Association of relapse-linked ARID5B single nucleotide polymorphisms with drug resistance in B-cell precursor acute lymphoblastic leukemia cell lines

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Abstract

Background

The genetic variants of the ARID5B gene have recently been reported to be associated with disease susceptibility and treatment outcome in childhood acute lymphoblastic leukemia (ALL). However, few studies have explored the association of ARID5B with sensitivities to chemotherapeutic agents.

Methods

We genotyped susceptibility-linked rs7923074 and rs10821936 as well as relapse-linked rs4948488, rs2893881, and rs6479778 of ARDI5B by direct sequencing of polymerase chain reaction (PCR) products in 72 B-cell precursor-ALL (BCP-ALL) cell lines established from Japanese patients. We also quantified their ARID5B expression levels by real-time reverse transcription PCR, and determined their 50% inhibitory concentration (IC50) values by alamarBlue assays in nine representative chemotherapeutic agents used for ALL treatment.

Results

No significant associations were observed in genotypes of the susceptibility-linked single nucleotide polymorphisms (SNPs) and the relapsed-linked SNPs with ARID5B gene expression levels. Of note, IC50 values of vincristine (VCR) (median IC50: 39.6 ng/ml) in 12 cell lines with homozygous genotype of risk allele (C) in the relapse-linked rs4948488 were significantly higher (p=0.031 in Mann–Whitney U test) than those (1.04 ng/ml) in 60 cell lines with heterozygous or homozygous genotypes of the non-risk allele (T). Furthermore, the IC50 values of mafosfamide [Maf; active metabolite of cyclophosphamide (CY)] and cytarabine (AraC) tended to be associated with the genotype of rs4948488. Similar associations were observed in genotypes of the relapse-linked rs2893881 and rs6479778, but not in those of the susceptibility-linked rs7923074 and rs10821936. In addition, the IC50 values of methotrexate (MTX) were significantly higher (p=0.023) in 36 cell lines with lower ARID5B gene expression (median IC50: 37.1 ng/ml) than those in the other 36 cell lines with higher expression (16.9 ng/ml).

Conclusion

These observations in 72 BCP-ALL cell lines suggested that the risk allele of the relapse-linked SNPs of ARID5B may be involved in a higher relapse rate because of resistance to chemotherapeutic agents such as VCR, CY, and AraC. In addition, lower ARID5B gene expression may be associated with MTX resistance.

Background

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common neoplasm in children. Recent genome-wide association studies (GWAS) on pediatric patients with BCP-ALL have identified common single nucleotide polymorphisms (SNPs) associated with the disease susceptibility [1,2]. Among them, SNPs located in intron 3 of the *ARID5B* gene (i.e. rs7923074 and rs10821936; Fig. 1) are the most

significant and recapitulated SNPs in various races, including Asian populations [3–17]. *ARID5B* belongs to the AT-rich interactive domain (ARID) family and acts as a transcription coactivator that binds to the 5'-AATA[CT]-3' core sequence [18,19]. Although the direct mechanism for leukemogenesis is not fully understood, the risk allele of susceptibility-linked SNPs in intron 3 of the *ARID5B* gene may alter the transcription network involved in normal lymphopoiesis by disrupting *ARID5B* expression [20]. Interestingly, further GWAS on pediatric ALL patients revealed that the other SNPs located in intron 2 of the *ARID5B* gene (i.e. rs4948488, rs2893881, and rs6479778; Fig. 1) were significantly associated with their relapse rate [21]. This clinical observation suggests that the genotype of these relapse-linked SNPs of *ARID5B* may be associated with the responses to chemotherapeutic agents. Nevertheless, few studies have focused on the association of ARID5B with drug sensitivities in BCP-ALL [22].

Therefore, to address this issue, we analyzed any association of *ARID5B* genotype with *ARID5B* gene expression and drug sensitivity in a series of BCP-ALL cell lines. We found that genotypes of the relapse-linked SNPs of *ARID5B* are associated with resistance to several chemotherapeutic agents.

Materials And Methods

Cell Lines

We used 72 BCP-ALL cell lines that were established from Japanese patients as described in detail previously [23]. Among the 72 cell lines, 13 cell lines were *BCR/ABL1*-positive, 13 cell lines were *TCF3/PBX1*-positive, 12 cell lines were *MLL* (*KMT2A*)-rearranged, 5 cell lines were *ETV6/RUNX1*-positive, and 3 cell lines were *TCF3/HLF*-positive. No hyperdiploid cell lines were included. Forty-six cell lines were sequentially established in our laboratory from 1980 to 2011, while 24 cell lines were provided by 10 institutes. Two additional cell lines were purchased from American Type Culture Collection (ATCC). All cell lines were maintained in RPMI1640 media with 10% fetal calf serum at 37°C under a 21% O_2 and 5% CO_2 atmosphere.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from each cell line using TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse transcription reactions were performed using a random hexamer (Amersham Bioscience, Buckinghamshire, UK) and Superscript II Reverse Transcriptase (Invitrogen). To remove unreacted mRNA, the samples were treated with RNase (Invitrogen) after the reaction. Real-time reverse transcription polymerase chain reaction (RT-PCR) analyses of *ARID5B* were performed using a TaqMan probe kit (Hs01382781_m1). Gene expression level of the beta-actin (*ACTB)* gene was also examined as an internal control using a TaqMan probe kit (Hs01060665_g1).

SNP Genotyping

Genomic DNA was extracted from each cell line using a PureLink Genomic DNA Mini Kit (Invitrogen). Genomic regions containing two representative susceptibility-linked SNPs (rs7923074 and rs10821936) and three representative relapse-linked SNPs (rs4948488, rs2893881 and rs6479778) of *ARID5B* in 72 BCP-ALL cell lines were amplified using primers described in Table 1. Then, genotypes of five SNPs in each cell line were determined after direct sequencing of each genomic PCR product using forward primers for rs7923074, rs4948488, and rs2893881 as well as a reverse primer for rs10821936 and rs6479778.

AlamarBlue Assay

Fifty percent inhibitory concentration (IC50) values of prednisolone (Pred), dexamethasone (Dex), vincristine (VCR), daunorubicin (DNR), L-asparaginase (L-Asp), cytarabine (AraC), methotrexate (MTX), mercaptopurine (6MP), and mafosfamide [Maf; active metabolite of cyclophosphamide (CY)] were determined using the alamarBlue cell viability assay (Bio-Rad Laboratories, Hercules, CA) as previously reported [24]. Cells ($1–4 \times 10^5$) were placed onto 96-well flat bottom plates in the presence or absence of seven separate concentrations of each drug in triplicate. The cells were cultured for 44 hours to determine the DNR, VCR and CY (Maf) sensitivities and for 68 hours to determine Pred, Dex, L-Asp, MTX, and 6MP; 20 μL of alamarBlue was then added. After incubation for an additional 6 h in the presence of alamarBlue, the optimal density was read on a spectrophotometer at 570 nm using 600 nm as a reference wavelength. Cell viability was calculated by the ratio of the optical density of the treated wells to that of the untreated wells as a percentage. The concentration of each agent required to reduce the viability of the treated cells to 50% of the untreated cells (IC50 value) was calculated and the median IC50 value of three independent assays was determined.

Statistics

We applied Fisher's exact test for comparison of allele frequencies between cell lines and Japanese population in HapMap project database (https://www.ncbi.nlm.nih.gov/variation/news/NCBI_retiring_HapMap/). Mann–Whitney U test was always applied for comparisons between two groups of cell lines using R (version 3.5.1) statistical software.

Results

Genotype of susceptibility-linked and relapse-linked SNPs of ARID5B in BCP-ALL cell lines

We first analyzed *ARID5B* genotypes in 72 BCP-ALL cell lines established from Japanese patients [23]. Our cell line bank contained 13 *BCR/ABL1*-positive, 13 *TCF3/PBX1*-positive, 12 *MLL* (*KMT2A*)-rearranged,

5 *ETV6/RUNX1*-positive, and 3 *TCF3/HLF*-positive cell lines, but no hyperdiploid cell lines. Thus, the majority of our cell lines had been established from BCP-ALL with high or intermediate risk karyotypes. We determined genotypes of two representative susceptibility-linked SNPs [21] (rs7923074 and rs10821936, Fig. 1) and three representative relapse-linked SNPs [21] (rs4948488, rs2893881, and rs6479778, Fig. 1) in each cell line after direct sequencing of each genomic PCR product. Allele frequencies of each SNP in BCP-ALL cell lines were in Hardy–Weinberg equilibrium. Due to linkage disequilibrium, genotypes of rs7923074 and rs10821936 were identical in 71 of 72 cell lines. Genotypes of rs2893881 and rs6479778 were also identical in 71 cell lines. In HapMap project database (Table 2), we compared the allele frequency of each SNP between our cell lines and the Japanese population, but no significant differences were observed in the genotypes of both the susceptibility-linked SNPs and the relapse-linked SNPs of *ARID5B*.

No association of susceptibility or relapse-linked SNPs of ARID5B with ARID5B expression

Since both the susceptibility-linked SNPs and the relapse-linked SNPs of *ARID5B* are located in intronic regions, we next performed expression quantitative trait locus (eQTL) analysis. We quantified *ARID5B* gene expression level in each cell line by real-time RT-PCR using *ACTB* gene expression as an internal control. However, in eQTL analysis of 72 BCP-ALL cell lines, neither genotypes of the susceptibility-linked rs7923074 and rs10821936 nor those of the relapse-linked rs4948488, rs2893881, and rs6479778 were significantly associated with *ARID5B* expression level (Fig. 2). These observations demonstrated that genotypes of both susceptibility-linked SNPs and the relapse-linked SNPs of *ARID5B* were not clearly associated with *ARID5B* expression levels in the BCP-ALL cell line.

Association of relapse-linked SNPs of ARID5B with drug sensitivity

We next verified whether genotype of the relapse-linked SNPs of *ARID5B* in BCP-ALL cell lines is associated with their sensitivities to chemotherapeutic agents. We performed an alamarBlue assay to determine IC50 values (concentration that needs to kill 50% of the cells) of nine representative agents used for ALL chemotherapy including Pred, Dex, VCR, DNR, L-Asp, AraC, MTX, 6MP, and CY (Maf). Of note, IC50 values of VCR (median IC50: 39.6 ng/ml) in 12 cell lines with homozygous genotype of risk allele (C) in the relapse-linked rs4948488 were significantly higher ($p = 0.031$ in Mann–Whitney U test) than those (1.04 ng/ml) in 60 cell lines with heterozygous or homozygous genotypes of non-risk allele (T) (Fig. 3a). In addition to VCR, sensitivities to CY (Maf) (Fig. 3b) and AraC (Fig. 3c) tended to be associated with the genotype of the relapse-linked rs4948488. Similar associations were observed in genotypes of rs2893881 and rs6479778 (Fig. 3a-c). Among the nine agents, IC50 values of six agents (Dex, Pred, DNR, L-Asp, MTX, and 6MP) were not significantly associated with genotypes of the relapse-linked rs4948488, rs2893881, and rs6479778 (Supplemental Fig. 1a-f).

We further analyzed association of the susceptibility-linked rs7923074 and rs10821936 with drug sensitivities. In contrast to the genotypes of the relapse-linked SNPs, no significant associations were observed in genotypes of rs7923074 and rs10821936 with sensitivities to VCR, CY and AraC (Fig. 3a-c) and the other six agents (Supplemental Fig. 1a-f). These observations suggest that the risk allele of

relapse-linked SNPs, but not susceptibility-linked SNPs, may be associated with a higher relapse rate in pediatric BCP-ALL patients due to reduced sensitivities to VCR, CY and AraC.

Association of ARID5B gene expression with drug sensitivity

We finally verified whether gene expression level of *ARID5B* is associated with drug sensitivities of BCP-ALL cell lines. To address this issue, we simply divided our 72 BCP-ALL cell lines into two groups—36 cell lines with higher gene expression levels than the median value and the other 36 cell lines with lower gene expression levels than the median value—and compared the IC50 values of each drug. Of note, the IC50 values of MTX in 36 cell lines with lower *ARID5B* expression (median IC50: 37.1 ng/ml) was significantly higher ($p = 0.023$ in Mann–Whitney U test) than those in the other 36 cell lines with lower expression (16.9 ng/ml) (Fig. 4a). In contrast, although the sensitivities to VCR, CY, and AraC were associated with genotypes in the relapse-linked SNPs of *ARID5B*, no significant differences were observed in the IC50 values of VCR, CY, and AraC between the two groups (Fig. 4b-d). Furthermore, although genotypes in the susceptibility-linked SNPs of *ARID5B* were associated with sensitivities to Pred and Dex, no significant differences were observed in the IC50 values of Pred and Dex between the two groups (Supplement Fig. 2a, b). In the IC50 values of the remaining three agents (DNR, L-Asp, and 6MP), there were no statistically significant differences between the two groups (Supplement Fig. 2c-e). These observations suggest that lower *ARID5B* expression may be a genetic marker for MTX resistance in BCP-ALL.

Discussion

In the present study, using a series of BCP-ALL cell lines, we tried to verify the significance of genotype in the susceptibility-linked and relapsed-linked SNPs of *ARID5B* with *ARID5B* gene expression and drug sensitivities. It should be noted that the karyotypes in our cell lines were highly biased in comparison with those in childhood BCP-ALL patients: 13 cell lines were *BCR/ABL1*-positive, 13 cell lines were *TCF3/PBX1*-positive, 12 cell lines were *MLL* (*KMT2A*)-rearranged, and 3 cell lines were *TCF3/HLF*-positive. Furthermore, we later discovered that 15 cell lines were positive for *MEF2D*-fusions (under review), which are recently identified fusion genes with a poor therapeutic outcome [25,26]. In contrast, only five cell lines were *ETV6/RUNX1*-positive, and no cell lines were hyperdiploidy. Thus, the majority of our cell lines were established from BCP-ALL with a poor prognosis.

Using these biased samples, we analyzed associations between genotypes of the relapsed-linked SNPs of *ARID5B* and the sensitivities to representative drugs for ALL treatment. Among the nine agents, sensitivities to VCR, CY, and AraC were associated with relapsed-linked SNPs; risk alleles of the relapsed-linked rs4948488, rs2893881, and rs6479778 were associated with resistance to these three agents. These relapsed-linked SNPs of *ARID5B* were located in intron 2 of the *ARID5B* gene, and their genotypes were not associated with *ARID5B* gene expression level. Moreover, *ARID5B* gene expression level was not associated with the sensitivities to VCR, CY, and AraC. Thus, although the underlying mechanism behind the association remains to be clarified, genotypes of the relapse-linked SNPs of *ARID5B* are associated with VCR, CY, and AraC sensitivities of BCP-ALL cell lines independent of their *ARID5B* expression levels.

Regarding the association of ARID5B with drug sensitivities in BCP-ALL cell lines, we also found that lower *ARID5B* gene expression level was associated with resistance to MTX. This finding seems to be partly consistent with a recent report by Xu et al [22] who found that *ARID5B* knockdown in ALL cell lines led to specific resistance to MTX and 6MP.

Conclusion

In summary, our observations in 72 BCP-ALL cell lines suggest that the risk allele of the relapse-linked SNPs of *ARID5B* may be involved in higher relapse rate because of resistance to chemotherapeutic agents such as VCR, CY, and AraC. Moreover, lower *ARID5B* expression may be associated with MTX resistance. Limitations of the present study include that the study sample was restricted to limited numbers of leukemic cell lines with biased karyotypes and that underlying biological mechanisms for the associations remain unclarified. Thus, further studies are needed before making a conclusion.

Declarations

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Authors' contributions

TI conceptualized and designed experiments. TI and KK performed cell culture. MT, KK, and MH performed PCR analyses. TI, MH, KK, MA, DH, TS, AW, KA and KG performed drug sensitivity assays. MT and TI analyzed and visualized data. MT and TI co-wrote the paper. HG, MM, SI, KS, TI, KK and KG established cell lines. TI supervised the study. All authors edited the paper.

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Data availability statement

The data used in present study are available from corresponding author on request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests

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Supplemental Figure Legends

Supplement Fig. 1 Association of relapse- and susceptibility-linked SNP genotypes with sensitivities to Pred (a), Dex (b), DNR (c), L-Asp (d), MTX (e), and 6MP (f). Vertical axis indicates log-scaled IC50 values of Pred (a), Dex (b), DNR (c), L-Asp (d), MTX (e), and 6MP (f). The IC50 values of cell lines with homozygous genotype of risk allele and those with heterozygous or homozygous genotypes of non-risk allele in each SNP were compared. P-value in Mann–Whitney U test is indicated at the top of each SNP.

Supplement Fig. 2 Association of *ARID5B* gene expression with sensitivities to Dex (a), Pred (b), DNR (c), LAsp (d), and 6MP (e). Vertical axis indicates log-scaled IC50 values of Dex (a), Pred (b), DNR (c), LAsp (d), and 6MP (e). The IC50 values of 36 cell lines with higher *ARID5B* expression and the other 36 cell lines with lower expression were compared. P-value in Mann–Whitney U test is indicated at the top of each SNP.

Tables

Due to technical limitations, Tables 1-2 are provided in the Supplemental Files section.

Figures

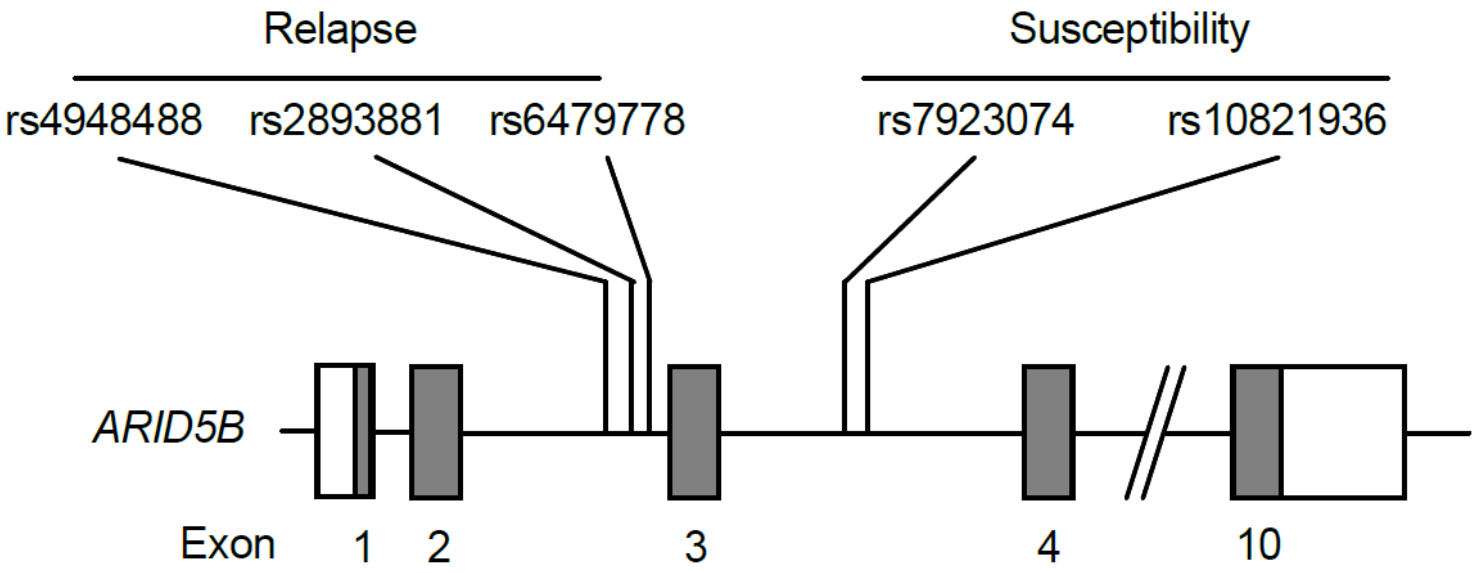

Figure 1

Locations of relapse- and susceptibility-linked SNPs of ARID5B.

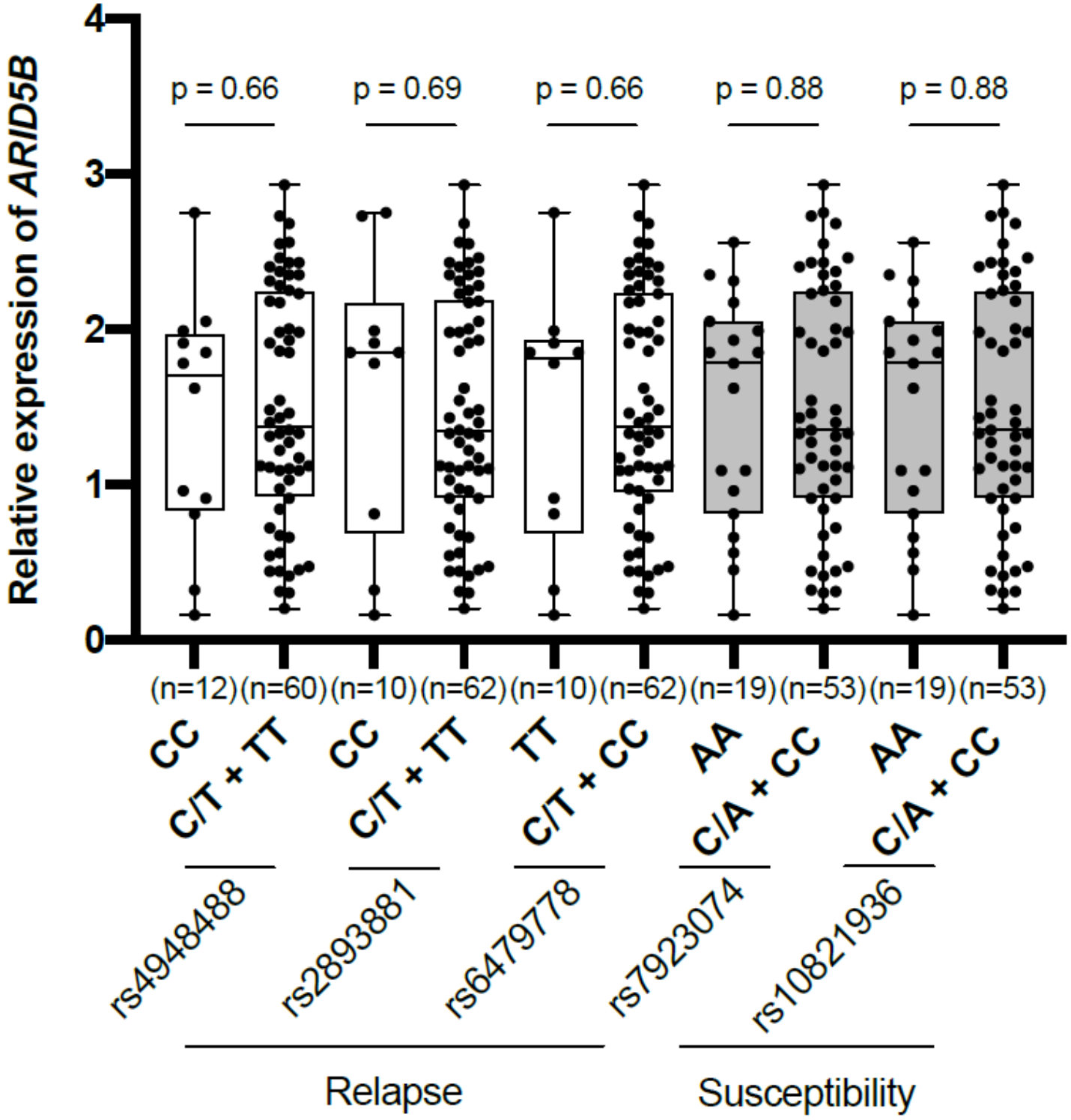

Figure 2

Association of relapse- and susceptibility-linked SNP genotypes with ARID5B gene expression. Relative ARID5B gene expression levels were compared between cell lines with homozygous genotype of risk allele and those with heterozygous or homozygous genotypes of non-risk allele in each SNP. P-value in Mann–Whitney U test is indicated at the top of each SNP.

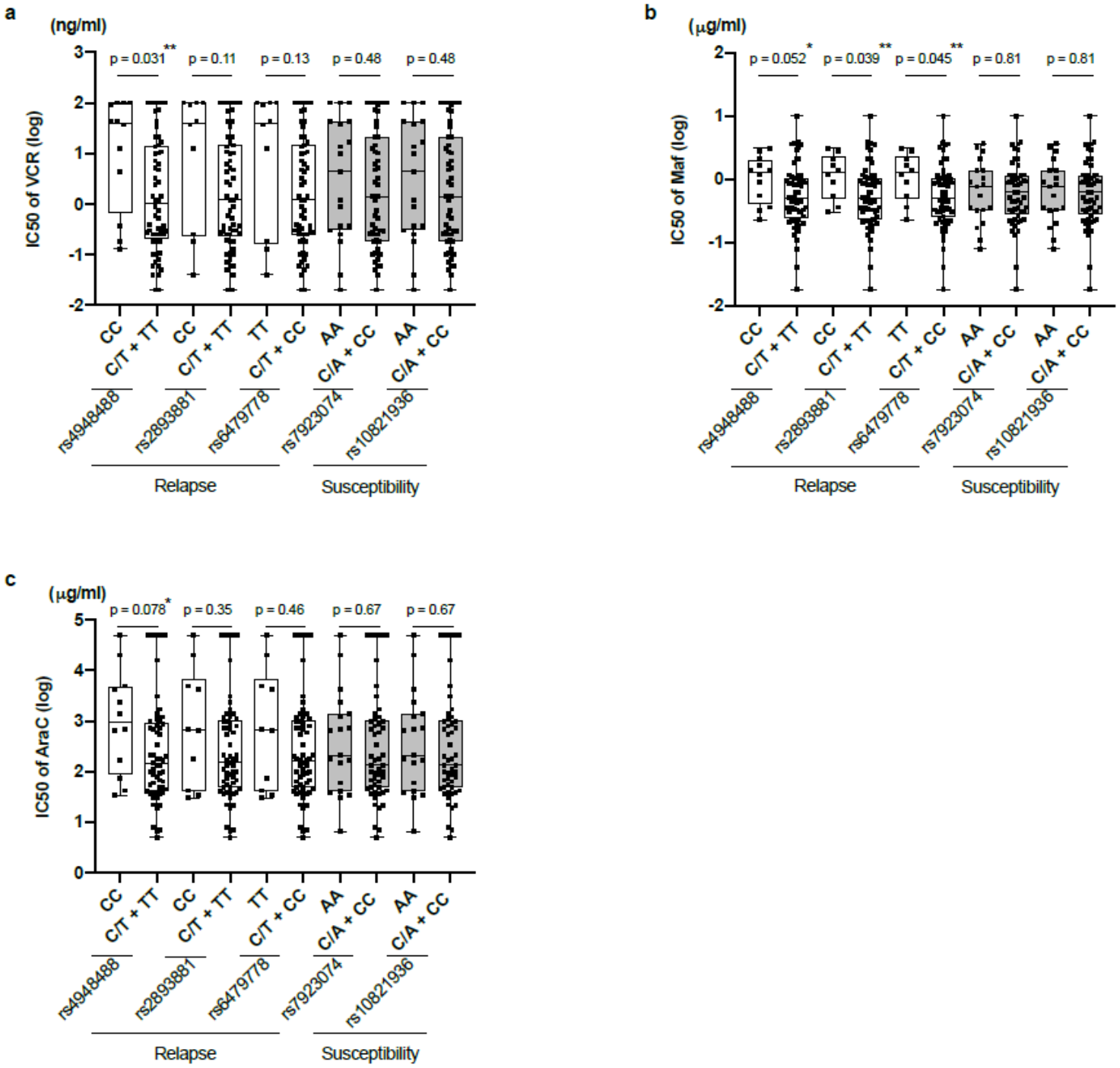

Figure 3

Association of relapse- and susceptibility-linked SNP genotypes with sensitivities to VCR (a), CY (b), and AraC (c). Vertical axis indicates log-scaled IC50 values of VCR (a), CY (Maf) (b), and AraC (c). The IC50 values of cell lines with homozygous genotype of risk allele and those with heterozygous or homozygous genotypes of non-risk allele in each SNP were compared. P-value in Mann–Whitney U test is indicated at the top of each SNP.

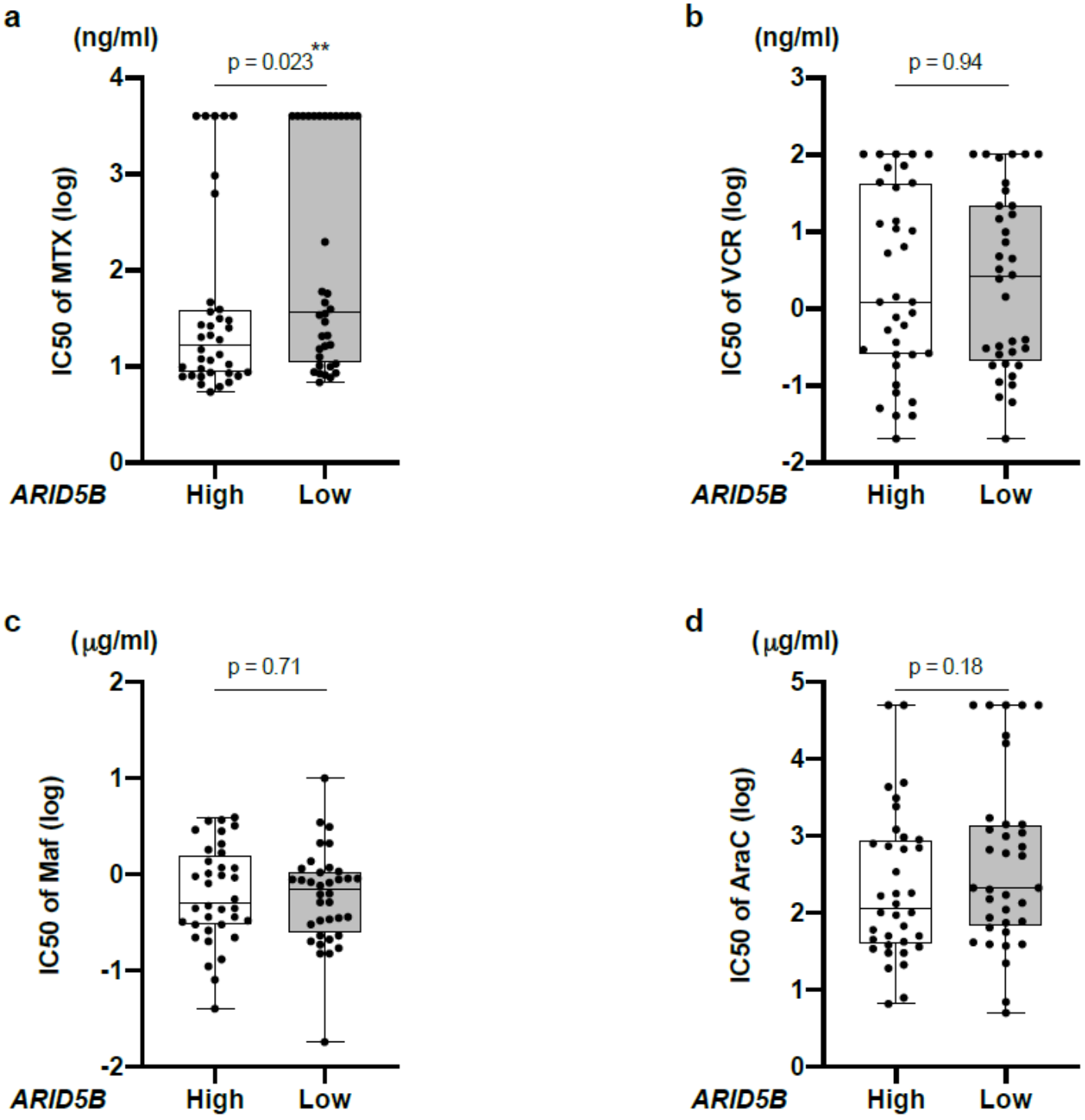

Figure 4

Association of ARID5B gene expression with sensitivities to MTX (a), VCR (b), CY (c), and AraC (d). Vertical axis indicates log-scaled IC50 values of MTX (a), VCR (b), CY (Maf) (c), and AraC (d). The IC50 values of 36 cell lines with higher ARID5B expression and the other 36 cell lines with lower expression were compared. P-value in Mann–Whitney U test is indicated at the top of each SNP.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.1.xls
- SupplementFig.2.pdf
- Table.2.xls
- SupplementFig.1.pdf