*CYP1A1*2B (Val)* allele is overrepresented in a subgroup of acute myeloid leukemia patients with poor-risk karyotype associated with *NRAS* mutation, but not associated with *FLT3* internal tandem duplication

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The etiology of acute myeloid leukemia (AML) is largely unknown. Biologic and epidemiologic data implicate exogenous toxicants, including cytotoxic drugs, benzene, radiation, and cigarette smoking. Allelic variation in genes encoding enzymes such as NADP(H) quinone oxidoreductase (NQO1) and glutathione *S*-transferase T1 (GSTT1) that metabolize environmental toxicants predispose to subtypes of AML, including therapy-related AML. We assayed *NRAS* oncogene mutation and *FLT3* internal tandem duplication in 447 AML patients with an abnormal karyotype treated in Medical Research Council (MRC) AML clinical trials. Functional allelic variant frequencies in genes encoding carcinogen-metabolizing enzymes *GSTT1*, *GSTM1*, *CYP1A1*, *CYP2D6*, *CYP2C19*, *SULT1A1*, and *NQO1* were previously determined for this cohort. *FLT3* internal tandem duplication (ITD) frequency was 17%, and *NRAS* mutation 12% for the entire cohort. The 2 mutations were found together in only 4 patients. No association was found between enzyme allelic variant frequencies and the presence of *FLT3* ITD for the entire cohort or within cytogenetic subgroups. *CYP1A1*2B (Val)* high-inducibility variant allele was overrepresented in patients with *NRAS* mutation compared with no mutation, for (1) the entire AML cohort (n = 8/53 vs 26/371; odds ratio [OR] = 2.36; 95% confidence interval [CI] 1.01-5.53) and (2) the poor-risk karyotype group (n = 6/14 vs 4/89; OR = 15.94; 95% CI 3.71-68.52) comprising patients with partial/complete deletion of chromosome 5 or 7, or abnormalities of chromosome 3. The *CYP1A1*2B* allele may predispose to the development of these subgroups of AML by augmented phase 1 metabolism to highly reactive intermediates of CYP1A1 substrates, including polycyclic aromatic hydrocarbons, or by generation of oxidative stress as a metabolic by-product. (Blood. 2003;101:2770-2774)

Introduction

The etiology of acute myeloid leukemia (AML) is unknown for the majority of cases. The identification of biologically well-defined subgroups on the basis of cytogenetic analysis has improved prognostic power and led to risk-directed therapeutic strategies. It is increasingly clear that the etiologic mechanisms underlying the "good-risk" translocational karyotypes t(15:17), inv(16), and t(8:21), which predominate in younger patients, will differ from the largely deletional abnormalities seen in "poor-risk" disease, which are more common in older patients. The karyotypic similarities between therapy-related AML (t-AML) and poor-risk de novo AML[1] suggest that an environmental etiology is more likely in the latter group than in the de novo good-risk group. Questionnaire-based case-control studies have suggested associations between exposure to specific environmental toxicants and the development of AML, but cannot define mechanisms of leukemogenesis.[2,3]

The cytochrome P450 enzymes are expressed predominantly in the liver and function to detoxify many environmental toxicants (xenobiotics). These phase 1 metabolic enzymes transfer electrons onto substrate toxicants to create highly reactive intermediates, which are then available for detoxification by a variety of phase 2 enzymes including glutathione *S*-transferases and sulfotransferases.

Several carcinogen-metabolizing genes with functional allelic variants are now known to predispose to t-AML[4,5] and also to de novo AML.[6-8] Furthermore, this predisposition may be greater or less in subgroups of AML defined by specific genomic damage at the karyotypic level. The paradigm for an AML-predisposition gene encoding a carcinogen-metabolizing enzyme is NADP(H) quinone oxidoreductase (NQO1). The *C609T* polymorphic variant, which confers reduced phase 2 metabolism, is associated with a predisposition to therapy-related AML[4] and selected cytogenetic subgroups of de novo AML.[6] Reduced phase 2 metabolism has the potential to result in an accumulation of reactive intermediates, which in turn oxidize DNA and protein, leading to DNA mutation and/or cell death. Phase 1 enzymes with functionally relevant allelic variants are also predisposition genes for AML and include cytochrome P450 2C19 (CYP2C19) and CYP2D6.[8] The proposed mechanism of predisposition for these 2 genes is a consequence of reduced phase 1 metabolism, presumably allowing the accumulation of nonmetabolized toxic carcinogens.

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We selected functional polymorphic variants in specific carcinogen-metabolizing enzymes for study. Enzymes were selected on the basis of an established role in the metabolism of known leukemogenic compounds (eg, benzene, cytotoxic chemotherapeutic drugs). We then selected only patients with well-characterized karyotypic abnormalities, to include abnormalities known to occur following genotoxic insult (eg, chromosome 5 and 7 abnormalities) and also good-prognosis translocations for which an environmental etiology may be less likely. Finally, we assayed the 2 most common molecular abnormalities in AML, namely, *FLT3* internal tandem duplication (ITD) and *NRAS* mutation; each of which is likely to arise from a different mechanism.

We demonstrate that the **2B* high-inducibility variant of *CYP1A1* is overrepresented in a subgroup of poor-risk AML patients with *NRAS* mutation and may represent a predisposition allele for these specific forms of genomic damage in AML.

Patients, materials and methods

Patients

We studied 447 patients with de novo or secondary AML (defined as secondary to chemotherapy, or following an antecedent hematologic disorder), and who were entered into the Medical Research Council (MRC) AML 10 (younger than 60 years, n = 109); AML 11 (older than 55 years, n = 115), and AML 12 (younger than 60 years, n = 223) clinical trials. This patient cohort overlaps with cohorts in previously published studies.[6] Median age was 46 years (range, 15-79 years). DNA was extracted from presentation bone marrow by dodecyltrimethylammonium bromide lysis[9] and stored at University College London (United Kingdom).

Cytogenetic classification

Most cytogenetic studies were done in local laboratories, all of them participants in a central quality control scheme (United Kingdom National External Quality Assessment Scheme). Where this was not available, cytogenetic analyses were done at a central facility in University College London. Standard methods of bone marrow culture were used; 20 metaphases were fully analyzed and reported in accordance with the International System for Human Cytogenetic nomenclature (ISCN) guidelines.[10] A hierarchical cytogenetic classification was used to assign all patients to a cytogenetic subgroup as previously described.[11] For patients with more than one chromosomal abnormality, the subgroup assigned was determined by the most important abnormality in the following order (with the most important listed first): established translocation, established deletion, established trisomy, nonestablished translocation, nonestablished deletion, nonestablished trisomy.[11] Data in this study are now presented for cytogenetic risk groups on the basis of the MRC hierarchical classification[12] and defined here as (1) good-risk, t(15;17), t(8;21), inv(16); (2) poor-risk, partial or complete deletion of chromosomes 5 and 7 (5q/7q), abnormalities of 3q; and (3) intermediate-risk, which includes +8, 11q23 abnormalities, +11, 17p−, +21, 20q−, 12p− (but excludes normal karyotype).

Carcinogen-metabolizing enzyme gene allelic variant analysis

Assays for allelic variants at the following loci have been previously described: *GSTM1* (null), *GSTT1* (null),[7] *CYP2D6* (poor/extensive metabolizer), *CYP2C19*2* (*681G→A*; aberrant splice), *CYP1A1*2B* allele (*2455A→G, Ile→Val*),[8] and *NQO1* (*609C→T*; *Pro→Ser*).[6] In addition, a polymorphism in *SULT1A1* (**2, 213G→ A*; *Arg→His*) was assayed by allelic discrimination by means of the polymerase chain reaction (PCR)–based TaqMan technology (PE Applied Biosystems, Foster City, CA). Primer and probe sequences for this assay were as follows: forward primer, 5′-GGTTGAGGAGTTGGCTCTGC-3′ (300 nM); reverse primer, 5′-ACGTGTGCTGAACCATGAAGT-3′ (300 nM) (annealing temperature, 62°C); wild-type (WT) probe, 5′-AGTTTGTGGGGCGCTCCCTG-3′ (100 nM) (Tet labeled); variant probe 5′-AGTTTGTGGGGCACTCCCTGC-3′ (200 nM) (Fam labeled).

FLT3 internal tandem duplication assay

Genomic DNA was amplified from exons 12 and 13 of the *FLT3* gene.[13] This produced a wild-type fragment of 328 bases plus a higher–molecular weight fragment containing internal tandem duplicated sequences (when present) of varying length as previously described.[13,14] Bands with higher molecular weight than wild type were cut from the gel and directly sequenced by means of a fluorescent primer adapted chain-termination method[15] on an ABI 3100 sequencer (PE Applied Biosystems) to determine the start site and size of each duplication.

NRAS mutational analysis

Mutation screen. Separate assays were developed for mutation detection at "hot spots" in codons 12/13 (exon 1) and codon 61 (exon 2). Oligonucleotide primers amplifying short fragments (241 base pair [bp], exon 1; 201 bp, exon 2) were designed for PCR as follows: N12/13 assay: forward, 5′-GACTGAGTACAAACTGGTGG-3′; reverse, 5′-TGCATAACTGAATGTATACCC-3′. N61 assay: forward, 5′-CAAGTGGTTATAGATGGTGAAACC-3′; reverse, 5′-AAGATCATCCTTTCAGAGAAAATAAT-3′. PCR products were then subjected to denaturing heteroduplex high-pressure liquid chromatography (HPLC) analysis (dHPLC) (Transgenomic WAVE, Crewe, United Kingdom). PCR conditions were as follows: (1) Exon 1, HotStarTaq (Qiagen, Valencia, CA), 0.625 U. Primers (12.5 pmol), N12/13 forward, N12/13 reverse. Denaturing, 95°C for 15 minutes and 94°C for 30 seconds; annealing, 55.5°C for 1 minute; extension, 72°C for 1 minute for 35 cycles; final cycle at 72°C for 10 minutes. (2) Exon 2, HotStarTaq (Qiagen), 0.625 U. Primers (12.5 pmol), N61F forward, N61R reverse. Denaturing, 95°C for 15 minutes; 94°C for 30 seconds; annealing, 55.5°C for 1 minute; extension, 72°C for 1 minute for 35 cycles; final cycle at 72°C for 10 minutes. Heteroduplexes were then generated by means of a thermal cycler as follows: 95°C for 5 minutes; 95°C, reducing at 1°C per 22 seconds, for 70 cycles. Then, 10 μL heteroduplexed PCR product per well was loaded from 96-well plates and analyzed by dHPLC under the following conditions: flow, 0.9 mL/min, 47% to 52% buffer (B) in 0.1 minutes, to 60% B in 4 minutes at 61°C. Representative dHPLC plots are shown in Figure 1.

Mutation confirmation. Samples exhibiting an abnormal dHPLC profile were confirmed as mutant by direct sequencing and/or PCR-restriction fragment length polymorphism mutation-sensitive digestion analysis for codons 12 and 13.[16,17] If these assays were insufficiently sensitive to confirm mutation, PCR products were cloned (Original TA Cloning Kit; Invitrogen, Groningen, the Netherlands) and sequenced.[15] The sensitivity of the dHPLC is such that 15% mutant DNA can be confidently detected in a sample containing wild-type and mutant sequence (M.E.F., manuscript in preparation).

Statistical analysis

CYP1A1 allellic variants were analyzed as dichotomous variables *(Ile/Val* plus *Val/Val,* versus *Ile/Ile).* Odds ratios (ORs) and 95% confidence intervals were calculated from 2 × 2 tables for mutant versus nonmutant *(NRAS* or *FLT3)* and *Ile/Val* plus *Val/Val,* versus *Ile/Ile.* Logistic regression was used to test these associations within the entire cohort and also between the 3 cytogenetic risk groups. Power calculations were prospectively computed to inform sample size for the case-control study reported elsewhere[6] and were based upon expected enzyme variant polymorphism frequencies. These prospective calculations were not done for *NRAS* or *FLT3* ITD frequency within the patient cohort alone. Retrospective power calculations were performed with the assumption of an expected *CYP1A1*2B* allele variant frequency *(Ile/Val* plus *Val/Val)* of 11%,[8] an *FLT3* ITD mutant frequency of 25%,[14] and an *NRAS* mutant frequency of 15%. The sample size of 427 patients was sufficient to provide 80% power at a *P* value less than .05 to detect an OR of 2.5 for the difference in *CYP1A1*2B* variant frequency between *FLT3* ITD mutant versus no ITD, and an OR of 2.9 for *NRAS* mutation versus no *NRAS* mutation.

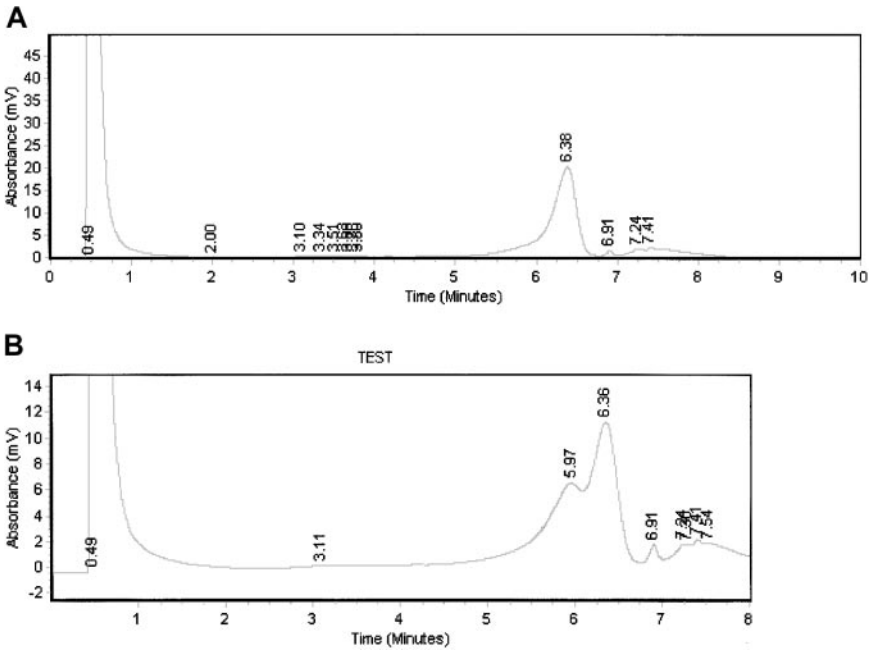

Figure 1. Denaturing HPLC profiles from representative AML patient DNA. (A) Denaturing HPLC profile from DNA containing wild-type codon 12/13 *NRAS* sequence only. (B) Denaturing HPLC profile from DNA containing wild-type plus mutant sequences. In this profile, wild-type homoduplexes are retained on the dHPLC column for longer (6.36 minutes) than mutant/wild-type heteroduplexes (5.97 minutes).

Results

FLT3 ITD frequency was 17% (77 of 447) and *NRAS* mutation was 12% (53 of 443) for the entire cytogenetically selected AML cohort. The 2 mutations were found together in only 4 patients, confirming previous observations.[18]

Presence of *FLT3* ITD is not associated with variant allele frequency in carcinogen-metabolizing enzymes

No differences in carcinogen-metabolizing enzyme variant allele frequency were found for *GSTT1*, *GSTM1*, *CYP2D6*, *CYP2C19*, *CYP1A1*, *SULT1A1*, or *NQO1*, either for the presence versus absence of *FLT3* ITD (data for *CYP1A1* variant alleles shown in Table 1) or in relation to start site or duplication size (data not shown).

Table 1. *CYP1A1* allele frequency in AML patients (all cases and subgroups) with or without *FLT3* internal tandem duplication

	CYP1A1 allele frequency			
	FLT3 ITDs no. (%)	ITDs no. (%)	OR	95% CI
All cases*				
Ile/Ile	67 (93)	326 (92)	0.84	0.31-2.25
Ile/Val or *Val/Val*	5 (7)	29 (8)		
Good-risk karyotypes				
t(15;17), t(8;21), inv(16)				
Ile/Ile	48 (94)	154 (91)	0.64	0.18-2.31
Ile/Val or *Val/Val*	3 (6)	15 (9)		
Intermediate karyotypes				
Ile/Ile	14 (100)	77 (94)	0.49	0.02-9.3
Ile/Val or *Val/Val*	0 (0)	5 (6)		
Poor-risk karyotypes				
5q/7q or 3q				
Ile/Ile	5 (71)	95 (91)	4.22	0.71-25.0
Ile/Val or *Val/Val*	2 (19)	9 (9)		

Ile/Ile, homozygous for *Ile* allele common among whites. *Ile/Val* or *Val/Val,* heterozygous or homozygous, respectively, for the **2B Val* allele. CI indicates confidence interval.

*n = 427.

*CYP1A1*2B* allele is overrepresented in AML with *NRAS* mutation

Patients with *NRAS*-mutant AML had a higher frequency of the *CYP1A1*2B* allele (combined *Ile/Val* plus *Val/Val*) compared with nonmutant AML cases ($z = 2.0$; $P < .045$) (Table 2). When the cohort was subdivided by cytogenetic risk group, this association was confined to the poor-risk karyotype group (Table 2), which was in turn statistically significantly different (logistic regression) from the good-risk group alone ($z = 2.78$; $P < .005$); the intermediate-risk group alone ($z = 2.0$; $P < .045$); and the combined good- plus intermediate-risk groups ($z = 3.026$; $P < .005$).

The poor-risk group comprised patients with abnormalities of chromosomes 5 and/or 7 (5q/7q) and also patients with abnormalities of 3q. The frequency of the **2B* allele in patients with 5q/7q abnormalities was 4 of 7 for patients with *NRAS* mutation versus 4 of 79 for patients without the mutation. Corresponding frequencies

Table 2. *CYP1A1* allele frequency in AML patients (all cases and subgroups) with or without *NRAS* mutation

	CYP1A1 allele frequency			
	Mutant *NRAS*,no. (%)	No mutant *NRAS*, no. (%)	OR	95% CI
All cases*				
Ile/Ile	45 (84.9)	345 (93)	2.36	1.01-5.53
Ile/Val or *Val/Val*	8 (15.1)	26 (7)		
Good-risk karyotypes				
t(15;17), t(8;21), inv(16)				
Ile/Ile	24 (96)	190 (88)	0.46	0.06-3.66
Ile/Val or *Val/Val*	1 (4)	17 (12)		
Intermediate karyotypes				
Ile/Ile	13 (93)	70 (93)	1.08	0.12-9.99
Ile/Val or *Val/Val*	1 (7)	5 (7)		
Poor-risk karyotypes				
5q/7q or 3q				
Ile/Ile	8 (57)	85 (96)	15.94	3.71-68.52
Ile/Val or *Val/Val*	6 (43)	4 (4)		

See Table 1 for abbreviations.

*n = 424.

for the 3q group were 2 of 7 for patients with the *NRAS* mutation versus 0 of 10 for those without.

No difference in variant allele frequency was found for *GSTT1, GSTM1, CYP2D6, CYP2C19, SULT1A1,* or *NQO1* when patient groups with and without the *NRAS* AML mutation were compared.

The *CYP1A1*2B* allele is not associated with a specific *NRAS* signature mutation

The *NRAS* mutation spectrum for patients with the *CYP1A1*2B* allele is presented in Table 3. Codon 12 mutations were most frequent; 6 of 7 of those mutations were at hot-spot sites, compared with 24 of 45 mutations at codon 12 among patients lacking the **2B* allele (not significant). The other base change at codon 33 is a novel *NRAS* mutation in a patient with trisomy 8.

Discussion

We have previously observed that the allele frequency for the *CYP1A1*2B* variant is no different in AML patients than in controls[8] and cannot therefore be considered a predisposition allele for the development of all types of AML. We now show that the *CYP1A1*2B* allele may indeed predispose patients to develop a subgroup of AML characterized by specific genomic damage, namely, *NRAS* mutation and poor-risk karyotype.

CYP1A1 is a phase 1 detoxification enzyme, expressed predominantly in extrahepatic tissue and with a wide variety of substrates, including the carcinogenic polycyclic aromatic hydrocarbons (PAHs). Baseline cellular expression of CYP1A1 is usually low, but high inducibility is mediated through the binding of inducers such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and PAH to a cellular protein, the aryl hydrocarbon receptor (AhR). AhR is in turn bound to heat-shock protein 90 (Hsp90) and releases this before translocation to the nucleus to associate with another protein, the Ah receptor nuclear translocator (Arnt). This complex binds an enhancer sequence activating transcriptional induction of CYP1A1 mRNA.[19] Thus, phase 1 detoxification capacity of CYP1A1 is influenced at 3 levels: baseline expression level, expression induction, and enzymatic activity.

Several polymorphic allelic variants have now been described within the *CYP1A1* gene (reviewed in Wormhoudt et al[20]). The most common variant allele, designated **2A*, introduces an *Msp*1 restriction site in the 3′ end of the gene. The next most common, and potentially functionally more significant, variant is a single nucleotide polymorphism in exon 7 (*A*→*G*) producing an Iso→Val substitution at amino acid 462 (allelic variant designated **2B*). These alleles are often, but not always, in linkage disequilibrium.[20] While the data linking the **2A* allele to high inducibility of CYP1A1 in human systems remain inconclusive,[21,22] the **2B* allele does appear to increase induced gene expression and enzymatic function in lymphocytes from healthy donors with a significant trend through heterozygotes to homozygotes for the variant *(Val)* allele.[22] In the Crofts et al[22] study, CYP1A1 expression was particularly high among smokers with at least one **2B* allele but was not elevated for smokers with at least one **2A* allele. Thus, augmented phase 1 metabolism resulting from high inducibility and increased expression of CYP1A1 upon exposure to environmental inducers and substrates will lead to accumulation of toxic metabolic intermediates should phase 2 metabolic pathways be saturated.

Table 3. Mutation spectrum for patients with *NRAS*-mutant AML and who have the *CYP1A1*2B* (*Val*) allele

Karyotype	*CYP1A1* allele	Mutation: codon-base substitution/ amino acid change
5q/7q	*Ile/Val*	12.2 G → A/Gly → Asp
5q/7q	*Ile/Val*	12.2 G → A/Gly → Asp
5q/7q	*Ile/Val*	61.1 C → A/Gln → Lys
5q/7q	*Val/Val*	12.2 G → A/Gly → Asp
3q	*Ile/Val*	12.1 G → A/Gly → Ser
3q	*Val/Val*	12.2 G → C/Gly → Ala
+8	*Ile/Val*	33.3 T → G/Asp → Glu
t(15;17)	*Ile/Val*	12.1 G → A/Gly → Ser

The high concentration of PAH in cigarette smoke has led to extensive studies of *CYP1A1* allelic variant frequencies in smoking-associated cancers. Although most studies are small, the **2B* allele may be overrepresented in smokers developing lung,[23] breast,[24] and colorectal[25] cancers. Several studies provide plausible epidemiologic support for the association between AML and substrates and inducers of CYP1A1. A cohort of subjects exposed accidentally to the potent CYP1A1 inducer TCDD have shown a 3.8-fold increased risk of myeloid leukemia with a latency of 15 years.[26] Exposure to cigarette smoke is a weak but reasonably consistent risk factor for AML in large case-control studies.[27,28] Smoking history data were unfortunately not available for most subjects in our study.

NRAS mutations in our patients with the *CYP1A1*2B* allele show no signature mutation, and this is perhaps not surprising given the diversity of putative environmental carcinogens in the etiology of AML. It is of note, however, that in those patients reported here and in our extended cohort of AML samples (data not shown), most *NRAS* mutations are transitions, as has previously been reported in AML and other hematologic malignancies.[29] Thus, although PAHs are the most attractive candidate carcinogenic substrate of CYP1A1 in the etiology of AML, on the basis of the mutation spectrum in our study, it is likely that the other substrates are involved. This may indeed be the case in other tumors, including lung cancer. Although a signature p53 mutation spectrum is evident in cohorts of smokers with lung cancer,[30] a recent study found a higher prevalence of p53 mutation in lung cancers from heavy smokers (compared with nonsmokers) with variant *CYP1A1* alleles (**2A/*2B*), but the usual signature mutation spectrum was not found in this subgroup.[31] This suggests that the mutation signature may be less evident in patients with variant *CYP1A1* alleles. Also in this study, smokers with a variant *CYP1A1* allele had an increased frequency of *KRAS* mutation but the authors do not comment upon the mutation spectrum. Cigarette smoke contains a multitude of carcinogenic compounds in addition to both TCDD and PAHs. Exposure to combinations of CYP1A1 inducers and substrates may therefore exacerbate the potential for genomic damage on the background of inheritance of the high-inducibility **2B* allele. A final alternative hypothesis is that reactive oxygen species known to be generated as a byproduct of CYP1A1-mediated carcinogen metabolism[32,33] may oxidize DNA. Oxidative DNA damage may generate a more diverse mutation spectrum, including transitions (eg, 5OH-cytosine G→A) and transversions (8OH-guanine G→T).

It remains unclear why the association between the CYP1A1*2B variant allele and *NRAS* mutation should be confined to the poor-risk karyotype groups. In the context of therapy-related AML, however, point mutations in critical regulatory genes are more common within the "deletional" karyotypes, namely, p53 mutation

associated with chromosome 5 deletion,[34] and *RAS* mutation associated with chromosome 7 deletion.[1] These data suggest that genomic instability leads to AML through different pathways,[1] and in the context of de novo AML, *CYP1A1* variant allele status may represent one determinant of this instability. It is not surprising that *FLT3* ITD is not associated with the *CYP1A1*2B* allele within these cytogenetic subgroups, as the ITD most likely arises from a mechanism different from that of point mutation, perhaps aberrant recombination or defective double-strand DNA repair.

In conclusion, our data suggest that the variant *CYP1A1*2B* allele may predispose to the development of a subgroup of AML patients characterized by poor-risk karyotypes and *NRAS* mutation. Epidemiologic data also suggest that substrates and inducers of CYP1A1 represent candidates for environmental carcinogens with potential to cause AML, but alternative mechanisms such as CYP1A1-induced oxidative stress are also proposed.

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