allogeneic HSCT.[1,10,11] In conclusion, pulmonary extramedullary hematopoiesis may be a more common finding in patients with myelofibrosis than has been previously reported. However, it is not a contraindication for allogeneic HSCT.

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Exon 8 splice site mutations in the gene encoding the E3-ligase *CBL* are associated with core binding factor acute myeloid leukemias

Acute myeloid leukemia (AML) is a heterogeneous disease with diverse genetic abnormalities and variable response to treatment. In the last decade the diverse genetic abnormalities have refined risk-stratification of AML.[1] Recently, mutations in the Casitas B-cell lymphoma gene encoding the E3-ligase CBL[2] were identified in *de novo* AML.[3,4] In one study, a single case with an inactivating point mutation in exon9 of the *CBL* gene was identified in a cohort of 150 *de novo* AML cases.[3] In a second study, exon8 missense mutations were demonstrated in 3 out of 12 randomly selected AML cases.[4] In an additional AML case, a DNA insertion/deletion mutation in intron7 of the *CBL* gene resulted in the expression of a *CBL* splice variant, i.e., a *CBL* mRNA lacking exon8.[4]

All published *CBL* mutations are located within the conserved linker region (LR) and ring finger (RF) of the CBL protein.[2] In fact, the mutant *CBL* splice variant without exon8 results in an in-frame deletion, which encodes a CBL protein lacking part of the LR, including two essential tyrosine residues, and almost the entire RF, which is critical for E3 activity.[2] This suggests that mutant CBL may act as a dominant negative protein by inhibiting proper downregulation of critical activated tyrosine kinases, such as KIT and FLT3 in AML.[5]

It is still not clear how frequently mutations in the *CBL* gene occur in newly diagnosed AML. In a diverse population of primary AML (n=319, Table 1) we assessed the frequency of CBL mutations, i.e., point mutations in exon8[4] and exon9[3], and mutations affecting proper splicing of CBL exon8.[4]

Patients had a diagnosis of primary AML, confirmed by cytological examination of blood and bone marrow, and were treated according to the HOVON protocols (*http://www.hovon.nl*). After obtaining patients' informed consent, bone marrow aspirates or peripheral blood samples were taken at diagnosis (n=319). CBL exon8 mRNA splice variants, as well as point mutations in exon8, were determined by cDNA amplification using the primer set CBLe6F 5'-AAACCTCTCTTCCAAGCACTG-3' and CBLe9R 5'-TCCCTCTAGGATCAAACGGA-3' or CBL-exon7-FOR 5'-GTGAACCAACTCCCCAAGAC-3' and CBL-exon9-REV 5'-GGACAGCCCTGACCTTCTG-3'. Mutations in genomic DNA were determined by amplification using CBL-intron7-FOR 5'-GGACCCAGACTA-GATGCTTTC-3' and CBL-exon8-REV 5'-GTGCACAT-GAGGTGTCCACAG-3' (mutations 5' of exon8) or CBL-exon7-FOR and CBL-exon9-REV (mutations 3' of exon8). (0.25 mM dNTP, 15 pmol primers, 2 mM $MgCl_2$, Taq polymerase and 1xbuffer (Invitrogen Life Technologies, Breda, The Netherlands); 1 cycle at 94°C for 5 mins., 35 cycles at 94°C for 1 min, 60°C for 1 min., 72°C for 1 min., and 1 cycle at 72°C for 7 mins.). Samples showing aberrant patterns were sequenced by using forward and reverse primers on the ABI PRISM3100 genetic analyzer

Table 1. Clinical and molecular characteristics of the 319 patients* with *de novo* acute myeloid leukemia.

	#	Percent
Gender		
Male	154	48
Female	165	52
FAB		
M0	11	3
M1	62	19
M2	69	22
M3	19	6
M4	52	16
M5	79	25
M6	6	2
not determined	21	7
Cytogenetic abnormalities°		
t(15;17)	19	6
t(8;21)	21	7
inv(16)/t(16;16)	22	7
+8	23	7
-5/-5(q)	13	4
-7/-7(q)	22	7
3q	10	3
t(6;9)	3	1
t(9;22)	3	1
t(11q23)	14	4
complex (>3 abn.)	18	6
other	77	24
normal	127	40
not determined	14	4
Molecular abnormalities		
FLT3 ITD	90	28
FLT3 TKD	33	10
N-*RAS*	26	8
K-*RAS*	0	0
CEBPA	17	5
KIT (exon8 and D816 mutations)	14	4

**median age 45 (range 15-75), median bone marrow blast count 65 percent (range 0 (for acute promyelocytic leukemia)-99), median white blood cell (WBC) count 32 (×10⁹/L) (range 0.3-349) and median platelet count 51 (×10⁹/L) (range 3-998). °All patients with a specific abnormality were considered, irrespective of the presence of additional abnormalities.*

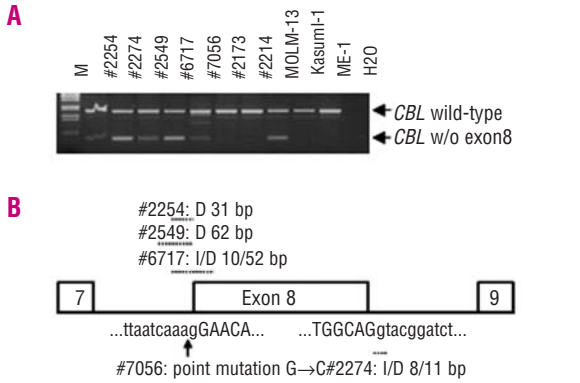

Figure 1. ***CBL*** **mRNA splice variant mutations in core-binding factor acute myeloid leukemia. (A) RT-PCR with primers *CBL*-exon7-FOR and *CBL*-exon9-REV of primary acute myeloid leukemia cases showing aberrant splice variants (#2254 (inv(16)), #2549 (t(8;21)), #2274 (inv(16)), #6717 (inv(16)), #7056 (t(8;21)), wild-type *CBL* (#2173 and #2214) and cell lines MOLM13 (*FLT3* internal tandem duplication (ITD)), Kasumi-1 (t(8;21)) and ME-1 (inv(16)). The presence of these CBL exon8 splice mutants in the primary AML were confirmed using various primer sets (*data not shown*). AML #7056 consistently expresses two additional CBL mRNA splice variants. Of note, both these *CBL* splice variants are in-frame ((exon7) – ACC CAG ATG GGC TCC (exon8) and (exon7) ACC CAG GAT GTA AAG (exon8)) (B) Schematic representation of exon 7-to-9 of the *CBL* gene to indicate the insertion (I)/deletion (D) mutations in the *CBL* gene of the 5 CBF acute myeloid leukemia cases (#2254, #2549, #2274, #6717, #7056).**

(Applied Biosytems, Foster City, CA, USA).

All AML cases were screened by RT-PCR for *CBL* exon8 splice variants, whereas a randomly selected subset of 183 out of the 319 AML cases was examined by sequence analysis for the presence of exon8[4] or exon9[3] point mutations. We did not find any point mutation in exon8 or exon9 of CBL in the subset of 183 AML cases. However, out of the 319 AML cases, we did identify two AML cases expressing a *CBL* mRNA splice variant (#2274 and #6717, Figure 1A), which lacked exon8. This aberrant CBL mRNA is similar to the splice variant previously identified in the leukemic cell line MOLM13 (Figure 1A).[4] *CBL* transcripts lacking exon8 were not present in 5 normal bone marrow samples and 3 fluorescence activated cell sorted CD34-positive progenitor cell samples (*data not shown*).

Interestingly, the two primary AML cases showing aberrant splicing carried an inversion of chromosome 16 (inv(16)) suggesting that *CBL* mutations might be associated with core-binding factor (CBF) leukemias, i.e., AML and inv(16) or t(8;21). In a selected screen of 39 inv(16) AML and 40 t(8;21) AML we did not detect point mutations in CBL exon8 or exon9. However, the *CBL* exon8 splice variant was present in 2 additional cases with a t(8;21) and one additional case with an inv(16) (Figure 1A). The *CBL* exon8 splice variant was absent in 4 independent remission samples of AML patient #2549 (*data not shown*).

By nucleotide sequencing of the flanking sequences of *CBL* exon8 in all AML cases expressing the CBL mRNA splice variant, we identified various insertion/deletion mutations in the CBL gene (Figure 1B). All insertion/deletion mutations affect the splice acceptor or donor sites of exon8 of CBL. In fact, the G to C point mutation in case #7056 is located within the splice acceptor site of *CBL* exon8, resulting in two additional aberrant in-frame *CBL* transcripts (Figure 1A).

These results indicate that there is a preferential association between *CBL* exon8 splice variant mutations and CBF leukemias. This raises the possibility of a functional association between impaired CBL function and the CBF-related fusion proteins CBFB-MYH11 and AML1-ETO. Activating point mutations in receptor tyrosine kinase KIT are strongly associated with CBF leukemias.[6-8] In fact, we recently screened 500 cases of AML for exon8/exon17 *KIT* mutations and mutations were present in 25 AML cases (5%). Of the KIT mutant cases, 88% carried a proven CBF mutation, such as t(8;21), inv(16) or t(3;21) (*data not shown*). CBL proteins mediate ubiquitination and degradation of KIT upon stimulation with stem cell factor.[9] Thus, the expressed mutant CBL protein, which is still able to bind KIT by its N-terminal tyrosine kinase binding domain, but impaired in downstream ubiquitination,[2] may act as a dominant negative protein. Expression of this dominant negative protein could result

in impaired routing of KIT and sustained activation, similar to KIT activating mutations in CBF leukemias.

No other AML-specific mutations, such as those affecting *FLT3, NRAS, KRAS, CEBPA* and *NPM1*, were present in the *CBL* mutant AML cases. Interestingly, however, *CBL* mutant t(8;21) AML case #2549 also carried a KIT D816 mutation. In this AML, impaired function of the CBL protein would potentially result in prolonged constitutive activation of KIT.

Our results demonstrate that *CBL* mutations are rare in AML. However, the strong association of these mutations with CBF leukemias suggests that there may be a co-operative activity of mutant CBL with the CBF-related fusion proteins CBFB-MYH11 and AML1-ETO in CBF leukemogenesis, most likely by impaired ubiquitination of KIT.

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Genetic variation in genes expressed in the germinal center and risk of B cell lymphoma among Caucasians

The germinal center reaction is integral to B-cell maturation, where class switch recombination (CSR) and somatic hypermutation (SHM) are targeted to the immunoglobulin (Ig) locus to facilitate antibody diversity.[1] Selection against B cells with auto-reactive or low affinity antigen receptors promotes the generation of highly effective B cells.

SHM is initiated following deamination of cytidine to uracil, resulting in U:G mismatches.[2] Direct removal of uracil by uracil-DNA glycosylase (UNG) can lead to mutation if DNA is replicated across these non-informative abasic sites. Uracil-containing mispairs can also be recognized by mismatch repair (MMR) and base excision repair (BER) machinery, key components of which include MLH1, MSH2, PMS2 and XRCC1.[2] Excision of a repair patch and DNA re-synthesis using error-prone DNA polymerases promotes mutation.[2] MMR also functions in CSR where mismatches are recognized by the MSH2/MSH6 heterodimer, ultimately resulting in the DNA double strand breaks integral to class switching.[2]

Reciprocal translocations involving the Ig locus arising during VDJ recombination or CSR are a hallmark of B-cell lymphomas, and can lead to the activation of proto-oncogenes such as *MYC, CYCLIN D1, BCL6* and *BCL2*. Point mutations occur in genes outside the immunoglobulin locus, including *BCL6* and *FAS*.[3,4] These data suggest that translocations and mutations in B-cell lymphomas can arise via mis-targeting of the CSR and SHM machinery specifically during the germinal center reaction, and may allow cells to bypass the normal processes regulating cell proliferation, differentiation and apoptosis.

We hypothesized that genetic variation in genes expressed in B cells during the germinal center reaction, and which are components of CSR, SHM or B-cell selection, may affect the risk of developing lymphoma. We, therefore, examined the frequency of nine common polymorphisms with putative functionality (allele frequency >0.02) in six genes (*PMS2, UNG, XRCC1, MSH2, MLH1* and *FAS*) in 884 patients with lymphoma and 1,019 population controls. Six hundred and forty-nine Caucasian cases of B-cell non-Hodgkin's lymphoma (NHL), 235 Caucasian cases of Hodgkin's lymphoma (HL) and 1,019 Caucasian controls were recruited to a study conducted in the north and southwest of England between January 1998 and July 2003.[5] The study was approved by the UK Multicentre Research Ethics Committee and all participants gave informed consent according to the Declaration of Helsinki.

DNA was genotyped using allelic discrimination single nucleotide polymorphism (SNP) assays (TaqMan, Applied Biosystems (ABI), Warrington, UK). Genotype clusters were ascertained independently by two researchers and genotypes designated only when there was consensus. Assay accuracy was verified in 30 randomly selected patient samples by direct DNA sequencing (100% concordance). Among the controls, all geno-