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t(14;18) translocations in lymphocytes of healthy dioxin-exposed individuals from Seveso, Italy

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Dioxin exposure has been associated with non-Hodgkin's lymphoma (NHL) in epidemiological investigations. The NHL-related t(14;18) translocations can be detected at a low copy number in lymphocytes from healthy subjects. Exposure to NHL-associated carcinogens, such as dioxin or pesticides, may cause expansion of t(14;18)-positive clones. We investigated prevalence and frequency of circulating t(14;18)-positive lymphocytes in 144 healthy subjects from a population exposed to dioxin [plasma TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) range: <1.7–475.0 parts per trillion (p.p.t.)] after the Seveso, Italy, accident of 1976. t(14;18) translocations were measured in DNA from peripheral blood lymphocytes by high-sensitivity real-time quantitative polymerase chain reaction. We found that the frequency, but not the prevalence, of t(14;18) translocation-positive cells increased with increasing plasma TCDD. Among t(14;18)-positive subjects ($n = 50$; 34.7%), the mean number of t(14;18) translocations/10^6 lymphocytes was 4.2 [95% confidence interval (CI), 2.9–6.2] in subjects with plasma TCDD < 10.0 p.p.t., 8.1 (95% CI, 4.9–13.3) in subjects with plasma TCDD between 10.0 and 50.0 and 12.5 (95% CI, 7.4–21.1) in subjects with plasma TCDD between 50.0 and 475.0 p.p.t. (P-trend = 0.003). As expected, t(14;18) frequency was associated with cigarette smoking and was highest in subjects who smoked for ≥16 years (mean = 12.6; 95% CI, 7.4–21.3; $P = 0.01$). Higher t(14;18) prevalence was found among individuals with fair hair color ($P = 0.01$) and light eye color ($P = 0.04$). No significant association between t(14;18)- and age was found. Our results show that dioxin exposure is associated with increased number of circulating t(14;18) positive cells. Whether this change in t(14;18) frequency is an indicator of elevated lymphoma risk remains speculative and needs further investigation for its potential impact on public health.

Abbreviations: BCL-2, B-Cell leukemia/lymphoma 2; CI, confidence interval; DL, detection limit; NHL, non-Hodgkin's lymphoma; PCR, polymerase chain reaction; RRT, relative retention time; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Introduction

The incidence of non-Hodgkin's lymphoma (NHL) has increased over the past decades in Western countries (1). More recently, this trend has leveled off in several countries, possibly reflecting decreased exposures to organic pollutants such as polychlorinated byphenyls (PCBs) or dioxins (2). The etiology of the most common NHL types remains elusive. Increased incidence of and mortality from NHL have been reported in several investigations conducted on subjects exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a major environmental contaminant and known human carcinogen (3–8), thus suggesting a role for dioxin in NHL development. TCDD is chemically related to other common environmental contaminants that have been often associated with increased risk of NHL, including pesticides (phenoxiacetic acid and chlorophenols), PCBs and other organohalogen compounds.

More than 80–90% of follicular and 20% of diffuse large lymphomas carry the t(14;18) translocation, the most frequent chromosomal translocation in human lymphoid malignancies (9). The t(14;18) translocation results from a reciprocal translocation that juxtaposes the *BCL-2* (B-Cell Leukemia/Lymphoma 2) gene on chromosome 18q21 near the immunoglobulin heavy chain (*IgH*) locus at chromosome 14q32 (10). In t(14;18), the anti-apoptotic *BCL-2* gene comes under the control of the *IgH* gene promoter, causing overexpression of the BCL-2 protein (11). Analysis of the junctional sequences has suggested that the translocation represents an early event in NHL development, occurring in pre-B cells when attempting IgH rearrangement in the bone marrow (12). In t(14;18) transgenic mice, the B-cell population has a prolonged survival and polyclonal B-cell hyperplasia is uniformly observed, but only 5–15% of the animals develop malignant lymphoma after a long latency (11).

In 30–80% of healthy individuals, highly-sensitive polymerase chain reaction (PCR) techniques have allowed for the detection of a low number of t(14;18) copies in peripheral blood lymphocytes and other normal lymphatic tissues (12–14). High prevalence and frequency of t(14;18) translocations in healthy subjects have been associated with individual characteristics, such as older age (15–17), hepatitis-C virus (HCV) infection (18) and smoking (19,20). Also, an association between t(14;18) translocation in B lymphocytes and ultraviolet-B (UV-B) levels estimated from sunlight records for the 3 weeks before blood sampling has been reported in a study conducted on a limited number of healthy subjects (21), while a later study by Roulland *et al.* (17) did not find any variation in t(14;18) prevalence correlated with daily sunlight

data on a similar number of agriculture workers. Subjects' pigmentation may have had a role in these associations. The significance of the t(14;18) translocations found in healthy subjects is unresolved. Progression toward the clinical disease may occur by clonal expansion of existing t(14;18)-positive cells, potentially promoted by intervening carcinogenic exposures and associated with additional molecular and cytogenetic events (22). The effect of a single chemical carcinogen specifically associated with NHL risk on circulating t(14;18)-carrying cells has never been tested.

In 1976, an industrial accident contaminated a residential area surrounding Seveso, Italy, with very high TCDD levels. In multistage models for chemical carcinogenesis, TCDD acts as a tumor promoter by reducing apoptosis (23) or increasing growth rate and clonal expansion of initiated cells (24). TCDD does not exhibit covalent binding to DNA, and tests for DNA damage have been mostly negative (3). As in other dioxin-exposed populations, NHL cases in excess of the expected number were observed in Seveso among the exposed individuals (7,8). Many studies have advocated the role of epigenetic alterations in cancer development (25). TCDD-mediated epigenetic disruptions may be the basis of TCDD–NHL association. In particular, TCDD may have caused the expansion of t(14;18)-positive clones in exposed subjects, leading to the development of clinical NHL. To test this hypothesis, we investigated whether body burdens of TCDD in healthy subjects from the Seveso population influenced the prevalence and frequency of circulating t(14;18)-positive cells. In addition, we evaluated whether other selected subjects' characteristics (age, sex, pigmentation and smoking) and exposures may have contributed to the generation and clonal expansion of t(14;18)-bearing cells.

Materials and methods

Study subjects

Subjects were selected from a population sample we described previously (26) that included 211 healthy subjects representative of the Seveso area and 101 individuals who had developed well-documented chloracne, the typical skin disorder consequent to intoxication from halogenated hydrocarbons, after the accident. This population included exposed and unexposed subjects and had dioxin levels that ranged from background levels to 475 parts per trillion (p.p.t). (26) Subjects for the present study were selected from the original study population so that two groups with high (>10 p.p.t.) or low (<10 p.p.t.) current dioxin levels were formed. Seventy-two Caucasian subjects (52 females and 20 males) with current high-TCDD plasma levels (plasma TCDD >10 p.p.t. lipid adjusted, equivalent to picograms/gram fat) and 72 subjects (41 females, 31 males) with low plasma TCDD (<10 p.p.t.) were included in the study. The cut-off of 10 p.p.t. is commonly considered to separate background from elevated TCDD plasma levels (27–29). Except for chloracne, the subjects did not have any history of major illnesses, including cancer, blood disorders, allergies and chronic infectious diseases. The high- and low-TCDD groups were frequency-matched by history of chloracne and cigarette smoking status (never, former or current smoker) (Table I). However, average duration of smoking in the low-TCDD group was 6.4 years longer than in the high-TCDD group. As expected, a higher number of individuals in the high-TCDD group were from the most contaminated zones (Zones A and B) of the Seveso area. Subjects in the high-TCDD group had slightly older age, both at the accident and at recruitment, than those in the low-TCDD group (Table I).

In the high-TCDD (>10 p.p.t.) group, current plasma TCDD concentrations were between 10.5 and 475.0 p.p.t., with a geometric mean of 44.5 p.p.t. [95% confidence interval (CI), 35.8–55.9). The low-TCDD (<10 p.p.t.) group included (i) 23 subjects with detectable TCDD levels (plasma TCDD between 4.3 and 9.7 p.p.t.; geometric mean of 6.1 p.p.t.; 95% CI, 5.6–6.8); (ii) 49 subjects with plasma TCDD below the detection limit (DL) of the analytical method, but with DL < 10 p.p.t. (DL range between 1.7 and 7.8 p.p.t., geometric mean of DL values = 5.1; 95% CI, 4.5–5.8). The TCDD assay has variable DL that depends, among other factors, on the amount of lipids that can be extracted from the samples. The amount of lipids is related to the amount of plasma available for the assay and on the blood lipid levels of the individuals tested. In addition, the recovery of the analyte from the lipids through the clean-up procedure can vary from sample to sample, affecting the DL for that sample (30).

To assess dose–response trends, we used a three-category plasma TCDD variable to test for the association between plasma TCDD and t(14;18) translocation or frequency, by considering as reference the low-TCDD group and dividing the high-TCDD group into two categories (plasma TCDD between 10.0 and 50.0 p.p.t., $n = 42$; plasma TCDD between 50.0 and 475.0 p.p.t., $n = 30$). Using the median value of 38.5 p.p.t. to divide the high-TCDD group into two subgroups of 36 subjects each gave similar results. We chose the 50 p.p.t. cut-off value to include a larger range of dioxin levels in the second category.

A questionnaire including data on demographics, lifestyle, foods consumed at the time of the accident, residential history and occupations was administered by trained interviewers, who also determined individual pigmentary characteristics. We obtained written informed consent from all participants. The local and U.S. National Cancer Institute's Institutional Review Boards reviewed and approved the study.

Real-time quantitative PCR

Whole blood was collected from donors into tubes treated with sodium heparin and processed to obtain cryopreserved lymphocytes as described by Landi *et al.* (31). DNA was extracted from lymphocytes, and real-time quantitative PCR was carried out for the t(14;18)-MBR-translocation as described by Dolken *et al.* (13) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). 5′-TGGTGGTTTGACCTTTAGAGA-3′ (forward primer), 5′-ACCTGAGGAGACGGTGAC-3′ (reverse primer) and 5′-CTCTGGGTGGGTCTGTGTTGAAACA-3' (probe) were used for the t(14;18)-MBR-PCR. The PCR mixture contained 400 nM of forward and reverse primer, 200 nM probe, 4 mM $MgCl_2$, 200 mM dATP, dCTP and dGTP, 400 mM dUTP, 1.25 U AmpliTaq Gold, 0.5 U AmpErase UNG (uracil-*N*-glycosylase) and 1× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), including 60 nM ROX in a total volume of 50 ml] and 0.6–1.0 μg DNA. All reagents were obtained from Applied Biosystems. In addition to spectrophotometric measurements of DNA concentration, the number of cells tested in a single PCR assay was determined by using the *K-ras* wild-type gene as a reference gene. 5′-CTTGTGGTAGTTGGAGCT-3′ (forward primer), 5′-ATCAAAGAATGGTCCTGC-3′ (reverse primer) and 5′-AAGAGTGCCT-TGACGATACAGC-3′ (probe) were used for *K-ras* real-time quantitative PCR. Both probes were labeled with the reporter dye FAM (6-carboxy-fluorescein) at the 5′ end and the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) at the 3′ end. After incubation at 50°C for 2 min to allow for UNG cleavage, AmpliTaq Gold was activated at 95°C for 10 min. Each of the 50 PCR cycles consisted of 15 s at 95°C and 1 min at 61°C for combined annealing/extension. Standard curves were established for quantification of t(14;18)-MBR-translocations and for *K-ras* wild-type gene, as described previously (13). The standard procedure for every DNA sample consisted of five replicates with 1 μg DNA for the detection of t(14;18)-MBR-translocations and three replicates with 0.05 μg DNA for *K-ras*. For samples with >5 μg DNA, the number of t(14;18) replicates was increased so that the maximum DNA content per replicate remained $\leq$1 μg. Dilutions of frozen aliquots of genomic DNA from the t(14;18)-MBR-positive cell line Karpas 422 served as standards for both the t(14;18)-MBR and the *K-ras* PCR. Each 96-well plate included two negative controls for each PCR assay. The primer and $MgCl_2$ concentration and the annealing temperature of the real-time PCR assay used in this study were optimized so that reaction conditions allowed only the amplification of specific translocation sequences. In addition, the TaqMan real-time PCR system combines a set of primers with a probe that hybridizes only and specifically to the target sequence.

Electrophoresis of PCR products

The amplification products of all t(14;18)-positive samples were analyzed by agarose gel electrophoresis to identify samples with more than one t(14;18)-positive clone. Sequencing analysis in an earlier study (13) confirmed that all PCR products that were detected on the agarose gel were specific t(14;18) translocation fragments.

Dioxin assay

TCDD was measured at the Centers for Disease Control and Prevention (CDC), using a high-resolution gas chromatographic mass spectrometric analysis performed on human plasma. All dioxin measurements were performed in the same testing facility by using the same assay, technology and standardized procedures, as described previously (32). Criteria for a positive TCDD determination were as follows: (i) signal/noise > 3/1 for both signals on ions 320 and 322; (ii) signal/noise > 10/1 for both signals on ions 332 and

Table I. Characteristics of the study subjects

	Plasma TCDD[a]				
	Low (<10 p.p.t.) ($n = 72$)		High (>10 p.p.t.) ($n = 72$)		P-value[b]
History of chloracne[c]					
No, *n* (%)	46	(63.9)	45	(62.5)	
Yes, *n* (%)	26	(36.1)	27	(37.5)	1.00
Cigarette smoking[c]					
No, *n* (%)	38	(52.8)	39	(54.2)	
Former, *n* (%)	18	(25.0)	16	(22.2)	
Current, *n* (%)	16	(22.2)	17	(23.6)	0.95
Smoking duration (years), mean (SD)	20.4	(15.9)	14.0	(10.2)	0.17
Cigarette number/day, mean (SD)	12.3	(9.2)	10.6	(6.1)	0.74
Zone of residence at the time of the accident[d]					
A, *n* (%)	10	(13.9)	41	(56.9)	
B, *n* (%)	7	(9.7)	27	(37.5)	
R, *n* (%)	24	(33.3)	2	(2.8)	
Reference, *n* (%)	31	(43.3)	2	(2.8)	<0.001
Age at the accident,[e] mean (SD)	22.7	(18.1)	24.2	(17.9)	0.39
Age at recruitment,[e] mean (SD)	41.7	(17.0)	43.2	(17.2)	0.50

[a]TCDD plasma concentrations measured at the time of the study, in parts per trillion (p.p.t.), lipid-adjusted.
[b]Test for differences between low- and high-TCDD groups.
[c]Subjects with low- and high-TCDD levels were frequency-matched by history of chloracne and smoking status.
[d]Delimited after the accident based on TCDD soil concentrations with decreasing levels from Zones A–R, and non-detectable in the non-contaminated Reference area.
[e]The Seveso accident occurred on July 10, 1976. The study subjects were recruited ~20 years later, from January 1993 through April 1998.

334 from the internal standard; (iii) observed retention times within ±1 scan of each other on ions 320 and 322 and the relative retention time (RRT) (to [13C12]-2,3,7,8-TCDD) within 2 parts-per-thousand of the RRT of the analytical standard; (iv) ratios of the intensities of the ion 320–322 and 332–334 within the 95% CIs established for these ratios.

Statistical analysis

We defined t(14;18) prevalence as the proportion of individuals with a t(14;18)-positive blood sample and t(14;18) frequency as the number of t(14;18) copies per 10^6 lymphocytes. Univariate analysis was used to evaluate the association of t(14;18) translocation with age, cigarette smoking and individual pigmentary characteristics. We used the Fisher's exact and the Wilcoxon (Mann–Whitney) tests to assess differences in the t(14;18) prevalence and frequency, respectively. Because of its skewed distribution, the number of t(14;18)-positive lymphocytes was expressed by using geometric means and 95% CIs. Age and smoking have been previously associated with both plasma TCDD levels (33,34) and t(14;18) translocations (15–17,19,20). Thus, analyses based on variables correlated with dioxin exposure (i.e. plasma TCDD, zone of residence or diagnosis of chloracne) were adjusted by age, smoking status at recruitment (current smoker, ex-smoker or never smoker) and duration of smoking. Because age at time of the Seveso accident has been related with differential TCDD-elimination rates (26), and age at recruitment was found to be associated with current TCDD plasma levels (34), we included both age-variables in multivariate models. In multivariable analysis, we calculated *P*-values and tests for trend for variations in t(14;18) prevalence by means of unconditional logistic regression models. Adjusted geometric means of t(14;18) frequency, 95% CIs and related *P*-values were computed by means of linear multivariable regression models (procedure adjust in Stata 9.0) that included the logarithm of the t(14;18) frequency as the dependent variable. We performed all analyses using Stata 9.0 (Stata Corp., College Station, TX). All reported *P*-values are two-sided.

Results

The t(14;18) translocation was found in peripheral blood lymphocytes from 50 (34.7%) of the 144 subjects evaluated. t(14;18) prevalence was not associated with age at the accident ($P = 0.86$), gender ($P = 0.14$), cigarette smoking ($P = 0.23$) and duration of smoking ($P = 0.58$) (Table II). Among t(14;18)-positive subjects, the frequency of t(14;18) translocation did not show significant differences by age at the accident ($P = 0.27$) and gender ($P = 0.75$). The mean number of translocations/10^6 lymphocytes was 5.7 (95% CI, 3.8–8.5) in never smokers, 6.2 (95% CI, 3.5–11.1) in ex-smokers and 9.2 (95% CI, 5.1–16.6) in current smokers (P-trend = 0.12). Subjects who reported a smoking duration of ≥16 years showed higher mean t(14;18) frequency (12.6/10^6 lymphocytes; 95% CI, 7.4–21.3) than subjects who had smoked for no more than 15 years (5.2/10^6 lymphocytes; 95% CI, 3.1–8.9) ($P = 0.01$). No significant association with the average lifetime number of cigarettes/day was seen ($P = 0.49$) (Table II). Also,

Table II. Prevalence and frequency of t(14;18) translocations by selected host characteristics

	t(14;18)-positive subjects		t(14;18) frequency[a]	
	%	(Positive/total)	Mean	(95% CI)
Age at the accident				
1–15 years	33.3	(24/72)	5.6	(3.7–8.2)
15–58 years	36.1	(26/72)	7.7	(5.3–11.3)
Gender				
Male	43.1	(22/51)	6.4	(4.2–9.8)
Female	30.1	(28/93)	6.7	(4.6–9.7)
Cigarette smoking				
Never	29.9	(23/77)	5.7	(3.8–8.5)
Ex-smoker	41.2	(14/34)	6.2	(3.5–11.1)
Current smoker	39.4	(13/33)	9.2	(5.1–16.6)
Cigarette number/day				
1–10	40.5	(15/37)	6.9	(5.2–9.2)
11–40	40.0	(12/30)	8.5	(5.2–13.9)
Duration of smoking				
1–15 years	40.0	(16/40)	5.2[b]	(3.1–8.9)
≥16 or more years	40.7	(11/27)	12.6[b]	(7.4–21.3)

[a]Geometric means and 95% CIs of the number of t(14;18) translocations/10^6 lymphocytes among t(14;18)-positive subjects;.
[b]$P = 0.01$, test for difference in mean t(14;18) frequency between smoking duration categories.

no association was found with being a smoker at the time of the accident and with the number of cigarettes smoked on the day before the study (data not shown).

The prevalence of t(14;18) translocation did not differ by plasma TCDD (Table III). The proportion of t(14;18)-positive subjects was 34.7% both in the low- and the high-TCDD group ($P = 0.69$). When the high-TCDD group was divided into two subgroups, the proportion of t(14;18)-positive subjects was 31.0% in the group with TCDD between 10.0 and 50.00 p.p.t. and 40.0% in the group with plasma TCDD between 50.0 and 475.0 p.p.t. (P-trend = 0.47 across the three plasma-TCDD categories). Nonetheless, among t(14;18)-positive subjects, the translocation frequency was significantly higher in subjects with elevated plasma TCDD levels (4.2 copies/10^6 lymphocytes, 95% CI, 2.9–6.2 in subjects with TCDD < 10.0 p.p.t.; and 9.9 copies/10^6 lymphocytes, 95% CI, 6.8–14.5 in subjects with TCDD between 10.0–475.0 p.p.t.; $P = 0.006$). When the subjects were categorized into three plasma TCDD categories, by subdividing subjects with elevated plasma TCDD into two groups, a trend in t(14;18) frequency across TCDD categories was observed (Figure 1). The mean number of translocations/10^6 lymphocytes was 4.2 (95% CI, 2.9–6.2) in subjects with plasma TCDD < 10.0 p.p.t., 8.1 (95% CI, 4.9–13.3) in subjects with plasma TCDD between 10.0 and 50.0 and 12.5 p.p.t. (95% CI, 7.4–21.1) in subjects with plasma TCDD levels between 50.0 and 475.0 p.p.t. (P-trend = 0.003).

Consistently, the t(14;18) prevalence did not vary according to the zone of residence at the time of the accident (P-trend = 0.89; and $P = 0.19$ for Zone R versus Reference), but a statistically significant trend was found when the translocation frequency was evaluated (P-trend = 0.04) (Table III). Past history of chloracne was associated with neither t(14;18) prevalence ($P = 0.77$) nor the frequency ($P = 0.84$) (Table III).

A higher proportion of subjects with detectable t(14;18) translocations was found among individuals with medium/light hair color (48.2%, 26 out of 54), relative to those with dark hair color (23.4%, 18 out of 71; $P = 0.01$). Similarly, t(14;18) prevalence increased in subjects with lighter eye color (24.6%, 14 out of 57 for dark; 39.3%, 22 out of 56 for medium; and 45.2%, 14 out of 31 for light eye color; P-trend = 0.03). The t(14;18) prevalence was higher, though not significantly, among individuals who reported that their skin usually burns after 1 h of sun exposure (36.3%, 4 out of 21) than in those who did not (19.0%, 41 out of 113) ($P = 0.14$). The t(14;18) frequency was not associated in t(14;18)-positive subjects with hair color ($P = 0.76$), eye color ($P = 0.77$) or skin response after 1 h in the sun ($P = 0.40$). In addition, the t(14;18) prevalence and frequency were not associated with presence of freckles, freckling after sun exposure and history of sunburns (data not shown). The association between pigmentary characteristics and t(14;18) did not change meaningfully after the analyses were adjusted for plasma TCDD levels, age and smoking status and duration.

Table III. Prevalence and frequency of t(14;18) translocations by plasma TCDD levels, zone of residence and diagnosis of chloracne

	t(14;18)-positive subjects		t(14;18) frequency[a]	
	%	(Positive/total)	Mean	(95% CI)
Plasma TCDD				
<10 p.p.t.	34.7	(25/72)	4.2[b]	(2.9–6.2)
10.0–475.0 p.p.t.	34.7	(25/72)	9.9[b]	(6.8–14.5)
Zone of residence at the time of the accident				
Reference	42.4	(14/33)	4.3[c]	(2.3–8.0)
R	26.9	(7/26)	4.9[c]	(2.2–10.7)
B	29.4	(10/34)	7.2[c]	(3.8–13.6)
A	37.3	(19/51)	9.3[c]	(5.8–14.8)
Chloracne after the accident				
No	35.2	(32/91)	6.2	(3.7–10.6)
Yes	34.0	(18/53)	6.7	(4.7–9.6)

[a]Geometric means and 95% CIs of the number of t(14;18) translocations/10^6 lymphocytes among t(14;18)-positive subjects, adjusted for age, smoking status (never, ex or current smoker) and smoking duration in multivariable analysis.
[b]$P = 0.006$, test for difference in mean t(14;18) frequency between plasma TCDD categories.
[c]$P = 0.04$, test for trend in mean t(14;18) frequency across residence zones.

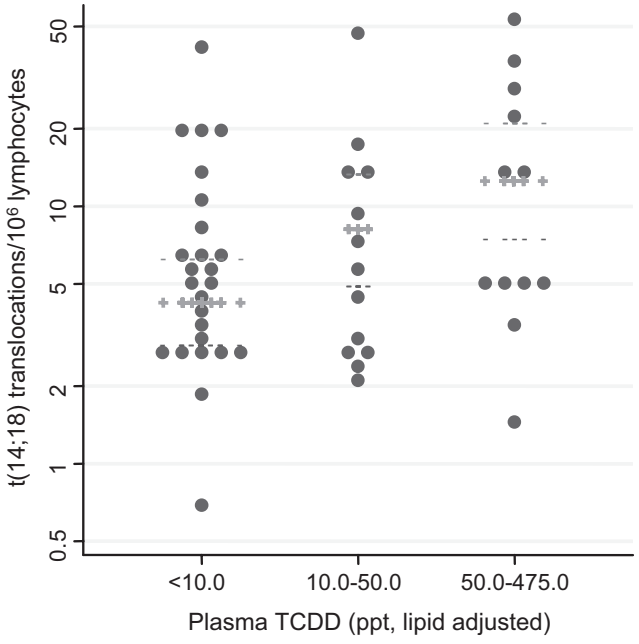

Fig. 1. t(14;18) translocation frequency in t(14;18)-positive healthy subjects ($n = 50$) from the Seveso, Italy, population, by plasma TCDD levels ($P = 0.003$, test for trend across TCDD categories). t(14;18) frequency is presented on a log-scale. Individual observations (dots), group means (plus) and 95% CIs (dotted lines) are shown.

Discussion

The finding that the t(14;18) translocation can be detected in non-malignant circulating lymphocytes of healthy subjects has fueled extensive debate on the role of the translocation in NHL and, at large, on the biological steps underlying NHL development. Although deregulated expression of the *BCL-2* gene resulting from the t(14;18) translocation may represent an early event in a pre-B stage cell contributing to cell tumorigenicity, additional events, possibly involving exposures to carcinogenic agents, are required for t(14;18)-positive cells to develop a fully malignant phenotype (22).

In the present study conducted on subjects exposed to TCDD after the Seveso accident, we found a significant association between dioxin levels and the frequency of t(14;18) translocation-positive cells. TCDD is a known cancer-promoting agent and has been associated with increased NHL risk in several epidemiology investigations (3–8). In our study

we found that the frequency of t(14;18) translocations increased in a dose-related manner with increasing levels of plasma TCDD, indicating that TCDD exposure may have promoted the expansion of existing t(14;18)-positive clones. The lack of association with prevalence of t(14;18)-positive cells may be related to the non-mutagenic properties of TCDD. It has been suggested that t(14;18)-positive cells in healthy individuals are under control of immunological mechanisms (14,21). TCDD has been shown to affect both cell-mediated and antibody immunity, and alterations of immune parameters have been documented in the Seveso population (35). Modulation of immune responses by TCDD may also have contributed to the TCDD-associated changes in the number of t(14;18)-bearing cells we observed in our study.

Because of the extremely long half-life of TCDD in humans ($\geq$7 years), TCDD plasma levels at the time of the study reflected both past exposure levels and current body burden. Thus, the increased number of t(14;18)-positive lymphocytes in our study may have resulted from the effect of current TCDD levels or long-lasting exposure to high body burdens of TCDD.

In our study, the frequency of t(14;18)-positive lymphocytes tended to be higher in smokers, particularly in those who had longer smoking duration. In healthy subjects, smoking has been previously correlated with higher frequency of t(14;18)-positive cells in the blood (19) and increased proportion of subjects with t(14;18)-positive bone-marrow samples (20). t(14;18)-positive peripheral blood lymphocytes have been found in eight out of eight subjects diagnosed with persistent polyclonal B-cell lymphocytosis, a rare immunological disorder observed mostly in women who are heavy smokers (36). Whether this finding indicates increased risks of NHL for smokers is uncertain.

We found a higher t(14;18) prevalence among individuals with fair hair and eye color. Increased incidence of lymphomas after UV-light exposure has been reported in mice (37,38), and increased NHL risk has been associated with UV exposure in some epidemiology investigations (39,40). However, recent investigations have shown opposite results (41,42). One of these studies (41) found reduced NHL risk in subjects with history of high UV exposure, while light hair color was associated with increased risk for follicular lymphoma. Unfortunately, we do not have data on sun exposure in our study; thus, we cannot verify the effect of sunlight exposure on translocations in subjects with different pigmentation characteristics. Our finding suggests that fair skin complexion may be associated with early NHL-carcinogenesis events related to t(14;18)-generation, and needs further investigation.

Although a slight increase by age was found, we could not observe any significant association between age and t(14;18)-positive lymphocytes. Some previous studies have shown progressively higher prevalence of t(14;18) translocations in older healthy subjects (15–17), but several others did not find any age-related change (20,43–45). Differences in sample size, age range and populations investigated may account for the conflicting results. In the Seveso population, TCDD exposure and differential elimination by age of the initial TCDD body burden may have reduced our capability to detect age-related t(14;18) variations.

In the present study, we detected the t(14;18) translocation in 34.7% of subjects. This proportion was similar to the average estimate (36%) obtained by Schuler *et al*. (12) in a meta-analysis of previous investigations that had evaluated t(14;18) in peripheral blood cells from healthy individuals either with no assessment of any specific exposure or in relation to radiation (46), domestic radon (47) or smoking (19). For t(14;18) detection, the sensitivity and, consequently, the number of subjects testing positive for t(14;18) is related to the amount of DNA used (12). In our study, we used an average 5 μg DNA and obtained a sensitivity of nearly 10^{-6} [i.e. capability to detect one t(14;18) copy in 10^6 lymphocytes]. Using higher DNA amounts, Fuscoe *et al.* (44) obtained a sensitivity for t(14;18) of 10^{-7} and detected the translocation in peripheral blood mononuclear cells of 30 out of 34 (88%) healthy subjects. On the basis of these data, it has been suggested that every healthy individual might carry circulating t(14;18)-positive cells that could be detected in peripheral blood if the sensitivity is high enough or if enriched B-cell fractions are used for DNA extraction (12). If this is the case, the difference among individuals may be more related to the frequency of positive cells than the simple detection of a positive cell in the sample. Similarly, the known association between age, HCV, smoking or UV-B light with prevalence of translocations (15–21) might be the result of misclassification of the translocations due to an assay with low sensitivity. The finding that differences in the t(14;18) frequency, but not in the t(14;18) prevalence, are associated with TCDD exposure may indicate that TCDD operates at later stages of NHL development by promoting proliferation or extending survival in t(14;18)-positive clones that have already expanded to a number of cells that exceeds the assay sensitivity threshold. However, the significance of our findings with respect to individual risk of NHL is uncertain. In our study, the frequency of t(14;18)-positive cells remained within the range found in healthy subjects by previous investigations (12,46), even in the highest plasma TCDD level group. In addition, PCR-based determination of t(14;18) frequency has been shown to be highly reproducible when the initial number of t(14;18) copies is $\geq$5, but greater between-replicate variations have been observed at lower copy numbers (13). Thus, our results should be used with caution, also considering that analyses were based on a relatively small sample size.

In conclusion, TCDD exposure was related to increased numbers of t(14;18)-positive circulating lymphocytes, but not to the proportion of t(14;18)-positive subjects, in healthy individuals from Seveso, Italy, suggesting that TCDD may promote clonal expansion of non-malignant cells carrying t(14;18). Other organohalogen compounds or pesticides whose use has been associated with NHL risk might have similar effects on lymphocytes. Whether this change in t(14;18) frequency is an indicator of an elevated lymphoma risk remains speculative, since prospective samples of dioxin-exposed subjects who developed NHL are not available. However, this finding needs further investigation for its potential impact on public health.

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