# Monoallelic Mutations of the Perforin Gene may Represent a Predisposing Factor to Childhood Anaplastic Large Cell Lymphoma

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**Summary:** Anaplastic large cell lymphoma (ALCL) accounts for approximately 15% of all pediatric non-Hodgkin lymphomas. It has distinct clinical features, including frequent involvement of extranodal sites and rare localization to the central nervous system. As some presenting features of ALCL are in common with the hemophagocytic syndrome, we previously analyzed a small series of patients with ALCL for *PRF1* mutations and found that 27% of them carried mutations. We now expanded our preliminary study by increasing the cohort of ALCL patients to a total of 84 consecutive cases, in whom we extended mutation analysis to the genes *SH2D1A*, *PRF1 e UNC13D*, all related to familial HLH. Furthermore, perforin expression in tumor cells was investigated on paraffin-embedded tissues by immunohistochemical analysis. Mutations were observed in 23/84 patients (27.4%). Twenty-one patients (25%) carried a total of 10 different mutations of *PRF1*; they were monoallelic in 20 patients, biallelic in 1. No mutations were found in the gene *SH2D1A*. Two additional patients had missense mutations of the *UNC13D* gene. These data show that monoallelic germline mutations of *PRF1* are frequent in patients with childhood ALCL, suggesting that partially impaired cytotoxic machinery may represent a predisposing factor for ALCL. Involvement is less frequent for *UNC13D* and absent for *SH2D1A*.

**Key Words:** monoallelic mutation, pediatric, anaplastic large cell lymphoma, *PRF1*, *UNC13D*

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Anaplastic large cell lymphoma (ALCL) was first described approximately 20 years ago by Stein et al.[1] The majority of cases in the past diagnosed as malignant histiocytosis,[2,3] and lymphohistiocytic lymphoma, and some rare cases of Hodgkin disease belong to this category. It is characterized by proliferation of neoplastic lymphoid cells that coexpress several activation antigens, such as CD30 (Ki-1), the epithelial membrane antigen, and interleukin-2 receptor.[2,4] With respect to lymphocyte lineage markers, it is widely accepted that most of the cases express a T-cell lineage if studied with an appropriate and extensive immunohistochemical panel; however, a few cases show a null phenotype.

According to the recent World Health Organization classification of lymphomas,[5] ALCL is now included as a subgroup of mature T-cell lymphoma. The majority of ALCL is associated with abnormalities of the anaplastic lymphoma kinase (*ALK*) gene, in particular with the chromosomal translocation t(2;5); (p23;q35).[6] This genetic aberration originates fusion of the nucleophosmin (NPM) gene on 5q35 to the ALK receptor tyrosine kinase gene on 2p23,[7] causing the expression of the NPM-ALK protein that can be detected by the ALK-1 monoclonal antibody.

ALCL accounts for 10% to 15% of childhood non-Hodgkin lymphoma.[8] Its clinical features include a predominance of systemic symptoms and an unusually high frequency of extranodal involvement, particularly of the skin. Hemophagocytosis is observed in a proportion of cases.[9–12]

The concept of immune-surveillance against cancer was proposed in 1970 by Burnet, who predicted that immune-deficient individuals, or those being treated with immunosuppressive drugs, would have an increased incidence of cancer, and that differences in immunologic host defense among healthy individuals might influence the occurrence of cancer.[13] Furthermore, natural cytotoxic activity of peripheral-blood mononuclear cells was assessed in 3625 adult Japanese individuals, between 1986 and 1990. After an 11-year follow-up survey of the cohort, analysis of cancer incidence demonstrated that high cytotoxic activity of peripheral-blood lymphocytes was associated with reduced cancer risk, whereas low activity was associated with increased cancer risk suggesting a role for natural immunologic host defense mechanisms against cancer.[14]

Perforin plays a key role in the cytotoxicity of natural killer (NK) and cytotoxic T lymphocytes (CTL). It is stored as an active protein in specialized secretory lysosomes, known as lytic granules, of NK and CTL. Upon target cell recognition, lytic granules polarize and release their contents at the immunologic synapse. Secreted perforin inserts into the lipid bilayer, polymerizing to form pores in the membranes of target cells, thus allowing the entry of a series of proteins which trigger apoptotic pathways in the target cells.[15–19] Biallelic perforin gene (*PRF1*) mutations

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have been associated with an autosomal recessive immune deficiency, familial hemophagocytic lymphohistiocytosis type 2 (FHL2, MIM 603553).[20] Some of the presenting clinical features of FHL2 are common to ALCL, including fever, lymphadenomegaly, skin rash, and hemophagocytosis.[21–23] Therefore, some connection between the 2 disorders has been hypothesized.[24,25] Furthermore, the association between the *PRF1* mutations and diseases other than FHL2, including lymphoma, autoimmune lymphoproliferative syndrome, Dianzani autoimmune lymphoproliferative disease, and multiple sclerosis have been also reported.[26–28]

These data indicate that some perforin amino acid changes, either alone or in combination with other mutations of genes involved in lymphocyte survival or functional activity, may be present in patients with lymphoma or with disorders related to cell apoptosis, supporting the concept that perforin also plays a key role in the mechanisms related to the development of tumor, and in particular of lymphoid tumors. In 2007 we investigated 44 pediatric ALCL and found a higher frequency of *PRF1* mutations in ALCL patients compared with healthy controls, suggesting a possible predisposing role for such *PRF1* abnormalities.[29]

Mutations in the *UNC13D* gene have been associated with FHL type 3, due to defect of Munc13-4, a protein which controls fusion of lytic granules with the plasma membrane.[30] Furthermore, patients with XLP1, caused by mutation of the *SH2D1A* gene, may develop variable clinical pictures including propensity to develop non-Hodgkin lymphoma.[31,32]

In this study, we expanded our previous preliminary study of *PRF1* mutations[29] by testing 40 additional consecutive patients with ALCL. The cumulative series of 84 patients was screened also for mutations in the *UNC13D* and *SH2D1A* genes. Perforin expression on tumor tissue was also investigated.

## MATERIALS AND METHODS

### Patients

A total of 84 pediatric ALCL patients, diagnosed according to the World Health Organization classification[5] and treated in the Italian Association of Pediatric Hematology and Oncology centers, were investigated for the presence of mutations in *PRF1*, *UNC13D*, and *SH2D1A*. Formalin-fixed paraffin-embedded tumor biopsies from all the ALCL cases were analyzed by immunohistochemistry using a wide panel of antibodies recognizing T-lineage and B-lineage markers (CD2, CD3, CD4, CD5, CD7, CD8, CD20, CD43, CD45RO, CD79a), NK markers (CD56, CD57), Alk-1, epithelial membrane antigen, and CD30. In all cases, histologic and immunohistochemical diagnoses were centrally reviewed. All patients were treated according to the international ALCL-99 protocol.[33]

The study was approved by ethics committee or by the internal review board of each participating institution and informed consent was obtained from parents or legal guardians before patient enrollment.

### Mutation Analysis

High–molecular weight genomic DNA was prepared from blood nucleated cells of each patient and control subject, isolated by differential lysis, using the QIAamp Tissue Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Coding sequences of the *PRF1* (NM_001083116.1), *SH2D1A* (NM_001114937.2), and *UNC13D* (NM_199242.2) were obtained from the National Center for Biotechnology Information. Primers were designed to amplify the coding exons and the flanking intron sequences. The primer sequences are available upon request. Amplification reactions were performed with 50 to 100 ng of DNA, 10 ng of each primer, 200 mM dNTPs, 1 × PCR reaction buffer, and 2.5 U Taq polymerase in a final volume of 25 μL. The cycling condition was initially denatured at 95°C for 15 minutes, followed by 30 cycles of denaturation at 95°C for 45 minutes, annealing at 58°C to 62°C for 45 minutes, extension at 72°C for 45 minutes, and a final extension at 72°C for 10 minutes. After purification, the PCR products were directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster City, CA). Sequences obtained by the ABI PRISM 3130 Sequence Detection System (Applied Biosystems) were analyzed and compared with the reported gene structure, using the dedicated software SeqScape (Applied Biosystems).

### In Silico Analysis

All variants of the sequence were searched in dbSNP (http://www.ncbi.nlm.nih.gov/snp/). For variants not reported, we used bioinformatics tools to predict whether an amino acid substitution could be benign or deleterious. We used 3 WebQuery tools: SIFT (Sorting Intolerant From Tolerant: http://sift.jcvi.org/), POLYPHEN (= *Poly*morphism *Phen*otyping: http://genetics.bwh.harvard.edu/pph/), and Pmut (http://mmb.pcb.ub.es/Pmut). SIFT predicts whether an amino acid substitution affects protein function. It is based on the degree of conservation of amino acid residues in multiple sequence alignments derived from closely related sequences. SIFT defines tolerated or nontolerated protein changes with a score ranging from 0 to 1. POLYPHEN is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative analyses. It defines a substitution as "probably damaging" with a score $>2$, "possibly damaging" 1.5 to 2, and "benign" $<1.5$. Pmut is based on the use of a variety of information to label mutations, and neural networks to process this information.[34] It provides a yes/no answer and a reliability index.

### Minimal Disseminated Disease (MDD)

All patients with NPM-ALK-positive tumor biopsy were analyzed for MDD. Total RNA obtained from bone marrow cells at diagnosis, was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. An amount of 1 μg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Milan, Italy) and random hexamers. For each sample, b2-microglobulin expression was assessed as a control for the presence of amplifiable RNA and the efficiency of reverse transcription. The 5′ and 3′ primers specific for the chimeric transcript NPM-ALK were TCCCTT GGGGGCTTTGAAATAACACC (5′NPM) and CGAGG TGCGGAGCTTGCTCAGC (3′ALK). Each reaction mixture contained 10 × buffer, 1.5 mM MgCl2, 1.6 mM dNTPs, 400 nM of each primer, 0.2 IU of Taq polymerase, and 5% of the RT product in a final 20 μL reaction volume. PCR reaction consisted of initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 minutes, 68°C for 15 minutes, 72°C for 30 minutes, and a final extension at 72°C for 10 minutes. PCR products were analyzed by 3% agarose gel electrophoresis and visualized under UV

illumination after ethidium bromide staining. Ladder 50 (Invitrogen, Milan, Italy) was used as a molecular weight marker.

### Detection of Antibody Response to ALK

Cytocentrifuge preparations of monkey epithelial COS-1 cells transiently transfected with a pcDNA3-based vector encoding NPM-ALK or with empty vector were prepared and stained with patient serum or plasma in an indirect immunoperoxidase-based assay, as previously described.[35]

The cytocentrifuge preparations were incubated with patient's serum and/or plasma diluted from 1:50 to 1:60750. The cut-off for a positive result was defined as the highest dilution giving a positive reaction with NPM-ALK transfectants.

### Perforin Immunohistochemistry

The evaluation of perforin immunoreactivity in tumor tissue was performed semiquantitatively by scoring cytoplasmic staining in cells of interest as follows: − = negative; + = positive in <30% of tumor cells; + + = positive in 30% to 60% of tumor cells; and + + + = positive in >60% of tumor cells.

### Statistical Analysis

The association of *PRF1* mutational status with specific clinical characteristics (ie, sex, stage, median age, skin, mediastinum, and visceral involvement) and biological characteristics (histologic subtype, MDD, and antibody titer) was analyzed by the $\chi^2$ test, or the Fisher exact test when the frequency of cases in a given subgroup was <5.

## RESULTS

In this ALCL series, all cases were ALK-1-positive according to the immunohistochemical analysis (Fig. 1).

Overall, 23 of the 84 patients (27.4%) showed mutations in *PRF1* or *UNC13D* genes, whereas none of the patients had mutations in the *SH2D1A* gene.

### PRF1

A total of 10 different mutations were identified in 21 unrelated patients (Table 1). The c.82C > T p.R28C mutation had not been previously described in patients with FHL2 and was not identified in the 100 healthy controls of our study. In silico analysis (Pmut, Polyphen, SIFT) predicts R28C as pathogenic. The c.1262T > G p.F421C, predicted as benign, was instead previously described in a patients with lymphoma[26] and a subsequent study showed its activity in downregulating protein expression.[41]

Four mutations, c.695G > A p.R232H, c.755A > G p.N252S (identified in 2 cases), c.1349C > T p.T450M, and c.632C > T p.A211V had been reported in patients with FHL2.[20,23] Finally, the c.272C > T p.A91V mutation, frequently reported in patients with FHL2, was found in 11 additional cases.

One patient, already described in our preliminary report,[29] showed biallelic mutations (c.272C > T p.A91V and c.695G > A p.R232H).

For 15 patients, DNA from lesional tissue was available. Of them, 6 had monoallelic mutation. In all cases, the results obtained on constitutional DNA were reproduced by analysis of the lesional tissue.

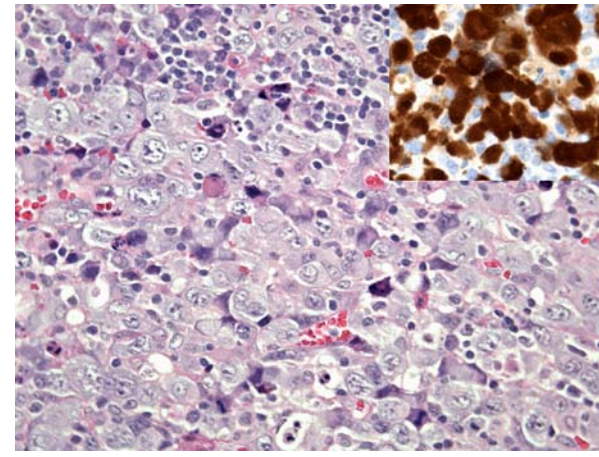

**FIGURE 1.** Anaplastic large cell lymphoma of the classic subtype. Insert: strong nuclear and cytoplasmic positivity for ALK-1.

### UNC13D

A total of *2* missense mutations were identified in 2 patients (2.3%). The c.2191G > A, p.V731M mutation was previously reported in patients with FHL3.[33] The c.1555A > T p.I519F mutation is novel, not found in over 100 healthy controls. In silico analysis (Pmut, Polyphen e SIFT) predicted that it is possibly pathogenic. Finally, the c.175G > A, p.A59T polymorphism was also found in 6 cases (7.1%), and the c.2782C > T p.R928C polymorphism in 2 additional patients.

### Correlation Between Mutational Status and Patient Characteristics

The bone marrow samples obtained at diagnosis were MDD-positive by RT-PCR in 68% of the patients (50/73) studied. Circulating antibodies recognizing the NPM-ALK protein were detected in 94% (51/54) of the plasma samples. We did not find any correlation between *PRF1* mutational status and MDD or anti-NPM-ALK antibody titer, nor with the main presenting clinical features including stage, mediastinal, visceral involvement, and age (Table 2).

In a subgroup of patients with available tumor tissue, expression of perforin was evaluated by immunohistochemistry (clone 5B10, Novocastra). Of the 29 cases tested, 23 (79%) showed high level of perforin expression (grading 2 +, 3 +) (Fig. 2), including 6 cases with *PRF1* mutation. Six of the 29 patients (21%) showed reduced or absent perforin expression (grading 0, 1 +) and 2/6 were mutated (Table 1, #69, #70).

## DISCUSSION

The association between PRF deficiency and spontaneous hematological malignancy is well recognized in mice. Thus, interest has been raised over the last years in exploring this association in humans. The present study was designed to confirm and expand our previous preliminary findings of *PRF1* monoallelic mutation in patients with childhood ALCL,[29] and to investigate additional genes involved in natural cell cytotoxicity. To address these issues we extended the investigation by studying 40 additional consecutive patients, with a total of 84 unselected cases of childhood ALCL. We now have the largest series ever reported on this topic.

**TABLE 1.** PRF1 and *UNC13D* Mutations Observed in 23 Children With Anaplastic Large Cell Lymphoma

| Case No. | Mutations | In Silico | Age/Sex | References | IHC |
|---|---|---|---|---|---|
| *PRF1* | | | | | |
| 1 | c.272C > T p.A91V* | Prob. Dam | 7/F | Santoro et al[36] | 3 + |
| 3 | c.272C > T p.A91V* | Prob. Dam | 11/F | Santoro et al[36] | NP |
| 4 | c.368G > A p.R123H | Poss. Dam. | 14/M | | NP |
| 10 | c.272C > T p.A91V* | Prob. Dam | 8/F | Santoro et al[36] | 2 + |
| 17 | c.272C > T p.A91V* | Prob. Dam | 10/M | Santoro et al[36] | 3 + |
| 24 | c.272C > T p.A91V* | Prob. Dam | 11/F | Santoro et al[36] | NP |
| 26 | c.272C > T p.A91V* | Prob. Dam | 7/M | Santoro et al[36] | 3 + |
| 27 | c.529C > T p.R177C | Prob. Dam | 1/M | | NP |
| 28 | c.1741G > A p.D491N | Prob. Dam | 12/M | van Montfrans et al[37] | NP |
| 40 | c.1262T > G p.F421C† | Benign | 13/M | Clementi et al[26] | NP |
| 43 | c.272C > T p.A91V* | Prob. Dam | 10/M | Santoro et al[36] | 2 + |
| 44 | c.272C > T p.A91V* | Prob. Dam | 13/F | Clementi and colleagues[26,36] | NP |
| | c.695G > A p.R232H* | Prob. Dam | | | |
| 72 | c.82C > T p.R28C‡ | Prob. Dam | 5/F | | NP |
| 49 | c.272C > T p.A91V* | Prob. Dam | 2/M | Santoro et al[36] | 3 + |
| 61 | c.272C > T p.A91V* | Prob. Dam | 4/M | Santoro et al[36] | NP |
| 65 | c.272C > T p.A91V* | Prob. Dam | 6/M | Santoro et al[36] | NP |
| 70 | c.368G > A p.R123H | Poss. Dam. | 2/M | | 1 + |
| 46 | c.632C > T p.A211V* | Benign | 2/M | Zhizhuo et al[38] | NP |
| 52 | c.755A > G p.N252S* | Benign | 4/F | Stepp et al[20] | NP |
| 54 | c.755A > G p.N252S* | Benign | 8/M | Stepp et al[20] | NP |
| 69 | c.1349C > T p.T450M* | Prob. Dam | 6/M | Ueda et al[39] | 1 + |
| *UNC13D* | | | | | |
| 35 | c.1555A > T p.I519F‡ | Prob. Dam | 6/F | | NP |
| 66 | c.2191G > A p.V731M* | Prob. Dam | 4/F | zur Stadt et al[40] | NP |

*Known in patients with FHL.
†Known in ALCL.
‡Novel mutation.
ALCL indicates anaplastic large cell lymphoma; IHC, immunohistochemistry; NP, not performed; Poss. Dam, possibly damaging; Prob. Dam, probably damaging.

The selection of genes investigated was driven by our experience in the field of FHL.[42–45] FHL2, due to *PRF1* mutations, accounts for about 40% of the patients with FHL in Italy; a comparable proportion of patients belongs to the FHL3 subtype, due to *UNC13D* mutations.[44] The third gene was selected because about 20% of patients with XLP1, due to *SH2D1A* mutations, are reported to present with B-cell non-Hodgkin lymphoma.[30]

Our study show that 27.4% of children with ALCL harbor monoallelic mutations in one of the genes related to cellular cytotoxicity and in particular with FHL. The involvement of the 3 genes investigated in this study appears to be remarkably different. Absence of constitutional *SH2D1A* mutations seems to exclude that this gene is directly implicated in the propensity to develop ALCL in our white, Southern European population; this may also indicate that proneness to develop B-cell non-Hodgkin lymphoma in patients with XLP depends on different pathogenic mechanisms compared to childhood ALCL. On the contrary, the predisposing role of *PRF1* mutations in children with ALCL that we had proposed in our previous study,[29] appears to be confirmed in this larger and unselected series of patients. The frequency of mutations in this cohort is definitely higher than observed in the general population, defined as 7.6% in a recent study by Trapani et al.[46]

Of note, only missense mutations were found, thus suggesting that minor impairment of perforin function, although far from causing the full-blown picture of FHL2, typical of patients with major or complete protein defect, may cause a moderate reduction of the cellular cytotoxicity function, paving the way to aberrant cell proliferation, as in ALCL development.

Although only in a subset of patients, including 6 patients with monoallelic mutation, we had the opportunity to compare the *PRF* genotype in lesional and constitutional DNA: our results suggest that mutation did not result from somatic evolution of the neoplastic cells, but were rather constitutional. Whether or not the pathogenesis of ALCL results from additional unfavorable genetic events, either constitutional or epigenetic, in yet unexplored genes, remain to be assessed.

Immunohistochemical analysis of perforin expression, although performed only in a subgroup of patients, provided some interesting results. Patients with the most frequent single mutation, p.A91V, showed normal/high expression of perforin. This finding, which might apparently suggest a neutral effect of the mutation, can be explained by the fact that the antibody used for the assay binds all isoforms (mature, intermediate, and immature) of the protein, thus masking the reduction of the active protein, as documented in A91V subjects.[47,48] This is confirmed by evidence that the use of a different antibody (clone $\Delta\gamma 9$) in cytofluorimetric assays allows to detect leak expression of $^{A91V}$perforin, even when it is carried at heterozygous state, as it binds specifically to the native form of perforin.[49] A91V is the most common variant found in the white population, with a frequency comprised between 7.5% and 10%.[29,46] Interestingly, it seems to be at a very low frequency in the African American subjects and sub-Saharan Africans, with no reported cases of the polymorphism in Japan, supporting the concept of a Mediterranean origin of the mutation. Thus, it is not surprising that A91V is the most frequent single mutation found in our study

**TABLE 2.** Statistical Analysis of the Correlations Between Clinical Data and Mutational Status

| | *PRF1* | | *P* | |
|---|---|---|---|---|
| **Characteristics** | **Not Mut** | **Mut** | **Fisher** | **$\chi^2$** |
| Sex | | | | |
| Female | 16 | 8 | | 0.5022 |
| Male | 40 | 14 | | |
| Stage | | | | |
| I + II | 7 | 2 | 1 | |
| III + IV | 49 | 20 | | |
| Risk group | | | | |
| SR | 19 | 4 | 0.2695 | |
| HR | 37 | 18 | | |
| Median age | | | | |
| < 10-31 | 29 | 10 | | 0.6148 |
| ≥ 10-31 | 27 | 12 | | |
| Age | | | | |
| < 10 | 25 | 10 | | 0.9483 |
| ≥ 10 | 31 | 12 | | |
| Histologic subtype | | | | |
| Other | 21 | 5 | | 0.2842 |
| Common type | 24 | 11 | | |
| Skin involvement | | | | |
| No | 43 | 15 | | 0.4336 |
| Yes | 13 | 7 | | |
| Mediastinum involvement | | | | |
| No | 30 | 8 | | 0.1712 |
| Yes | 26 | 14 | | |
| Lung involvement | | | | |
| No | 43 | 20 | 0.3266 | |
| Yes | 12 | 2 | | |
| Liver involvement | | | | |
| No | 49 | 21 | 0.6658 | |
| Yes | 6 | 1 | | |
| Spleen involvement | | | | |
| No | 47 | 17 | 0.5216 | |
| Yes | 9 | 5 | | |
| AB titer | | | | |
| ≤1/750* | 17 | 8 | | 0.7232 |
| > 1/750* | 21 | 8 | | |
| MDD | | | | |
| Negative | 18 | 5 | | 0.4622 |
| Positive | 35 | 15 | | |
| Median LDH | | | | |
| < 564 | 13 | 4 | 1 | |
| ≥ 564 | 24 | 9 | | |
| B symptoms | | | | |
| No | 14 | 4 | 0.5614 | |
| Yes | 38 | 17 | | |

*1/750 is the cut-off value with best sensitivity and specificity compared with other antibody levels.

AB indicates antibody; HR, high risk; LDH, lactate dehydrogenase; MDD, minimal disseminated disease; mut, mutated; SR, standard risk.

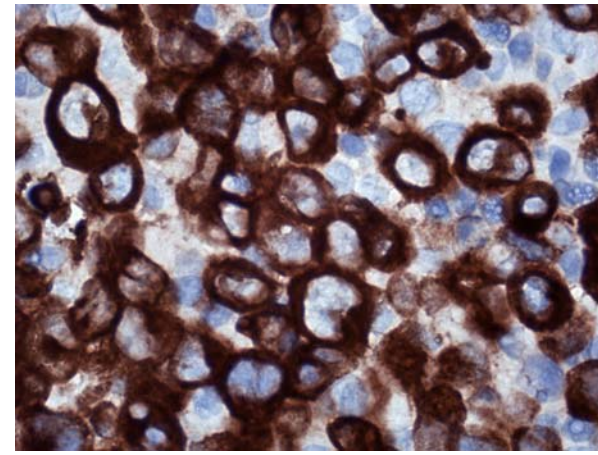

**FIGURE 2.** Strong cytoplasmic positivity for perforin in most of the tumor cells of a classic anaplastic large cell lymphoma (score +3).

population. The behavior of this mutation, which detrimental effect has been widely documented, appears intriguing: on one side it is associated with neoplastic or autoimmune disorders[26–29,46]; on the other side, given its wide diffusion at least in Southern European population, whether it may have provided any advantage to the carriers over human evolution, remains unclear.

Interestingly, immunohistochemistry revealed a substantial reduction of expression in samples carrying monoallelic p.R123H and p.T450M mutations. Unfortunately, we could not test some samples with other interesting mutations, such as the novel p.R28C. Another intriguing finding is that 4 samples, wild-type for *PRF1* mutations, showed a significant reduction of protein expression. Further investigation is warranted to clarify whether this may depend on an antibody artifact, or whether a yet unknown regulatory factor may be involved.[49]

Although there is an increasing interest on the role of the immune response in tumor development, it is becoming evident that an individual immune response to a tumor relies on multiple factors. Expression of immunosuppressive molecules by ALK-positive ALCL cells, which may contribute to downregulation of the immune response (eg, CD274) has been reported.[50] Furthermore, tumor cells may escape immune recognition by downregulating MHC class II expression, as shown for diffuse large B-cell lymphoma.[51] In addition, the protective effect against tumor relapse of anti-ALK antibody titer,[52,53] and the favorable impact of both CTLs and CD4-T helper (Th) cell responses (both being of primary importance in tumor immunity), have been identified in ALK-positive ALCL.[54,55] Thus, a multiplicity of immunologic mechanisms involved in ALK-positive ALCL pathogenesis may explain why we failed to demonstrate a direct association between mutational status and some relevant clinical and biological characteristics. On the basis of the currently available information, we cannot evaluate a possible impact of *PRF* mutations on the long-term outcome, and in particular on the risk of relapse in our patients, which needs to be assessed in long-term follow-up.

Another gene, *UNC13D*, is frequently involved in FHL in our country, accounting for a comparable proportion of cases than *PRF1*. Yet, the frequency of its mutation in patients with childhood ALCL appears to be greatly inferior. This might suggest that subjects who are heterozygous for *UNC13D* mutations apparently have other escape mechanisms to prevent the development of ALCL. Whether or not this may occur also in subjects who develop ALCL during adulthood remains to be assessed.

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