Agricultural pesticide exposure and the molecular connection to lymphomagenesis

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The t(14;18) translocation constitutes the initiating event of a causative cascade leading to follicular lymphoma (FL). t(14;18) translocations are present in blood from healthy individuals, but there is a trend of increased prevalence in farmers exposed to pesticides, a group recently associated with higher risk of t(14;18)+ non–Hodgkin's lymphoma development. A direct connection between agricultural pesticide use, t(14;18) in blood, and malignant progression, however, has not yet been demonstrated. We followed t(14;18) clonal evolution over 9 yr in a cohort of farmers exposed to pesticides. We show that exposed individuals bear particularly high t(14;18) frequencies in blood because of a dramatic clonal expansion of activated t(14;18)+ B cells. We further demonstrate that such t(14;18)+ clones recapitulate the hallmark features of developmentally blocked FL cells, with some displaying aberrant activation–induced cytidine deaminase activity linked to malignant progression. Collectively, our data establish that expanded t(14;18)+ clones constitute bona fide precursors at various stages of FL development, and provide a molecular connection between agricultural pesticide exposure, t(14;18) frequency in blood, and clonal progression.

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Abbreviations used: AID, activation-induced cytidine deaminase; CSR, class switch recombination; FL, follicular lymphoma; GC, germinal center; HI, healthy individuals; ICV, intraclonal variation; LR-PCR, long-range PCR; *mbr*, major breakpoint region; NHL, non-Hodgkin's lymphoma; SHM, somatic hypermutation; SR-PCR, short-range PCR.

Agricultural pesticide use has been repeatedly, but not consistently, associated with risk of non–Hodgkin's lymphoma (NHL; Blair et al., 1992; Dreiher and Kordysh, 2006). Over the past years, it has become clear that NHL heterogeneity is setting many hurdles to this connection and that pertinent biomarkers must be identified according to malignant subtypes (Harris et al., 1994). Among those, t(14;18) translocation constitute the genetic hallmark and early initiating event of follicular lymphoma (FL), a germinal center (GC)–derived malignancy representing one of the most common adult NHLs (Cleary et al., 1986; Kuppers, 2005). However, the t(14;18) is also present in the peripheral blood of a significant proportion of healthy individuals (HI; Limpens et al., 1995; Dolken et al., 1996; Roulland et al., 2003). Although the significance of t(14;18) in blood from HI is as yet unclear, we have previously reported trends to higher

t(14;18) prevalence among farmers occupationally exposed to pesticides (Roulland et al., 2004). Since then, recent epidemiological data have established that pesticide exposure is strongly associated with the risk of t(14;18)+ NHL development but not with t(14;18)-negative cases, suggesting that pesticides may act through a t(14;18)-dependant pathway (Chiu et al., 2006). Nonetheless, a direct mechanistic link between environmental exposure and the t(14;18) during FL pathogenesis remains to be conclusively demonstrated. Because securing this correlation between a biomarker of risk and a carcinogen exposure could have major impact on cancer prevention and public health, we sought to determine the molecular links of this connection.

J. Agopian and J.-M. Navarro contributed equally to this paper.

RESULTS AND DISCUSSION

To address this issue, we used a prospective agricultural cohort representing a total of 753 individuals, including adult family members living/working on a farm. We performed a longitudinal cohort study in a sample of 128 farmers from this cohort defined as pesticide users on crops at enrollment (the "exposed" population). As a reference, an independent cohort of 25 unexposed nonfarmers recruited from the same geographical area (the "control" population) was similarly followed, as detailed previously (Roulland et al., 2003; Roulland et al., 2004).

The evolution of t(14;18) frequency was followed over 9 yr in both cohorts. Strikingly, although t(14;18) frequency slowly increased in the control population (+87%; P = 0.03), mostly as the result of aging (Roulland et al., 2006a), a dramatic increase was observed for the exposed cohort (+253%; P < 0.0001 using the paired t test; Fig. 1 A). Contrary to
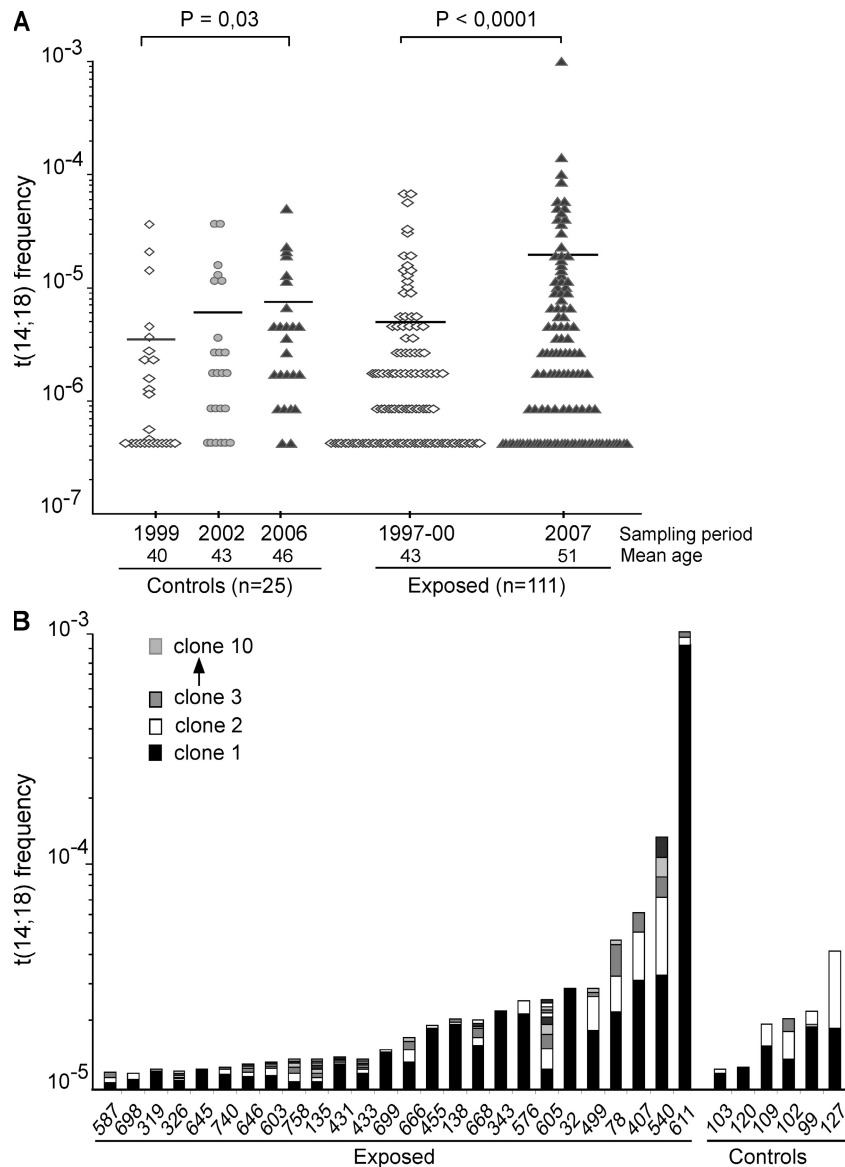

Figure 1. Agricultural exposure drives a dramatic increase of t(14;18)+ clones in blood. (A) Evolution of t(14;18) frequency in PBMCs from control and exposed populations over time. The mean age at enrollment was 40 yr ($\pm$9 yr) for the controls and 43 yr ($\pm$9 yr) for the exposed individuals, with an average follow-up time of 7 and 9 yr, respectively. t(14;18) frequency was assessed at each time point by fluctuation SR-PCR. Horizontal lines indicate mean frequencies. p-values were calculated using the Mann-Whitney test. (B) Clonality analysis. Exposed (n = 26/51, 51%) and control (n = 6/46, 13%) samples with a high t(14;18) frequency (>10^{-5}) in the last sampling period are shown and ordered according to increasing t(14;18) frequencies (the total number of clones in each individual for which more than one positive amplicon was found is summarized in Table S1). *BCL2/J$_H$* breakpoints (Table I and unpublished data) were identified by the cloning/sequencing of SR-PCR products ($n \approx$ 1,000) amplified from PBMCs, and were used to monitor the extent of polyclonality within t(14;18)+ cells in each individual. For each individual, independent *BCL2/J$_H$* clones are pictured in distinct shades and are represented from bottom to top according to increasing frequency.

Table I. Phenotypic and genotypic characterization of *BCL2/IGH* junctions in isolated B cell subsets from both exposed and control HI

B cell subsets			PCR[a]	RAG–mediated activity			AID–mediated activity			GC transit[b]
IgD[c]	CD10	CD27[d]	Nb clones	*BCL2-mbr* (5′ to 3′)[e]	N nucleotides (5′ to 3′)	J_H	CSR	SHM[f]	Rate (%)	
Exposed[g]										
78	+	+	−	SR (1)	CAGTGGTGCT TACGCTCCACCA	CCCCTGAGGCAGAT	J6	n.a.	n.a.	−	−
	n.d.	+	+	SR(3)/LR(1)	AAGAAAGCAGGAA[e]	GAACGTTACCTCAGGGG ACTTCCCCGCGCCCCCG	J6	γ1	9/821	10.9	yes
	+	+	−	SR (1)	CAGACCTCCCCCGGC	∅	J6	n.a.	n.a.		−
	+	−	+	LR (1)	CAGTGGTG CTTACGCTCC	TTCGGGTGTGATCGGTCGG	J6	μ	3/1,262	2.4	yes
	+	−	+	LR (1)	AAGAAAGCAGGAA[e]	GAACGTTACCTCAGGGG ACTTCCCCGCGCCCCCG	J6	γ1	8/821	9.8	yes
	+	−	+	LR (1)	CAGTGGTG	TTGTAGTAGGAAA	J6	μ	1/1,262	0.8	yes
	+	−	+	LR (1)	CAGTGGTG	TTGTAGTAGGAAA	J6	γ1	1/474	2.1	yes
138	+	+	−	SR (1)	CAGTGGTGCTTACGCT	ATCGCGACGGGCTA	J_{3-6}	n.a.	n.a.		
	+	−	−	LR (1)	ACAAGTCCTTTA (*ICR)	GATCGGGGTCCCCCGGGA	J6b	γ2	4/900	4.4	yes
	+/−[h]	−	+	LR (3)	CAGACCTC	TTGTGAGGGGCCATGGTTA TCCCAGGACGTCGTCGCCGA	J6b	γ1	44/4,176	10.5	yes
326	+	+	−	SR (1)	CAGTGG[e]	ATACTTGGGACAG	J4	n.a.	n.a.		−
	+	+	−	SR (1)	CAGTGGTGCTTA	TCAGGTAGGGGG	J6	n.a.	n.a.		−
	−	−	+	LR (1)	CAGTGG[e]	ATACTTGGGACAG	J4	γ1	1/687	1.5	yes
	+	−	+	LR (2)	CCTCCTGCCCTCC	GGTAGACTAGACCC	J4	μ	6/2,524	2.4	yes
431	+	+	−	SR(7)/LR(2)	CAGTGGTGCTTA[e]	AATTTAGTCT	J6	μ	6/2,524	2.4	yes
	+	−	+	LR (2)	CAGTGGTGCTTA[e]	AATTTAGTCT	J6	μ	3/2,276	1.3	yes
433	+	+	−	SR (1)	CAGACCT CCCCCGGCGG	TTTTTCGAAGACGATATCAGGGT	J6	n.a.	n.a.		−
	+	−	+	LR (1)	CAGACCTCCCCC	CCGAGCG	J4	μ	10/753	13.5	yes
576	+/−	−	+	LR (3)	CCTCCTGCCCTC	GTACGATTTTTGGAGTGGTCT	J6	μ	4/2,046	1.9	yes
605	+	+	−	SR (1)	CCTCCTGCCCTCCTTC[e]	GGGTTTACTCCCCGGGTTTT	J5	n.a.	n.a.		−
	+/−	+	+	SR(1)/LR(1)	TGCTTTACGT	TGCTC	J6	μ	1/1,262	0.8	yes
	+	+	+	SR (1)	AAGAAAGCAGGA[e]	CCCCCACGGAG	J6	n.a.	n.a.		yes
	+	+	−	LR (1)	TCAGGGAACAGAA	CCCCTAGATAGTGGGAGCTCCAT	J6b	μ	0/1,262	0	−
	−	−	+	LR (1)	CAGTGGTGCTTA	AGGAAGAAGTCG	J4	μ	2/1,262	1.6	yes
	+	−	+	LR (1)	AAGAAAGCAGGA[e]	CCCCCACGGAG	J6	μ	n.d.	n.d.	yes
	+	−	+	LR (1)	CCTCCTGCCCTCCTTCC[e]	TCGAGAGAAG	J5	μ	5/1,262	4	yes
611	+	+	−	LR (3)	CAGTGGTGC[e]	CGAATGG	J6	μ	20/3,500	5.7	yes
	n.d.	+	+	SR (3)	CAGACCTCCCCCGG[e]	TTAGAGTTTGTGGGGCCCGTTGGGACG	J6	n.a.	n.a.		yes
	n.d.	+	+	SR (1)	CAGTGGTGCTTACG	TGCCGACCAAA	J3	n.a.	n.a.		yes
	+	+	−	SR (2)	AAGAAAGCAG	AGGGCCCCACCCTG	J6	n.a.	n.a.		−
	n.d.	+	+	SR (4)	AAGAAAGCA	AGGCAGGGCTTCGAGGG	J6	n.a.	n.a.		yes
	+/−	−	+	LR (4)	CAGTGGTGC[e]	CGAATGG	J6	μ	21/5,048	4.2	yes
	+/−	−	+	LR (3)	CAGTGGTGC[e]	CGAATGG	J6	γ1	2/95	−[i]	yes

Table I. Phenotypic and genotypic characterization of *BCL2/IGH* junctions in isolated B cell subsets from both exposed and control HI *(Continued)*

B cell subsets			PCR[a]	RAG–mediated activity			AID–mediated activity			GC transit[b]
IgD[c]	CD10	CD27[d]	Nb clones	*BCL2–mbr* (5′ to 3′)[e]	N nucleotides (5′ to 3′)	J$_H$	CSR	SHM[f]	Rate (%)	
−	−	+	LR (1)	CAGACCTCCCCCGG[e]	TTAGAGTTTGTGGGGCCCGTTGGGACG	J6	γ3	23/1,109	20.7	yes
−	−	+	LR (1)	CAGACCTCCC	GAGATAGGGTTCAGGGGC	J6	γ3	11/667	16.5	yes
+	−	+	LR (1)	CAGTGGTGCTTAC	CGCCCTAGCTGC	J6	γ1	7/857	8.1	yes
Anonymous blood donors[f]										
33	n.d.	+	n.d.	LR (1)	CTTTACGTGGCCT[e]	TAGTACCAGCTGCCCCCTA	J6	μ	n.d.	n.d.	−
	n.d.	+	n.d.	SR (3)	CAGTGGTGC[e]	CAGCTGCCCCCCCCCGCCT GGCGGTGGGTTGTGCGGCCCC	J1	n.a.	n.a.		−
	n.d.	+	n.d.	LR (1)	CAGTGGTGC	CCTCGCCATATGTTC GTAACCCCGTCACCACC	J6	γ3	2/521	3.8	yes
	n.d.	−	+	SR (1)	CTTTACGTGGCCT[e]	TAGTACCAGCTGCCCCCTA	J6	n.a.	n.a.		yes
	n.d.	−	+	SR (7)	CAGTGGTGC[e]	CAGCTGCCCCCCCCCGCCT GGCGGTGGGTTGTGCGGCCCC	J1	n.a.	n.a.		yes
	n.d.	−	+	SR(13)/ LR(3)	CAGTGG	GGGT	J6	μ	5/1,262	4	yes
37	n.d.	+	n.d.	LR (1)	CCTCCTGCCCTCCTT	AAACCA	J4	μ	13/1,282	11.1	yes
	n.d.	+	n.d.	SR(5)/LR (1)	CCTCCTGCCCTCCTTCC[e]	CCTTGGGGGGCT	J6	μ	3/1,448	2	yes
	n.d.	+	n.d.	SR (1)	CAGTGGTGCTT[e]	GATTAGAGG	J5	n.a.	n.a.		−
	n.d.	+	n.d.	LR (1)	CAGACCTCCCCG[e]	AGAACCCCGCATAAA	J6	μ	1/1,526	0.7	−
	n.d.	−	+	SR(4)/LR (1)	CCTCCTGCCCTCCTTCC[e]	CCTTGGGGGGCT	J6	μ	8/2,793	2.9	yes
	n.d.	−	+	SR(1)/LR (1)	CAGTGGTGCTT[e]	GATTAGAGG	J5	μ	2/2,966	0.7	yes
	n.d.	−	+	SR (4)	CAGACCTCCCCG[e]	AGAACCCCGCATAAA	J6	n.a.	n.a.		yes
Controls[f]										
118	+	n.d.	+	LR (1)	CTCCTTCCGCGGG	A	J4	μ	5/2,620	1.9	yes
	+	n.d.	+	LR (1)	CAGTGGTGCTTA	GTGAATAGCGGGGCTC	J3	μ	3/1,262	2.4	yes
92	+	n.d.	+	LR (1)	GCTTTCTCATGG	TTCTTTTTCGGTAGGGTTGAGCACG	J5	μ	0/1,262	0	yes
	+	n.d.	+	LR (1)	CAGTGGTGCTTAC	TGACCTCGGTCGA	J4	μ	2/1,262	1.6	
111	+	n.d.	+	LR (2)	CAGTGGT	TTCTTTCGGGG–(D$_H$3-9/D$_H$3-10)–GTTAGG	J5	μ	17/2,524	6.8	yes
100	+	n.d.	+	LR (1)	CAGTGGTG	GATATTTTCGGGGGCAACG ACCGGGAGGATTGTCTCAAAA	J4	μ	1/1,262	0.8	yes
103	+	n.d.	+	LR (1)	CAGACCTCCCCGGC	CCCCCCCCCAGAA	J6	μ	2/1,262	1.5	yes
				LR (4)	CAGACCTCCCCGGC	CCCCCCCCCAGAA	J6	γ1	7/5,048	1.4	yes
102	+	n.d.	+	LR (4)	CCTCCTGCCCTCCTTCCG	TTCCTGTCCAAAAAG	J6	μ	5/5,048	0.9	yes
	+	n.d.	+	LR (1)	CAGACCTCCCCGGC	TGGGTTAGG	J5	γ1	3/645	4.6	yes
	−	n.d.	+	LR (1)	CCTCCTGCC CTCCTTCCGCGG	CCGTTGTGGGGACTCA	J6	μ	5/1,262	4	yes
127	+	n.d.	+	LR (2)	CAGACCTCCCCGG	TATCGCTAAACACGAT	J6	γ2	20/1,932	10.3	yes
	−	n.d.	+	LR (3)	CAGACCTCCCCGG	TATCGCTAAACACGAT	J6	γ2	110/7,329	15	yes
	−	n.d.	+	LR (3)	CAGTGGTGCTTA	AGGTGGTC	J6	γ1	n.d.	n.d.	yes

n.a., not applicable; n.d., not determined.

controls, neither age nor aging effects were found in the exposed cohort at enrollment or follow up (P = 0.8 or 0.47, respectively, using the Spearman rank test). However, the relation with age was restored in farmers who stopped all agricultural activity (P = 0.05 using the Spearman rank test; Fig. S1). Collectively, our data firmly establish that cumulative agricultural pesticide exposure is associated with a dramatic increase of t(14;18) frequency in blood.

We next sought to define how environmental exposure might be causally connected to this increase. Pesticides are often regarded as genotoxic chemicals generating DNA breaks, thereby favoring the accumulation of chromosomal translocations (Garry et al., 1996). We thus monitored the extent of polyclonality of t(14;18) breaks by analyzing $BCL2/J_H$ junctions. Surprisingly, although some exposed individuals indeed displayed an unusual accumulation of distinct t(14;18) translocations, most individuals carried one to two dominant clones (Fig. 1 B and Table S1). Furthermore, upon cell sorting of the peripheral B cell subsets, most clones displayed markers of GC transit: sCD27 and/or class switch recombination (CSR)/somatic hypermutation (SHM; Klein and Dalla-Favera, 2008), including the previously described "allelic paradox" (Roulland et al., 2006b) that stands as a hallmark of FL (simultaneous presence of sIgM+D+ on the productive allele and of CSR on the non-functional allele; Table I; and Figs. S2 and S3). Note that the presence of this allelic paradox is in sharp contrast with the features of the peripheral IgM+IgD+CD27+ B cell subset (being devoid of CSR both on the productive and the nonproductive alleles), and adds to the evidence of GC-expanded clones, distinct from a putative marginal zone B cell origin (Weller et al., 2004; Roulland et al., 2006b). This indicates that the t(14;18) increase observed in exposed farmers results far more from an immunogenic effect of agricultural pesticide exposure, leading to an extensive clonal expansion of activated t(14;18)+ B cells, than from a genotoxic effect. Importantly, expansions of activated t(14;18)+ B clones (>10−5) could also be observed among a few control individuals (Fig. 1 B and Table I). Although the expansions were more moderate, this suggests that a complex set of environmental factors (possibly including infectious agents such as the hepatitis C virus) can give rise to the activation of t(14;18)+ B clones (Giannelli et al., 2003;

Roulland et al., 2008), with agricultural pesticide exposure provoking a particularly pronounced immunogenic effect.

Is this clonal expansion of activated t(14;18)+ cells connected to a high risk of malignant transformation? Upon antigen encounter, normal activated B cells reach the GCs, where the activation-induced cytidine deaminase (AID)–mediated program of affinity maturation randomly modifies their receptors through CSR and SHM (Muramatsu et al., 2000). In the vast majority of cases, this process leads to a decrease in receptor affinity and B cells die by neglect in situ. In FL, three successive steps lay out the lymphomagenesis pathway: (a) t(14;18)-enforced ectopic expression of BCL2 in GC-activated B cells, leading to their illegitimate rescue from cell death (McDonnell et al., 1989; Kuppers, 2005; Klein and Dalla-Favera, 2008); (b) consequent maturation arrest and accumulation as centrocyte/centroblast–like cells with ongoing AID activity (Aarts et al., 2000; Smit et al., 2003); and (c) consequent accumulation of complementary oncogenic hits, partly because of AID-mediated genomic instability (Ramiro et al., 2006; Bende et al., 2007; Pasqualucci et al., 2008). We thus sought to determine where the activated t(14;18)+ B cells stood in this cascade of events leading to FL genesis.

Although t(14;18) carried by HI are morphologically undistinguishable from those found in FL tumors (Dolken et al., 1996; Jager et al., 2000; Roulland et al., 2006b), the expression of $BCL2/IGH$ fusion transcripts (Cleary et al., 1986; Seto et al., 1988) has to date never been functionally demonstrated in HI, partly because of the experimentally challenging issue of detecting transcripts at such low frequencies. We thus set up a sensitive RT-PCR assay (Fig. S4) and studied a set of control ($n = 15$) and exposed ($n = 32$) individuals with low (<10−5) or high (>10−5) frequencies (Fig. 2 A, triangles). $BCL2/IGH$ quantification clearly shows that transcription levels closely parallel translocation frequencies (Fig. 2 A, histograms). Furthermore, in purified B cell subsets from a given individual, only t(14;18)-positive fractions allowed $BCL2/IGH$ detection (Fig. S5). We next assessed how $BCL2/IGH$ transcription levels compared with those in FL patients. To account for the large difference in t(14;18) frequency between FL and HI (Fig. 2 B, triangles), we calculated a normalized ratio of transcription level per t(14;18)+ cell. Our data clearly show that $BCL2/IGH$ expression is comparable in the t(14;18)+ cells of FL and HI (Fig. 2 B, histograms).

Table I. Phenotypic and genotypic characterization of $BCL2/IGH$ junctions in isolated B cell subsets from both exposed and control HI *(Continued)*

[a]Type of PCR assay in which the fragment was obtained (number of positive amplicons of the given immunogeno-/phenotype; Fig. S6 B).
[b]Based on CD27+ and/or CSR+ and/or SHM+.
[c]See Fig. S3 for evaluation of the IgM status.
[d]CD27 is a pertinent GC marker only when present, as the loss/absence of CD27 after GC transit has been shown to occur in recently identified memory B cell subsets (Ehrhardt et al., 2005; Wirths and Lanzavecchia, 2005; Tangye and Good, 2007) and is variably expressed on FL cells.
[e]$BCL2/J_H$ junctions detected in both CD10+ and CD10− fractions.
[f]SHM corresponds to the number of somatic mutations/total length of Sμ analyzed (bp). Background mutation levels were calculated to be 0.55‰ (Fig. S2 A).
[g]Sorted according to CD10, CD27, and IgD for exposed samples (Fig. S6 A, top); according to CD10 and CD27 into CD10+ and CD10−CD27+ for anonymous blood donors (Fig. S6 A, bottom); and according to CD27 and IgD for controls (only the CD27+ is shown). Eight additional anonymous blood donors were screened and excluded from the analysis due to the absence of the t(14;18) detection.
[h]+/− indicates PCR fragments detected in both positive and negative sorted B cell fractions.
[i]The mutation rate was not estimated due to the partial deletion of the Sμ region.

Collectively, these findings demonstrate that similar to tumor cells in FL patients (Seto et al., 1988), t(14;18)$^+$ cells in HI are actively transcribing *BCL2* from the translocated allele, allowing their rescue from apoptosis in the GCs.

Malignant FL cells are frequently released from the founder follicle to the bloodstream, and undergo extensive trafficking between organs, thereby favoring tumor dissemination (Oeschger et al., 2002). However, consistent with their status as frozen centrocyte/centroblast-like cells, most nodal and at least a fraction of circulating FL tumor cells retain some GC markers such as CD10 (CD10$^{+/-}$, REAL classification; Harris et al., 1994). To determine if t(14;18)$^+$ clones from HI also represent released centrocyte/centro-

blast-like cells, CD10$^+$ and CD10$^-$ cell populations were sorted (Fig. S6 A). In all individuals, t(14;18) could be found in both the CD10$^+$ and CD10$^-$ subsets (50 vs. 66 amplicons, respectively; Table I and Fig. S6 C). In most cases, CD10$^+$ and CD10$^-$ clones (with identical *BCL2/IGH*) coexisted in peripheral blood after GC transit (Table I, note e). This indicates that similar to FL patients, at least a fraction of the circulating t(14;18)$^+$ cells from HI also retained the CD10 marker normally lost upon GC exit (Kuppers, 2005). One additional seminal feature of FL tumor cells that stands as a direct consequence of their status as frozen GC B cells is the sustained AID expression (Smit et al., 2003; Bende et al., 2007), normally down-regulated upon GC exit. We thus
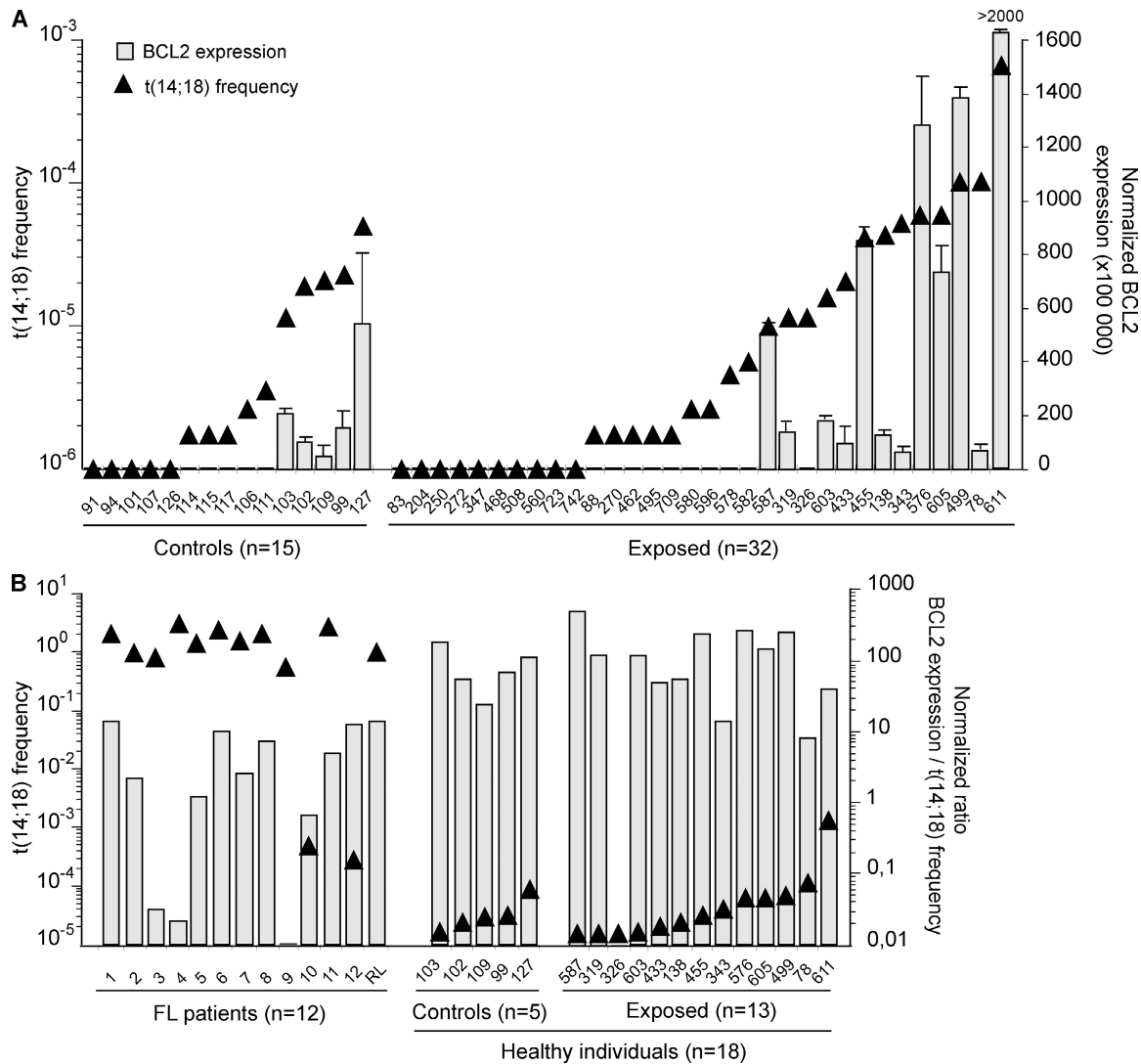
Figure 2.   t(14;18)$^+$ cells in HI are actively transcribing BCL2 from the translocated allele. (A) Superposition of t(14;18) frequency (triangles; left y axis) and the relative *BCL2/J$_H$* expression levels (histograms; right y axis) in PBMC samples from controls (n = 15) and exposed individuals (n = 32) evaluated by real-time quantitative PCR. The absolute *BCL2/J$_H$* expression data were normalized to *ABL*, and the value is arbitrarily set as ×100,000. Each bar represents the mean of replicate wells. The results shown were obtained from two independent PCRs performed in duplicate (B) *BCL2/J$_H$* expression levels in FL biopsies (n = 12) compared with samples from HI (n = 18) with respect to their t(14;18) frequency. A normalized ratio of BCL2 expression per t(14;18)$^+$ cell was calculated by dividing the normalized *BCL2/J$_H$* transcription level of a given sample by the corresponding t(14;18) frequency (as determined by real-time PCR).

first attempted to evaluate AID expression in t(14;18)$^+$ cells. Because AID transcripts are not different in translocated and normal cells, we sought to assess an expression difference of AID between individuals with low ($<10^{-5}$) and high ($>10^{-5}$) t(14;18) frequencies. Results show that detection of AID expression in total PBMCs correlates remarkably well with increasing t(14;18)$^+$ frequencies (Fig. 3 A, shaded histograms). Moreover, AID levels could be enriched through fractionating total blood into t(14;18)–positive B cell subsets (Fig. 3 B). A similar tendency (but less significant because of high background levels; Ye et al., 1997) was observed with BCL6 (Fig. S7). To next functionally assess if, similar to FL, t(14;18)$^+$ clones from HI also maintained AID activity and kept evolving with time, we sought to analyze filiations between t(14;18)$^+$ cells. The prediction was that if AID were off during clonal expansion, identical clones (in terms of CSR and SHM) should be generated, whereas if AID were on, clonal expansion should lead to a population of clonal SHM/CSR variants. We thus obtained large blood samples from control ($n = 7$) and exposed ($n = 8$) individuals, and analyzed switched versus unswitched purified B cell subsets for both SHM and CSR events on the translocated allele (Fig. S6 B). For most *BCL2/IGH* clones issued from clonal expansions, we were able to build genealogical trees based on intraclonal variation (ICV) of the Sμ region on the translocated allele (Nagaoka et al., 2002), taking into account both CSR and SHM (Fig. 4). Strikingly, the vast majority of subclones displayed ICV: out of 69 amplicons (and despite the fact that the expected rate of SHM is much lower in the Sμ region than in the IgVH region), only 2 were found to be strictly identical (case #103, B1/B2).
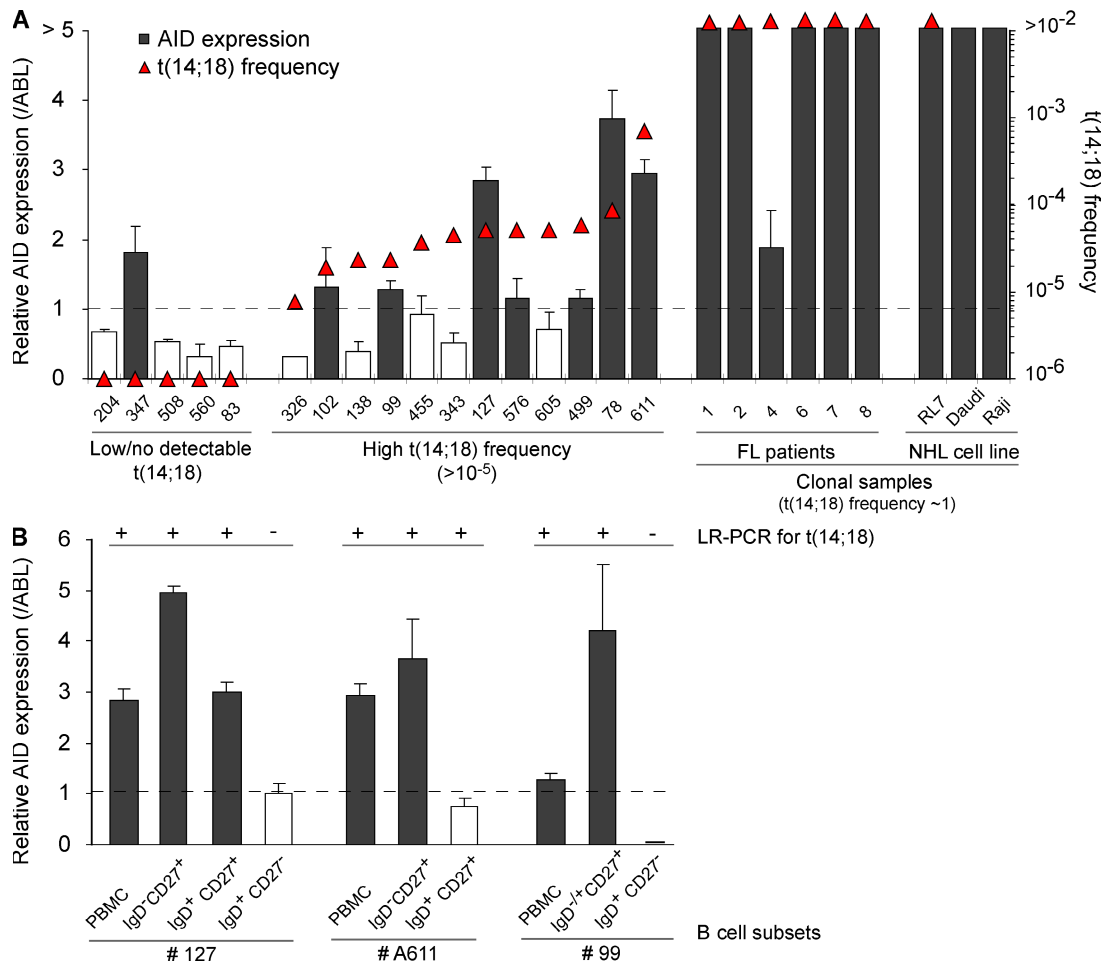

Figure 3. **AID expression correlates with increasing t(14;18) frequencies in HI.** (A) Superposition of t(14;18) frequency (triangles; left y axis) and the relative *AID* expression levels (histograms; right y axis) in PBMC samples from HI ($n = 18$), FL patient samples ($n = 6$), and lymphoma cell lines (RL7, FL; Daudi and Raji, Burkitt lymphoma) evaluated by real-time quantitative PCR. The expression data were normalized to *ABL*. As AID expression is assumed to be low/negative in naive cells, RT-PCR data are represented relative to the levels obtained in the sorted naive (CD19$^+$CD27$^-$IgD$^+$) B cell fraction and are considered positive (shaded histograms) above this arbitrary threshold of 1 (dashed line). The results shown were obtained from two independent PCRs performed in duplicate (except for clonal FL samples). (B) AID expression is enriched in t(14;18)$^+$ fractions. Comparison of t(14;18) detection (+/−, as determined by LR-PCR) and *AID* expression levels (shaded histograms) in isolated B cell subsets (based on CD27 and IgD markers) from two HI. The results shown were obtained from one PCR performed in duplicate (except for PBMC samples analyzed two times in duplicate). Data were normalized as in Fig. 3 A.
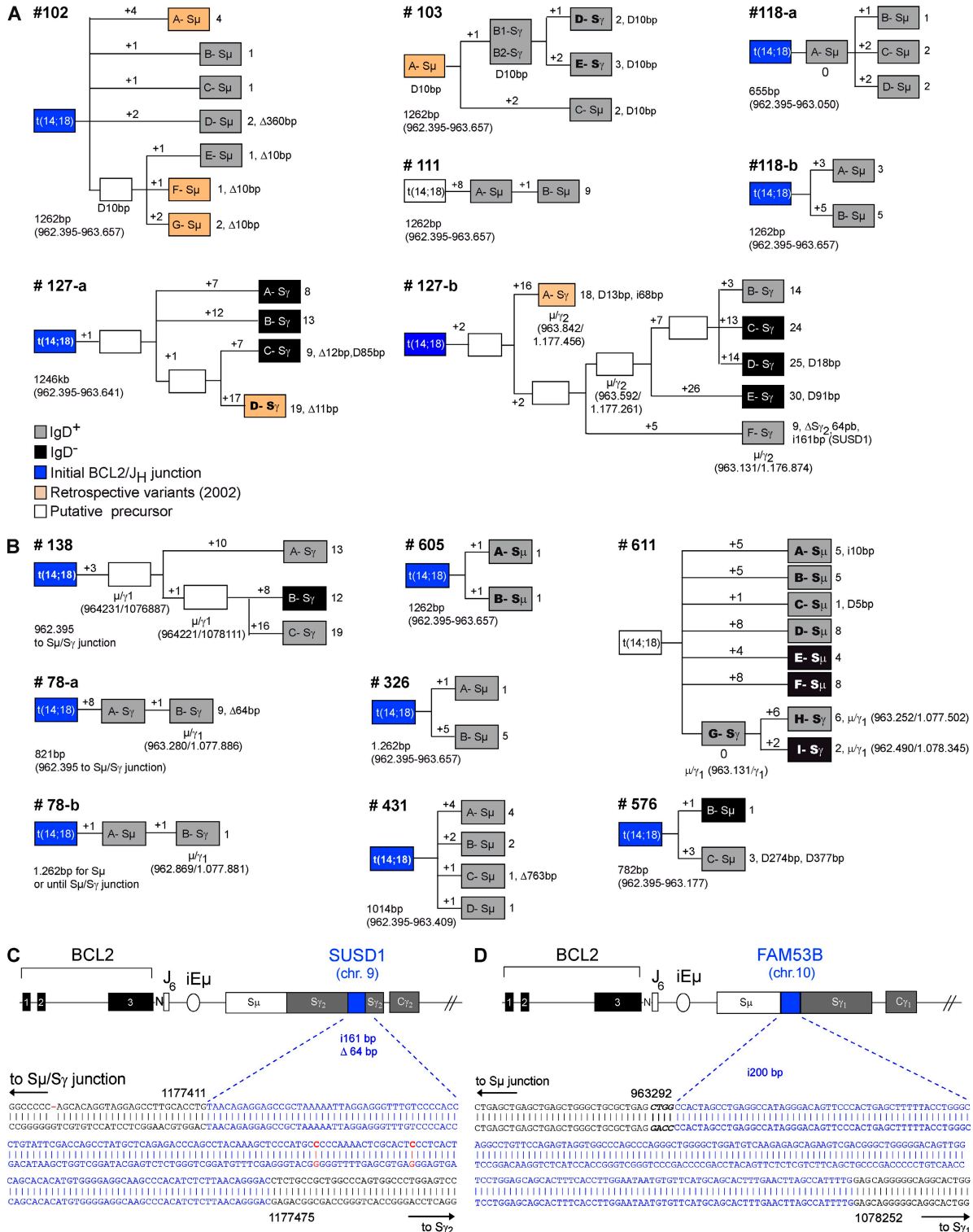
Figure 4. AID mediates the evolution of t(14;18)⁺ activated clones in HI. (A and B) Genealogic trees generated from healthy controls ($n = 5$) and exposed individuals ($n = 7$) for which several molecular subclones derived from the same *BCL2/J$_H$* junction (labeled A to G in boxes) were obtained. Trees are organized based on ICV, including SHM in the Sμ region and CSR on both the expressed (gray boxes, sIgD⁺; black boxes, sIgD⁻) and the translocated (indicated as Sμ or Sγ in boxes) alleles. For most trees, the *BCL2/J$_H$* junction was already identified at enrollment (blue boxes, ICV information not available). Six LR-PCR amplicons were obtained from the retrospective PBMC samples (orange boxes) and could be branched in four distinct trees. Stepwise accumulation of mutations is indicated by numbers above the branches (+1 to +26). Total mutations, insertions (i), duplications (D), and deletions (△) are summarized at the end of each branch. Shared mutations were used to define putative intermediate or precursor cells (open boxes). The length of the

This quasisystematic presence of ICV clearly indicates that cell division is constantly associated with SHM and/or CSR events, and therefore that t(14;18)+ clones have maintained AID expression over time. Together with the expression of CD10, the presence of ongoing AID-mediated activity demonstrates that t(14;18)+ cells in HI indeed represent circulating counterparts of "frozen" GC B cells.

In lymphomas, sustained AID activity provides a high propensity for the acquisition of further oncogenic aberrations. This mostly includes CSR-mediated translocations (Ramiro et al., 2006), aberrant CSR (Lenz et al., 2007), and the occurrence of SHM in non-Ig target genes (Pasqualucci et al., 2001; Takizawa et al., 2008). Remarkably, we found that almost 30% (19 out of 69) of the *BCL2/IGH* clones tested in the Sμ region display unusually large intra-Sμ deletions (up to 763 bp) or Sμ tandem duplications interspersed with mutations in AID hotspots (Fig. 4; and Fig. S2 A, note e), suggesting that CSR is functionally impaired in these clones (Reina-San-Martin et al., 2003; Lenz et al., 2007). Furthermore, the insertion of an irrelevant DNA fragment from a distinct chromosome into a Sμ deletion site has been recently observed in a diffuse large B cell lymphoma sample, and proposed as a surrogate marker of AID-mediated genomic instability and malignant progression (Lenz et al., 2007). Strikingly, two of such rare insertion events were found in our samples (Fig. 4, C and D). Although probably not directly relevant to tumor progression, such events demonstrate the occurrence of illegitimate AID-mediated events and highlight the propensity of some t(14;18)+ clones to withstand a complementary oncogenic hit over time.

Collectively, our results clearly demonstrate that the expanded t(14;18)+ clones, which are particularly prominent in farmers exposed to pesticides, constitute bona fide FL precursors standing at various stages of tumor progression. Together with the recent findings that pesticide exposure is specifically associated with a higher risk of t(14;18)+ NHL development (Chiu et al., 2006), our data provide the first direct connection between t(14;18) frequency in blood and malignant progression. Remarkably, the dynamics of clonal evolution observed in HI are strikingly similar to those observed in FL (Ruminy et al., 2008) and transformed-FL in diffuse large B cell lymphoma (Carlotti et al., 2009), suggesting that the selection process operating in malignant clones is already at work at early development stages (Fig. 4 and Fig. S8).

The most pressing question in our report is how to assess which of those individuals are more likely to develop FL in the near future. Some individuals displayed unusual t(14;18) frequencies (e.g., # 611, 1 in 1,000 PBMCs) reaching levels observed in some FL patients, and others displayed aberrant CSR events (e.g., #127-b, ectopic insertions) that indicate a predisposition to chromosomal translocations (Lenz et al.,

2007). Our current challenge is to determine how the combined assessment of t(14;18) frequency and surrogate markers of malignant progression could be helpful in the identification of individuals at "high" risk for transformation to overt FL. Besides the identification of at-risk individuals, the precise understanding of the immunogenic effect described in this paper on t(14;18)+ cells might also provide important targets for its disruption in preventive approaches (Staudt, 2007; Roulland et al., 2008).

MATERIALS AND METHODS

Human samples. A population of 753 farmers/farm workers were included in an agricultural cohort that has been previously described (Roulland et al., 2004). After enrollment (1997–2000), a subset was selected to control for potential confounding factors based on the following criteria: males, no smoking history, and current pesticide applicators on open-field crops. Among the 144 individuals matching these criteria, 128 provided DNA/blood samples (∼50 ml) for analysis at enrollment (Roulland et al., 2004), and 111 participated in the average 9-yr follow up (response rate = 89%; Table S2). Large blood samples (∼250 ml) were obtained from eight of them at follow up. As controls, blood samples (∼50–250 ml) were taken from a group of 25 healthy adults issued from a followed-up cohort (Roulland et al., 2006a), coming from the same geographical area as the exposed group and defined as "subjects who have never lived/worked on a farm and were not exposed to pesticides." Large blood samples were also collected from 10 anonymous bank donors. 2 out of the 10 were screened highly positive for t(14;18) frequency and subjected to cell sorting and molecular analysis (see B cell isolation…). In addition, 20 tumor biopsies were obtained from FL patients at diagnosis (Jager et al., 2000; Ruminy et al., 2008). This study was approved by the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale #99-07, Caen, France), and all participants of the study provided their written informed consent.

Pesticide exposure assessment. A face-to-face comprehensive standardized questionnaire was administrated to all enrolled subjects at each time point. The interviews included detailed information on agricultural practices and pesticide exposure, including the timing and duration of farming activities, the years of exposure, the personal handling of specific pesticides, the average annual number of days of pesticide use on crops, the type of crops and average acres grown, the use of personal protective equipment, the nature of chemical classes, and the spraying equipment (Table S2). Exposed and control individuals (excluding anonymous blood blank donors) were also asked to provide information about sociodemographic characteristics, tobacco and alcohol use, dietary habits, medical history, and lifetime occupational exposure (Roulland et al., 2004).

B cell isolation, cell sorting, and FACS analysis. PBMCs were obtained by Ficoll-density gradient centrifugation. For large blood samples, enriched B cells were isolated from PBMCs by negative selection using a B cell isolation kit (Invitrogen and/or Miltenyi Biotec). The resulting purified B cells were then stained for CD19-allophycocyanin (APC) or PE-Cy7 (clone HIB-19; BD or eBioscience), IgD-FITC (Invitrogen), IgM-APC (G20-127; BD), and CD27-PE (clone O323; eBioscience) mAbs. For control samples, the naive (IgD+CD27−) and memory (IgD+CD27+, IgD−CD27+) B cell subsets were isolated by FACS sorting, as previously performed (Roulland et al., 2006b). 10 large blood samples from HI (8 from the exposed cohort and 2 from anonymous blood donors) showing translocation frequencies >10−5

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analyzed Sμ region is indicated below each tree and varied slightly in case of CSR. (C) Sequence analysis of an AID-mediated foreign DNA insertion (200 bp from the *FAM53B* gene, chr 10, in blue) into an Sμ/Sγ switch junction of an activated t(14;18)+ clone from sample #138. (D) Sequence analysis of a foreign DNA insertion (161 bp from the *SUSD1* gene, chr 9, in blue) into an intra-Sγ2 deletion (△64 bp) from an activated t(14;18)+ clone from sample #127-b (see clone F-Sγ in A). Numbers refer to germline *IGH* available from GenBank/EMBL/DDBJ under accession no. NG_001019.

were further stained with CD10-PeCy5 (clone HI10a; BD) and used to sort the CD19$^+$CD10$^+$ (4–7% of B cells) and CD19$^+$CD10$^-$CD27$^+$ (25–35% of B cells) B cell fractions. In the exposed cohort, the CD19$^+$CD10$^+$ fraction was further split according to CD27 and IgD expression (Fig. S6 A). Cell sorting experiments were performed on a FACSAria (BD), and each sorted fraction was reanalyzed on a flow cytometer (LSR; BD) with FlowJo software (Tree Star, Inc.) showing at least 95% purity.

Fluctuation PCR amplification of *BCL2/IGH* translocations. Genomic DNA was extracted from PBMCs, FL biopsies, and/or sorted fractions using DNA blood or a tissue mini kit (QIAGEN). A fluctuation two-step short-range PCR (SR-PCR) assay was used to amplify *BCL2/J$_H$* junctions in HI, as previously described (Roulland et al., 2006b). Amplification of *BCL2/Cμ* and *BCL2/Cγ* fragments by long-range (LR-PCR) was performed using the same fluctuation approach and as previously described (Roulland et al., 2006b). In brief, nested primers flanking the *BCL2*–major breakpoint region (*mbr*) were used in combination with 3′ primers from the Cμ/Sμ region or the consensus Cγ/Sγ regions (Fig. S6 B). The PCR products were visualized by electrophoresis on a 0.8% agarose gel and ethidium bromide staining. All amplicons were gel purified from each independent PCR replicate, cloned into pGEMT-easy vector (Promega), and sequenced. Primers and probes are listed in Table S3.

Quantitative RT-PCR. RNA was extracted from PBMCs, FL biopsies, and/or sorted fractions with the RNAqueous-4 PCR kit (Applied Biosystems), and 1 μg RNA was converted into cDNA using the high capacity RT kit (Applied Biosystems). To prevent the potential contamination of the RNA samples with genomic DNA, all preparations were DNaseI treated. To accurately measure *BCL2/J$_H$* and *ABL* gene expression from different origins, we developed an absolute quantitative PCR strategy (Fig. S4). During the translocation process, most *BCL2* breakpoints arise in the *mbr*, located in the 3′ untranslated region (UTR) of *BCL2* exon III. This generates a long *BCL2/IGH* fusion transcript carrying the complete *BCL2* open reading frame (Cleary et al., 1986; Seto et al., 1988). Recombinant RNA (recRNA) was generated (Fig. S4, left) to build standard calibration curves. RT- and real-time PCR steps were performed in parallel on recRNA standards and human RNA samples in a PCR System (ABI PRISM 7500; Applied Biosystems). Quantitative PCR was performed with 1/10th of the RT-PCR product (∼100 ng cDNA) using primers flanking the 5′ *BCL2-mbr* (3′ UTR region) in combination with the 3′ *J$_H$* consensus reverse sequence from the *IGH* locus (Fig. S4, right) and a TaqMan MGB probe (Applied Biosystems) matching the *mbr* region. For absolute quantification, the samples were analyzed in two independent PCR reactions, each performed in duplicate and normalized to the signal obtained for the *ABL* copy number. PCR for AID and BCL-6 transcripts was performed with specific TaqMan gene expression assays (Hs00757808_m1 and Hs00277037_m1, respectively; Applied Biosystems) and normalized to the expression of the endogenous *ABL*. The results were analyzed according to the ΔΔCt comparative method, normalizing to 1 the expression of AID and BCL6 in the naive IgD$^+$CD27$^-$ fraction of the R127 control.

Sμ SHM analysis. The presence and patterns of mutations accumulating on the 5′ Sμ region (Nagaoka et al., 2002) from the *IGH* locus on the der(14) translocated allele were examined by sequencing cloned *BCL2/Sμ* and *BCL2/Sγ* LR-PCR amplicons. The analysis was performed in an ∼1,250-bp sequence upstream of and overlapping the repetitive Sμ region using two consecutive primers (label 4; Fig. S6 B). To evaluate the PCR error rate introduced by the *Expand Taq* DNA polymerase (Roche) and exclude polymorphic variants from analysis, the germline Sμ region of each individual was amplified using primers 3+5 and sequenced (Fig. S6 B). Mutations corresponding to polymorphic variants were excluded, and the mean baseline mutation rate was evaluated to be 0.55‰ (Fig. S2). Sμ deletions, mutations, duplications, and insertions were then organized into genealogical trees of the stepwise accumulation of these events into individual t(14;18)$^+$ molecular clones (Fig. 4).

Statistical analysis. To examine the relationships between t(14;18) and agricultural pesticide use, we first compared the evolution of t(14;18) frequency over time among exposed and control groups using the paired *t test*. Normality was tested and rejected because of the skewness of the distribution of t(14;18) frequencies. Relationships between t(14;18) frequency and age were made with the Spearman rank test. For mutation analysis, Fisher's exact test was used to compare Sμ mutation frequencies between various B cell subpopulations as compared with FL cells, and the χ^2 tests were used to compare mutation distribution patterns.

Online supplemental material. Fig. S1 shows the restoration of the age effect in farmers who stopped agricultural pesticide use at follow up. Fig. S2 shows the Sμ mutation patterns in normal and t(14;18)$^+$ B cells from HI and FL patients. Fig. S3 shows that the majority of t(14;18)$^+$ cells are IgD$^+$M$^+$. Fig. S4 illustrates the absolute quantitative RT-PCR strategy to accurately measure the *BCL2/J$_H$* transcripts levels in t(14;18)$^+$ B cells from HI as compared with FL. Fig. S5 shows that *BCL2/J$_H$* transcript expression strictly correlates with t(14;18)$^+$ fractions. Fig. S6 depicts a representative cell sorting experiment to isolate peripheral B cell subsets according to CD27, CD10, and IgD cell-surface markers, and a diagram showing the positions of primers used to characterize *BCL2/IGH* translocations by SR-PCR and/or LR-PCR assays. Fig. S7 shows BCL6 expression in PBMCs and B cell subsets from HI with various t(14;18) frequencies. Fig. S8 illustrates the two kinds of dynamics than can be modelized from circulating FL precursor cells in HI. Table S1 provides a summary of the *BCL2/J$_H$* polyclonality in individuals for which more than one positive amplicon was found. Table S2 describes the demographic and exposure characteristics of the farmer population at enrollment and follow up. Table S3 list the primers used for PCR and quantitative RT-PCR reactions. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082842/DC1.

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