doi:10.4149/neo_2010_06_507

Better detection of Ig heavy chain and TCRγ gene rearrangement in plasma cell-free DNA from patients with non-Hodgkin Lymphoma

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Received January 18, 2010

Plasma cell-free DNA is the soluble DNA and tumor-derived DNA in plasma which has the same mutation as the tumor cellular DNA. This study aimed at comparing the properties of plasma cell-free DNA with the biopsy's DNA in order to evaluate the clinical significance of IgH and TCRγ gene rearrangement in plasma cell-free DNA from patients with non-Hodgkin's Lymphoma. A total of 360 samples were studied. IgH (FR3A/VLJH) and TCRγ (TVG/TJX) were amplified by PCR. Results of plasma cell-free DNA were compared with biopsy's DNA and mononuclear cellular DNA respectively. Plasma cell-free DNA were successfully extracted from 288 cases of newly diagnosed, refractory and relapsed NHL in total 360 patients (80%).But nothing was found in the other 72 remittent patients. The positive percentage of IgH rearrangement in patients with B-NHL was 81% in plasma cell-free DNA and 77% in biopsy's DNA (P>0.05). As to the ratio of TCRγ rearrangement in patients with T-NHL, the former was 44%, and the latter was 39% (P>0.05). These results show tumor-derived DNA could be detected in tumor loaded plasma, even of underlying cancer patients. For NHL patients, detecting IgH and TCRγ gene rearrangement of plasma cell-free DNA has the same clinical significance as biopsy's DNA. Moreover, it's more simple, convenient and non-invasive.

Key words: Lymphoma non-Hodgkin, plasma, cell-free DNA, gene rearrangement, immunoglobulin, heavy-chain gene, T-cell receptor.γ

In 1977, Leon firstly found that the concentration of plasma cell-free DNA in cancer patients was much higher than that in normal people [1]. A number of research results indicate that tumor-derived DNA could be released into the circulation and enriched in plasma. Furthermore, it has the same gene mutation as the tumor cellular DNA [2, 3]. Hence it could stand as evidence for the presence of tumor. Using plasma cell-free DNA as sample would be a new, convenient and non-invasive diagnostic method for tumors because it may come from each organ and be associated with special mutation of many different kinds of cancers [4]. Testing this suggestion could be an interesting and seemed easy to undertake by the development of molecular analysis. Different detection methods have been used, such as microsatellite typing to show the presence of LOH and specific mutation analysis such as p53.

Lymphoma is one kind of therioma that derived from lymph nodes or other lymph tissues. It's one of the ten most common malignant tumors in China. Some of these patients don't have superficial lymph nodes intumesce, but have tumor-cells attacked inner core of certain organ or body structure. So these people are often delayed or misdiagnosed for absence of pathological proof. Due to the lack of effective screening tools, early discovery of lymphoma is the biggest challenge we're facing. It's of great importance to establish sensitive and reliable methods for earlier diagnosis of lymphoma. Both IgH and TCRγ gene rearrangement are the specific markers of lymphocytes malignant proliferation. Lymphoma cells could liberate soluble DNA into plasma. Thus detection IgH and TCRγ rearrangement by plasma cell-free DNA should have potentially clinically important effect on diagnose and prognosis of NHL. To determine the incidence and magnitude of this phenomenon we detected the rearrangement of IgH and TCRγ gene in total 360 samples via PCR. It become clear that monoclonal IgH DNA and TCRγ DNA could be amplified from cell-free blood sample of some NHL patients with different stages and histological types. Furthermore, it's one of prognostic factors. It's proved that detection of rearrangement of IgH and TCRγ in plasma is an effective approach for early diagnosis of NHL and forecasting prognosis.

Materials and methods

Patients and samples. From Nov. 2007 to May 2009, 360 patients (180 males and 180 females with a M vs F ratio of 1:1)

consecutively admitted, newly diagnosed NHL patients in our hospital were enrolled in this study. The median age of them was 45 years with a range from 12 years to 76 years. The World Health Organization classification of Neoplastic diseases of the hematopoietic and lymphoid tissues were performed for the diagnosis and sub-classification of all patients. 190 patients were diagnosed as B-NHL including 79 cases of DLBL (Diffuse large B-cell lymphoma), 25 MCL (Mantle cell lymphoma), 22 FL (Follicular lymphoma), 9 BL (Burkitt's lymphoma), 25 MZL (Marginal zone lymphoma), 17 B-SLL (B-small lymphocytic lymphoma), 13 B-LBL (B-lymphoblastic lymphoma). 170 patients were diagnosed as T-NHL including 21 cases of AITCL (Angioimmunoblastic T-cell lymphoma), 22 PTCL (Peripheral T-cell lymphoma), 12 ALCL (Anaplastic large cell lymphoma), 12γ/δHSTCL (Hepatosplenic gamma/delta T-cell lymphoma), 57 NK/T (NK/T-cell lymphoma), 20 T-SLL (T- small lymphocytic lymphoma), 26 T-LBL (T-lymphoblastic lymphoma). The normal control group consisted of 360 healthy volunteers. The EDTA PB samples were drawn and then spun down. Plasma and cells were collected respectively and frozen until DNA was extracted.

DNA extraction. Cellular DNA was extracted using the SBS DNA extraction kit (SBS, Beijing, China) according to the manual. Plasma DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manual. Briefly, 200μl of thawed plasma was added to a mixture of 20μl proteinase K and 200μl AL buffer (Qiagen) and incubated at 56°C for 30min.Then 200μl of 100% ethanol was added to the mixture followed by the elution of the mixture using the QIAamp spin column in two equivalent increments. The column was then washed with three different buffers (AW1, AW2, AE; Qiagen). DNA was eluted to a volume of 50μl with the last buffer.

PCR. The DNA products from both the cells and plasma were analyzed for clonotypic DNA. The exquisite sensitivity of the polymerase chain reaction meant DNA contamination could ruin an entire experiment. Tidiness and adherence to the strict set of protocols advised by Kwok [5] could avoid the disaster. PCR reactions were set up in a total volume of 25μl that were composed of 12.5μl premix Taq (TaKaRa, Dalian, China), 0.2μl 25μmol/L each of two primers (Invitrogen, Shanghai, China), 4.0μl DNA template (0.8μg) and 8.3μl dH_2O. They are cycled in a Thermal Cycler (Bio-RAD, American). To show intact DNA, a 196bp globin DNA fragment was amplified using primers 5'-GATCTGTCCACTCC TGATGCTG-3' and 5'-ATCAAGCGTCCCATAGACTCAC-3'. Cycling conditions were: 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 59°C for 30seconds, 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. For amplification of rearranged CDRIII of IgH gene, a semi-nested PCR strategy was used: Thirty cycles of a first-round PCR with a FR3A primer 5'-ACACGGCCGTGTAT TACTGT-3' and a LJH primer 5'-TGAGGAGACGGTGA CC-3' were followed by 20 cycles using the same FR3A primer combined with a nested VLJH primer 5'-GTGACCAGGGTTCCTTGGCCCCAG-3'. 2μl of the first-round PCR product was used in the second- round PCR. Thermocycling conditions were: First turn(FR3A/LJH): 95°C,3 min; 94°C, 30 sec; 55°C, 30 sec; 72°C, 40 sec, 30 cycles with final extended at 72°C for 10 min; second turn(FR3A/VLJH): 95°C,3 min; 94°C, 30 sec; 59°C, 30 sec; 72°C, 30 sec, 20 cycles with final extended at 72°C for 10 min. Raji cell line was taken as the positive control. Cellular DNA of normal people and no template DNA were taken as double negative controls. The targeting fragment was 80-120bp. Primers for TCRγ portion of the assay were: TVG5'-AGGGTTGTGTTGGAATCAGG-3', TJX5'-CGTCGACAACAAGTGTTGTTCCAC-3'. Touchdown PCR consisted of an initial denaturation step of 3 minutes at 95°C; followed by five cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds from 60°C to 55°C decreasing by 1°C every cycle, extension at 72°C for 40 seconds; and then 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 40 seconds at 72°C, with a final extension step of 10 minutes at 72°C. Jurkat cell line was taken as the positive control. Negative controls were the same as described previously. The targeting fragment was 160-190bp.10μl of the final PCR product was run on a 30g/L agarose gel. DNA was visualized by ethidium bromide staining. A sample was defined as monoclonal, if the assay showed distinct and single band in relevant area. If there was no band or smear band, the sample was taken as polyclonal.

Statistics. Chi-square test wwas used to compare qualitative variables. The rates of PCR-positive samples in patients with various clinical parameters of tumor load were compared by the two-tailed spearman rank correlation.

Results

Extractions of cell-free DNA from plasma. Extractions of plasma cell-free DNA were identified by electrophoresis results of Globin that's the genomic housekeeping gene (Fig. 1). Plasma cell-free DNA were successfully obtained from 288 cases (161 B-NHL cases and 127 T-NHL cases) of incipient, refractory and relapsed NHL out of total 360 patients (80%). But no plasma cell-free DNA was found in the other 72 patients of remission that had completed several phases of chemotherapy. In the 288 patients, there were 240 patients had neither BM nor PB attacked by lymphoma, and 48 patients had BM and/or PB attacked. While there were no plasma cell-free DNA extracted from the normal control group.

Monoclonal rearrangement of IgH and TCRγ gene using plasma cell-free DNA. Results of Monoclonal rearrangement of IgH gene and TCRγ gene using plasma cell-free DNA were shown in Table1, Fig.2 and Fig.3. Out of 161 B-NHL cases, 130 cases showed IgH rearrangement (81%) and none showed TCRγ rearrangement. In 127 T-NHL cases, 56 showed TCRγ rearrangement (44%). The incidence rate of TCRγ rearrangement was low in NK/T, AITCL and ALCL. And 2 cases out of 50(NK/T) had IgH gene rearrangement synchronously. IgH, TCRγ rearrangement could be found in patients of every stage, especially in some stage I patients. There were several patients in stage I just fever, night sweat and fleshless. And no patholog-

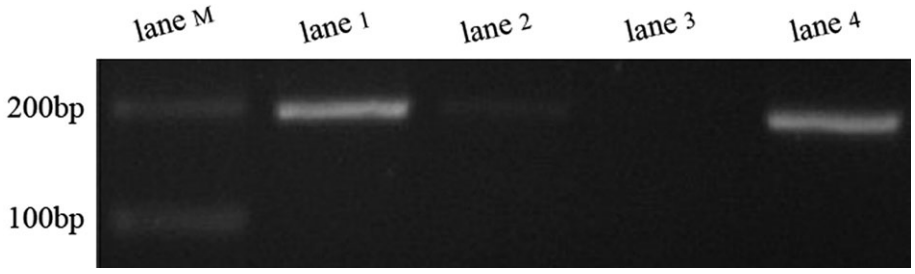

Fig. 1. Detection of Globin DNA in the plasma of patients with NHL. Lane M, DNA marker. Lane 1~4,PCR product of plasma cell-free DNA in NHL patients (196bp).

ical changed tissue was found. For these people, cell-free DNA and clonotypic rearrangement would be detected in plasma too. 47 of the 288 patients were followed up. In 19 of them, clonotypic rearrangement had been tested when they're incipient, but nothing was found when remission. In 11 of them, IgH and TCRγ rearrangement were detected before relapse. 17 of them were refractory and had constantly PCR positive results.

Comparison of IgH and TCRγ monoclonal rearrangement results of plasma cell-free DNA and biopsy's DNA. PCR amplification for IgH and TCRγ rearrangement of 210 patients were performed both in plasma cell-free DNA and biopsy's DNA. In the other 78 patients did not, or could not be performed biopsy. Collecting the same patient, sampling at the same time, using the same ways and primers, producing the same size of fragments, all of them was to make sure the consistency operation. Out of 107 B-NHL cases, there were 87 cases in plasma cell-free DNA (81%) and 82 cases in biopsy samples (77%) were positive (χ^2 =0.55, P=0.458). 17 patients were detected IgH rearrangement not from biopsy's DNA but from plasma cell-free DNA. 12 patients were positive only in biopsy. There were 8 patients negative both in plasma and in biopsy.

Table 1. Results of IgH and TCRγ rearrangement in plasma cell-free DNA

Histological subtype	Total n	IgH positive n	TCRγ positive n
B-NHL	161	130	0
DLBL	75	66	0
MCL	21	18	0
FL	18	14	0
BL	4	4	0
MZL	21	15	0
B-SLL	13	8	0
B-LBL	9	5	0
T-NHL	127	2	56
AITCL	13	0	6
PTCL	15	0	10
ALCL	8	0	5
γ/δ HSTCL	9	0	6
NK/T	50	2	12
T-SLL	13	0	7
T-LBL	19	0	10

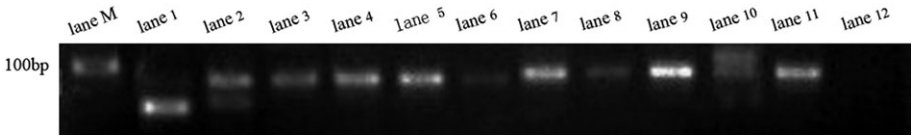

Fig. 2. Detection of IgH DNA in the plasma of patients with B-NHL. Lane M, DNA marker. Lane 1~9,PCR product of plasma cell-free DNA of B-NHL patients (80bp-120bp). Lane 10, PCR product of cellular DNA of normal people. Lane 11, Raji-positive control. Lane 12, no template negative control.

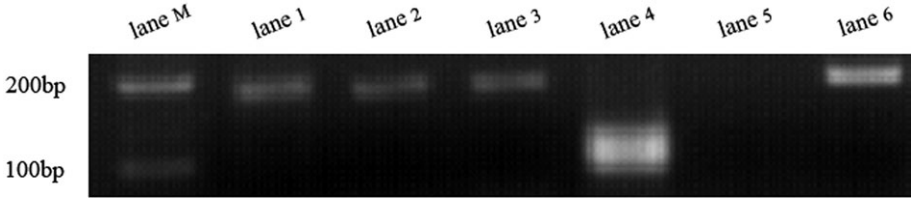

Fig. 3. Detection of TCRγ DNA in the plasma of patients with T-NHL. Lane M, DNA marker. Lane 1~3,PCR product of plasma cell-free DNA of T-NHL patients (160bp- 190bp). Lane 4, PCR product of cellular DNA of normal people. Lane 5, no template negative control. Lane 6, Jurkat- positive control.

Table 2. Patients' characteristics associated with CR

clinical parameters	N (%)	CR N (%)	P-value
Ann Arbor stage			
I-II	100(35)	92(92)	0.000
III-IV	188(65)	88(47)	
Serum LDH level			
N or less	128(44)	100(78)	0.000
Greater than N	160(56)	91(57)	
Serum β_2M level			
N or less	125(43)	93(74)	0.001
Greater than N	163(57)	91(56)	
IPI scores			
0-1	71(25)	59(83)	0.000
≥2	217(75)	110(51)	
Monoclonal IgH or TCRγ			
Positive	180(63)	86(48)	0.000
Negative	108(38)	95(88)	

LDH, lactic dehydrogenase; β_2M, β_2-microglobin; CR, complete remission

Table 3. Relevancy of IgH or TCRγ rearrangement with Patients prognostic variables

clinical parameters	N (%)	Rearranged (%)	P-value
Ann Arbor stage			
I-II	100(35)	23(23)	0.000
III-IV	188(65)	157(84)	
Serum LDH level			
N or less	128(44)	33(26)	0.000
Greater than N	160(56)	147(92)	
Serum β_2M level			
N or less	125(43)	31(25)	0.000
Greater than N	163(57)	149(91)	
IPI scores			
0-1	71(25)	5(7)	0.000
≥2	217(75)	175(81)	

While in 103 T-NHL patients, there were 45 cases in plasma cell-free DNA (44%) and 40 cases in biopsy (39%) were positive(χ^2=1.23, P=0.267). 9 patients got TCRγ rearrangement in plasma cell-free DNA and not in biopsy sample's DNA. 4 patients got TCRγ rearrangement in biopsy sample's DNA and not in plasma cell-free DNA. 54 patients got TCRγ rearrangement neither in plasma cell-free DNA nor in biopsy sample's DNA.

Comparison of IgH and TCRγ monoclonal rearrangement results of plasma cell-free DNA and mononuclear cellular DNA. In the 161 B-NHL patients there were 130 cases in plasma cell-free DNA (81%) were positive, and 32 cases of them were positive in mononuclear cellular DNA at the same time. While in 127 T-NHL patients, there were 56 cases in plasma cell-free DNA (44%) positive, and 25 out of 56 were positive in mononuclear cellular DNA synchronously.38 cases of the 57 patients which showed gene rearrangement in mononuclear cellular DNA had bone marrow attacked by lymphoma cells.

*Implications for various clinical parameters of tumor load in terms of NHL prognosis.*On the basis of clinical and instrumental findings, the International Prognostic Index (IPI) score, which represents a predictive model of outcome for patients with aggressive NHL based on age, disease stage, performance status, number of extranodal sites of disease, and LDH level, was also determined in this study. IPI score was also used to inertia NHL, but its precision was lower. By spearman rank correlation analysis, stage of disease, LDH, β_2M, IPI score and monoclonal IgH or TCRγ DNA were the prognostic variables (Table 2). And besides, monoclonal rearrangement correlated with Stage of disease, LDH, β_2M and IPI score (Table 3).

Discussion

The study presented here indicates that tumor-derived DNA can be frequently recovered from plasma of patients with NHL whose tumor cells are informative on PCR analysis. Plasma cell-free DNA can't be detected in normal people or is under the threshold of detection (<100μg/ml) [6]. There're reports of successful amplification of genomic or tumor-associated DNA from fresh or archival plasma samples, supporting our observation [7]. Plasma cell-free DNA might be associated with proliferation of tumor cells. We successfully obtain plasma cell-free DNA from 288 cases of incipient, refractory and relapsed NHL out of total 360 patients, but found nothing in the other 72 patients of remission. Treatment of patients with cytotoxic drugs was followed by rapid clearance of DNA from peripheral blood, suggesting soluble tumor-derived DNA could only be tested within limited disease stages. It's probably highly predictive for resistance to treatment and impending relapse [8].Degradation of clonal DNA by nucleases in vitro was shown to be one cause of false-negative PCR results. This technical drawback can be relieved by adding a nuclease inhibitor like EDTA. So we should use EDTA- anticoagulant tubes.

Lymphoma generates from malignant cloned hyperplasia of immature lymphocytes, and has IgH gene or TCR gene rearrangement in its transformation. Therefore, PCR amplification of IgH or TCRγ would be helpful to make a judge for lymphoma's diagnose and prognosis. If DNA shows distinct band of the same electrophoresis mobility, the sample is defined as monoclonal. Normal DNA and reactive DNA hyperplasia are polyclonal with smear bands [9]. It's said that about 80% of B-NHL have IgH monoclonal rearrangement and 30% of T-NHL have TCR monoclonal rearrangement [10].The latest study reports that positive ratio could be up to 99% (B-NHL)and 94% (T-NHL) using multiplex primers [11]. There were several patients with stage I high-grade lymphoma positive in plasma while negative in their peripheral blood cells. And their pathological tissues were hard to be obtained. For some of these patients, plasma cell-free DNA is important in early diagnosis. As pointed out by Tohda et al. [12, 13], tumors

release enough soluble DNA to be detected in plasma, and the positive ratio of IgH rearrangement is up to 86%. In our study, there were 81% of B-NHL with IgH rearrangement and 44% of T-NHL with TCRγ rearrangement by analyzing 288 patients with NHL. It's consistent with Tohda [12]. Two cases of T-NHL had IgH gene and TCRγ gene rearrangement synchronously. The phenomenon is called lineage promiscuity. Furthermore, we found out that testing gene rearrangement using plasma cell-free DNA as sample correlated with pathology. The significant correlation between plasma cell-free DNA and histopathologic examination seen in this study suggests that using plasma cell-free DNA as sample has high accuracy and crucial value. It's undoubtedly that plasma cell-free DNA is senior to mononuclear DNA. It seems that T cell lymphoma is more likely to attack bone marrow and PB than B cell lymphoma.

However, even when excluding methodological pitfalls such as interference by chemotherapy, there're false-negative PCR results. The proportion is low in LBL, SLL, MCL (<5%). Because somatic mutation hardly occurs in that stage. If B cells have already contacted with antigen such as FL, DLBL, BL, they may have high frequency of somatic mutation. So false-negative ratio will be high (15%-50%). In this assay, the positive ratio of TCRγ gene rearrangement is low. One explanation for this would be that NK/TCL has low incidence rate of TCRγ rearrangement. It's a cause of concern that malignant cells of AITCL are in early stage and don't have gene rearrangement [14].

As we know, many factors influence NHL patients' prognosis, such as histologic subtype, stage of disease, LDH level and IPI score. The study shows that there is a prevalence of monoclonal IgH and TCRγ DNA relate to complete remission, and also correlate with stage, LDH, β_2M and IPI. In addition, clonal rearrangement from plasma cell-free DNA could be detected in newly diagnosed, relapse and refractory subgroups, but not in remission. Thus it can be seen that IgH and TCRγ gene rearrangement in plasma cell-free DNA plays important role in terms of NHL diagnosis and prognosis including every single group.

In conclusion, plasma cell-free DNA should be added to the list of markers that can be used to monitor cancer patients. Using it as samples to test IgH and TCRγ rearrangement is more convenient, non-invasive. And besides, it has the same clinical meaning as biopsy sample.

Ackowledgement.We acknowledge the cooperation of all patients, nurses, and physicians who contributed to the collection of samples for this study.

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