

## Original Article

# Perforin gene mutations in 77 Chinese patients with lymphomas

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**BACKGROUND:** Perforin gene (*PRF1*) mutations have been reported in patients with lymphoma, but the prevalence and characteristics of *PRF1* mutation have not been identified in Chinese patients with lymphoma.

**METHODS:** Seventy-seven patients with lymphoma, including 6 patients with Hodgkin lymphoma and 71 patients with non-Hodgkin lymphoma, were recruited. DNA samples from peripheral blood were used for *PRF1* mutation detection by the PCR-sequencing method.

**RESULTS:** Eleven novel *PRF1* mutations were found in 8 of the 77 patients with lymphoma. Biallelic or compound monoallelic missense mutations in 3 patients indicated the severe impairment of perforin function, monoallelic missense mutations in 3 patients possibly contributed a genetic predisposition to malignancies, and synonymous mutations in 2 patients showed unknown significance.

**CONCLUSIONS:** The frequency of EBV infection was similar in lymphoma patients with *PRF1* mutations and those without the mutations. The same *PRF1* mutations were also found in DNA samples from nails or hair follicles from 4 patients with *PRF1* mutations, suggesting that these mutations may be of germ-line origin.

**KEY WORDS:** Perforin; Mutation; Lymphoma

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## INTRODUCTION

Lymphoma is a panel of diseases characterized by uncontrolled clonal proliferation of mature or immature B cells, T cells or natural killer (NK) cells. Because of the various maturities of the malignant cells, lymphoma can be classified based on their corresponding differential stage. Lymphoma is a group of bewildering diseases in their limited effective therapy and untoward prognosis. Various approaches have been applied to clarify the pathogenesis of lymphoma, such as mRNA transcriptome,<sup>[1]</sup> miRNA targetome,<sup>[2]</sup> cell signaling,<sup>[3]</sup> gene translocation,<sup>[4]</sup> and Epstein-Barr virus infection,<sup>[5,6]</sup> etc. However, our understanding in development of neoplastic lymphoid cells is largely insufficient.

Perforin is a membrane-disruptive protein secreted

by cytotoxic T lymphocytes (CTLs) and NK cells, which is essential for killing virus-infected and transformed cells through the granule exocytosis pathway.<sup>[7]</sup> One outstanding finding in perforin gene (*PRF1*) knockout mice is the predisposition of spontaneous malignancy from distinct lymphoid cell lineages.<sup>[8]</sup> In addition to familial hemophagocytic lymphohistiocytosis due to *PRF1* mutations, human lymphomas were also found to harbor *PRF1* mutations in some cases.<sup>[9,10]</sup> The presence of *PRF1* gene mutations may be different in different geographic regions and ethnic groups,<sup>[11,12]</sup> and little is known about the mutations in Chinese populations. Here we report our findings of mutations in the *PRF1* gene and the association with EBV infection in Chinese patients with lymphoma.

## METHODS

### Patients

We recruited 77 unrelated Chinese patients with lymphomas (43 males and 34 females; their age ranged from 2 to 73 years) treated in the two hospitals during the period of 2007–2010. They were sporadic cases with complete clinical data and confirmative diagnosis complying with the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues, 2008. Six cases were Hodgkin's lymphoma, including nodular lymphocyte predominant type 2 cases, mixed cellularity classical type 2 cases, and nodular sclerosis classical type 2 cases; and 71 cases were non-Hodgkin lymphoma, including diffusion large B cell lymphoma (DLBCL) 30 cases, follicular lymphoma (FL) 15 cases, anaplastic large cell lymphoma (ALCL) 10 cases, T lymphoblastic lymphoma 6 cases, NK/T lymphoma 3 cases, splenic marginal zone lymphoma (SMZL) 2 cases, mucosa-associated lymphoid lymphoma 2 cases, B lymphoblastic lymphoma 2 cases, and transformation of FL to DLBCL 1 case. Genomic DNA samples from 63 healthy medical students were used as controls. Informed consent was obtained from all participants or their guardians. The Medical Ethics Committee of Peking University First Hospital and the Institutional Review Board of General Hospital of Armed Police approved the research proposal.

### Sample preparation

Peripheral blood obtained from the patients was centrifuged and separated into plasma and cell fractions. Mononuclear cells from the cell fraction were separated by Ficoll-Paque density gradient method (Pharmacia, Piscataway, NJ, USA). Genomic DNA was extracted from  $(2-3) \times 10^6$  mononuclear cells using the QIAamp Blood Kit (Qiagen, Hilden, Germany) and re-suspended in 50  $\mu$ L elution buffer. DNA sample from nails or hair follicles was extracted by using the QIAamp DNA Investigator Kit (Qiagen).

### PRF1 mutation analysis

The coding region of the *PRF1* gene (exon 2 and exon 3) was amplified from genomic DNA using polymerase chain reaction (PCR). The primers 2F (5'-CCTTCCATGTGCCCTGATAA) and 2R (5'-GCCAGGATTGCAG TTTCTTC) were used for the amplification of exon 2, the primers 3aF (5'-CCCTGGGTTCCAGTCC TAGT) and 3aR (5'-GCCCTGTCCGTCAGGTACT) were used for the amplification of 5' part of exon 3, and the primers

3bF (5'-GTGGACTACACCCTGGAACC) and 3bR (5'-CTGGTCCTTTCCA AGCTCAC) were used for the amplification of 3' part of exon 3. PCR was performed in a 50  $\mu$ L mixture containing 2  $\times$  PCR mixture 25  $\mu$ L, primers 10 pmol/each, and genomic DNA 125 ng, with the thermal cycle condition of 95  $^{\circ}$ C 5 minutes for pre-denaturation, 35 cycles at 95  $^{\circ}$ C 30 seconds, 60  $^{\circ}$ C 30 seconds and 72  $^{\circ}$ C 45 seconds, followed by extension at 72  $^{\circ}$ C for 7 minutes. PCR products were sequenced from both directions using the same primers for PCR.

### Evaluation of serum EBV copy number by quantitative real-time PCR and EBV infection in lymphoma by *in situ* hybridization

A primer set to amplify a DNA fragment in EBV genome and a fluorogenic TaqMan probe was used to measure serum EBV DNA (EBV quantification kit, Da-An, Guangzhou, China). The amount of EBV DNA was expressed as EBV copies/mL of serum. A fluorescein-conjugated RNA probe was used to detect EBER1 RNA in lymphoma sections by *in situ* hybridization, and the signals were examined under a microscope.

### Statistical analysis

Allele frequency of a SNP = (cases of homozygote  $\times 2$  + cases of heterozygotes)/total cases  $\times 2$ . Genotype and allele frequencies of a SNP in lymphoma and normal controls were assayed for Hardy-Weinberg equilibrium. The Chi-square test or Fisher's exact test was used for the comparisons of genotype and allele frequencies of a SNP between patients and controls, patients with and without *PRF1* mutation, and EBV infection positive and negative patients.

## RESULTS

### Eleven novel mutations identified in 8 of the 77 Chinese patients with lymphoma

Table 1 summarizes the results of *PRF1* mutation detection in the 77 lymphoma patients. We found 11 novel mutations in 8 lymphoma patients (Table 1), and none of them were detected in healthy controls. Three patients had one or two biallelic missense mutations (cases 2 and 7), or two monoallelic missense mutations (case 8), indicating that their perforin function was severely impaired. Three patients had one monoallelic missense mutation (cases 1, 3 and 4), suggesting that their perforin function may be partially impaired. We found 3 synonymous mutations in 3 patients (cases 5, 6 and 7), of which case 7 also had two biallelic

**Table 1.** Eleven novel missense and/or synonymous mutations in the *PRF1* gene identified in 8 Chinese patients with lymphoma

Case no.	Sex/age (y)	Mutation	Lymphoma immunohistochemistry	Primary involvement site	Diagnosis	Bone marrow infiltration	Hemophagocytosis	EBER 1 in lymphoma	Therapy	Survival period
1	M/10	c.17T>C, (L6P)[m]	CD3+, CD43+, Ki-67+, CD15-, CD20-, CD79a-	Neck lymph node	Hodgkin, mixed cellularity type	+	-	-	Chemo + radio	4 y
2	M/53	c.374T>A, (I125N)[b]	CD3+, CD10-, CD45 RO+, CD79a-, CD57-, CD20-	Spleen	T cell lymphoma	+	-	+	Chemo	6 m
3	M/39	c.563C>T, (P188L)[m]	CD30+, CD43+, CD20-, CD3-, CD15-	Neck lymph node	ALCL	-	-	-	Auto-HSCT	>3 y
4	M/72	c.1153C>T, (R385W)[m]	CD22+, CD19+, CD10+, CD34+, CD13+, HLA-DR+, CD45dim+	Submandibular lymphnode	Transformation of FL to DLBCL	-	-	-	Chemo + radio	9 y
5	F/60	c.9C>T, (A3A)[b]	CD20+, CD10+, Bcl-6+, MUM-1+, CD3-, CD68-	Submandibular lymphnode	DLBCL	-	-	-	Chemo	>1 y
6	F/60	c.180A>G, (P60P)[m]	CD3+, CD56+, Ki-67+, CD20-, CD5-	Grz+, Left eye	NK/T	-	+	-	Chemo + radio	5 m
7	M/59	c.32T>C, (L11P)[b], c.216C>A, (T72T)[b], c.491A>T, (Q164L)[b]	CD5+, CD20+, CD79a+, CD43-, CD68-	Spleen	SMZL	+	-	-	Chemo	1.5 y
8	F/02	c.326C>G, (A109G)[m], c.506T>C, (F169S)[m]	CD19+, CD10+, CD20+, CD79a+, CD3-	Right eye	DLBCL	+	-	-	Chemo	3 m

[m]: monoallelic mutation; [b]: biallelic mutation; Chemo: chemotherapy; radio: radiotherapy; auto-HSCT: auto hematopoietic stem cell transplantation

missense mutations. The 11 missense and synonymous mutations are not found in the Human Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>) nor in the SNP database (<http://www.ncbi.nlm.nih.gov/snp/?term=PRF1>).

To identify whether these mutations were of germline origin or somatic mutations only limited in tumour cells, we collected nails and/or hair follicles from 4 patients (cases 3, 4, 6 and 7) with missense or synonymous mutations, and extracted DNA from these samples by using a special DNA extraction kit. Target DNA fragment could be amplified and sequenced from these samples except the nail DNA of case 4. All of them were found to have mutations in nails (cases 3 and 7) or hair follicles (cases 4 and 6) same as those in peripheral blood. Therefore, these mutations seem to be of germline origin.

#### No differences in the 4 SNPs in the *PRF1* gene between lymphoma patients and healthy controls

The genotypes of SNPs rs885821 (in exon 3), rs885822 (in exon 3), rs10999427 (in intron 2), and rs10999426 (in exon 2, closely linked to rs10999427) were also identified during the mutation detection. There were no differences in genotype and allele frequencies in the 4 SNPs between lymphoma patients and controls (data not shown).

#### No significant difference in EBV infection between lymphoma patients with *PRF1* mutation and those without the mutation

We measured serum EBV copy number and detected EBV RNA in lymphomas by *in situ* hybridization in the 8 lymphoma patients with *PRF1* mutations. One of the 8 patients (case 2) was found to have positive serum EBV DNA ( $5 \times 10^2$  copies/mL) and positive EBV RNA in lymphoma sections, indicating EBV infection in lymphoma. We also measured serum EBV copy number in 25 lymphoma patients without *PRF1* mutations. Three patients were found to have positive serum EBV ( $2.3 \times 10^3 - 2.7 \times 10^4$  copies/mL). Subsequently, the prevalence of EBV infection seems to be similar between lymphoma patients with *PRF1* mutation and those without mutation.

## DISCUSSION

To date, there are about 60 mutations in *PRF1* documented in several clinical diseases, such as in familial hemophagocytic lymphohistiocytosis and lymphoma.<sup>[13]</sup> The prevalence and characteristics of *PRF1* mutation may be different in different populations.<sup>[11,12]</sup> In this study, we found 11 novel mutations in 8 of 77 Chinese patients with lymphoma. We collected nails and/or hair follicles from 4 patients with *PRF1* mutations. The 6 mutations (4 missense mutations and two synonymous mutation) detected in

peripheral blood in the 4 patients were also found in their nails and/or hair follicles, suggesting the germ-line origin of the 6 mutations. Consequently, genomic mutations in the *PRF1* gene may be important factors in the pathogenesis of lymphoma in some lymphoma patients. Nine of the 11 novel mutations were located in exon 2. Of the 8 missense mutations in 6 patients, 5 were located in the first low homology domain of perforin protein (cases 2, 3, 7 and 8), 2 were located in the signal peptide region (cases 1 and 7), and 1 in the EGF-like domain (case 4). Therefore, mutations in exon 2 and in the first low homology domain of *PRF1* protein may be the hotspots for *PRF1* mutations in Chinese lymphoma patients. We did not find mutations in the amphipathic alpha helix and the second low homology domain, which were frequently reported in lymphoma patients in western countries.<sup>[9,10,13,14]</sup>

Functional perforin plays a critical role in immune surveillance of viral infected cells and abnormal transformed cells.<sup>[15,16,17]</sup> Perforin mutations resulting in the decrease or absence of the protein or its activity cause the uncontrolled cellular proliferation or lymphoproliferative disorders.<sup>[7,18]</sup> EBV infection is closely correlated to lymphoma in some cases.<sup>[19]</sup> To evaluate whether uncontrolled EBV infection due to *PRF1* mutations is correlated to the emerging of lymphoma in patients with *PRF1* mutations, we assayed serum EBV DNA and EBER1 expression in lymphoma in the 8 lymphoma patients with *PRF1* mutation and 25 patients without *PRF1* mutation. The prevalence of EBV infection was similar in lymphoma patients with *PRF1* mutation and those without the mutation, suggesting that the uncontrolled EBV infection may not be the leading cause of lymphoma in patients with *PRF1* mutation. Moreover, *PRF1* mutation may not be associated with the higher prevalence of EBV infection.<sup>[11,20,21]</sup>

In summary, 11 novel *PRF1* mutations were found in 8 of the 77 lymphoma patients. These mutations may be of germ-line origin, and may therefore be inherited from their parents. Three patients (cases 2, 7 and 8) had biallelic or compound monoallelic missense mutations, which may be the direct cause of lymphoma. Another 3 patients (cases 1, 3 and 4) carried monoallelic missense mutation. Monoallelic *PRF1* mutation usually has normal phenotype, but may contribute a genetic predisposition factor to develop malignant diseases, and may become one of the pathogenesis factors in the presence of other detrimental factors or gene variations. Three patients had monoallelic (case 6) or biallelic (cases 5 and 7) synonymous mutations in *PRF1* exon 1, of which the

significance is yet unknown.

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**Conflicts of interest:** The authors declare that there is no conflict of interest.

**Contributors:** Ding Q proposed the study, analyzed the data and wrote the first draft. All authors contributed to the design and interpretation of the study and to further drafts.

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