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TOXICOGENOMICS

EPHX1 rs1051740 T>C (Tyr113His) is strongly associated with acute myeloid leukemia and *KMT2A* rearrangements in early age

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Abstract

Experimental and epidemiological data have shown that acute myeloid leukemia in early-age (i-AML) originates prenatally. The risk association between transplacental exposure to benzene metabolites and i-AML might be influenced by genetic susceptibility. In this study, we investigated the relationship between genetic polymorphisms in *CYP2E1, EPHX1, MPO, NQO1, GSTM1* and *GSTT1* genes, and i-AML risk. The study included 101 i-AMLs and 416 healthy controls. Genomic DNA from study subjects was purified from bone marrow or peripheral blood aspirates and genotyped for genetic polymorphisms by real-time PCR allelic discrimination, Sanger sequencing and multiplex PCR. Crude and adjusted odds ratios (OR, $_{adj}$OR, respectively) with 95% confidence intervals (95% CI) were assessed using unconditional logistic regression to estimate the magnitude of risk associations. *EPHX1* rs1051740 T>C was associated with i-AML risk under the co-dominant ($_{adj}$OR 3.04, $P=0.003$) and recessive ($_{adj}$OR 2.99, $P=0.002$) models. In stratified analysis, *EPHX1* rs1051740 was associated with increased risk for i-AML with *KMT2A* rearrangement ($_{adj}$OR 3.06, $P=0.045$), i-AML with megakaryocytic differentiation ($_{adj}$OR 5.10, $P=0.008$), and i-AML with type I mutation ($_{adj}$OR 2.02, $P=0.037$). *EPHX1* rs1051740-rs2234922 C-G haplotype was also associated with increased risk for i-AML ($_{adj}$OR 2.55, $P=0.043$), and for i-AML with *KMT2A* rearrangement ($_{adj}$OR 3.23, $P=0.034$). Since EPHX1 enzyme is essential in cellular defense against epoxides, the diminished enzymatic activity conferred by the variant allele C could explain the risk associations found for i-AML. In conclusion, *EPHX1* rs1051740 plays an important role in i-AML's genetic susceptibility by modulating the carcinogenic effects of epoxide exposures in the bone marrow.

Keywords *EPHX1* · *KMT2A*/*MLL*-r · Infant AML · Genetic polymorphisms

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease, characterized by malignant transformation and deregulated proliferation of myeloid precursor cells (Langmuir et al. 2001). In Brazil, it accounts for about 15–20% of all childhood leukemia cases, with age-adjusted incidence rate of 10.4 cases per million (de Souza Reis et al. 2011). There is a relevant incidence peak of infant AML (i-AML), reaching about 18 cases per million (de Souza Reis et al. 2016; Howlader et al. 2017). Although it is a rare malignancy, there are enough evidence that infant leukemia (lymphoid and myeloid) originates prenatally during fetal hematopoiesis (Greaves and Wiemels 2003), as illustrated by reports of concordant leukemia in twins sharing unique abnormal gene rearrangements of lysine

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N-methyltransferase 2A gene (*KMT2A*/*MLL*-r) (Ford et al. 1993), and the detection of *RUNX1–RUNX1T1* gene fusion in neonatal blood spots of children who developed leukemia during early age (Wiemels et al. 2002).

Epidemiological studies have demonstrated that several environmental agents, e.g. maternal and/or child exposures to tobacco smoke, alcohol, pesticides, diet rich in topoisomerase-II (topo-II) inhibitors, and air pollutants, have been associated with childhood AML risk (Puumala et al. 2013). Considering that chemical carcinogens are commonly metabolized into more reactive compounds to exert toxic effects, genetic polymorphisms in metabolic pathways of xenobiotic substances may modulate childhood leukemia susceptibility. To date, very few investigations have evaluated the associations between genetic variants and i-AML risk (Brisson et al. 2015).

Benzene is a well-known carcinogen, associated with hematological disorders in adults, throughout environmental exposures studies (IARC 2012). Proxies of benzene exposure were also associated with increased risk for AML in children, e.g. paternal tobacco smoking during preconceptional or gestational period (Metayer et al. 2016), residence proximity to highways, gas stations or repair garages (Brosselin et al. 2009), and high density of air pollutants (Heck et al. 2014). Benzene metabolism is held by phase I and II enzymes, mainly represented by cytochrome P450 2E1 (CYP2E1), microsomal epoxide hydrolase (EPHX1), myeloperoxidase (MPO), NADP(H) quinone dehydrogenase 1 (NQO1), and glutathione S-transferases (GST) (Kim et al. 2006). Genetic polymorphisms related to those metabolic enzymes were associated with susceptibility to benzene hematotoxic effects among exposed workers (Dougherty et al. 2008).

Recently, in a population-based study, an 8.24-fold positive association was found between maternal occupational exposure to chemicals and i-AML risk ($P = 0.01$) in Brazil (de Souza Reis et al. 2017). Additionally, the molecular characterization of pediatric AML, which included 703 AML cases, revealed that *KMT2A*–r was the most frequent genetic abnormality identified (67.6%) and associated with i-AML (Andrade et al. 2016). Unlike infant acute lymphoblastic leukemia, in which *AFF1* is the most common *KMT2A* fusion partner, among i-AML cases, *KMT2A* is frequently fused to *MLLT3, MLLT10* and *ELL*. (Meyer et al. 2013). Those differences could underlie distinct molecular pathways according to oncogenic transformation when deoxyribonucleic acid (DNA) damage occurs after chemical exposure during pregnancy. With the premise that early-age leukemia arises from somatic clonal cells originated during fetal life due to transplacental chemical exposures, our aim was to investigate whether common genetic polymorphisms of *CYP2E1* (rs3813867), *EPHX1* (rs1051740, rs2234922), *MPO* (rs2333227), *NQO1* (rs1800566), *GSTM1*, and *GSTT1* would be associated with i-AML risk.

Materials and methods

Study population and samples

One hundred and forty-five samples at diagnosis of infants with de novo AML (aged < 2 years old) were collected between January 1st, 2002 and December 31st, 2014 in the Pediatric Hematology-Oncology Research Program at Instituto Nacional de Câncer (INCA) at Rio de Janeiro, Brazil. Cases were forwarded from several Brazilian medical institutions located in regions covered by population-based cancer registries. Cases with secondary AML, genetic syndromes (e.g. Down, Ataxia Telangiectasia, Bloom, Noonan, monosomy 7) or whose mothers have not agreed to provide child's biological sample were not included. Because of availability of good quality biological material for molecular tests, 101 cases (70%) were included in this study. AML subtypes were characterized as recommended by the World Health Organization (WHO) classification criteria (Swerdlow et al. 2008).

Unmatched controls consisted of 416 umbilical cord blood (UCB) samples randomly selected from the National Umbilical Cord and Placental Blood Bank at INCA. Those samples were collected from healthy newborns at birth, whose mothers donated UCB samples, attending to pre-established criteria: (a) maternal age greater than 18 years, (b) had at least two documented prenatal visits, (c) gestational age greater than 35 weeks, and (d) no history of genetic syndromes, neoplastic, infectious or hematological diseases. Final UCB donation was done when the child was alive at 2 months of age. Demographic variables, such as sex, race/skin color and age at diagnosis of i-AML were obtained from on-line completed forms (http://www.inca.gov.br/phop). The criteria for race/skin color was based on child's parent- or guardian-reported classification as White, Brown, Black, Yellow, and Indigenous, as recommended by the Instituto Brasileiro de Geografia e Estatística (http://www.ibge.gov.br). For purposes of this study, race/skin color was categorized as Blacks/Non-Blacks, which best represents the racial admixture of the Brazilian population (Pena et al. 2011).

Molecular characterization of i-AML cases

Total ribonucleic acid (RNA) was purified from bone marrow (BM) aspirates of i-AML cases using TRIzol® reagent (Invitrogen, CA, USA), according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized by transcriptase reverse enzyme and verified by *GAPDH* gene

amplification. AML's recurrent somatic abnormalities, i.e. *RUNX1–RUNX1T1, CBFβ–MYH11, PML–RARα*, and *KMT2A*–r, were identified by reverse transcriptase polymerase chain reaction or fluorescent in situ hybridization, as described elsewhere (Andrade et al. 2016). Type I mutations, referred as *FLT3, NRAS, KRAS, KIT*, and *PTPN11* mutations were identified in DNA purified from BM or peripheral blood (PB) samples using TRIzol® reagent (Invitrogen, CA, USA), QIAmp DNA Blood Mini Kit (Qiagen, CA, USA) or HiYield Genomic DNA Mini Kit (Real Biotech Corporation, Taiwan) according to manufacturer's instructions. *FLT3* D835 point mutation in tyrosine kinase domain (TKD) and internal tandem duplication (ITD) were identified by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), as described elsewhere (Andrade et al. 2016). *NRAS* and *KRAS* mutations (in codons 12 and 13), *KIT* mutations (in exons 8 and 17), and *PTPN11* mutations (in exon 3) were detected by Sanger sequencing (Andrade et al. 2016).

Genotyping

Cases and controls were genotyped using DNA purified from BM, PB or UCB samples by TRIzol® reagent (Invitrogen, CA, USA), QIAmp DNA Blood Mini Kit (Qiagen, CA, USA) or HiYield Genomic DNA Mini Kit (Real Biotech Corporation, Taiwan). *CYP2E1* rs38138867 G>C, *EPHX1* rs1051740 T>C and rs2234922 A>G, and *NQO1* rs1800566 C>T single nucleotide polymorphisms (SNPs) were detected by real-time PCR allelic discrimination technique using TaqMan® Drug Metabolism Genotyping Assays (C___2431875_10, C_____14938_30, C__11638783_30, and C___2091255_30, Applied Biosystems, CA, USA). Experimental procedures were carried out according to manufacturer's instructions. Briefly, 10 ng of genomic DNA were used for amplification in the ViiA™ 7 Real-Time System (Applied Biosystems, CA, USA). Thermal cycler conditions were as follows: 95 °C for 10 min, and 50 cycles of denaturation at 92 °C for 15 s and annealing/extension at 60 °C for 90 s.

MPO rs2333227 G>A polymorphism was investigated by Sanger sequencing. Initially, 100 ng of genomic DNA was used for the PCR amplification of a 324-bp fragment using a pair of forward (5′-AATCTTGGGCTGGTAGTGCT-3′) and reverse (5′-TGTGCAATGGTTCAAGCGAT-3′) primers. Amplification was carried on Veriti® Thermal Cycler (Applied Biosystems, EUA) under the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Twenty microliters of PCR product were purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK), according to manufacturer's instructions. Purified PCR products were quantified using Low Mass DNA Ladder (Applied Biosystems, EUA) as a reference, in a 2% agarose gel stained with GelRed™ (Biotium, EUA). Sequencing reaction was set up using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, EUA). Samples were precipitated by the isopropanol–ethanol method and denatured with formamide Hi-Di™ (Life Technologies, EUA) at 95 °C for 5 min. Capillary electrophoresis was held in ABI 3500 Genetic Analyzer (Applied Biosystems, EUA), and sequences were analyzed using Chromas 2.32 (Technelysium, Australia) and Mutation Surveyor (Softgenetics, EUA).

GSTM1 and *GSTT1* homozygous deletions were detected by multiplex PCR, and the exon 7 of *CYP1A1* was amplified as an internal control, as described elsewhere (Abdel-Rahman et al. 1996). By this technique, we were able to distinguish two *GSTM1* or *GSTT1* genotypes: (1) null genotype, in which both copies of the gene are deleted and (2) non-null genotype, in which one or both copies of the gene are present.

Statistical analysis

Demographic variables (age, sex and race/skin color) as well as allele and genotype frequencies were compared between cases and controls on goodness-of-fit tests by Pearson's chi-squared (χ^2) test to assess for differences in frequency distributions among categories. Hardy–Weinberg equilibrium (HWE) was calculated by χ^2 test with one degree of freedom for all genetic polymorphisms, except for *GSTM1* and *GSTT1* deletions, comparing observed with expected genotype frequencies. Crude odds ratios (OR) and skin color-adjusted OR ($_{adj}$OR) and their 95% confidence intervals (95% CI) for selected variables (i-AML subtypes and somatic genetic alterations) were assessed using unconditional logistic regression to estimate the magnitude of risk associations. For the three-genotype polymorphisms, dominant (A/B + B/B vs. A/A), recessive (B/B vs. A/A + A/B), and multiplicative (log-additive, allele B vs. allele A) models were considered in addition to the classic co-dominant model (A/B vs. A/A and B/B vs. A/A), except for *CYP2E1*, for which recessive model was not considered because of the very low frequency of homozygous variant genotype. Linear trend was tested by Mantel–Haenszel test (*P*-trend). Analyses were performed using the statistical package Statistical Product and Services Solutions (SPSS Inc., Chicago, IL, USA), version 18.0.

Additionally, based on maximum likelihood estimates, we have calculated the haplotypic frequencies of *EPHX1* polymorphisms (rs1051740 and rs2234922) in cases and controls, and tested for linkage disequilibrium (LD) between both alleles by the calculation of D' and r^2 values using the web platform SNPStats (available at http://bioin

fo.iconcologia.net/en/SNPStats_web, by the Institut Català d'Oncologia, ICO, Spain). *EPHX1* haplotypes were also tested for risk associations with i-AML by the calculation of OR and 95% CI. For all analyses, P values < 0.05 were considered as statistically significant.

Results

Study population characteristics

The demographics and frequency distributions of i-AML subtypes are shown in Table 1. i-AML cases were aged from 1 day to 23.9 months with a median age of 12.5 months at the diagnosis. Cases and controls did not differ in relation to sex distribution ($P = 0.647$) with predominance of males in both groups. Regarding race/skin color categories, cases and

Table 1 Demographic characteristics of controls and i-AML cases, and frequencies of AML subtypes and type I mutations, Brazil (2002–2014)

	Controls, *n* (%)	i-AML, *n* (%)	*P* value
Total	416 (100.0)	101 (100.0)	
Age (median 12.5, range 0–23.9 months)			
< 12 months	416 (100.0)[a]	46 (45.5)	
≥ 12 months	0 (0.0)	55 (54.5)	
Sex			
Male	237 (57.0)	55 (54.5)	0.647
Female	179 (43.0)	46 (45.5)	
Skin color			
Black	97 (23.3)	10 (9.9)	**0.002**
Non-black	297 (71.4)	88 (87.1)	
Unknown	22 (5.3)	3 (3.0)	
AML subtypes			
AML with recurrent genetic abnormalities			
AML with *KMT2A*–r	NA	35 (34.7)	
AML with *RUNX1–RUNX1T1*	NA	8 (7.9)	
AML with *CBFB–MYH11*	NA	3 (3.0)	
Acute promyelocytic leukemia, *PML–RARA*	NA	2 (2.0)	
AML (megakaryoblastic) with *RBM15–MKL1*	NA	1 (1.0)	
AML, NOS			
AML with minimal differentiation (M0)	NA	4 (4.0)	
AML without maturation (M1)	NA	4 (4.0)	
AML with maturation (M2)	NA	3 (3.0)	
Acute myelomonocytic leukemia (M4)	NA	12 (11.9)	
Acute monoblastic/monocytic leukemia (M5)	NA	12 (11.9)	
Acute erythroid leukemia (M6)	NA	3 (3.0)	
Acute megakaryoblastic leukemia (M7)	NA	14 (13.9)	
Type I mutations[b]			
KRAS (G12 or G13)	NA	6/99 (9.1)	
NRAS (G12 or G13)	NA	7/81 (8.6)	
KIT (exon 8 or 17)	NA	4/56 (7.1)	
PTPN11 (exon 3)	NA	4/61 (6.6)	
FLT3 (ITD or D835)	NA	3/95 (3.2)	

(i-)AML (infant) acute myeloid leukemia, *NOS* not otherwise specified, *ITD* internal tandem duplication, *NA* not applicable

Statistic significance value is in bold ($p < 0.05$)

[a]Control samples were collected at birth and the child was followed until 2 months of age

[b]Not all samples had information regarding type I mutations, and frequencies are shown as percentages of the total number of samples that were analyzed for each mutation

controls significantly differed within Blacks and non-Blacks subgroups ($P=0.002$), although both cases and controls were mostly categorized as non-Blacks. The most frequent AML subtype was AML with *KMT2A*–r (34.7%), followed by megakaryoblastic (AML-M7, 13.9%), and myelomonocytic/monoblastic subtypes (i-AML-M4/M5, 11.9% each). Other less frequent molecular subtypes observed were AML with *RUNX1–RUNX1T1* (7.9%), AML with *CBFB–MYH11* (3.0%), acute promyelocytic leukemia (2.0%) and AML with *RBM15–MKL1* (1.0%). Concerning additional somatic mutations (Type I mutations), 13 cases presented *RAS* mutations, 4 cases presented *KIT* and *PTPN11* mutations each, and 3 cases presented *FLT3* mutations.

Genotype frequencies of all genetic polymorphisms were in accordance with HWE among controls (*CYP2E1* rs3813867, $P=0.101$; *EPHX1* rs1051740, $P=0.467$; *EPHX1* rs2234922, $P=0.425$; *MPO* rs2333227, $P=0.095$; *NQO1* rs1800566, $P=0.342$; *GSTM1* and *GSTT1*, not calculated). Genetic polymorphisms were tested for independence of skin color/ethnicity by χ^2 test in the control group, and, with the exception of *NQO1* rs1800566, all polymorphisms were shown to be independent of skin color/ethnicity (*CYP2E1* rs3813867, $P=0.411$; *EPHX1* rs1051740, $P=0.592$; *EPHX1* rs2234922, $P=0.167$; *MPO* rs2333227, $P=0.224$; *NQO1* rs1800566, $P=0.028$; *GSTM1,* $P=0.099$; *GSTT1,* $P=0.305$).

There were no differences between cases and controls' minor allele frequencies of *CYP2E1* rs3813867 (0.10 and 0.07, $P=0.116$), *EPHX1* rs2234922 (0.19 and 0.21, $P=0.647$), *MPO* rs2333227 (0.25 and 0.28, $P=0.458$) and *NQO1* rs1800566 (0.22 and 0.27, $P=0.160$). In contrast, *EPHX1* rs1051740 minor allele was overrepresented in cases (C allele frequency, 0.31 in cases and 0.22 in controls, $P=0.007$). Regarding *GSTM1*, the null genotype occurred in 36.8% of cases and 40.3% of controls ($P=0.536$), while for *GSTT1*, the null genotype was present in 25.3% of cases and 22.7% of controls ($P=0.591$).

Risk associations between genetic polymorphisms and i-AML

In crude analysis, *EPHX1* rs1051740 was associated with i-AML risk under the co-dominant (CC vs. TT, OR 3.24, 95% CI 1.58–6.63, $P=0.001$), recessive (CC vs. TT + TC, OR 3.11, 95% CI 1.56–6.22, $P=0.001$), and multiplicative (OR 1.54, 95% CI 1.11–2.15, $P=0.011$) models (Supplementary Table 1). Adjusting for skin color, risk associations of *EPHX1* rs1051740 with i-AML remained statistically significant, under the co-dominant (CC vs. TT, $_{adj}$OR 3.04, 95% CI 1.47–6.32, $P=0.003$), recessive (CC vs. TT + TC, $_{adj}$OR 2.99, 95% CI 1.47–6.08, $P=0.002$), and multiplicative ($_{adj}$OR 1.46, 95% CI 1.04–2.06, $P=0.029$) models (Table 2). Indeed, there was a significant trend for increasing risk among *EPHX1* genotypes (P-trend = 0.010). Also, we observed that the frequency of *CYP2E1* rs3813867 variant genotype GC was increased in cases (18.0%) in relation to controls (11.6%), but risk associations did not reach statistical significance in crude (OR 1.68, 95% CI 0.93–3.03, $P=0.085$, Supplementary Table 1) or adjusted analysis ($_{adj}$OR 1.40, 95% CI 0.75–2.61, $P=0.294$, Table 2). For *NQO1* rs1800566, there was a borderline association of variant genotypes with i-AML risk in adjusted analysis (CT + TT vs. CC, $_{adj}$OR 0.64, 95% CI 0.40–1.02, $P=0.062$, Table 2).

In stratified analysis, we have considered the three most frequent i-AML subtypes that occurred in our cohort (*KMT2A*–r, M7, and M4/M5). We observed increased risk associations between *EPHX1* rs1051740 and i-AML *KMT2A*–r, and i-AML-M7 in both crude (Supplementary Table 1) and adjusted analyses (Table 3). Associations with *KMT2A*–r were significant considering the co-dominant model (CC vs. TT, OR 3.24 95% CI 1.09–9.57, $P=0.043$, and $_{adj}$OR 3.06, 95% CI 1.03–9.14, $P=0.045$). While for i-AML-M7, risk associations were significant considering co-dominant (CC vs. TT, OR 4.40, 95% CI 1.28–15.14, $P=0.031$, and $_{adj}$OR 4.18, 95% CI 1.21–14.51, $P=0.024$) and recessive (CC vs. TT + TC, OR 4.86, 95% CI 1.48–15.94, $P=0.020$, and $_{adj}$OR 5.10, 95% CI 1.53–16.98, $P=0.008$) models. No significant associations were found between any genetic polymorphism and risk for i-AML-M4/M5 (Supplementary Table 2).

We have also tested for risk associations between genetic polymorphisms and i-AML harboring type I mutations, i.e. *FLT3, KRAS, NRAS, KIT* and/or *PTPN11* mutations. We observed that *EPHX1* rs1051740 variant genotypes (TC/CC) were more frequent among cases (59.1%) than among controls (38.9%), and risk associations reached statistical significance under the multiplicative model in both crude (OR 1.95, 95% CI 1.04–3.64, $P=0.043$, Supplementary Table 1) and adjusted analysis ($_{adj}$OR 2.02, 95% CI 1.06–3.85, $P=0.037$, P-trend = 0.033, Table 3). Similarly, the frequency of *GSTT1*-null genotype was increased among cases (40.9%) than in controls (22.7%), showing borderline associations with i-AML with any type I mutation in crude (OR 2.36, 95% CI 0.98–5.70, $P=0.050$, Supplementary Table 1) and adjusted analysis ($_{adj}$OR 2.38, 95% CI 0.94–6.07, $P=0.068$, Table 3).

EPHX1 haplotypes

Using the SNPStats web tool, we have estimated haplotypes' frequencies and LD between the two SNPs of *EPHX1*, rs1051740 C>T and rs2234922 A>G, respectively (Supplementary Table 3). The T–A haplotype was estimated to be the most frequent (58.5%), followed by C–A (21.0%), T–G (17.5%), and C–G (2.9%) haplotypes. LD analysis showed that *EPHX1* alleles are not considered to be in LD

Table 2 Genotype frequencies of genetic polymorphisms in *CYP2E1, EPHX1, MPO, NQO1, GSTM1* and *GSTT1* in cases and controls and risk associations with i-AML, Brazil (2002–2014)

Polymorphism	Controls ($n=416$) *n* (%)	i-AML ($n=101$) *n* (%)	$_{adj}$OR[a] (95% CI)	*P* value	*P* for trend[b]
CYP2E1 rs3813867					
GG	362 (87.4)	81 (81.0)	1.00[c]		
GC	48 (11.6)	18 (18.0)	1.40 (0.75–2.61)	0.294	
CC	4 (1.0)	1 (1.0)	1.20 (0.12–11.73)	0.874	0.128
Dominant model			1.38 (0.75–2.54)	0.297	
Multiplicative model			1.32 (0.76–2.29)	0.340	
EPHX1 rs1051740					
TT	253 (61.1)	51 (52.6)	1.00[c]		
TC	138 (33.3)	31 (32.0)	1.02 (0.62–1.69)	0.929	
CC	23 (5.6)	15 (15.5)	**3.04 (1.47–6.32)**	**0.003**	**0.010**
Dominant model			1.31 (0.83–2.06)	0.253	
Recessive model			**2.99 (1.47–6.08)**	**0.002**	
Multiplicative model			**1.46 (1.04–2.06)**	**0.029**	
EPHX1 rs2234922					
AA	258 (62.3)	63 (63.6)	1.00[c]		
AG	141 (34.1)	34 (34.3)	1.07 (0.66–1.74)	0.771	
GG	15 (3.6)	2 (2.0)	0.55 (0.12–2.50)	0.440	0.638
Dominant model			1.02 (0.64–1.63)	0.934	
Recessive model			0.54 (0.12–2.43)	0.421	
Multiplicative model			0.96 (0.64–1.45)	0.840	
MPO rs2333227					
GG	206 (50.9)	46 (56.8)	1.00[c]		
GA	175 (43.2)	30 (37.0)	0.81 (0.49–1.35)	0.415	
AA	24 (5.9)	5 (6.2)	1.02 (0.36–2.88)	0.968	0.442
Dominant model			0.84 (0.51–1.36)	0.472	
Recessive model			1.12 (0.41–3.07)	0.833	
Multiplicative model			0.90 (0.60–1.35)	0.610	
NQO1 rs1800566					
CC	227 (54.8)	61 (61.6)	1.00[c]		
CT	154 (37.2)	33 (33.3)	0.67 (0.41–1.09)	0.109	
TT	33 (8.0)	5 (5.1)	0.51 (0.19–1.37)	0.180	0.169
Dominant model			0.64 (0.40–1.02)	0.062	
Recessive model			0.58 (0.22–1.54)	0.271	
Multiplicative model			0.69 (0.47–1.01)	0.048	
GSTM1					
Non-null	237 (59.7)	60 (63.2)	1.00[c]		
Null	160 (40.3)	35 (36.8)	0.84 (0.52–1.35)	0.469	
GSTT1					
Non-null	307 (77.3)	71 (74.7)	1.00[c]		
Null	90 (22.7)	24 (25.3)	1.17 (0.69–2.00)	0.564	

i-AML infant acute myeloid leukemia, $_{adj}$*OR* adjusted odds ratio, *95% CI* 95% confidence interval

Statistic significance values are in bold ($p < 0.05$)

[a]Adjusted by skin color

[b]Mantel–Haenszel test for linear trend across the three genotypes

[c]Reference genotype

(D' 0.4036, r^2 0.0131, $P=0.0002$). Then, we tested for the associations between *EPHX1* haplotypes and i-AML risk, and observed that the C–G haplotype was associated with an increased risk for i-AML overall, although with a lower OR than what was observed for SNP rs1051740 alone (C–G vs. T–A, OR 2.55, 95% CI 1.03–6.28, $P=0.043$), as well

Table 3 Risk associations between *CYP2E1, EPHX1, MPO, NQO1, GSTM1* and *GSTT1* polymorphisms and i-AML subgroups, Brazil (2002–2014)

Polymorphism	Controls ($n=416$) n (%)	i-AML, *KMT2A*–r ($n=35$)				i-AML, megakaryoblastic[a] ($n=20$)				i-AML with type I mutations[b] ($n=24$)			
		n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]	n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]	n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]
CYP2E1 rs3813867													
GG	362 (87.4)	28 (80.0)	1.00[e]			15 (78.9)	1.00[e]			21 (87.5)	1.00[e]		
GC	48 (11.6)	6 (17.1)	1.58 (0.62–4.06)	0.338		4 (21.1)	1.56 (0.43–5.66)	0.497		3 (12.5)	0.71 (0.16–3.15)	0.651	
CC	4 (1.0)	1 (2.9)	3.71 (0.37–37.12)	0.265	0.164	0 (0.0)	-	0.999	0.388	0 (0.0)	-	0.999	0.894
Dominant model			1.72 (0.71–4.18)	0.231			1.46 (0.40–5.29)	0.563			0.66 (0.15–2.92)	0.584	
Multiplicative model			1.73 (0.81–3.71)	0.180			1.31 (0.40–4.28)	0.670			0.64 (0.16–2.66)	0.510	
EPHX1 rs1051740													
TT	253 (61.1)	17 (50.0)	1.00[e]			10 (55.6)	1.00[e]			9 (40.9)	1.00[e]		
TC	138 (33.3)	12 (35.3)	1.23 (0.57–2.65)	0.605		4 (22.2)	0.53 (0.14–1.98)	0.348		10 (45.5)	2.16 (0.83–5.63)	0.113	
CC	23 (5.6)	5 (14.7)	**3.06 (1.03–9.14)**	**0.045**	0.063	4 (22.2)	**4.18 (1.21–14.51)**	**0.024**	0.131	3 (13.6)	3.89 (0.96–15.87)	0.058	**0.033**
Dominant model			1.48 (0.73–3.00)	0.275			1.06 (0.39–2.84)	0.913			2.41 (0.97–5.96)	0.058	
Recessive model			2.82 (0.99–8.05)	0.052			**5.10 (1.53–16.98)**	**0.008**			2.74 (0.74–10.08)	0.130	
Multiplicative model			1.63 (0.96–2.77)	0.078			1.58 (0.77–3.23)	0.230			**2.02 (1.06–3.85)**	**0.037**	
EPHX1 rs2234922													
AA	258 (62.3)	26 (74.3)	1.00[e]			10 (52.6)	1.00[e]			19 (79.2)	1.00[e]		
AG	141 (34.1)	8 (22.9)	0.63 (0.27–1.43)	0.267		8 (42.1)	1.49 (0.54–4.11)	0.440		5 (20.8)	0.57 (0.21–1.63)	0.306	
GG	15 (3.6)	1 (2.9)	0.67 (0.08–5.26)	0.700	0.196	1 (5.3)	1.83 (0.22–15.42)	0.579	0.392	0 (0.0)	-	0.999	0.080
Dominant model			0.63 (0.29–1.40)	0.255			1.54 (0.58–4.08)	0.390			0.52 (0.19–1.46)	0.216	
Recessive model			0.77 (0.10–6.05)	0.804			1.58 (0.20–12.73)	0.668			-	0.999	
Multiplicative model			0.72 (0.36–1.45)	0.340			1.43 (0.65–3.16)	0.390			0.51 (0.20–1.34)	0.140	
MPO rs2333227													
GG	206 (50.9)	20 (66.7)	1.00[e]			6 (42.9)	1.00[e]			10 (58.8)	1.00[e]		
GA	175 (43.2)	9 (30.0)	0.56 (0.25–1.27)	0.165		7 (50.0)	1.42 (0.47–4.33)	0.534		5 (29.4)	0.62 (0.21–1.87)	0.399	

Table 3 (continued)

Polymorphism	Controls ($n=416$) n (%)	i-AML, *KMT2A*–r ($n=35$)				i-AML, megakaryoblastic[a] ($n=20$)				i-AML with type I mutations[b] ($n=24$)			
		n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]	n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]	n (%)	$_{adj}$OR[c] (95% CI)	P value	P for trend[d]
AA	24 (5.9)	1 (3.3)	0.49 (0.06–3.90)	0.502	0.107	1 (7.1)	1.52 (0.17–13.36)	0.705	0.576	2 (11.8)	1.91 (0.39–9.39)	0.427	0.888
Dominant model			0.55 (0.25–1.21)	0.139			1.44 (0.49–4.25)	0.506			0.78 (0.29–2.09)	0.617	
Recessive model			0.59 (0.08–4.57)	0.614			1.29 (0.16–10.32)	0.813			2.34 (0.50–11.06)	0.282	
Multiplicative model			0.56 (0.27–1.16)	0.100			1.32 (0.56–3.10)	0.520			1.00 (0.45–2.26)	0.990	
NQO1 rs1800566													
CC	227 (54.8)	20 (58.8)	1.00[e]			11 (57.9)	1.00[e]			13 (56.5)	1.00[e]		
CT	154 (37.2)	12 (35.3)	0.72 (0.33–1.55)	0.396		8 (42.1)	0.97 (0.36–2.63)	0.956		8 (34.8)	0.59 (0.22–1.61)	0.304	
TT	33 (8.0)	2 (5.9)	0.63 (0.14–2.85)	0.550	0.593	0 (0.0)	-	0.998	0.459	2 (8.7)	0.94 (0.20–4.40)	0.936	0.944
Dominant model			0.71 (0.34–1.47)	0.350			0.81 (0.30–2.18)	0.673			0.66 (0.27–1.64)	0.373	
Recessive model			0.69 (0.16–3.02)	0.621			-	0.998			1.11 (0.25–5.00)	0.896	
Multiplicative model			0.77 (0.43–1.40)	0.390			0.70 (0.30–1.62)	0.380			0.79 (0.39–1.63)	0.520	
GSTM1													
Non-null	237 (59.7)	23 (74.2)	1.00[e]			12 (66.7)	1.00[e]			14 (63.6)	1.00[e]		
Null	160 (40.3)	8 (25.8)	0.51 (0.22–1.17)	0.111		6 (33.3)	0.79 (0.28–2.18)	0.646		8 (36.4)	0.91 (0.36–2.30)	0.846	
GSTT1													
Non-null	307 (77.3)	26 (83.9)	1.00[e]			13 (72.2)	1.00[e]			13 (59.1)	1.00[e]		
Null	90 (22.7)	5 (16.1)	0.70 (0.26–1.90)	0.489		5 (27.8)	1.46 (0.50–4.26)	0.494		9 (40.9)	2.38 (0.94–6.07)	0.068	

i-AML infant acute myeloid leukemia, *$_{adj}$OR* adjusted odds ratio, *95% CI* 95% confidence interval

Statistic significance values are in bold ($p < 0.05$)

[a]All megakaryoblastic cases are grouped together, including those with *KMT2A*–r and *RBM15–MKL1* ($n = 5$)

[b]*KRAS, NRAS, KIT, PTPN11* or *FLT3* mutations

[c]Adjusted by skin color

[d]Mantel–Haenszel test for linear trend across the three genotypes

[e]Reference genotype

as for i-AML *KMT2A*–r, with comparable OR value as for rs1051740 alone (C–G vs. T–A, OR 3.23, 95% CI 1.10–9.51, $P = 0.034$) (Table 4).

Discussion

In this study, we have selected genetic polymorphisms involved in benzene metabolism, *CYP2E1* (rs3813867), *EPHX1* (rs1051740, rs2234922), *MPO* (rs2333227), *NQO1* (rs1800566), *GSTM1* and *GSTT1* (null genotypes), to test for risk associations with i-AML in Brazil. The rationale was based on previous studies that demonstrated strong risk associations between maternal exposures during pregnancy and infant leukemia (Andrade et al. 2014; Ferreira et al. 2012, 2013). Here, we reported that *EPHX1* rs1051740 was associated with an increased risk for i-AML overall, and mainly for i-AML with *KMT2A*–r, which accounts for a high proportion of i-AMLs. Interestingly, risk associations were also found for i-AML with type I mutation and for acute megakaryoblastic leukemia, which represented the second most frequent AML subtype among our cohort.

The *EPHX1* gene encodes for microsomal epoxide hydrolase, which neutralizes epoxides originated from oxidative metabolism of aromatic compounds, including environmental pollutants such as benzene and polycyclic aromatic hydrocarbons resulted from incomplete combustion of organic materials, e.g. tobacco smoke (Decker et al. 2009). Microsomal epoxide hydrolase catalyzes the conversion of epoxides, which are strong electrophiles that readily react with nucleophilic portions of nucleic acids and proteins, into less-reactive vicinal diols, protecting cells from epoxide's carcinogenic effects (Decker et al. 2009). Two common non-synonymous genetic polymorphisms of *EPHX1*, rs1051740 T>C and rs2234922 A>G, have been associated with cancer susceptibility, e.g. lung, prostate and colorectal cancer (Kiyohara et al. 2006; Mittal and Srivastava 2007; Ulrich et al. 2001).

EPHX1 rs1051740 consists of a T>C substitution in exon 3 that results in a change from tyrosine to histidine amino acid residue at position 113 (Tyr113His) of epoxide hydrolase protein, while the *EPHX1* rs2234922 consists of an A>G substitution within exon 4, changing histidine to arginine residue at position 139 (His139Arg) (Hassett et al. 1994). An in vitro study showed that the 113His isoform has 39% reduced enzymatic activity, while the 139Arg isoform presents 125% increased enzymatic activity, both compared to the wild-type construct 113Tyr–139His (Hassett et al. 1994). Thus, the associations found between rs1051740 and i-AML risk overall and among molecular subgroups are justified by the effect of T allele (113His) on reducing cell's ability to neutralize epoxides, becoming more susceptible to damage.

Table 4 Haplotype frequencies of *EPHX1* and risk associations with i-AML, Brazil (2002–2014)

EPHX1 haplotypes[b]	Controls ($n = 414$)	i-AML ($n = 99$)			i-AML, *KMT2A*–r ($n = 34$)			i-AML, megakaryoblastic[a] ($n = 19$)		
	n (%)	*n* (%)	OR (95% CI)	*P* value	*n* (%)	OR (95% CI)	*P* value	*n* (%)	OR (95% CI)	*P* value
T–A	245 (59.3)	54 (55.0)	1.00[c]		20 (60.2)	1.00[c]		9 (46.3)	1.00[c]	
C–A	83 (20.1)	26 (25.8)	1.32 (0.89–1.97)	0.170	9 (25.2)	1.16 (0.63–2.17)	0.630	5 (27.4)	1.68 (0.73–3.89)	0.230
T–G	77 (18.5)	13 (13.5)	0.79 (0.47–1.32)	0.370	2 (6.6)	0.34 (0.12–1.00)	0.051	4 (20.2)	1.43 (0.57–3.62)	0.450
C–G	9 (2.1)	6 (5.7)	**2.55 (1.03–6.28)**	**0.043**	3 (8.1)	**3.23 (1.10–9.51)**	**0.034**	1 (6.2)	3.59 (0.74–17.40)	0.110

i-AML infant acute myeloid leukemia, *OR* crude odds ratio, *CI* confidence interval

Statistic significance values are in bold ($p < 0.05$)

[a]All megakaryoblastic cases are grouped together, including those with *KMT2A*–r and *RBM15-MKL1* ($n = 5$)

[b]rs1051740 and rs2234922, respectively

[c]Reference genotype

This result suggests that microsomal epoxide hydrolase is quite important in driving epoxide detoxification pathways in infants and even in fetuses. In corroboration, Omiecinski et al. (1994) showed that fetal liver already expresses this enzyme since the first trimester of gestation (Omiecinski et al. 1994). Additionally, using a murine model, Kim (1995) showed that maternal hepatic microsomal epoxide hydrolase levels decreased during gestation, reaching 20% of normal adult levels at the end of gestational period (Kim 1995), a finding that highlights the importance of fetal microsomal epoxide hydrolase for protecting the fetuses from epoxide effects resulted from in utero exposures. Also, O'Shaughnessy et al. (2011) showed that fetuses' microsomal epoxide hydrolase can be induced by maternal smoking exposure (O'Shaughnessy et al. 2011). These findings would corroborate with the evidence that modulation of toxic effects of environmental exposures by genetic polymorphisms influences in utero initiation of infant acute leukemia.

Specifically regarding the risk associations between *EPHX1* rs1051740 and i-AML with *KMT2A*–r, it is important to highlight that this subtype comprehends a high proportion of cases, and thus, could respond for the risk associations found for i-AML overall. Benzene metabolites, mainly 1,4-benzoquinone, are able to inhibit topo-II activity, which stimulates the occurrence of DNA-strand breaks and potentially induces *KMT2A*–r (Lindsey et al. 2005). Indeed, several chromosomal abnormalities were observed in humans exposed to benzene, including structural and numerical alterations (Zhang et al. 2002). In this context, the *EPHX1* rs1051740 polymorphism could influence susceptibility to the occurrence of genetic abnormalities once it could favor the accumulation of benzene oxide in BM cells, increasing the generation of oxygen-reactive species, and possibly favoring the production of 1,4-benzoquinone, which is the main reactive metabolite responsible for benzene hematotoxicity (Smith 2010). Thus, the lower activity of epoxide hydrolase resulted from rs1051740 could contribute to a higher production of topo-II inhibitors in the BM. To date, *EPHX1* polymorphisms were underexplored regarding childhood AML susceptibility. Only two case–control studies conducted in France, one of them included 51 AMLs and 549 controls (2003–2004), and the other included 28 non-lymphoblastic acute leukemia cases and 105 controls (1995–1999), investigated risk associations of rs1051740 and rs2234922 with AML risk in children up to 15 years old, but no significant associations were found (Bonaventure et al. 2012; Clavel et al. 2005).

For all other genetic polymorphisms, no significant associations were observed. Few studies have addressed the role of *CYP2E1* rs3813867 in childhood AML risk, and the results were controversial (Aydin-Sayitoglu et al. 2006; Bolufer et al. 2007; Bonaventure et al. 2012). For *NQO1* rs1800566, the lack of association was corroborated with previous studies (Bonaventure et al. 2012; Eguchi-Ishimae et al. 2005; Gra et al. 2008; Sirma et al. 2004), with the exception of one study that showed a protective association with i-AML ≤ 12 months (de Aguiar Goncalves et al. 2012). For *GSTM1* and *GSTT1* deletions, previous studies have shown controversial results (Aydin-Sayitoglu et al. 2006; Balta et al. 2003; Clavel et al. 2005; Davies et al. 2000; Gra et al. 2008; Lopes et al. 2015).

This study has limitations concerning the statistical power, though i-AML is an extremely rare disease and it can be arduous to gather such a wide cohort. Also, risk associations found in the subgroup analysis, mainly for i-AML-M7 and i-AML with type I mutations, should be interpreted carefully, since the number of cases is very small, and we cannot rule out that results could be due to chance. The study lacked environmental exposure data that could be included in the study model to estimate the effects of gene–environment interactions. However, it was the first study that addressed the role of genetic polymorphisms in i-AML risk spotting the distinct subtypes, taking into account the molecular and morphological heterogeneity of AML. We gathered a cohort of i-AML cases, which were extensively characterized by recurrent genetic abnormalities. Herein, we have shown that *EPHX1* rs1051740 was associated with i-AML risk overall and within i-AML subgroups (*KMT2A*–r, M7, and with type I mutations). For a comprehensive understanding of i-AML's etiology, well-designed experimental toxicological models as well as risk assessments based on adverse outcome pathways (Pelkonen et al. 2017) would reinforce the contribution of genetic polymorphisms in i-AML susceptibility. We are aware that the results generated in this study are a starting point for further collaborations aiming to reinforce statistically significant results through the aggregation of more cases to this early-age and well-characterized AML cohort as recommended by WHO.

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Author contributions GDB wrote the manuscript. GDB, BAL and BAAG performed genotyping assays of cases and controls, and contributed to data collection and DNA samples preparation of controls. BAL contributed to experimental procedures set ups and statistical analyses. FGA, FVSB, ISC and GDB worked on the molecular characterization of cases. ETG worked on cases' diagnosis. FHPB provided controls samples. MSPO contributed to the conception and critical analysis of the study and the manuscript.

Compliance with ethical standards

Ethical standards The study was performed in accordance with the 1964 Declaration of Helsinki and its later amendments. The Ethics and Scientific Committee of Instituto Nacional de Câncer and all collaborating Brazilian institutions have approved the study (CEP/CONEP #186.688; CEP/CONEP #626.268).

Conflict of interest The authors declare that they have no conflict of interest.

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