluctuant, ill-defined soft tissue mass of approximately 2 cm in diameter was identified on the left frontal region. The underlying bone appeared to be indented on palpation, and the overlying skin appeared normal. She had no significant past medical history. Lymphadenopathy or skin rash was not found. The liver and spleen were not palpable. Other physical examination and laboratory tests were unremarkable. A plain skull roentgenogram (Figure 1D, anteroposterior view; Figure 1E, lateral view) revealed a well-demarcated osteolytic lesion in the left frontal bone. Computed tomographic (CT) scan also showed a localized osteolysis of the frontal bone with erosion of the inner and outer tables, along with suspected involvement of the adjacent soft tissue (Figure 1F). As in Case 1, surgery with intraoperative frozen section and squash smears was performed. The primary cytological diagnosis of LCH was further confirmed by the tissue sections. The ancillary tests, including immunohistochemistry, EBER-ISH and BRAF V600E mutational analysis, were performed. The postoperative course was uneventful, and the patient left the hospital 4 days later. No recurrence over the last 6 months was apparent.

Materials and methods

Squash cytological preparation

For squash cytology, biopsy samples obtained at the time of surgery were transported immediately in small vials containing isotonic saline for processing in the pathology departments' cryolaboratory. One to 2 millimeters of the biop-
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Figure 2. Squash cytological findings of LCH. A. LCs displayed centrally or eccentrically located, oval or retiform, grooved or contorted nuclei (black arrows), and were admixed with inflammatory background. B. They had abundant, dense cytoplasm and round or oval cell shape. Their nuclei possessed fine granular chromatin and delicate nuclear membranes and intranuclear cytoplasmic pseudoinclusion (blue circle). C. A prominent folded configuration manifested as nuclear grooves (yellow circle) and pseudoinclusions (blue circle). D. An eccentrically located nucleus showed a large pseudoinclusion (blue circle). E. The background consisted predominantly of numerous eosinophils with bilobed nuclei (white arrows). F. Some neutrophils, scattered small lymphocytes and multinucleated giant cells were accompanied.

Immunohistochemistry

Immunohistochemical staining was performed by using standard reagents and techniques, as previously described [18-24]. Briefly, the sections were deparaffinized with Bond Dewax solution (Leica Biosystems, Bannockburn, IL, USA) and an antigen retrieval procedure was performed using Bond Epitope Retrieval solution 1 (Leica Biosystems) for 30 min at 100°C. Endogenous peroxidases were blocked by incubation with hydrogen peroxide for 5 min and the sections were incubated for 15 min at ambient temperature with primary antibodies (Table 1). Staining was performed using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system with a Leica Bond-Max automated immunostainer (Leica Microsystems), and the slides were visualized with 3, 3’-diaminobenzidine (DAB) solution (1 mm DAB, 50 mm Tris-HCl buffer [pH 7.6] and 0.006% H₂O₂). In addition, the nuclei were counterstained with hematoxylin, and the slides were dehydrated through a series of graded alcohols (70%, 90% and 100%), cleared in xylene (Sigma-Aldrich, St. Louis, MO, USA) and sealed.
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EBV-encoded RNA in situ hybridization and CD3/CD20 double staining

The formalin-fixed, paraffin-embedded sections obtained from excised specimens were used for EBER-ISH and CD3 or CD20 double staining. The sections of LCH tissue were deparaffinized with xylene (Sigma-Aldrich), pretreated with proteinase K for 20 min and incubated with fluorescein isothiocyanate (FITC)-conjugated EBER oligonucleotide probes (Novocastra Laboratories, Newcastle upon Tyne, UK) at 55°C for 2 h. The sections were rinsed in water and incubated with horseradish peroxidase-conjugated anti-FITC antibody for 15 min before adding fresh 3, 3-DAB color substrate to produce an alcohol-insoluble brown intranuclear stain in EBV-positive cells. Immediately following the EBER-ISH, immunohistochemical staining with CD3 and CD20 was performed on the Leica Bond-maX autostainer (Leica Microsystems) using standard reagents and techniques to produce fast red membranous stain in either CD3- or CD20-positive cells. We used EBV-negative lymphoid tissues processed using the hybridization mixture without EBER oligonucleotides as negative controls.

Detection of BRAF V600E mutation

For the detection of BRAF V600E mutation, nucleic acids from fresh tissue were isolated using a DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Briefly, isolated nucleic acids were mixed with a PCR master mix from a Seeplex-BRAF V600E ACE detection kit (Seegene Inc., Seoul, Republic of Korea). The mixed samples were immediately placed in a preheated (94°C) thermal cycler for 15 min, and PCR was carried

Figure 3. Histological (A-C) and immunohistochemical (D-F) findings of LCH. (A) At low power, diffuse infiltration of LCs was noted. (B) LCs were admixed with eosinophils, neutrophils, lymphocytes and multinucleated giant cells (C). Consistent with squash cytological findings, characteristic nuclear indentations (yellow circles) and grooves were detected. Immunohistochemically, the LCs were strongly reactive for (D) CD1a, (E) S-100 and (F) langerin.
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out using the recommended program in a GeneAmp PCR 9700 system (Applied Biosystems, Foster City, CA, USA). The cycling amplification program consisted of 35 cycles: denaturation for 30 s at 94°C, annealing for 30 s at 63°C and extension for 1 min at 72°C. The amplified PCR products were loaded onto a 2% agarose gel and visualized with Safe View Stain (Applied Biological Materials Inc., Richmond, BC, Canada). The BRAF V600E mutation was detected with a Gel Documentation system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Results

Smears were highly cellular and showed numerous histiocytes as the predominant cell type scattered singly or in loosely cohesive clusters with an admixture of a polymorphic population of inflammatory cells (Figure 2A). The histiocytes were large cells with abundant, dense cytoplasm and round or oval cell shape (Figure 2B). Their nuclei, eccentrically or centrally located, had characteristic features, consisting of fine granular chromatin, delicate nuclear membranes and a distinctive, prominent folded configuration manifested as nuclear grooves (with a coffee bean appearance; Figure 2C), indentations and pseudoinclusions (Figure 2D). The nucleoli were inconspicuous to small, but occasionally, were prominent. Mitotic figures were rare. The cytological features of these histiocytes were consistent with LCs. The background of the smears consisted of numerous eosinophils with bilobed nuclei (Figure 2E), neutrophils, scattered small lymphocytes, plasma cells and multinucleated giant cells (Figure 2F). Cytological diagnosis of LCH was made.

Macroscopically, the specimen revealed a gray-white, soft mass that involved the skull bone. Microscopically, a diffuse infiltrate of LCs

Figure 4. Findings of immunohistochemical staining for viruses (A-F and I) and EBER-ISH (G and H). LCs did not demonstrate immunoreactivity for (A) adenovirus, (B) CMV, (C) HHV-8, (D) HPV, (E) HSV and (F) SV 40. Double staining for EBER-ISH and CD3 or CD20 showed that neither LCs, (G) CD3-positive T-lymphocytes nor (H) CD20-positive B-lymphocytes exhibited EBV positivity. (I) LCs displayed scattered, weak to moderate nuclear p53 immunoreactivity, indicating wild-type expression pattern (absence of TP53 mutation).
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(Figure 3A) was admixed with eosinophils, polymorphonuclear leukocytes, lymphocytes and multinucleated giant cells (Figure 3B). The LCs were large-sized and polygonal, with abundant glassy eosinophilic cytoplasm. Round to oval, grooved nuclei displayed finely granular or slightly vesicular chromatin (Figure 3C). Multinucleation was evident. Mitotic figures were rarely seen. No necrosis was detected. Immunohistochemically, the LCs were strongly reactive for CD1a (Figure 3D), S-100 (Figure 3E) and langerin (Figure 3F), confirming the diagnosis of LCH.

Based on the recent data that suggests that viral infection is associated with the pathophysiology of LCH [3-6, 25], we further investigated whether viruses were present in the LCH lesions. However, LCs did not demonstrate immunoreactivity for any of the following antibodies: adenovirus (Figure 4A), cytomegalovirus (CMV; Figure 4B), human herpesvirus-8 (HHV-8; Figure 4C), human papilloma virus (HPV; Figure 4D), herpes simplex virus (HSV; Figure 4E) and simian virus 40 (SV 40; Figure 4F). We also performed double staining for EBER-ISH and CD3/CD20 to investigate whether EBV infects bystander T- or B-lymphocytes. However, neither LCs, T-lymphocytes (Figure 4G) nor B-lymphocytes (Figure 4H) exhibited EBV positivity.

We assessed the status of 2 known molecular abnormalities in LCH, i.e., p53 protein expression and the presence of BRAF V600E mutation in order to find evidence of neoplastic pathogenesis [11, 26]. However, no mutant pattern of p53 expression was observed in LCs (Figure 4I). No LCH tissue samples exhibited BRAF V600E mutation.

Discussion

The diagnosis of LCH in our patients was made on the basis of characteristic cytomorphology on squash cytological preparations. This was corroborated by appropriate radiological and clinical findings. In the present cases, both MRI and CT scans showed lytic lesions in the skull bones with sharp borders and punched-out appearances. In Case 2, destruction of both inner and outer tables resulted in a double-contour or a beveled-edge appearance, which is a typical roentgenographic finding in the diagnosis of LCH [4, 27].

This report highlights the usefulness of squash cytological preparations for rapid intraoperative diagnosis. Intraoperative frozen section is an important component of surgical management of tumors. Critical decision regarding treatment and the extent of surgical resection can sometimes depend on an appropriate intraoperative cytological diagnosis [28]. The cytological findings were similar to those described in fine needle aspiration samples of LCH in other organs [29-34]. In the present cases, the squash smears displayed a high cellularity consisting of isolated or loosely aggregated LCs with eccentrically located, oval to reniform nuclei showing delicate nuclear membranes and prominent nuclear grooves, indentations and pseudoinclusions. These were admixed with a polymorphic population of inflammatory cells with a predominance of eosinophils. The key to the diagnosis is identification of LCs through characteristic nuclear features, i.e., nuclear grooves and pseudoinclusions. However, occasionally, the LCs are few or nuclear grooves not very prominent. Furthermore, they can show variable degree of pleomorphism and mitotic activity [31, 32, 35, 36]. Similarly, the degree of eosinophilic infiltration varies in different areas of the LCH lesion, thus their number can vary from scant to abundant in cytology smears [35]. Nevertheless, the presence of eosinophils can help attract attention to the diagnosis of LCH. There have been several case reports on the cytologic features of LCH, but most of these cases were taken from the bone or thyroid as aspiration cytology of LCH, but most of these cases were taken from the bone or thyroid as aspiration cytology [2, 29-34]. There is only one previous Japanese case demonstrating squash cytologic features of LCH of the skull [12]. Together with previous reports, we demonstrated that squash cytomorphology closely reflects histomorphology.

The cytological diagnosis may be missed due to lack of familiarity with its cytomorphology among pathologists or due to the lack of characteristic cytological findings resulting from a sampling error. Therefore it is prudent for the pathologist to consider this diagnosis only in an appropriate clinical and radiological setting. It is also necessary to be familiar with cytological features of other differential diagnoses. The most common differential diagnoses of cranial LCH include Ewing sarcoma, non-Hodgkin lymphoma and osteomyelitis with abundant histiocytes. Ewing sarcoma and non-Hodgkin lym-
phoma are characterized by a monomorphic population of small round blue cells. In acute osteomyelitis, numerous neutrophils form a prominent component of inflammatory lesion. The observed reactive histiocytes can be easily distinguished due to the absence of characteristic features of LCs. Plasma cells and lymphocytes predominate in chronic osteomyelitis. Neutrophils and plasma cells can be present, but infrequent in LCH.

A possible relationship between the development of LCH and expression of some human herpes viruses has been reported [25]. The granulomatous nature of LCH lesions and the generally benign morphology of pathologic LCs have suggested possible infectious, environmental or autoimmune pathogeneses [3, 5]. In particular, EBV infects monocytes and LCs and is associated with the pathophysiology of LCH according to some studies [3, 5-7, 25]. However, other studies failed to replicate these findings, and the possible causative role of EBV in LCH is debated [37-39]. Shimakage and colleagues [25] showed positive signals for EBER-ISH in 17 cases of LCH, in contrast, McClain and colleagues [37] failed to find evidence of genomes for EBV in 56 LCHs. Slacmeulder and colleagues [40] demonstrated the absence of HHV-8 DNA in 12 LCH biopsy specimens. Similarly, herpes viruses such as EBV, CMV and HHV-6 have been detected in clinical samples [41], but differences in the overall prevalence or titers of antibodies against these viruses were not significant between LCH patients and controls. Consistent with earlier reports that were unable to document an association between herpes viruses and LCH, we did not observe immunoreactivity for all viruses examined [37-40, 42]. Double staining for EBER-ISH and CD3 or CD20 revealed the absence of EBV in B- and T-lymphocytes. However, we could not definitively exclude an association of EBV infection with LCH because of a number of EBV-infected cells infiltrating LCH lesion, suggestive of an EBV-infected B-lymphocytes and LC interaction that might contribute to the development of LCH [40, 41]. Jeziorski and colleagues [41] observed that EBV-positive cells were labeled with CD20 or CD79a, but not with CD1a, CD3 or CD68, indicating that EBV infects bystander B-lymphocytes and not LCs. Thus, EBV-infected B-lymphocytes in the lesion may trigger the activation of LCs and eventually the development of LCH in some cases.

We did not observe the mutation pattern (either diffuse and strong positivity or complete absence of immunoreactivity) of p53 protein expression. BRAF V600E mutation was absent. Some previous studies have shown TP53 mutation and p53 overexpression in LCH [11, 43], raising the possibility that p53 may be a primary contributor to a hyperproliferative, transformed phenotype through its inability to induce apoptosis [26]. Furthermore, the demonstration of BRAF V600E mutation in more than a half of LCH samples examined provides the recurrent genomic abnormality that is required, in addition to clonality, to assign LCH a neoplastic origin [11]. However, p53 overexpression can be a normal secondary response to a proliferative stimulus. There are several examples of benign, reactive proliferative disorders that are characterized by high levels of p53 expression [44-46]. Likewise, BRAF mutation alone may be necessary but not sufficient for the development of LCH; several additional genetic abnormalities are required for tumorigenesis. Much effort is required to identify additional genetic abnormalities in LCH and to understand the molecular basis of its pathogenesis.

In conclusion, the presence of a mixed population of inflammatory cells, including abundant eosinophils, and a high content of histiocytes with characteristic cytomorphology on the intraoperative squash smears, along with radiologic evidence and appropriate clinical findings, is highly suggestive of LCH. Nuclear features including grooves and pseudo-inclusions were evident in squash cytological smears, closely reflecting histomorphology of LCH. Squash smear cytology can serve as a useful tool for the intraoperative diagnosis of LCH. Awareness of cytological features of LCH, its differential diagnoses and causes of diagnostic pitfall is necessary. In addition, our results did not support an association between LCH and viral infection or genetic abnormalities. Although the precise etiology remains to be elucidated, our understanding of LCH has been greatly enhanced by the application of advanced genomic technologies to the analysis of primary human material. Further investigation is required to clarify the molecular pathogenesis of LCH.

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Disclosure of conflict of interest

None.

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