

A proportion of patients with lymphoma may harbor mutations of the perforin gene

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Perforin mutations have been demonstrated in a proportion of patients diagnosed with the familial form of hemophagocytic lymphohistiocytosis (HLH). In the present study, we evaluated whether some patients with lymphoma sharing clinical characteristics with HLH might harbor mutations of the perforin gene. We analyzed 29 patients and found that 4 patients, who developed either Hodgkin or non-Hodgkin lymphoma, had biallelic mutations of the perforin gene. One of

these 4 patients, a 19-year-old female with T-cell lymphoma, had a brother carrying the same mutations who developed HLH. In 2 of the 4 patients with biallelic mutations of the perforin gene, we evaluated perforin expression by flow cytometry and natural killer (NK) activity and both were found to be absent. Moreover, we documented the presence of monoallelic mutations of the perforin gene in 4 more patients. One of these 4 latter patients also carried a mutation of the *Fas*

gene. These data indicate that perforin deficiency, either alone or in combination with other mutations of genes involved in lymphocyte survival or functional activity, may be present in patients with lymphoma. These findings suggest that perforin also plays a key role in the mechanisms of immune surveillance that prevent tumor growth and/or development. (Blood. 2005;105:4424-4428)

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Introduction

Perforin is a cytolytic protein of 555 amino acids, expressed mainly in activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, which plays a critical role in cell-mediated lytic function and immune surveillance, and is essential for killing via non-Fas-mediated mechanisms.^{1,2} In CTLs and NK cells, perforin is stored in intracytoplasmic granules. Following cell activation, perforin, in the presence of calcium, polymerizes into transmembrane tubules to form pores and allows cytolytic enzymes, such as granzyme, to enter the target cells, this resulting in cell death by osmotic lysis and apoptosis.³

In humans, biallelic mutations of the perforin gene, leading to either complete lack or severe deficiency of the encoded protein, have been found in a proportion of patients with familial hemophagocytic lymphohistiocytosis (HLH).⁴⁻⁷ HLH is a life-threatening disease, usually occurring in early childhood, associated with profound immune derangement and characterized by impaired T-cell and NK cell granule-mediated cytotoxic activity.⁸ The main features of HLH are fever, hepatosplenomegaly, pancytopenia, hypertriglyceridemia, hypofibrinogenemia, and, frequently, central nervous system involvement.⁹ Knockout mice for the perforin gene are immunodeficient and, similarly to patients with HLH, lack NK cell granule-mediated cytotoxicity. Perforin-deficient mice do not

usually develop HLH although they do develop a disease mimicking HLH when infected, the lymphocytic choriomeningitis virus (LCMV),^{10,11} with CD8⁺ cells and interferon- γ being essential for development of LCMV. Moreover, these knockout mice have a high incidence of lymphoproliferative disorders, which occur late in their life, especially when also Fas deficient (lymphoproliferation [lpr]).^{10,12} In view of these experimental findings, we reasoned that some patients with lymphomas might also harbor mutations of the perforin gene and thus we investigated the presence of either biallelic or monoallelic mutations of the gene in a group of lymphoma patients with predefined clinical characteristics.

Patients, materials, and methods

Patients

Twenty-nine unrelated Italian patients with lymphomas (16 males, 13 females; age range 3 to 51 years) were included in this study. We decided to study patients, mainly of young age, diagnosed with lymphoma who presented with either pathologic or clinical signs of hemophagocytosis or had concomitant liver disease, reasoning that similar clinical manifestations occurred in patients with HLH. In view of the same consideration, we also

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chose to investigate cases of lymphoma characterized by an unexplained aggressive course. Clinical characteristics of the patients enrolled in this study are detailed in Table 1. The study was approved by the Institutional Review Board of Pediatric Hematology/Oncology, Unit of the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy. The informed consent was provided according to the Declaration of Helsinki.

Analysis of the perforin gene mutations and of protein expression

The coding region of the perforin gene (exon 2 and exon 3) was amplified using standard polymerase chain reaction (PCR) conditions from peripheral blood mononuclear cells (PBMCs) and sequenced according to a previously published method.⁴ PCR products were purified using Millipore Microcon (Bedford, MA) and then sequenced in an automatic DNA analyzer (ABI 3730XL; Applied Biosystems, Foster City, CA).

Perforin expression was evaluated by fluorescence-activated cell sorter (FACS) analysis. PBMCs were first stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 or anti-CD56 (Becton Dickinson PharMingen, San Diego, CA) for detection of surface membrane antigens. Following fixation and permeabilization using Cytofix-Cytosperm (Becton Dickinson PharMingen), for cytoplasmic staining the cells were then stained intracellularly with phycoerythrin (PE)-conjugated antiperforin antibody (Becton Dickinson PharMingen).

Analysis of the Fas gene

In 3 patients with monoallelic mutations of the perforin gene, mutation analysis of the *Fas* gene (Mendelian Inheritance in Man [MIM] 134637; TNFRSF6) was performed by DNA genomic sequencing. DNA was extracted from PBMCs with standard methods. Eight fragments spanning the 5' untranslated region (UTR) and the 9 exons of the gene were amplified

in 20 μ L final volume containing 12 pmol of each primer, 50 ng of genomic DNA, and 0.5 units of AmpliTaq (Applied Biosystems). The used primers are listed in Table 2. Amplification was performed in a Gene Amp PCR System 9700 (PE Applied Biosystems). PCR conditions were as follows: 1 cycle at 96°C for 3 minutes; 35 cycles at 94°C for 35 seconds, melting temperature (T_m) for 35 seconds, 72°C for 35 seconds; 1 cycle at 72°C for 5 minutes. The PCR products were sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom).

NK activity assay

NK activity of PBMCs in both patients and controls was assessed by standard ⁵¹Cr-release assays using the NK-sensitive K562 cell line as target.¹³ Results were expressed as percent specific lysis.

Results

Biallelic mutations of the perforin gene were found in 4 of the 29 patients examined (Table 1). Details of the clinical characteristics of these 4 patients are briefly summarized.

A 19-year-old female (Pt 1) had been successfully cured of her T-cell lymphoblastic lymphoma and 2 years later her 22-year-old brother developed HLH. These 2 siblings have been previously reported in detail¹⁴ as affected by adult-onset HLH with atypical findings at time of presentation, the relationship between HLH, lymphoma, and defect of the perforin protein being at that time unrecognized. The 2 siblings shared the same 2 mutations. The first one, in exon 2 (272C>T), changes alanine at position 91 into valine; the second one, in exon 3 (1122G>A), changes tryptophan

Table 1. Patient characteristics

Patient	Age at diagnosis, y	Diagnosis	Sex	Evidence of hemophagocytosis	Liver disease	Biallelic perforin mutations	Monoallelic perforin mutations
1	21	NHL	M	Yes	No	Yes	No
2	7	HD/B-NHL	M	Yes	Yes	Yes	No
3	7	T-NHL	F	Yes	Yes	Yes	No
4	18	T-NHL	F	Yes	No	Yes	No
5	6	HD/B-NHL	F	No	No	No	No
6	10	B-NHL	F	No	Yes	No	Yes
7	3	B-NHL	M	Yes	Yes	No	No
8	17	T-NHL	F	Yes	No	No	No
9	10	T-NHL	M	No	No	No	Yes
10	7	T-NHL	M	No	No	No	No
11	27	B-NHL	M	No	Yes	No	Yes
12	22	T-NHL	M	No	Yes	No	No
13	5	T-NHL	F	No	Yes	No	No
14	19	HD	M	No	No	No	Yes
15	17	T-NHL	F	No	No	No	No
16	58	T-NHL	M	No	No	No	No
17	6	B-NHL	F	No	No	No	No
18	7	T-NHL	M	No	No	No	No
19	10	B-NHL	F	No	Yes	No	No
20	8	B-NHL/HD	M	No	No	No	No
21	30	T-NHL	M	No	No	No	No
22	40	HD	M	No	No	No	No
23	38	T-NHL	M	Yes	No	No	No
24	51	B-NHL	F	No	No	No	No
25	10	B-NHL	F	No	No	No	No
26	40	NHL	F	No	Yes	No	No
27	15	B-NHL	M	No	No	No	No
28	6	B-NHL	M	No	Yes	No	No
29	50	NHL	F	No	No	No	No

NHL indicates non-Hodgkin lymphoma; M, male; HD, Hodgkin disease; B-NHL, B-cell non-Hodgkin lymphoma; T-NHL, T-cell non-Hodgkin lymphoma; and F, female.

Table 2. Primers used for the analysis of the *Fas* gene

	Primer sequences	Size of PCR product, bp	Annealing temperature, °C
5'UTR - Exon 1		322	64
Primer forward	5'-AGCTCGTCTCTGATCTCGC-3'		
Primer reverse	5'-TATCCCCGGGACTAAGACG-3'		
Exon 2		291	58
Primer forward	5'-ACACTGGTTTACACGTTGCT-3'		
Primer reverse	5'-CTATGTGCTACTCCTAATG-3'		
Exon 3		304	58
Primer forward	5'-GTCATCCCTCTATACTTCCC-3'		
Primer reverse	5'-ACAGTAGGCCCAATTTCAA-3'		
Exon 4		272	63
Primer forward	5'-AATCCATGCAGCTCCTGCC-3'		
Primer reverse	5'-AGTCAGTGTACTTCCCTAGGA-3'		
Exon 5-exon 6		388	58
Primer forward	5'-AAGGAATTATTCTGCCAGGC-3'		
Primer reverse	5'-ATCTTTGTGAAGTACTTCCCA-3'		
Exon 7		216	58
Primer forward	5'-GAAAGTATGTTCTCACATGCA-3'		
Primer reverse	5'-AGCCAAATCACTAATTTCTCTA-3'		
Exon 8		279	58
Primer forward	5'-GGCCGGAACCTTTCAGAATA-3'		
Primer reverse	5'-ATTGGCTATTACTCTAAAGG-3'		
Exon 9		481	63
Primer forward	5'-CATGGTTTTCACCTAATGGG-3'		
Primer reverse	5'-CAAGCAGTATTTACAGCCAGC-3'		

at position 374 (within the endothelial growth factor [EGF]-like domain) into a stop codon. Both siblings had null NK activity and absent perforin expression on cell surface. Notably, the association of these 2 mutations has been observed in 3 additional patients with early onset HLH in whom NK activity was null and the perforin molecule was not expressed on lymphocyte cell surface (R.C., unpublished personal data, March 2002).

The second patient (Pt 2), a 7-year-old male, was diagnosed with stage III Epstein-Barr virus (EBV)-positive Hodgkin lymphoma. After having been successfully cured of Hodgkin disease, 3 years later he developed a large B-cell non-Hodgkin lymphoma with multiple sites of involvement, diagnosed through biopsy of a cervical node. Six months after the onset of this second lymphoproliferative disorder, during treatment with intensive chemotherapy associated with the anti-CD20 monoclonal antibody (rituximab), the patient developed liver disease and severe hemophagocytosis, which responded to treatment with etoposide and cyclosporine A. An allograft of hematopoietic stem cells from an HLA-identical unrelated volunteer was then performed. Sustained remission of both non-Hodgkin lymphoma and hemophagocytosis was obtained and now the patient is alive and disease-free 22 months after transplantation. Two mutations were identified in the perforin-coding region of this patient. The first one, in exon 3 (1304C>T), changes threonine at position 435 into methionine. The second one, in the same exon (1349C>T), results in a change from threonine at position 450 to methionine. Both mutations occur in the C2 domain, the former having been recently described.¹⁵

The third patient (Pt 3), a female, presented at age 7 years with multiple reddish nonulcerated subcutaneous nodules and B symptoms (fever and weight loss) of 2-month duration. Two surgical lesion biopsies were performed on trunk and leg, respectively, and a rare form of subcutaneous, panniculitis-like T-cell lymphoma was diagnosed. The patient's condition rapidly deteriorated and she developed pancytopenia and hepatosplenomegaly. Marrow trephine did not show lymphoma localization but revealed hemophagocytosis by histiocytes with normal morphology. The patient received chemotherapy as well as systemic steroids and cyclosporine

A for control of hemophagocytosis. Despite these therapies, the patient died due to progression of disease 5 months after diagnosis. Sequencing of the coding exons of the perforin gene disclosed the following 2 mutations: the first one, in exon 2 (272C>T), changes alanine at position 91 into valine; the second one, in exon 3 (1262T>G), changes phenylalanine at position 421 into cysteine.

An 18-year-old female (Pt 4) presented with fever, malaise, and massive hepatosplenomegaly. Peripheral T-cell lymphoma associated with bone marrow infiltration was diagnosed. Marrow trephine also showed the presence of evident hemophagocytosis. The patient was treated with intensive, multiagent chemotherapy specific for T-cell lymphoma of high-grade malignancy. Unfortunately, she did not obtain clinical and hematologic sustained remission. Thus, 6 months after diagnosis she received a transplant from an HLA-identical sibling. Complete remission with engraftment of donor cells was obtained and now the patient is alive and disease-free 7 months after the allograft. This patient had 2 mutations in exon 3: the first one (914G>A) changes glycine at position 305 into aspartic acid, the second one (1066C>T) changes arginine at position 356 in tryptophan (Figure 1A).

For each patient found to have biallelic mutations of the perforin gene, both mutations found were confirmed, each in the heterozygous status, in the parents.

Four more patients had monoallelic mutations of the perforin gene (Table 1). In detail, 3 patients had the 272C>T mutation in exon 2, and 1 patient had a point mutation in exon 3 (755G>A) resulting in a change at position 252 from a medium-sized and polar amino acid (asparagine) to a small-sized and polar amino acid (serine).

This last patient (Pt 11 of Table 1 with liver disease) was a 27-year-old man who presented with cervical lymphadenopathy, fever, weight loss, and itching; lymph node biopsy was diagnostic for a T-cell/histiocyte-rich large B-cell lymphoma. Disease staging demonstrated multiple sites of involvement so that a diagnosis of stage IV disease, age-adjusted International Prognostic Index 4, was performed. During treatment with chemotherapy the patient developed liver failure that precluded the administration of further

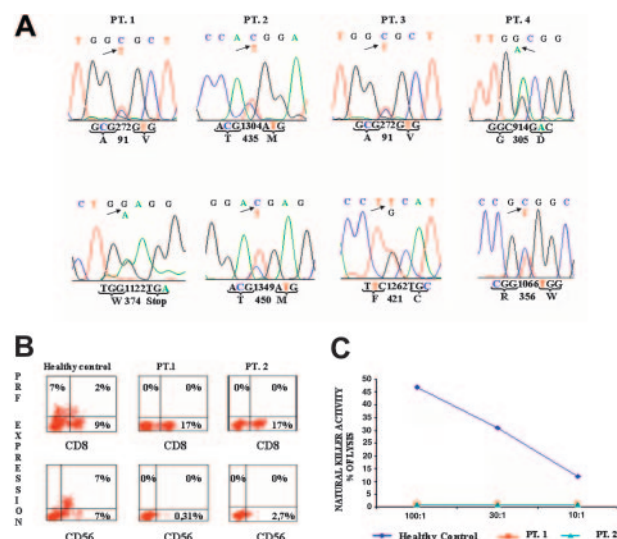


Figure 1. Molecular analysis of the perforin gene, perforin expression, and NK activity. (A) Molecular analysis of the perforin gene in the 3 patients who had the biallelic mutations. Study of the perforin gene was performed as reported by Stepp et al⁴ sequencing exon 2 and 3. The Blast program¹⁶ was used to compare the obtained sequences to the reported gene structure gene index ([GI] 190339). Red identifies thymine (T); black identifies guanine (G); blue identifies cytosine (C); green identifies adenine (A); corresponding initials are shown above the curves. (B) Perforin (PRF) expression in CD8⁺ and CD56⁺ cells was detected by FACS analysis after membrane staining with CD8 FITC and CD56 FITC monoclonal antibodies (mAbs; Becton Dickinson [BD]) and intracytoplasmic staining with perforin phycoerythrin (PE) (BD). Patients 1 and 2 (PT.1 and PT.2) were tested. (C) In vitro NK activity tested against the K562 cell line as target and expressed as percent specific ⁵¹Cr release at the different effector-target ratios of 100:1, 30:1, and 10:1 of Pt 1 and Pt 2. Results are compared with that of a healthy control.

cycles of intensive chemotherapy. He died due to disease progression, despite treatment also with rituximab. This patient had a history of previous splenomegaly, intestinal obstruction due to mesenteric lymphadenopathy, and severe thrombocytopenia requiring splenectomy. At age 25 years he had developed cervical adenopathy. In view of the clinical symptoms associated with an increase in peripheral CD4 and CD8 double-negative T-cell receptor α/β^+ lymphocytes and defective in vitro Fas-mediated apoptosis, a diagnosis of autoimmune lymphoproliferative syndrome (ALPS) had been made, and the patient had undergone exeresis of the cervical lymph nodes. Lymph node examination disclosed reactive follicular hyperplasia; bone marrow biopsy was negative for lymphoma localization. In this patient, *Fas* gene sequencing demonstrated a novel point mutation in intron 7 affecting a canonical splicing site (IVS7nt1G>A). The *Fas* mutation was inherited from his healthy father and was also present in a healthy brother, whereas the perforin gene mutation was detected in his healthy mother. Further details on this patient have been reported elsewhere.¹⁷ After having identified this double mutation of the perforin and *Fas* genes in this patient, we also tested 3 of the remaining 4 patients with monoallelic mutations of the perforin gene and causal mutations were not found in either the *Fas* gene-coding sequence or the intron-exon boundaries.

We investigated the presence of the mutations that we found in patients also in healthy controls. We found the A91V mutation in 10 of 492 control alleles (2%), the difference between patients and controls being statistically significant ($P < .004$). The mutation N252S was present in 1 of 660 alleles (0.2%) tested in our laboratory (492 alleles) and in another laboratory (164 alleles). This is not statistically significant due to the rarity of the event in the cohort of controls. The other mutations were not found in the controls.

In addition to the molecular analysis of the perforin gene (Figure 1A), perforin expression and NK activity were evaluated in

2 of the 4 patients with biallelic mutations of the perforin gene (Pt 1 and Pt 2). Figure 1B shows that in contrast to healthy controls, in whom perforin expression was present in both CD8⁺ and CD56⁺ lymphocytes, patients did not show perforin expression in the CD8⁺ or the CD56⁺ cells. As previously observed in early onset HLH-patients,⁴ lack of perforin expression was associated with undetectable NK activity (Figure 1C).

We also studied NK activity in 2 of the 4 patients with monoallelic mutations and it was present and comparable to that of healthy subjects.

Discussion

The rejection of many experimental cancers by CTLs and NK cells is dependent on the pore-forming protein perforin,^{12,18,19} and previous work with perforin-deficient mice demonstrated that perforin plays an important role in maintaining immune surveillance,²⁰ which protects against development of lymphoproliferative disorders in vivo.^{10,12} In these experimental animal models, the increased incidence of lymphoma associated with perforin deficiency is conceivably due to a defective cytotoxic mechanism physiologically involved in maintaining immune homeostasis, favoring the uncontrolled proliferation and expansion of premalignant lymphoid cells, which, in turn, may acquire a malignant phenotype.

In 1999, Stepp et al⁴ first suggested that, in humans, perforin deficiency is responsible for an acute immune dysregulation leading to HLH. Other mutational analyses and linkage analyses of subsets of consanguineous families have provided evidence that perforin mutations account for 20% to 40% of HLH.⁵⁻⁷ The results of our study, as well as previously published data on atypical HLH phenotypes,¹⁴ point to a more complex dysregulation of the immune system when perforin is absent, which may be associated with quite different clinical presentations, some patients developing HLH and others lymphoproliferative disorders. The observation of biallelic mutations of the perforin gene in patients with lymphoma and the presence in the same family of lymphoma and HLH provide further support to the hypothesis that the resulting effect of perforin mutations is likely to be more complex than previously anticipated and may vary among individuals, possibly depending on additional genetic and/or environmental factors.²¹ In this regard, it is also important to note that in the 4 patients reported here, the lymphomas associated with biallelic mutations of the perforin gene are of different types, including B-cell and T-cell non-Hodgkin lymphomas as well as Hodgkin disease.

The patients who had either biallelic or monoallelic mutations of the perforin gene were aged between 7 and 29 years (Table 1), suggesting that functional deficiency of the lytic activity of perforin or dysregulation of immune response associated with mutations of this pore-forming protein may be involved in the pathogenesis of lymphoproliferative disorders occurring in young people. The limited cohort of our patients does not allow for conclusions to be drawn on either the possible role of mutations of the perforin gene in the pathogenesis of the different types of lymphoma in elderly people or the real incidence of this genetic event in patients with lymphoproliferative disorders.

While frame-shift mutations or mutations of the perforin gene leading to stop codons have an unquestionable causative role in the loss of functional activity, mutations causing a single amino acid substitution have to be interpreted with more caution, being often unproved whether they are associated or not with a loss of functional activity. In particular, the role of the A91V substitution is still debated, as some researchers believe that it represents a simple

polymorphism, and because it has never been found in a homozygous state in patients.^{7,22} The finding that NK activity was null and perforin expression was absent in Pt 1 and her brother,¹⁴ as well as in 3 other patients with a confirmed diagnosis of HLH having recurrent re-exacerbations of the disease (R.C., unpublished personal data, March 2002), is in favor of a causative role of this mutation rather than supporting it as a simple polymorphism. In these 4 HLH patients, the A91V mutation, found in compound heterozygosity with a second mutation (W374Stop in 2 cases; R232H and del283L in 1 case each), was associated with either absent or very low perforin expression, as well as with profound impairment of NK activity. In order to be cured of their disease, 3 of these 4 patients required hematopoietic stem cell transplantation. Finally, it has been recently reported that in a rat basophilic leukemia cell line this mutation results in a marked decrease of lytic activity in comparison to the wild-type line.²³

The finding that 4 of the 29 patients studied had a monoallelic mutation of the perforin gene raises the question of whether and how this genetic event increases susceptibility to the development of lymphoma. One interpretative hypothesis is that monoallelic mutations of the perforin gene may act as inherited risk factors, which, in addition to other genetic variations (either somatic or constitutional) and/or in presence of environmental factors, predispose to the development of lymphomas. One piece of evidence supporting this hypothesis is represented by case 11 in Table 1, who had an additional mutation of the *Fas* gene.¹⁷ A pathogenetic role for defects in the *Fas* system in the development of lymphomas has been suggested by the identification of clonal somatic *Fas* mutations in B-cell lymphoproliferative disorders.²⁴ It is thus possible to speculate on a synergistic effect of the mutations in the perforin and *Fas* gene in our patient. Indeed, defective *Fas*-mediated apoptosis might have favored prolonged survival of lymphocytes, which may then be targets for transforming events, and the combined defects in

2 major pathways of cell-mediated cytotoxicity (ie, *Fas*-mediated killing and the granule-exocytosis pathway) could have blunted the capacity of the immune system to eliminate transformed cells. As we did not find any mutation of the *Fas* gene in the 3 more patients with monoallelic mutation of the perforin analyzed, we hypothesize that additional still undiscovered genetic defects, or possibly even environmental factors, could contribute, together with the impairment of perforin function, to the development of lymphoma. In this regard, candidate molecules to be investigated could be *Fas* ligand and caspases 8 and 10; it cannot be excluded that patients with somatic mutations of *Fas* are at risk for cancers originating in *Fas*-defective hematopoietic cells.^{25,26}

As immune surveillance is believed to play a major role in preventing tumor growth and/or development of autoimmune disorders, the range of clinical manifestations associated with perforin mutations may be wider than currently thought.²¹ In particular, our study suggests that perforin-dependent granule-mediated cytotoxicity plays a protective role against the development of lymphoproliferative disorders not only in mice^{10,12} but also in humans. Future studies should extend the analysis of the frequency of mutations of this gene in larger cohorts of patients with lymphoma in order to obtain more precise information on the incidence of this genetic event and should evaluate the potential role of perforin in controlling the development and growth of other tumors arising in nonlymphoid tissues, such as melanoma, where the role played by failure of immune surveillance in the pathogenesis of the disease is believed to be crucial.

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