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Cell-free circulating DNA in Hodgkin's and non-Hodgkin's lymphomas

S. Hohaus*, M. Giachelia, G. Massini, G. Mansueto, B. Vannata, V. Bozzoli, M. Criscuolo, F. D'Alò, M. Martini, L. M. Larocca, M. T. Voso & G. Leone

Istituto di Ematologia e di Anatomia Patologica, Università Cattolica S. Cuore, Rome, Italy

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Background: Levels of cell-free circulating DNA have been correlated to clinical characteristics and prognosis in patients with cancers of epithelial origin, while there are no data on patients with B-lymphoproliferative diseases.
Patients and methods: Cell-free DNA levels in the plasma samples of 142 patients with lymphomas [45 with Hodgkin's lymphoma (HL), 63 with diffuse large B-cell non-Hodgkin's lymphoma (DLBCL), 24 with follicular, and 10 with mantle cell non-Hodgkin's lymphoma (NHL)] at diagnosis and of 41 healthy individuals were determined using a quantitative PCR for the *β-globin* gene.
Results: Levels of circulating DNA in patients with HL, DLBCL, and mantle cell NHL were significantly higher than in controls ($P < 0.01$ for all). Increased levels of plasma DNA were associated with advanced stage disease, presence of B-symptoms, elevated lactate dehydrogenase levels, and age >60 years ($P = 0.009$; <0.0001; <0.0001; 0.04, respectively). In HL, histological signs of necrosis and grade 2 type of nodular sclerosis were associated with increased plasma DNA. Elevated plasma DNA levels were associated with an inferior failure-free survival in patients with HL ($P = 0.01$) and DLBCL ($P = 0.03$).
Conclusion: Quantification of circulating DNA by real-time PCR at diagnosis can identify patients with elevated levels that are associated with disease characteristics indicating aggressive disease and poor prognosis.
Key words: circulating DNA, Hodgkin's lymphoma, non-Hodgkin's lymphoma, prognosis, quantitative PCR

introduction

Extracellular circulating DNA can be found in small amounts in the plasma of healthy individuals. Increased levels of cell-free DNA have been reported in a number of clinical conditions as malignant and autoimmune diseases, myocardial infarction, trauma, and pregnancy-associated complications [1]. Circulating DNA in the blood of patients with cancer contains tumor-derived DNA, as shown by the presence of concordant genetic and epigenetic alterations both in the primary tumor and in plasma, offering a noninvasive mean to obtain surrogate tumor material [2].

While the presence of circulating DNA has been widely studied in solid tumors, there are only few descriptive reports on cell-free DNA in hematologic malignancies. Ras mutations, FLT3 internal tandem duplications, and loss of heterozygosity have been observed in cell-free DNA of patients with acute myeloid leukemia and myelodysplastic syndromes, indicating that plasma is a reliable alternative source to detect molecular abnormalities [3–5]. Little information is available for patients with lymphoproliferative diseases [6–9]. Frickhofen et al. detected clonotypic DNA from a rearranged immunoglobulin heavy chain locus in 86% of plasma samples of patients with B-cell malignancies at diagnosis. Detection of the bcl-2/IgH rearrangement in plasma may be a marker for minimal residual disease in patients with follicular lymphoma (FL) [8]. Kornacker et al. detected clone-specific DNA in the serum of a patient with Hodgkin's lymphoma (HL) both at diagnosis and at relapse, as proof of principle for the presence of circulating tumor-derived DNA in this disease.

Quantitation of circulating DNA has been proposed as a diagnostic biomarker for the detection of malignant epithelial diseases, but its prognostic role in these diseases is controversial [10–14]. There are no quantitative data on hematologic neoplasias and in particular, its prognostic role has to our knowledge not been defined. Circulating DNA has been detected using qualitative and semiquantitative methods; more recently, the introduction of real-time quantitative PCR for housekeeping genes, as β-actin or *β-globin*, has greatly improved the detection methods [15].

We used an accurate and reproducible real-time quantitative PCR for the *β-globin* gene to determine plasma DNA concentrations at diagnosis in 142 patients with B-cell-derived lymphomas and analyzed for associations with clinical characteristics and prognosis.

Correspondence to: Dr S. Hohaus, Istituto di Ematologia, Universita' Cattolica S. Cuore, L.go A. Gemelli 1, 00168 Rome, Italy. Tel: +39-06-30154180; Fax: +39-06-35503777; E-mail: stefan.hohaus@rm.unicatt.it

patients and methods

patients and controls

We studied plasma samples obtained at the time of initial diagnosis of 142 lymphoma patients, treated at our institution from February 2004 to January 2008. Patient characteristics are detailed in Table 1. Standard treatment for patients with stage II–IV non-Hodgkin's lymphoma (NHL) was for six to eight cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), administered every 14 days, in combination with Rituximab (R-CHOP) [16]. Patients with stage I disease without risk factors received four R-CHOP cycles, followed by radiotherapy. Initial treatment was R-CHOP-14 in 80 of 97 NHL patients, 21 of them also received consolidation therapy with three cycles of R-MICMA (mitoxantrone, carboplatin, cytosine arabinoside, and methylprednisolone) and high-dose therapy with peripheral blood stem-cell transplantation [13 patients with diffuse large B-cell lymphoma (DLBCL) and age-adjusted international prognostic index (IPI) ≥ 2 and eight patients with mantle cell lymphoma (MCL)] [17]. Twelve patients (eight patients with DLBCL and four patients with FL) received alternative regimens [cyclophosphamide, vincristine, and prednisolone (CVP), five patients; fludarabine, mitoxantrone, dexamethasone (FND), two patients; chlorambucil one patient; radiotherapy only, two patients; high-dose methotrexate, two patients], while five patients with FL were followed on a watch-and-wait strategy. For 45 patients with HL, therapy consisted of doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD, 24 patients), MOPP (mechlorethamine, vincristine, procarbazine, and prednisone)-containing regimens (five patients), and bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone [BEACOPP escalated in 16 patients with advanced-stage disease (stage IIB with bulky disease—IV) and <60 years of age] [18]. Radiotherapy was included for consolidation in patients with limited stage or initial bulky disease.

A control group consisting of 41 healthy individuals (19 males, 22 females; median age 32 years, range 19–74) was included in order to determine the normal range of circulating DNA in plasma. Informed consent was obtained from patients and controls according to institutional guidelines, and blood sample collection was approved by our institutional ethical committee.

Table 1. Patient characteristics

Variable	Total (n = 142)	DLBCL (n = 63)	MCL (n = 10)	FL (n = 24)	HL (n = 45)
Age, years					
Mean (range)	52 (15–92)	58 (15–92)	58 (44–75)	62 (35–77)	37 (15–83)
<60	82	28	6	8	40
>60	60	35	4	16	5
Gender					
Female	76	32	2	13	29
Male	66	31	8	11	16
Stage					
I–IIA	61	30	1	3	27
IIB–IV	77	30	9	21	17
Missing	4	3	0	0	1
B-symptoms					
No	101	46	6	21	28
Yes	39	16	4	3	16
Missing	2	1	0	0	1
Bulky disease					
No	93	45	6	14	28
Yes	44	17	4	9	14
Missing	5	1	0	1	3
LDH					
Normal	98	32	8	19	39
Elevated	37	30	1	2	4
Missing	7	1	1	3	2
IPI age adjusted					
0/1	66	39	8	19	–
2/3	27	22	1	4	–
Missing	4	2	1	1	–
IPS					
0–2	–	–	–	–	29
≥3	–	–	–	–	7
Missing	–	–	–	–	9
Therapy					
Standard[a]	82	42	2	14	24
Intensified[b]	38	13	8	1	16
Other	20	6	0	9	5
Missing	2	2	0	0	0

[a]Standard: R-CHOP in NHL and ABVD in HL.
[b]Intensified: R-CHOP followed by high-dose chemotherapy in NHL and BEACOPP in HL.
DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; HL, Hodgkin's lymphoma; LDH, lactate dehydrogenase; IPI, international prognostic index; R-CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone in combination with Rituximab; NHL, non-Hodgkin's lymphoma; ABVD, doxorubicin, bleomycin, vinblastine, and dacarbazine; BEACOPP, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone.

sample collection and DNA isolation

All plasma samples were collected at diagnosis, before cytotoxic therapy start. EDTA was used as anticoagulant, and samples were stored at +4°C and processed within 8 h [19]. In order to avoid contamination by blood cells, plasma was isolated by two consecutive centrifugations at 2500 *g* for 10 min at 4°C without brake. Supernatants were carefully removed, and aliquots were stored at −80°C. DNA was isolated from 400–800 µl plasma samples using the QIAamp UltraSens Virus Kit (QIAGEN GmbH, Hilden, Germany) to facilitate the collection of fragmented DNA, eluted in 50–100 µl low-salt buffer, according to the manufacturer's instructions, and stored at −20°C until use.

quantification of cell-free DNA in plasma

Plasma DNA was quantified using a SYBR green-based real-time PCR assay for the *β-globin* gene. Amplifications were set up in a reaction volume of 25 µl, containing 5 µl of the extracted plasma DNA, iQ SYBR Green Supermix 2X (BioRad Laboratories, Hercules, CA, USA), and 400 nM of the forward and reverse primers [5′-GTGCACCTGACTCCTGAGGAGA-3′ and 5′-CCTTGATACCAACCTGCCCAG-3′ (GenBank Sequence accession number U01317)]. Amplification was carried out on i-Cycler (BioRad) and consisted of 10-min initial activation at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A melting curve analysis was carried out at the end of each PCR assay to control for specificity of the amplified PCR products. A calibration curve obtained using a serial 10-fold dilutions of DNA from an healthy individual (30 000–3000–300–30–3 genome equivalent, respectively) was run in parallel with each PCR. Both samples and standards were analyzed in triplicate. A conversion factor of 6.6 pg of DNA per cell was used to quantify results as ng/ml [15]. The coefficients of variability intra- and interassay were both <0.01. Amplification data were analyzed by the i-Cycler-iQ software version 3.0a.

statistical analysis

Wilcoxon rank sum test was used to compare plasma levels between patients and controls. Comparisons between controls and different patient subgroups were adjusted for multiple comparisons using the Bonferroni method. The receiver operating characteristic (ROC) approach was applied to evaluate the diagnostic performance of plasma DNA concentrations. Each unique DNA value was used as a cutpoint to calculate sensitivity and specificity values defining the curve and the area under the curve. Since the distribution of plasma DNA levels obtained in our population revealed a departure from normality that was mitigated using a logarithmic transformation, the log of the concentration was used for testing purposes. However, untransformed values were used for reporting results. DNA concentrations were analyzed both as continuous variable following logarithmic transformation and as dichotomic variable using as cut-off point, the upper normal limit of controls. Univariate comparisons between patient characteristics and the logarithmic DNA concentrations were carried out by the Wilcoxon rank sum test. Multivariate regression models were used to examine the relationships between the dependent variable (DNA concentration) and potential predictor variables, both as continuous and as dichotomic variables, including age, lactate dehydrogenase (LDH) levels, and stage of disease. We used the age-adjusted IPI to dichotomize the patients with NHL into low- and high-risk patients, as we use this parameter for treatment decisions in younger and elderly patients, respectively.

The primary survival end point was freedom from treatment failure (FFTF), with progression during treatment, lack of complete remission at the end of first-line treatment, and relapse and death from any cause counted as adverse events. Survival curves were estimated using the Kaplan–Meier product limit method. Kaplan–Meier plots presented are unadjusted survival curves. Log-rank tests were used to analyze for differences in FFTF. The effect of the logarithmic transformed DNA concentrations on FFTF was evaluated using the proportional hazards model. Hazard ratios (HRs) and 95% confidence intervals (CIs) were adjusted for multiple prognostic factors using the Cox proportional hazards model. All analyses were stratified for kind of chemotherapy. Computations were carried out using the Stata 10.0 software (Stata Corp., College Station, TX).

results

cell-free circulating DNA concentration in plasma of healthy individuals and lymphoma patients

The median cell-free DNA concentration was 12.1 ng/ml (mean 13.9 ng/ml, range 3.0–34.7 ng/ml) in 41 healthy individuals. In patients with lymphoma, it was significantly higher ($n = 142$, median 24.1 ng/ml, $P < 0.0001$), with great variability among patients (mean 63.5 ng/ml, range 4.0–940.5 ng/ml). Elevated DNA levels were observed in patients with DLBCL ($n = 63$, median 26.9 ng/ml, mean 91.6 ng/ml, $P < 0.004$), MCL ($n = 10$, median 23.1 ng/ml, mean 74.1 ng/ml, $P = 0.04$), and HL ($n = 45$, median 25.7 ng/ml, mean 43.3 ng/ml, $P < 0.004$), while they did not significantly differ from controls in patients with FL ($n = 24$, median 14.7, mean 22.9 ng/ml, $P = 0.96$) (Figure 1).

Using the ROC analysis, we tested the diagnostic performance of the real-time quantitative PCR to discriminate between healthy controls and lymphoma patients. The area under the ROC curve was 0.81 (95% CI 0.72–0.90) and 0.79 (95% CI 0.71–0.88) for HL and DLBCL, respectively, suggesting a moderate discrimination power. At a cut-off of 18.5 ng/ml, the sensitivity and specificity were 73% and 71% for HL and 70% and 71% for DLBCL, respectively.

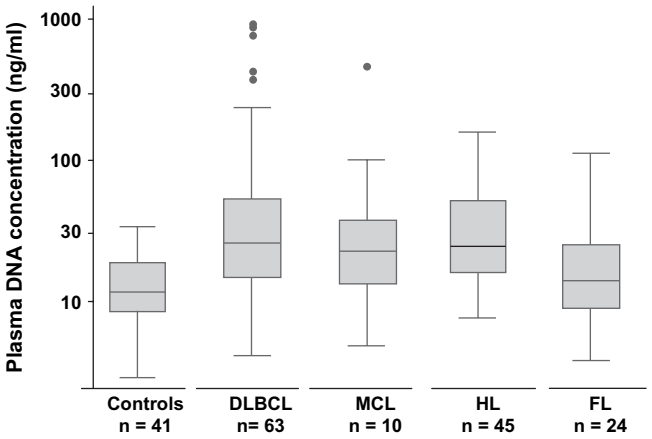

Figure 1. Box-plots of cell-free DNA plasma concentrations in healthy controls and patients with lymphoma at diagnosis. DNA plasma concentrations were determined using a real-time PCR for the *β-globin* gene: The upper border of the box indicates the upper quartile (75th percentile) while the lower border indicates the lower quartile (25th percentile), and the horizontal line in the box the median. The vertical lines are the whiskers indicating the upper and lower adjacent values. Controls, DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; HL, Hodgkin's lymphoma.

As a consequence, elevated plasma DNA levels were associated with a higher lymphoma risk. When analyzed as a continuous variable, a unit increase in plasma DNA (ng/ml) increased the lymphoma risk by 8.5% (odds ratio 1.085; 95% CI 1.039–1.133).

concentration of cell-free DNA and histopathological features

Forty-two HL cases were evaluated for the presence of necrosis, which was present in 14 cases (33%). Patients with HL and histological signs of necrosis had higher DNA plasma levels (median 42.1 versus 21.5 ng/ml, $P = 0.03$). HL cases of the nodular sclerosis type were subclassified into two grades according to the British National Lymphoma Investigation system [20]. Cases classified as grade 2 had significant higher levels of DNA in the plasma (median 35.2 versus 20.0 ng/ml, $P = 0.008$). Histological grading was available for 20 FL cases (grade1, $n = 5$; grade 2, $n = 6$; and grade 3, $n = 9$), but it did not correlate to DNA levels (data not shown).

correlation of plasma DNA concentrations to patient characteristics

Plasma DNA concentrations correlated to several known adverse prognostic factors. Age >60 years, advanced stage of disease (stage III/IV), and LDH levels above normal range were associated with increased levels of plasma DNA both when analyzing the total lymphoma group and when restricting the analysis to patients with either DLBCL or HL (Table 2). In patients with DLBCL, also the presence of B-symptoms and bulky disease correlated to high plasma DNA concentrations. As a consequence, patients with an

adverse prognostic score had higher levels of plasma DNA, which was significant in patients with DLBCL (age-adjusted IPI > 2, $P = 0.0002$), while only a trend for international prognostic score (IPS) scores >2 was observed in HL ($P = 0.07$).

We next included prognostic variables into a multivariate logistic regression analysis, using 29.4 ng/ml as cut-off between normal and elevated plasma DNA levels (means + 2 standard deviations of plasma concentrations in the control group). This cutpoint was chosen to identify patients with elevated DNA plasma levels with a high specificity (97.5%), while sensitivity was 40% in the ROC analysis. Increased LDH continued to be the strongest predictor for increased plasma DNA levels in the multivariate analysis ($P = 0.001$) (Table 3).

Table 2. Associations between patient characteristics and plasma cell-free DNA levels

Variable	All patients ($n = 142$)	DLBCL ($n = 63$)	HL ($n = 45$)
Age, years			
<60	21.3	20.7	24.7
>60	29.0	35.0	67.7
P	**0.018**	**0.009**	**0.03**
Gender			
Female	25.1	27.3	28.5
Male	22.7	26.8	18.8
P	0.66	0.73	0.07
Stage			
I–IIA	20	20.3	22.5
IIB–IV	26.7	33.7	42.9
P	**0.03**	**0.005**	0.07
B-symptoms			
No	21.3	22.4	24.7
Yes	37.6	57.9	32.1
P	**0.0005**	**0.0009**	0.34
Bulky disease			
No	22.5	22.9	26.9
Yes	24.4	41.9	19.9
P	0.18	**0.04**	0.36
LDH			
Normal	19.6	18.9	22.5
Elevated	64.0	55.4	142.7
P	**0.0001**	**0.0001**	**0.002**
Prognostic score[a]			
Low risk	18.9	21.2	25.7
High risk	55.4	55.4	49.2
P	**0.0001**	**0.0002**	0.07

P values of Mann–Whitney rank sum tests are given.
P-vales below 0.05 are shown in bold.
[a]The age-adjusted IPI score was used for patients with non-Hodgkin's lymphomas (DLBCL, MCL and FL) [24] and the IPS score according to Hasenclever for patients with Hodgkin's lymphoma [25]. Age-adjusted IPI scores 0 and 1 were considered low risk, age-adjusted IPI 2 and 3 as high risk, while with IPS score 0–2 were considered as low risk, and ≥3 as high in HL patients.
DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; LDH, lactate dehydrogenase; IPI, international prognostic index; MCL, mantle cell lymphoma; FL, follicular lymphoma.

concentration of cell-free DNA and prognosis

The role of the circulating DNA plasma levels at diagnosis as a prognostic marker was separately analyzed for the two major diagnostic entities included in this study, patients with DLBCL and HL. Ninety patients (DLBCL, $n = 52$; HL, $n = 38$) had completed their treatment and were available for follow-up. The calculated probability of FFTF at 2 years was 69% (95% CI 47%–84%) and 81% (95% CI 65%–91%), respectively, for patients for DLBCL and HL. Patients with DLBCL and elevated plasma DNA had a 2-year probability of FFTF of only 40% (95% CI 9%–71%), while patients with normal DNA levels had an 88% (95% CI 68%–96%) 2-year probability of FFTF ($P = 0.03$; Figure 2A). The 2-year probability of FFTF in patients with HL and elevated plasma DNA levels was 62% (95% CI 35%–81%), while patients with normal DNA levels had a 95% (95% CI 71%–99%) 2-year probability of FFTF ($P = 0.01$; Figure 2B). The difference became evident both when analyzing plasma DNA levels as continuous and as dichotomized variable (Table 4). In addition, age >60 years, advanced stage disease, and elevated LDH were prognostic markers for FFTF in univariate analyses (Table 4). Including the prognostic relevant parameters for HL from univariate analysis into a multivariate analysis (logarithmic concentrations of plasma DNA, LDH levels, and stage), the DNA plasma concentration maintained its

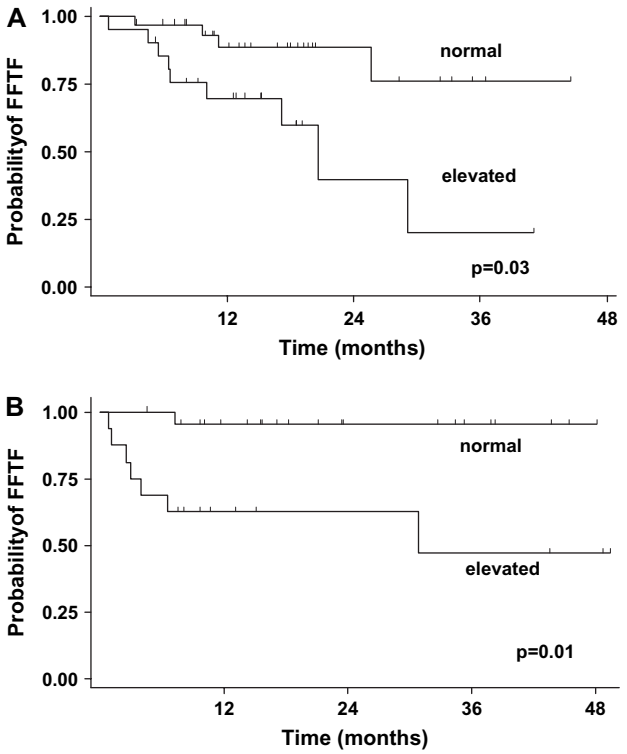

Figure 2. Unadjusted Kaplan–Meier plots of freedom from treatment failure (FFTF) according to the DNA plasma concentration (elevated, >29.4 ng/ml). *P* values of log-rank tests adjusted for type of chemotherapy regimen are given. (A) FFTF in 52 patients with diffuse large B-cell lymphoma (normal, 31 patients, four events; elevated 21 patients, eight events). (B) FFTF in 38 patients with Hodgkin's lymphoma (normal, 22 patients, one event; elevated 16 patients, seven events).

Table 3. Multivariate logistic regression analysis of patient characteristics as risk factors for elevated cell-free DNA plasma levels

Variable	Odds ratio (95% CI)		
	All patients ($n = 126$)	DLBCL ($n = 59$)	HL ($n = 37$)
Age > 60 years	2.25 (0.96–5.29)	5.82 (1.41–24.1)	3.26 (0.16–65.3)
	0.062	**0.015**	0.77
Male gender	0.93 (0.40–2.16)	1.27 (0.31–5.24)	0.11 (0.01–1.06)
	0.86	0.74	0.057
Advanced stage	1.48 (0.63–3.46)	1.99 (0.52–7.61)	1.39 (0.25–7.73)
	0.37	0.32	0.71
B-symptoms	2.48 (0.98–6.26)	2.40 (0.44–13.2)	1.76 (0.27–11.3)
	0.054	0.32	0.55
Bulky disease	0.71 (0.29–1.78)	0.84 (0.17–4.13)	0.25 (0.04–1.72)
	0.47	0.83	0.16
Elevated LDH	5.39 (2.7–14.0)	7.05 (1.45–34.2)	[a]
	0.001	**0.015**	

P-vales below 0.05 are shown in bold.
[a]Elevated LDH levels perfectly predicted elevated DNA levels and were therefore dropped in the multivariate analysis.
CI, confidence interval; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; LDH, lactate dehydrogenase.

Table 4. Univariate risk model for freedom from treatment failure

Variable	DLBCL ($n = 52$)	HL ($n = 38$)
DNA plasma level (>29.4 ng/ml)	4.27 (1.05–17.4)	9.59 (1.13–81.6)
	0.04	**0.04**
DNA plasma level, log (continuous)	4.51 (1.56–13.0)	111 (4.24–2902)
	0.005	**0.03**
Age > 60 years	16.2 (1.75–5.24)	2.23 (0.30–16.4)
	0.01	0.4
Advanced stage	3.32 (0.77–14.4)	9.07 (0.90–91.0)
	0.11	0.06
Elevated LDH	1.97 (0.50–7.79)	40.1 (7.0–230)
	0.335	**0.001**
B-symptoms	1.57 (0.45–5.42)	0.81 (0.17–4.0)
	0.5	0.8
Bulky disease	1.34 (0.29–6.27)	2.76 (0.25–30.8)
	0.7	0.4
Prognostic score[a]	2.02 (1.06–3.86)	1.22 (0.17–8.97)
	0.03	0.8

P-vales below 0.05 are shown in bold.
All analyses were stratified for kind of chemotherapy. Analysis in the total patient group was also stratified for lymphoma type.
[a]Prognostic scores were defined as in Table 2.
CI, confidence interval; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; LDH, lactate dehydrogenase.

prognostic significance (HR 62.6; 95% CI 1.4–2854.4; $P = 0.03$). In patients with DLBCL, the multivariate analysis including the age-adjusted IPI showed that logarithmic plasma DNA concentration was an independent prognostic factor of borderline significance (HR 3.5; 95% CI 0.94–13.3; $P = 0.06$).

discussion

Our study demonstrates that lymphoma patients frequently have elevated levels of cell-free circulating DNA at diagnosis, which can be accurately quantified by real-time PCR and which correlates with clinical parameters and prognosis.

Recently, quantification of circulating DNA has been proposed for cancer screening [10]. Using ROC analysis to determine the performance as a diagnostic test for lymphoma detection, we found that the maximum sensitivity and specificity of this method did not exceed 75%, making it unlikely to be an adequate test for lymphoma screening.

The analysis of histological subsets revealed that DNA levels varied according to lymphoma subtype. Elevated DNA levels were found in DLBCL, MCL, and HL. On the other hand, circulating DNA levels were similar to controls in FL. We did not observe differences according to histological grading, although our FL patient group might be insufficient for this analysis. It will be of interest to study whether other indolent lymphomas are as well characterized by low circulating DNA levels.

We observed an association between DNA concentrations and a number of clinical parameters indicating a worse prognosis, as older age, advanced stage of disease, presence of B-symptoms, and elevated LDH, suggesting that circulating DNA may reflect an active proliferating disease. In the same line, elevated DNA plasma concentrations have been reported to correlate with advanced-stage disease and tumor burden in epithelial tumors and in a human tumor xenograft model in mice [21, 22].

Circulating DNA does not reflect only tumor burden but also reflects dynamic states including proliferation, necrosis, and apoptosis: in this line, we found a strong association with LDH levels in all lymphoma types and with the presence of necrosis in patients with HL. Similarly, Gautschi et al. [13] found elevated LDH levels to be correlated with DNA plasma levels in patients with lung cancer, indicating a common mechanism for release of LDH and DNA from the tumor tissue.

The origin and mechanism of release of circulating DNA is still matter of debate. In healthy individuals, it is believed that nucleic acids enter the circulation via apoptosis of lymphocytes and other nucleated cells since normal plasma DNA exhibits on electrophoresis band sizes equivalent to multiple nucleosomal DNA fragments (185–200 bp), hallmarks of the apoptotic process [7, 21]. On the other hand, also active release of DNA by tumor cells has been proposed in patients with large or advanced/metastatic tumors. In patients with colorectal cancer with distant metastases, meanly 11% (range 2%–27%) of the total APC gene fragments were mutated, versus 0.9% in patients without metastases, indicating also that most of the circulating DNA did not derive from the neoplastic cells themselves, but from their engulfment by macrophages [21]. Macrophages engulfing necrotic cells release digested DNA into the medium, whereas macrophages engulfing apoptotic cells do not [23]. As necrosis involves killing of neoplastic cells and surrounding stromal and inflammatory cells within the tumor, the DNA released from necrotic regions is likely to contain both tumor-specific and normal DNA. This is particularly interesting in HL, which is characterized by only a small

fraction of tumor cells, the Reed–Sternberg cells, surrounded by a massive inflammatory infiltrate. In this line, in our patients with HL cell-free DNA levels were similar to those of patients with DLBCLs, where the content of lymphoma cells in the tumor mass is certainly much higher.

We found that the plasma DNA level is a strong and independent prognostic predictor. To our knowledge, there are no previous studies on the prognostic role of plasma DNA in patients with lymphoma. In solid tumors, some authors have reported on an association between plasma DNA levels and survival in lung and colon cancer or disease recurrence in prostate cancer [11–14]. Although our data need to be confirmed on larger and independent patient series, quantitation of circulating cell-free plasma DNA by real-time PCR may become a useful prognostic biomarker in DLBCL and HL.

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