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Differential allelic expression in leucoblast from patients with acute myeloid leukemia suggests genetic regulation of *CDA, DCK, NT5C2, NT5C3* and *TP53*

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Running title: Differential allelic expression in AML

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Abbreviations: AML: Acute Myeloid Leukemia; DAE: Differential Allelic Expression; rSNP: regulatory SNP; cSNP: exonic SNP

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ABSTRACT

mRNA expression levels of certain genes have shown predictive value for the outcome of cytarabine-treated AML-patients. We hypothesized that genetic variants play a role in the regulation of the transcription of these genes. We studied leucoblasts from 82 AML patients and observed various extent and frequency of differential allelic expression in the *CDA*, *DCK*, *NT5C2*, *NT5C3* and *TP53* genes. Our attempts to identify the causative regulatory SNPs by a bioinformatics approach did not succeed. However, our results indicate that genetic variations are at least in part responsible for the differences in overall expression levels of these genes.

INTRODUCTION

The deoxynucleoside analogue cytarabine (1-β-D-arabinofuranosylcytosine, araC) is a major component of the chemotherapeutic treatment of patients with acute myeloid leukemia (AML). The most important limitation for the use of deoxynucleoside analogues in the clinic is the presence of primary or acquired resistance. Several studies have identified clinically relevant mechanisms of resistance in patients with leukemia or other malignant diseases (Jordheim and Dumontet, 2007). In particular, mRNA expression level of several genes has been correlated to the outcome of the treatment with cytarabine or gemcitabine (2'-2'-difluorodeoxycytidine, dFdC), another analogue of deoxycytidine. In all these studies, large variations in the expression levels of genes involved in cytarabine metabolism have been observed between patients, suggesting the presence of important regulatory mechanisms. In addition to differences in levels and activities of transcription factors and stability of mRNA, variations in the genomic sequence of the gene and its regulatory elements can influence the mRNA level. In fact, at least 25-35% of interindividual differences in gene expression are supposed to be due to *cis*-acting variations (Pastinen and Hudson, 2004).

When a heterozygous genetic variation induces a difference in mRNA expression level, the two corresponding alleles are expressed at different levels. This is called differential allelic expression (DAE) or allelic expression imbalance (Pastinen and Hudson, 2004). Currently, DAE is studied in samples heterozygous for an exonic variation (cSNP) used as a marker to determine the relative amount of transcripts from the two alleles. This method allows the distinction between *cis*- and *trans*-acting effects since the cellular environment and mRNA extraction are exactly the same (Stamatoyannopoulos, 2004). The cSNP used for the assessment of DAE is not necessarily responsible for the allelic expression imbalance, and further investigations are needed to identify the functional regulatory variant (rSNP) or the underlying epigenetic modification (Milani et al., 2007).

Specific mRNA expression levels can be used to predict the outcome of cancer patients treated with chemotherapy. Since genetic variants are partially responsible for variations in gene expression, these could potentially be used as more precise markers for this prediction (Stamatoyannopoulos, 2004; Abraham et al., 2006). For AML, the use of reliable predictive markers would substantially increase the treatment success rate and the overall management of the cancer patients. Our main goal

in this study was to investigate whether interindividual variations in mRNA expression levels of genes involved in the cellular response to cytarabine could, at least in part, be due to genetic polymorphisms. As a secondary goal, we tried to identify rSNPs responsible for these differences. We focused on genes with a key role in the metabolism and mechanism of action of cytarabine and with strong evidence of clinical relevance. We chose the genes for the equilibrative nucleoside transporter 1, *SLC29A1*, deoxycytidine kinase, *DCK*, cytidine deaminase, *CDA*, cytosolic 5'-nucleotidases II and III, *NT5C2* and *NT5C3*, and the tumor suppressor gene *TP53*. We used a method based on high resolution melting curve analysis to assess the DAE of these genes, determined their relative expression level and tried to identify causative rSNPs in leucoblasts from 82 patients with AML.

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METHODS

Biological samples were obtained from 82 patients with AML at diagnosis before initiation of therapy, all followed in the Hematology Department of Edouard Herriot Hospital in Lyon, France. Approval was obtained from Lyon Protocol Review Board and written informed consent was provided according to the Declaration of Helsinki. Mononuclear cells including leukemic cells were isolated by Ficoll-Hypaque sedimentation from peripheral blood (n=33) and bone marrow (n=49). Median percentages of blast cells in peripheral blood and in bone marrow were 61% (range: 13%-99%) and 69% (range: 20%-95%) respectively.

Total RNA and genomic DNA were extracted with TRIzol® Reagent (Invitrogen, Cergy Pontoise, France) and cDNA synthesis was performed with 1 µg total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and oligo(dT) primers.

SNPs were genotyped by high resolution melting curve analysis of PCR products in presence of LCGreen® Plus+ (Idaho Technologies, Utah, USA) (Reed and Wittwer, 2004) or a fluorescent probe (Crockett and Wittwer, 2001), using a LightScanner® Instrument (Idaho Technologies). All PCR primers and cycling conditions are described in supplemental tables.

DAE was assessed with the same technique as the genotyping using cDNA and genomic DNA based on a previously described method (Ware et al., 2006). All samples were amplified 5 times. Melting curves were analyzed with the HR-1™ Instrument Analysis Software (Idaho Technologies), peak heights were measured manually and the ratio of the two peaks corresponding to the two alleles was calculated for cDNA and genomic DNA. Allelic expression imbalance of each sample was calculated as the ratio (mean ratio for cDNA) / (mean ratio for gDNA). A sample was considered to have DAE if this ratio was <0.8 or >1.2 and if the difference between the mean ratios was statistically significant at $p<0.01$ as calculated with Student's *t*-test. The linearity of the method for the assessment of DAE was validated using mix of genomic DNA from common and rare homozygous samples containing 20-80% of each allele. R^2 values were between 0.9918 and 0.9979 for *CDA, NT5C2, NT5C3* and *TP53*. For *NT5C3*, PCR products of PCR1 were digested with *Pvu*II to avoid interference with mRNA from pseudogene *NT5C3P1* as described earlier (Marinaki et al., 2001).

SNPs situated in putative transcription factor binding sites, or rSNPs, were identified in the region situated upstream the start codon of genes of interest using the public databases RAVEN (http://www.cisreg.ca/cgi-bin/RAVEN/a), CONSITE (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Their evolutionary conservation was verified by comparative sequence analysis with mouse, dog and cow using seqcom comparisons in FamilyRelations II (http://family.caltech.edu/) with a windowsize of 20 bp and threshold of 0.8. Putative rSNPs for *DCK* and *SLC29A1* were selected from the literature.

mRNA expression levels were determined by relative quantitative RT-PCR on an ABI PRISM 7900 sequence detection system (Applied Biosystems, France) using *GAPDH* as internal standard and the comparative C_T method as described in the user's guide. TaqMan® Gene Expression Assays were Hs00156401_m1 (CDA), Hs00176127_m1 (DCK), Hs00366992_m1 (NT5C2), Hs00826433_m1 (NT5C3), Hs00191940_m1 (SLC29A1) and Hs00153349_m1 (TP53).

Student's t-test was used for statistical analysis of DAE (differences in mean of ratios between cDNA and gDNA) and of the association between genotype and mRNA expression (differences in mean mRNA expression between genotype groups).

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RESULTS AND DISCUSSION

The DAE protocol can only be applied to samples that are heterozygous for a marker cSNP in the target gene. Genotyping *CDA*, *DCK*, *NT5C2*, *NT5C3* and *TP53* in our series of 82 patients revealed that 13 to 41 (15.9-51.3%) were heterozygous for selected cSNPs with theoretically high frequency, and thereby suitable for the DAE experiments (Table 1). For *NT5C2*, two highly frequent cSNPs were genotyped, but only rs3740387 in exon 18 was retained for DAE-assessment. *SLC29A1* was excluded from the analysis because only one heterozygote for the cSNP was found. This is consistent with the reported low frequencies of cSNPs in *SLC29A1* in Europeans (Osato et al., 2003). Statistically significant DAE was observed in 57.7%, 50.0%, 8.7%, 38.5% and 16.7% of positive samples for *CDA*, *DCK*, *NT5C2*, *NT5C3* and *TP53* respectively (Fig. 1 and table 1). The extent of imbalanced allelic expression varied from 20% up to mono-allelic expression of *NT5C3* in four samples. The percentage of DAE positive samples for each gene was not different between samples from peripheral blood and bone marrow. DAE has been reported to be tissue-dependent (Wilkins et al., 2007), but here we studied the same cells (leucoblasts) in two different environments (peripheral blood and bone marrow). Detection of DAE provides strong evidence that *cis*-genetic variation is involved in the determination of the expression level of these genes. We observed DAE in leucoblasts which are the target cells for the cytarabine-based treatment of AML. *Cis*-regulation of these genes could therefore have a direct impact on the efficiency of the chemotherapeutic drug used for treatment of AML. Since DAE was assessed in samples heterozygous for a marker cSNP only and the linkage disequilibrium with the causative SNP is unknown, it is difficult to estimate the rate of DAE in the whole population of 82 AML patients. The observed DAE could be due to genetic or epigenetic variants in transcription factor binding sites or by non-sense mediated mRNA decay. We did not have biological material to study NMD, but continued our research on putative rSNPs.

Eighteen putative rSNPs in the 5'-region of our genes of interest were identified by bioinformatics tools or selected from the literature (Shi et al., 2004; Fitzgerald et al., 2006; Gilbert et al., 2006; Joerger et al., 2006; Myers et al., 2006; Sugiyama et al., 2007). As compared to sequencing of large upstream regions of genes, this method allows screening thousands of kb *in silico* to make a selection of potential rSNPs in either proximal promoters or more distant candidate enhancers

(Wasserman and Sandelin, 2004). The different databases identified various SNPs situated in potential transcription factor binding sites within the regulatory elements and for which the two alleles potentially did not have the same affinity for transcription factors (data not shown). Sequence conservation through mouse, dog and cow was more or less constant. After genotyping of these SNPs, their comparison with the DAE status did not show any correlation (Table 1). This result might be explained by the limited power of the study of some SNPs (few heterozygote samples) or reflect the fact that the studied SNPs does not intervene in the regulation of the expression of these genes. Functional rSNPs in our genes of interest can be SNPs we did not chose to genotype, be situated in sequences not reported on the public available databases or be situated elsewhere than upstream the start codon. In addition, the analysis might have been complicated by the presence of several rSNPs in the same gene.

Median values (and ranges) for relative mRNA expression in leucoblasts from 67 patients were 8.1 (0-215.3) for *CDA*, 12.6 (2.5-162.6) for *DCK*, 8.0 (0-88.7) for *NT5C2*, 0.8 (0.1-6.0) for *NT5C3*, 1.0 (0-18.2) for *SLC29A1* (n=65), and 1.0 (0-6.6) for *TP53*. This result confirmed our previous publications reporting large inter-individual variations in gene expression between AML-patients (reviewed in (Jordheim and Dumontet, 2007)). If the role of the functional rSNP is important compared to other regulating parameters, genotypes should be correlated to the mRNA expression of the regulated gene, with heterozygous samples between the two groups of homozygote. Comparison between the genotype groups of cSNPs and rSNPs did not show statistically significant differences (Table 1). However, trends were seen for rs2072671 in *CDA* (p=0.07 for comparison between heterozygote (19.16, n=36) and rare homozygous (5.75, n=11) samples) and for rs12261294 in *NT5C2* (p=0.10 for comparison between the pool of common homozygous and heterozygote (13.23, n=56) samples and the rare homozygous (4.96, n=6) samples). We did not observe higher expression for *SLC29A1* in samples with C-alleles for rs747199 as previously reported (Myers et al., 2006). Lower promoter activity has been shown for T-alleles of rs532545 in *CDA* which is consistent with our mRNA data in TT-samples for this variant (Fitzgerald et al., 2006; Gilbert et al., 2006). When subgroups of samples were compared for their correlation between genotypes and mRNA expression, statistically significant differences were observed in some cases. This was the case for example for

rs4316067 in *NT5C3* (p=0.005 for comparison between heterozygote (n=11) and rare homozygous (n=4) samples from peripheral blood only) and rs1042522 in *TP53* (p=0.001 for comparison between common homozygous (n=22) and heterozygotes (n=14) samples from blood marrow only). Comparison of gene expression levels between samples with or without DAE showed no differences (data not shown), thus eliminating low expression of the target gene as a bias of DAE-assessment (Pastinen and Hudson, 2004).

This work provides proof that genes involved in the cellular response to cytarabine are subject to genetic or epigenetic regulation in leukemic blasts. The fact that a patient shows differential allelic expression in a cytarabine-related gene would not impact on the response to the treatment. However, this clearly indicates that interindividual differences in gene expression with predictive power in cohorts of AML patients treated with cytarabine can at least partially be explained by genetic variations. In addition to providing an explanation to previous data available in this field, our results strongly encourage the search of causative variants for the differences in expression levels.

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FOOTNOTES

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Figure legend

Figure 1. Allelic-specific expression of CDA (A), DCK (B), NT5C2 (C), NT5C3 (D) and TP53 (E) in leucoblasts from AML patients. All samples heterozygous for cSNPs in *CDA (*rs2072671*), DCK (*rs11544786*), NT5C2 (*rs10883841*), NT5C3 (*rs3750117*)* and *TP53 (*rs1042522*)* were assessed for DAE (5 replicates) and statistical significance was calculated using Student's *t*-test for comparison of the mean of the ratios of cDNA versus genomic DNA as explained in the Methods section. The Y-axis shows the value of the ratio of mean cDNA ratios over mean gDNA ratios. Samples without DAE have a ratio of 1. Horizontal bars indicate the lower (0.8) and upper (1.2) limits of the ±20% zone. X-axis shows sample IDs. *: Samples with ratio lower than 0.8 or higher than 1.2 and with $p<0.01$ using Student's *t*-test; **: Samples with monoallelic expression of NT5C3; BM: Samples from blood marrow; PB: Samples from peripheral blood.

Table 1. Genotype, DAE and quantitative RT-PCR data for all studied cSNPs and rSNPs.

Two to six SNPs were genotyped in each gene and correlated to mRNA expression level as determined by quantitative RT-PCR and the DAE status.

Gene	SNP information				Genotyping results				Quantitative RT-PCR results			DAE results				
	SNP ID	cSNP / rSNP	Nucleotide position[a]	Nucleotide change	HH[b] (%)	Hh[b] (%)	hh[b] (%)	Neg	mRNA HH[c] (n)	mRNA Hh[c] (n)	mRNA hh[c] (n)	Hh DAE+[d]	Hh DAE-[d]	HH / hh DAE+[d]	HH / hh DAE-[d]	Neg genotype DAE+ or DAE-[d]
CDA	rs2072671	cSNP	+79	A>C	28 (35.00)	41 (51.25)	11 (13.75)	2	19.33 ± 21.68 (19)	19.16 ± 7.67 (36)	5.75 ± 3.14 (11)	15	11	-	-	-
CDA	rs6690069	rSNP	-1172	G>A	62 (86.11)	7 (9.72)	3 (4.71)	10	17.54 ± 9.11 (51)	35.46 ± 40.15 (4)	6.95 ± 8.08 (3)	1	2	12	8	3
CDA	rs10916823	rSNP	-897	C>A	71 (100.00)	0 (0.00)	0 (0.00)	11	18.23 ± 8.47 (58)	-	-	0	0	13	10	3
CDA	rs532545	rSNP	-451	C>T	23 (32.86)	35 (50.00)	12 (17.14)	12	21.25 ± 25.61 (16)	20.28 ± 9.42 (29)	8.38 ± 3.92 (12)	12	8	1	2	3
CDA	rs12095662	rSNP	-378	T>C	71 (100.00)	0 (0.00)	0 (0.00)	11	18.23 ± 8.47 (58)	-	-	0	0	13	10	3
CDA	rs602950	rSNP	-92	A>G	25 (35.21)	33 (46.48)	13 (18.31)	11	21.67 ± 24.07 (17)	19.64 ± 9.86 (28)	8.32 ± 3.96 (12)	12	7	1	2	4
DCK	rs11544786	cSNP	+28624	C>T	69 (85.15)	13 (15.85)	0 (0.00)	0	19.18 ± 6.02 (56)	21.58 ± 8.14 (11)	-	5	5	-	-	-
DCK	SNP-360	rSNP	-360	C>G	79 (97.53)	2 (2.47)	0 (0.00)	1	19.15 ± 5.35 (64)	30.54 ± 34.90 (2)	-	0	0	5	5	0
DCK	SNP-243	rSNP	-243	G>T	81 (100.00)	0 (0.00)	0 (0.00)	1	19.49 ± 5.27 (66)	-	-	0	0	5	5	0
DCK	rs2306744	rSNP	-201	C>T	80 (100.00)	0 (0.00)	0 (0.00)	2	19.74 ± 5.32 (65)	-	-	0	0	5	5	0
NT5C2	rs10883841	cSNP	+7	T>C	61 (79.22)	16 (20.78)	0 (0.00)	5	15.71 ± 4.56 (49)	9.40 ± 4.27 (15)	-	0	7	2	13	1
NT5C2	rs3740387	cSNP	+85248	C>T	30 (38.46)	27 (34.62)	21 (26.92)	4	12.16 ± 4.56 (26)	15.34 ± 7.53 (22)	14.32 ± 6.66 (19)	2	21	-	-	-
NT5C2	rs12781668	rSNP	-19360	T>A	81 (100.00)	0 (0.00)	0 (0.00)	1	13.82 ± 3.54 (67)	-	-	0	0	2	21	0
NT5C2	rs7917650	rSNP	-2486	C>G	51 (66.23)	21 (27.27)	5 (6.49)	5	12.09 ± 3.38 (39)	18.15 ± 9.80 (19)	5.02 ± 1.64 (4)	1	9	1	10	2
NT5C2	rs12261294	rSNP	-238	G>A	35 (45.45)	34 (44.16)	8 (10.39)	5	13.04 ± 4.49 (26)	13.39 ± 4.50 (30)	5.00 ± 2.06 (6)	1	15	1	3	3
NT5C3	rs3750117	cSNP	+14603	C>T	38 (46.34)	32 (39.02)	12 (14.63)	0	1.31 ± 0.44 (33)	1.12 ± 0.37 (25)	1.10 ± 0.96 (9)	10	16	-	-	-
NT5C3	rs13228639	rSNP	-26881	A>G	36 (45.57)	36 (45.57)	7 (8.86)	9	1.27 ± 0.42 (33)	1.14 ± 0.47 (21)	0.88 ± 0.40 (4)	10	13	0	0	3
NT5C3	rs7778958	rSNP	-6937	G>A	37 (45.68)	34 (41.98)	10 (12.35)	1	1.27 ± 0.43 (33)	1.16 ± 0.40 (25)	1.15 ± 1.08 (8)	9	14	0	2	1
NT5C3	rs4723239	rSNP	-6441	A>G	76 (95.00)	4 (5.00)	0 (0.00)	2	1.26 ± 0.31 (61)	0.65 ± 0.62 (4)	-	0	4	9	12	1

NT5C3	rs4316067	rSNP	-5933	T>C	35 (43.75)	32 (40.00)	13 (16.25)	2	1.10 ± 0.42 (25)	1.25 ± 0.47 (29)	1.51 ± 0.74 (11)	5	9	5	6	1
SLC29A1	rs8187641	cSNP	+3312	T>C	78 (98.73)	1 (1.27)	0 (0.00)	3	1.56 ± 0.62 (66)	1.12 ± 0.00 (1)	-	-	-	-	-	-
SLC29A1	rs747199	rSNP	-706	G>C	54 (67.50)	24 (30.00)	2 (2.50)	2	1.71 ± 0.92 (43)	1.24 ± 0.54 (20)	1.64 ± 1.44 (2)	-	-	-	-	-
TP53	rs1042522	cSNP	+441	G>C	49 (60.49)	23 (28.40)	9 (11.11)	1	1.29 ± 0.35 (40)	1.57 ± 0.58 (21)	1.17 ± 0.61 (6)	3	15	-	-	-
TP53	rs17885803	rSNP	-12565	G>A	64 (84.21)	11 (14.47)	1 (1.32)	6	1.29 ± 0.27 (53)	1.77 ± 1.24 (9)	0.81 ± 0.00 (1)	2	3	1	11	1
TP53	rs17883670	rSNP	-11805	C>G	75 (100.00)	0 (0.00)	0 (0.00)	7	1.36 ± 0.29 (62)	-	-	0	0	3	13	2

[a]Position with respect to ATG as +1

[b]Genotyping results; HH: frequent homozygote; Hh: heterozygote; hh: rare homozygote

[c]Mean values for mRNA expression level ± 95% confidence interval; HH: frequent homozygote; Hh: heterozygote; hh: rare homozygote

[d]Data only concerning samples with determined DAE status

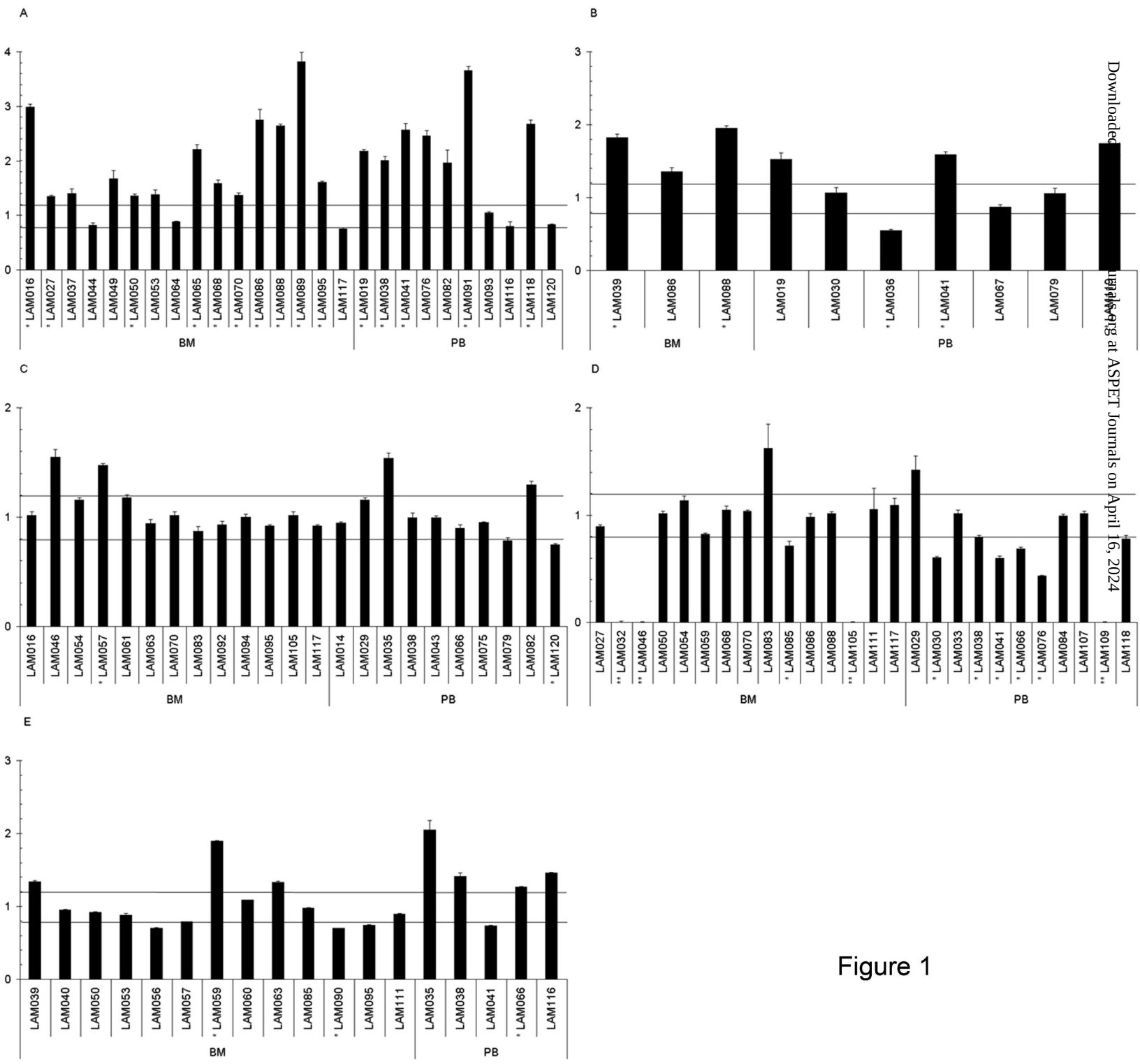

Figure 1