Glucocorticoid receptor gene mutations confer glucocorticoid resistance in B-cell precursor acute lymphoblastic leukemia

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\section*{A B S T R A C T}

Glucocorticoid (GC) is a key drug in the treatment of B-cell precursor acute lymphoblastic leukemia (BCP-ALL), and the initial GC response is an important prognostic factor. GC receptors play an essential role in GC sensitivity, and somatic mutations of the GC receptor gene, \textit{NR3C1}, are reportedly identified in some BCP-ALL cases, particularly at relapse. Moreover, associations of somatic mutations of the CREB-binding protein (\textit{CREBBP}) and Wolf-Hirschhorn syndrome candidate 1 (\textit{WHSC1}) genes with the GC-resistance of ALL have been suggested. However, the significance of these mutations in the GC sensitivity of BCP-ALL remains to be clarified in the intrinsic genes. In the present study, we sequenced \textit{NR3C1}, \textit{WHSC1}, and \textit{CREBBP} genes in 99 BCP-ALL and 22 T-ALL cell lines (32 and 67 cell lines were known to be established at diagnosis and at relapse, respectively), and detected their mutations in 19 (2 cell lines at diagnosis and 15 cell lines at relapse), 26 (6 and 15), and 38 (11 and 15) cell lines, respectively. Of note, 14 BCP-ALL cell lines with the \textit{NR3C1} mutations were significantly more resistant to GC than those without mutations. In contrast, \textit{WHSC1} and \textit{CREBBP} mutations were not associated with GC resistance. However, among the \textit{NR3C1} unmutated BCP-ALL cell lines, \textit{WHSC1} mutations tended to be associated with GC resistance and lower \textit{NR3C1} gene expression. Finally, we successfully established GC-resistant sublines of the GC-sensitive BCP-ALL cell line (697) by disrupting ligand binding and DNA binding domains of the \textit{NR3C1} gene using the CRISPR/Cas9 system. These observations demonstrated that somatic mutations of the \textit{NR3C1} gene, and possibly the \textit{WHSC1} gene, confer GC resistance in BCP-ALL.

\section*{1. Introduction}

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most prevalent malignancy in children. Recently, the prognosis of BCP-ALL patients has dramatically improved: the 5-year overall survival rate exceeds 90\% in the majority of contemporary regimens [1–3]. However, the prognosis of the relapsed cases remains poor due to acquired resistance to standard chemotherapeutic agents [4–6]. To characterize the genetic mechanisms for chemoresistance in the relapsed pediatric ALL cases, comprehensive genomic analyses have recently been undertaken. As a result, the acquired mutations in the genes involving drug sensitivities have been clarified in the leukemia cells at relapse [7–10].

Glucocorticoid (GC) is an essential therapeutic agent in the treatment of BCP-ALL patients [11,12], and resistance to GC-monotherapy is one of the most important poor prognostic factors [13,14]. In the anti-leukemic activities of GC, glucocorticoid receptor (GR), encoded by the \textit{NR3C1} gene, plays a central role. GR translocates from cytosol to the nucleus through GC binding, and functions as a transcriptional regulator [15].
Once GR binds to its responsive DNA elements, GR transcribes pro-apoptotic genes such as BIM. Conversely, GR represses anti-apoptotic genes such as BCL2 and BCL-XL [16,17]. The acquisition of the NR3C1 gene mutation in GC-resistant mechanisms in the relapsed ALL cases has recently been reported [7,18–20]. The significance of the NR3C1 mutations in the GC-sensitivity of ALL cells has been evaluated using all cell lines transfected with the expression vectors containing the wild-type or mutated-types of the NR3C1 cDNA [7,18,19]. The human NR3C1 gene has four splicing variants besides the functional isoform GRα, and their significance in GC sensitivity of ALL has been widely discussed [21,22]. Thus, to properly evaluate the significance of the NR3C1 mutations in the GC-sensitivity of ALL cells, the GC-resistant phenotype needs to be verified in the ALL cells with the intrinsic NR3C1 mutations. However, this issue still remains to be clarified.

As with other possible genetic mechanisms involved in GC-resistance in all cell lines, the CREB-binding protein (CREBBP) and Wolf-Hirschhorn syndrome candidate 1 (WHSC1) genes have also been reported [7,23–25]. The CREBBP gene mutations have reportedly induced impairment of histone acetylation ability, and subsequently disrupted transcriptional regulation of the target genes including the GC response genes [23]. WHSC1 is a histone methyltransferase specific for H3K27me2. The WHSC1 gene mutations have reportedly altered the GC transcriptional response, resulting in deactivation of pro-apoptotic BIM and BFM [26]. However, the significance of the CREBBP and WHSC1 gene mutations in GC-sensitivities of ALL cells still remains to be elucidated in their intrinsic genes.

In the present study, to verify the possible association of the NR3C1, CREBBP, and WHSC1 mutations with GC-resistance in their intrinsic genes, we analyzed a large series of BCP-ALL and T-ALL cell lines. We also developed GC-resistant sublines of the ALL cell lines by knocking out the NR3C1 gene using the CRISPR-Cas9 system.

2. Materials and methods

2.1. Cell lines

We used 99 BCP-ALL cell lines that were established from BCP-ALL patients (Supplement Table 1). Among the 99 cell lines, 18 cell lines were MEF2D fusion-positive, 18 cell lines were BCR/ABL1-positive, 16 cell lines were TCF3/PBX1-positive, 14 cell lines were MLL (KMT2A) rearrangement-positive, 6 cell lines were ETV6/RUNXI-positive, 4 cell lines were TCF3/HLF-positive, 2 cell lines were BCR/ABL1-like, 2 cell lines were DUX4 fusion-positive, 1 cell line was PAX5/ETV6-positive, 12 cell lines were B-others, and 6 cell lines of their karyotypes were not known. No hyperdiploid cell lines were included. We also analyzed 22 T-ALL cell lines (Supplement Table 2), which were precisely described in our previous report [27].kop, kocil, yamn, and yacil series of cell lines were sequentially established in our laboratory from 1980 to 2012 [28–31]. YCUB and KCB series of cell lines were sequentially established at Yokohama City University and Kanagawa Children’s Medical Center [32] and provided in 2014 (Dr. H. Goto). THP series of cell lines, L-KUM, L-ASK, L-MAT, and L-KAW were sequentially established at Tohoku University [33] and provided in 2014 (Dr. M. Minegishi). MB series of cell lines were sequentially established at Mie University Graduate School of Medicine [34] and provided in 2014 (Dr. S. Iwamoto). Kasumi series of cell lines were sequentially established at Hiroshima University [35] and provided in 2016 (Dr. T. Inaba). HBL-3 [36] was established at Fukushima Medical University and provided in 2016 (Dr. H. Hojo). SU-PH2 [37] was established at Kindai University Faculty of Medicine and provided in 2010 (Dr. Y. Maeda). TCC [38] was established at Tochigi Cancer Center and provided in 2011 (Dr. Y. Sato). HMLD01 [28] was provided in 1997 (Dr. A. T. Look at Dana-Farber Cancer Institute, Boston, MA). SK9 [39] was established at Tokyo Medical University and provided in 2012 (Dr. S. Okabe). Endokun [28] was established at Iwate Medical University and provided in 1997 (Dr. M. Endo). SCMLC1 and SCMLC2 [40] were established at Saitama Children’s Medical Center and provided in 2014 (Dr. J. Takita). P30/OHK [41] and Nalm27 [42], CCRF-SB [43], NAGL1 [44], LC4-L1 [45], PALL-2 [46], and TMD5 [47] were purchased from ATCC in 2012 and 2020, respectively. All cell lines were maintained in RPMI1640 media with 10 % fetal calf serum (FCS) at 37 °C under a 21 % O2 and 5 % CO2 atmosphere.

2.2. Target-exon sequences of the NR3C1, CREBBP, and WHSC1 mutations

A panel of primers for NR3C1, WHSC1, and CREBBP gene was designed using Ion AmpliSeq™ Designer v7.4.6.5 (Thermo Fisher Scientific, Waltham, MA). The pool of amplicons covered 100 %, 99.4 %, and 100 % of NR3C1, CREBBP, and WHSC1 genes, respectively. Genomic DNA was extracted from each cell line using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Libraries were constructed using an Ion AmpliSeq Library Kit v2.0 and Ion Xpress Barcode Adaptors Kit (Thermo Fisher Scientific). After Agencourt AMPure XP purification (Beckman Coulter, Brea, CA), individual libraries were amplified. The libraries were then processed with an Ion Chef System using an Ion PG Hi-Q Chef Kit (Thermo Fisher Scientific). Next-generation sequencing (NGS) was performed by using an Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific) and 850 flows on an Ion 318 Chip Kit v2 (Thermo Fisher Scientific). After sequencing, single processing and base calling were performed using Torrent Suite 5.0.2 (Thermo Fisher Scientific). Results were analyzed using the Ion Reporter™ 5.16.0.2. Then, Variant Call Format (VCF) files were converted to Mutation Annotation Format (MAF) files using the vcf2maf utility (https://github.com/mskcc/vcf2maf). Downstream analysis was performed using maftools (https://bioconductor.org/packages/release/bioc/html/maftools.html) [48] and circize (https://github.com/jokergoo/cirize) [49] in R software (version 4.0).

2.3. AlamarBlue assay

Fifty percent inhibitory concentration (IC50) values of dexamethasone (Dex) and prednisolone (Pred) were determined using the ala

marBlue cell viability assay (Bio-Rad Laboratories, Hercules, CA) as previously reported [50]. Cells (0.1–4 × 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 66 h to determine Dex and Pred sensitivities, and 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.

2.4. Cell apoptosis analysis

To detect apoptotic cell death, cells were incubated for 72 h in the presence or absence of dexamethasone (Dex) and prednisolone (Pred), and stained with fluorescein isothiocyanate (FITC) conjugated Annexin V and 7AAD (MBL, Nagoya, Japan). Data was analyzed using flow cytometry (FACSCelesta, BD Biosciences, San Jose, CA), FACS Diva software (v8.0.1.1, BD Biosciences), and FlowJo software (v10.6.1, LLC, Ashland, OR).

2.5. Real-time reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from each cell lines with TRizol reagent (Invitrogen, Waltham, MA), and reverse transcription was performed by using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Real-
time reverse transcription polymerase chain reaction (RT-PCR) analyses of \( NR3C1 \) gene were performed using a TaqMan Probe Kit (Hs00353740_m1). As an internal control, gene expression levels of beta-actin (ACTB) were also examined using a TaqMan Probe Kit (Hs99999903_m1).

2.6. \( NR3C1 \) gene mutations introduced by CRISPR/Cas9

A guide RNA sequence targeting human \( NR3C1 \) gene exon 4 was designed using Benchling software (https://www.benchling.com). Self-complementary oligos (5′-CAGCGATTTTCTAGATCGGCGTCT-3′ and 5′-AACACGATCGGCGTATCGAAATCT-3′) containing the \( NR3C1 \) gene guide sequence and Bsf ligation adaptors were purchased from IDT (https://www.idtdna.com). Those oligos were ligated into pSpCas9(BB)-2A-GFP (Addgene Plasmid 48138). A GC-sensitive cell line (697) was transfected with the plasmid using the Neon electroporation system (Thermo Fisher Scientific) at pulse voltage of 1500 V, pulse width 20 ms, and pulse number 1. Transfected cells were subsequently treated with Pred for 7 days. Then, induced mutations in \( NR3C1 \) gene exon 4 were examined. Briefly, genomic DNA was extracted from the cell using a PureLink Genomic DNA Mini Kit (Invitrogen). RT-PCR reaction was performed using a pair of primers (5′-CTCAAGGCTATGTTGCGT-3′ and 5′-GGCGAGACATTATTGCTAGTAAG-3′), and direct sequencing of RT-PCR products was performed using the forward primer.

3. Results

3.1. \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) mutations in BCP-ALL cell lines

In 99 BCP-ALL cell lines, we performed target-exon sequences of the \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) genes, in which the covering exon was 100 %, 99.4 %, and 100 %, respectively. The median sequence coverage in the analyses was 311 (range: 65.5–1117). Identified mutations are plotted in Fig. 1a as a heatmap with establishment information of each cell line including the types of fusion genes. Among 99 BCP-ALL cell lines, mutations of the \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) genes were detectable in 14, 24, and 20 cell lines, respectively. Two cell lines had mutations in all of the three genes, three cell lines had mutations in both the \( CREBBP \) and \( WHSC1 \) genes, and another two cell lines had mutations in both the \( NR3C1 \) and \( CREBBP \) genes. Among 15 \( NR3C1 \) mutations identified in 14 cell lines, 6 were missense mutations, 4 were frameshift insertions, 3 were nonsense mutations, 1 was a frameshift deletion, and 1 was an in-frame insertion (Fig. 1b). Six mutations were located in the glucocorticoid receptor domain, four were in the ligand-binding domain, and two were in the DNA binding domain (Fig. 1b, Table 1). Among 32 \( CREBBP \) mutations identified in 24 cell lines, 19 were missense mutations, 7 were nonsense mutations, 3 were frameshift deletions, 2 were frameshift insertions, and 1 was an in-frame deletion (Fig. 1c, Table 2). Five mutations were located in the KAT11 catalytic domain and three were in the Cred binding domain. Among 20 \( WHSC1 \) mutations identified in 20 cell lines, 19 were missense mutations and 1 was a splice-site mutation (Fig. 1d, Table 3). Eleven mutations were located in the SET domain.

Finally, we evaluated the association of \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) mutations with karyotypes in 99 BCP-ALL cell lines and compared with that in the clinical samples using PeCan database (https://pecan.stjude.cloud). Among 14 cell lines with the \( NR3C1 \) mutations, the incidences of \( MEF2D \) fusion (18 % vs 29 %) and \( TCF3-HLF \) (4 % vs 21 %) tended to be higher, while number of patients with \( NR3C1 \) mutations in PeCan database was too small (\( n=5 \)) to verify the association (Supplement Fig. 1a–d). Among 24 cell lines with the \( CREBBP \) mutations, the incidence of \( MEF2D \) fusion (18 % vs 29 %) tended to be higher, while that of \( MLL \) fusion (14 % vs 4 %) tended to be lower (Supplement Fig. 1a, e). Consistently, in the PeCan database, no case with \( MLL \) fusion was included (Supplement Fig. 1b, f). Among 20 cell lines with the \( WHSC1 \) mutations, the incidence of \( TCF3-PBX1 \) (16 % vs 35 %) and \( ETV6-RUNXI \) (6.1 % vs 10 %) tended to be higher (Supplement Fig. 1a, g). Consistently, in the PeCan database, the incidence of \( TCF3-PBX1 \) and \( ETV6-RUNXI \) were relatively higher as previously reported (Supplement Fig. 1b, h) [51,52]. Of note, although statistically insignificant, the \( NR3C1 \) gene mutations tended to be more frequently observed in the cell lines established at relapse (10/55 cell lines; 18 %) than those established at diagnosis (2/31 cell lines; 6 %) (Fig. 2a and b). In contrast, the \( CREBBP \) and \( WHSC1 \) gene mutations were almost equally observed in the cell lines established at relapse (16 % and 20 %, respectively) and in those established at diagnosis (31 % and 19 %, respectively) (Fig. 2a and b). These results indicated that the mutations of the \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) genes were relatively common in BCP-ALL cell lines and that the \( NR3C1 \) mutation tended to be more frequently observed in the cell lines established at relapse.

3.2. Association of \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) mutations with GC sensitivities in BCP-ALL cell lines

Next, we analyzed the possible association of the \( NR3C1 \), \( CREBBP \), and \( WHSC1 \) mutations with GC sensitivities in 98 BCP-ALL cell lines, except for HBL3 due to its poor proliferation in regular culture condition. As we previously reported in 72 BCP-ALL cell lines [53], the IC50 values of dexamethasone (Dex) and prednisolone (Pred) were significantly correlated with each other (\( R^2 = 0.67, p < 0.0001 \) (Supplement Fig. 2).

In 98 BCP-ALL cell lines, median IC50 values of Dex and Pred were 92.5 nM and 3.19 \( \mu \)M, respectively. Based on the previously reported median lethal concentration 50 % (LC50) values of GCs in clinical childhood ALL samples [54], 50 cell lines (IC50 of Dex < 200 nM) and 49 cell lines (IC50 of Pred < 3.5 \( \mu \)M) were considered as Dex and Pred-sensitive cell lines, respectively. Of note, the Dex-resistant phenotype was significantly more common in the \( NR3C1 \) mutated cell lines (\( p = 0.0035 \) in Fisher’s exact test); 12 out of 14 \( NR3C1 \) mutated cell lines were resistant to Dex, whereas 36 out of 84 \( NR3C1 \) unmutated cell lines were resistant to Dex.

As a result, the IC50 values of Dex in 14 \( NR3C1 \) mutated cell lines (median IC50; > 250 nM) were significantly higher than those in 84 \( NR3C1 \) unmutated cell lines (median IC50; 26.8 nM) (\( p = 0.0083 \) in Mann–Whitney U test) (Fig. 3a). Similarly, although the association of the Pred-resistant phenotype with the \( NR3C1 \) mutated cell line was statistically insignificant (10/14 mutated cell lines vs 39/84 unmutated cell lines; \( p = 0.15 \) in Fisher’s exact test), the IC50 values of Pred in 14 \( NR3C1 \) mutated cell lines (median IC50; 52.4 \( \mu \)M) were significantly higher than those in 84 \( NR3C1 \) unmutated cell lines (median IC50; 0.31 \( \mu \)M) (\( p = 0.012 \) (Fig. 3b). These results indicated that the \( NR3C1 \) gene mutation is clearly associated with the GC-resistant phenotype of BCP-ALL cell lines in their intrinsic gene.

Next, we analyzed the association between the \( WHSC1 \) mutation and GC sensitivities. In contrast to the \( NR3C1 \) mutation, no statistically significant differences were observed in the IC50 values of Dex or Pred between 20 \( WHSC1 \) mutated cell lines and 78 \( WHSC1 \) unmutated cell lines (Fig. 3c, d). Since mutation status of the \( NR3C1 \) gene was highly associated with the GC sensitivities in BCP-ALL cell lines, we focused next on 84 \( NR3C1 \) unmutated cell lines. Although statistically insignificant, 18 \( WHSC1 \) mutated cell lines were more resistant to Dex (\( p = 0.060 \) in Mann–Whitney U test) and Pred (\( p = 0.077 \)) than 66 \( WHSC1 \) unmutated cell lines (Fig. 3e, f). We finally analyzed the association of the \( CREBBP \) mutation with GC sensitivities. However, no statistically significant differences were observed in the IC50 values of Dex or Pred between 24 \( CREBBP \) mutated cell lines and 74 \( CREBBP \) unmutated cell lines (Fig. 3g, h). Moreover, even when focusing on the 84 \( NR3C1 \) unmutated cell lines, no statistically significant differences were observed in the IC50 values of Dex or Pred between 20 \( CREBBP \) mutated cell lines and 64 \( CREBBP \) unmutated cell lines (Fig. 3i, j). These results suggested that the mutation of the \( WHSC1 \) gene, but not the \( CREBBP \) gene, is associated with the GC-resistant phenotype in the \( NR3C1 \)
Fig. 1. Mutations of the NR3C1, CREBBP, and WHSC1 genes identified by target-exon sequencing in 99 BCP-ALL cell lines. (a) Heatmap of the NR3C1, CREBBP, and WHSC1 genes (rows) across 99 BCP-ALL cell lines (columns). In the main panel, types of mutation are indicated by colors. In the right panel, the prevalence for each gene mutation is shown as bar graph. In the bottom panel, types of fusion gene and status of establishment are indicated by colors. (b–d) Schematics of mutation locations within NR3C1 (b), CREBBP (c), and WHSC1 (d) protein structures. Horizontal axes indicate amino acid position and vertical axes indicate number of cell lines. Types of mutations are indicated by colored circles and functional domains are indicated by colored boxes.
Dexamethasone sensitivities in T-ALL cell lines

3.3. Association of NR3C1, CREBBP, and WHSC1 mutations with IC50 values of Dex and Pred.

We also analyzed the associations of the NR3C1, CREBBP, and WHSC1 mutations with IC50 values of both Dex and Pred. The NR3C1 mutation in T-ALL cell lines showed weak negative correlation with both IC50 values (Figure 4a, b). Among 22 T-ALL cell lines, only 3 cell lines were considered as Dex-sensitive (IC50 of Dex < 200 nM). As a result, all of the 5 NR3C1 mutated cell lines, 13 out of 14 CREBBP mutated cell lines, and all of the 6 WHSC1 mutated cell lines were resistant to Dex. Thus, most of T-ALL cell lines were resistant to Dex regardless of the NR3C1, CREBBP, and WHSC1 mutational status.

3.4. Association of the NR3C1 gene expression levels with GC sensitivities in BCP-ALL cell lines

We quantified the NR3C1 gene expression levels in 98 BCP-ALL cell lines except for HBL3 by RT-PCR with the TaqMan probe targeting at exons 4-5 that are commonly transcribed in all of the 4 splice variant forms of GR [53]. The NR3C1 gene expression levels in 98 BCP-ALL cell lines showed weak negative correlations with the IC50 values of both Dex (r² = 0.14, p = 0.0001) and Pred (r² = 0.15, p < 0.0001) (Fig. 4a, b).

Table 1
Characteristics of NR3C1 Mutations in BCP-ALL Cell Lines.

| Cell Lines | Mutations | Mutation Types | Amino Acid Changes | Amino Acid Changes | Frequency (%) |
|------------|-----------|----------------|-------------------|-------------------|---------------|
| HAL-O1     | c.1432C > G | Missense_Mutation | p.R474G           | p.Arg474Gly       | 43.61         |
| KB6        | c.1728T > C | Missense_Mutation | p.C576R           | p.Cys576Arg       | 48.89         |
| KCO3      | c.598C > T  | Missense_Mutation | p.T200M           | p.Thr200Met       | 80.40         |
| KOPN83     | c.624C > A  | Missense_Mutation | p.L208S           | p.Leu208Ser       | 49.72         |
| YAMN74     | c.366G > A  | Missense_Mutation | p.V122L           | p.Val122Leu       | 38.16         |

Table 2
Characteristics of CREBBP Mutations in BCP-ALL Cell Lines.

| Cell Lines | Mutations | Mutation Types | Amino Acid Changes | Amino Acid Changes | Frequency (%) |
|------------|-----------|----------------|-------------------|-------------------|---------------|
| HAL-O1     | c.6299 T > G | Missense_Mutation | p.F2100C          | p.Phe2100Cys      | 49.45         |
| KB6        | c.3718 T > C | Missense_Mutation | p.C1240R          | p.Cys1240Arg      | 56.3          |
| KCO3      | c.598C > T  | Missense_Mutation | p.Q200G           | p.Gln200Ter       | 67.61         |
| KOPN83     | c.624C > A  | Missense_Mutation | p.L208S           | p.Leu208Ser       | 48.89         |
| YAMN74     | c.366G > A  | Missense_Mutation | p.V122L           | p.Val122Leu       | 38.16         |

unmutated BCP-ALL cell lines.

3.3. Association of NR3C1, CREBBP, and WHSC1 mutations with Dexamethasone sensitivities in T-ALL cell lines

We also analyzed the associations of the NR3C1, CREBBP, and WHSC1 mutations with Dex sensitivity in 22 T-ALL cell lines. Among 22 T-ALL cell lines, mutations of the NR3C1, CREBBP, and WHSC1 genes were detectable in 5 (23 %), 14 (64 %), and 6 (27 %) cell lines, respectively (Supplement Tables 3–5). Three cell lines had mutations in the all of the three genes, and the other three cell lines had mutations in two of three genes. Of note, incidence of CREBBP mutation in T-ALL cell lines was significantly higher than that in BCP-ALL cell lines (p = 0.0006 in Fisher’s exact test). Although majority of T-ALL cell lines with information were established at relapse (12/13 cell lines), all NR3C1 mutated T-ALL cell lines with information were established at relapse (4/4 cell lines). Among 22 T-ALL cell lines, only 3 cell lines were considered as Dex-sensitive (IC50 of Dex < 200 nM). As a result, all of the 5 NR3C1 mutated cell lines, 13 out of 14 CREBBP mutated cell lines, and all of the 6 WHSC1 mutated cell lines were resistant to Dex. Thus, most of T-ALL cell lines were resistant to Dex regardless of the NR3C1, CREBBP, and WHSC1 mutational status.

3.4. Association of the NR3C1 gene expression levels with GC sensitivities in BCP-ALL cell lines

We quantified the NR3C1 gene expression levels in 98 BCP-ALL cell lines except for HBL3 by RT-PCR with the TaqMan probe targeting at exons 4-5 that are commonly transcribed in all of the 4 splice variant forms of GR [53]. The NR3C1 gene expression levels in 98 BCP-ALL cell lines showed weak negative correlations with the IC50 values of both Dex (r² = 0.14, p = 0.0001) and Pred (r² = 0.15, p < 0.0001) (Fig. 4a, b).
The NR3C1 gene expression levels in 50 Dex-sensitive cell lines (median relative expression level; 1.0) were significantly higher (p < 0.0001 in Mann–Whitney U test) than those in 48 Dex-resistant cell lines (0.59) (Fig. 4c). Similarly, the NR3C1 gene expression levels in 14 Pred-sensitive cell lines (median relative expression level; 1.01) were significantly higher (p < 0.0001 in Mann–Whitney U test) than those in 49 Pred-resistant cell lines (0.62) (Fig. 4d). The NR3C1 gene expression levels in 49 unmutated cell lines (0.86) (Fig. 4e) were significantly lower (p = 0.078 in Mann–Whitney U test) than those in 84 NR3C1 unmutated cell lines (0.86) (Fig. 4e). When focused on the 84 NR3C1 unmutated cell lines, the NR3C1 gene expression levels in 48 Dex-sensitive and 45 Pred-sensitive cell lines were significantly higher than those in the 36 Dex-resistant (p < 0.0001) and 39 Pred-resistant (p = 0.0001) cell lines, respectively (Fig. 4f, g). These results indicated that mutations and lower gene expression levels of the NR3C1 gene were independently associated with GC resistance in BCP-ALL cell lines.

Next, we evaluated the association of the WHSC1 and CREBBP gene mutations with the NR3C1 gene expression level. Interestingly, consistent with their relatively lower GC sensitivities, the NR3C1 gene expression levels in 20 WHSC1 mutated cell lines (median relative expression level; 0.55) tended to be lower (p = 0.078 in Mann–Whitney U test) than those in 84 NR3C1 unmutated cell lines (0.86) (Fig. 4e). When focused on the 84 NR3C1 unmutated cell lines, the NR3C1 gene expression levels in 48 Dex-sensitive and 45 Pred-sensitive cell lines were significantly higher than those in the 36 Dex-resistant (p < 0.0001) and 39 Pred-resistant (p = 0.0001) cell lines, respectively (Fig. 4f, g). These results indicated that mutations and lower gene expression levels of the NR3C1 gene were independently associated with GC resistance in BCP-ALL cell lines.

3.5. Establishment of GC-resistant sublines of BCP-ALL cell lines by disrupting the NR3C1 gene

Since most of the NR3C1 mutations (12/15; 80 %) observed in this study affected either the ligand binding or DNA binding domains (Fig. 1b), we tried to establish GC-resistant sublines of the GC-sensitive BCP-ALL cell line by disrupting ligand binding and DNA binding domains of the NR3C1 gene using the CRISPR/Cas9 genome editing system (Fig. 5a). We electroporated the plasmid containing both Cas9 cDNA and sgRNA targeting at the PAM site adjacent to codons 473–474 (hotspot mutation sites located in the middle of exon 4 that encodes the ligand binding domain) of the NR3C1 gene. We used the GC-sensitive 697 cell line (IC50 values of Dex and Pred; 3 nM and 3.6 nM, respectively), which has no mutation in the NR3C1, CREBBP, and WHSC1 genes. After 10-day expansion of the transfected cells in the absence of GCs, the cells were cultured in the presence of 90 nM of Pred (25 times higher concentration than the IC50). Following 7-day selection with Pred, the Pred-resistant subline was expanded. Sanger sequencing of genomic PCR products of the subline, which were subcloned into the TA-cloning system (Fig. 5a), revealed various insertion or deletion at the target site resulting in one to four amino acids insertion or frameshift mutations (Fig. 5b). The obtained subline showed a highly GC-resistant phenotype compared with its parental cell line; the IC50 values of Dex and Pred were > 250 nM and > 54.1 μM, respectively. When treated with 200 nM of Dex or 3.5 μM of Pred, more than 90 % of the parental cells underwent cell death. In contrast, no significant apoptotic cell death was induced in the obtained subline. These results demonstrated that 1–4 amino acid insertions and frameshifts in exon 4 adjacent to codons 473–474 induced GC resistance in the BCP-ALL cell lines.

4. Discussion

In the present study, we performed target exome sequencing of the NR3C1, CREBBP, and WHSC1 genes in 99 BCP-ALL cell lines, and found their mutations in 14, 24, and 20 cell lines, respectively. These mutations were not mutually exclusive: two cell lines had mutations in all of the three genes, and five cell lines had mutations in two of the three genes. Among 87 BCP-ALL cell lines with establishment information, mutations of the NR3C1, CREBBP, and WHSC1 genes were observed in 6 %, 31 %, and 19 % of the 32 cell lines established at diagnosis, whereas 18 %, 16 %, and 20 % of the 55 cell lines were established at relapse, respectively.

Thus, although statistically insignificant, the NR3C1 gene mutation was relatively more common in the cell lines established at relapse. In the previous study of paired clinical samples obtained at diagnosis and at relapse in 67 BCP-ALL cases, mutations of the NR3C1, CREBBP, and WHSC1 genes were observed in 4.5 %, 17.9 %, and 1.5 % of the samples at diagnosis compared to 9 %, 23.9 %, and 4.5 % of the samples at relapse, respectively [8]. Thus, the incidence of the CREBBP mutations in the BCP-ALL cell lines were almost similar to that in the clinical
samples. In contrast, incidence of the NR3C1 and WHSC1 mutations in the BCP-ALL cell lines seemed to be higher than that in the clinical samples. In particular, the WHSC1 mutation was observed approximately 10 times more commonly in the BCP-ALL cell lines than in the clinical samples, suggesting that the WHSC1 mutation may be somehow associated with growth advantage in vitro.

In the anti-leukemic activities of GC, GR plays a central role [11,12]. Indeed, in the present analysis of 98 BCP-ALL cell lines, lower NR3C1 gene expression level was significantly associated with GC resistance, as we previously reported in 72 BCP-ALL cell lines [53]. In addition to the lower NR3C1 gene expression level, the NR3C1 gene mutations were significantly associated with GC resistance, since the IC50 values of Dex
and Pred in 14 NR3C1 mutated cell lines were significantly higher than those in 84 NR3C1 unmutated cell lines. Consistent with the GC-resistance of the NR3C1 mutated cell lines in the present study, it has been reported that the introduction of mutated-types of the NR3C1 cDNA with expression vector rendered GC-resistance in the GC-sensitive ALL cell lines [18, 19]. In this context, it should be noted that the human NR3C1 gene has four splicing variants besides the functional isoform GRα [53]. Under these circumstances, previous analyses using expression vectors evaluated the mutated cDNAs of the main GRα isoform only. Importantly, in the present study, we confirmed the GC-resistance in the majority of NR3C1 mutated cell lines, in which various splicing isoforms of the NR3C1 gene are expressed [53]. Thus, this is the first direct confirmation regarding the impact of the NR3C1 gene mutation on the GC sensitivities of BCP-ALL in the intrinsic NR3C1 gene.

Among 14 NR3C1 mutated BCP-ALL cell lines, P30/OHK with C644R (IC50 of Dex and Pred; 5.2 nM and 7.9 nM, respectively) and THP5 with S377Lfs*5 (11.4 nM and 1.8 nM, respectively) were relatively sensitive to GCs. Of note, the majority (8 cell lines) of the 12 GC-resistant mutated cell lines had homozygous mutations while both P30/OHK (mutated sequences; 48.6 %) and THP5 (47.7 %) had the heterozygous mutation. Thus, wild-type GR is produced by an intact allele and may be involved in partial GC sensitivity of P30/OHK and THP5. In a protein function prediction tool (PROVEAN, http://provean.jcvi.org/) [55], C644R is predicted to be functionally deleterious (PROVEAN score; −10.3 < −2.5 cutoff value). Moreover, S377Lfs*5 is also deleterious due to a loss of the C-terminal domain of GR as a result of the frameshift mutation. Thus, heterodimers of the mutated and intact GR as well as homodimers of the mutated GR are likely to be non-functional in these two cell lines. In this context, other acceleration factors involving GC sensitivity may also contribute to partial GC sensitivity of these two cell lines in spite of the heterozygous mutation of the NR3C1 gene.

In contrast to the NR3C1 mutations, no statistically significant associations with GC-resistance were observed in BCP-ALL cell lines with the WHSC1 and CREBBP mutations. Since the NR3C1 mutation status was strongly associated with the GC-resistant phenotype, we further focused on the 84 NR3C1 unmutated cell lines. Of note, among 84 NR3C1 unmutated cell lines, the WHSC1 mutated cell lines tended to be more resistant to GC in comparison with the unmutated cell lines. Moreover, the NR3C1 gene expression levels in the WHSC1 mutated cell lines were significantly lower than those in the unmutated cell lines. These our observations in BCP-ALL cell lines were supported by a recent paper by Li J et al., in which introduction of WHSC1 mutation by genome editing into RCH-ACV cell line with the wild type WHSC1 sequence induced Dex-resistant phenotype, while revision of the WHSC1 mutation to wild type sequence by genome editing in the Dex-resistant ALL cell lines with the E1099 K mutation conferred Dex sensitivity [56]. Although there was no direct evidence for the association of WHSC1 and NR3C1 in GC resistance in clinical samples, recent report by

![Fig. 3. Association of the NR3C1 (a-b), WHSC1 (c-f), and CREBBP (g-j) gene mutations with the GC sensitivities in BCP-ALL cell lines. In panels (a), (b), (c), (d), (g), and (h), the IC50 values of Dex (a, c, and g) and Pred (b, d, and h) were compared between the cell lines with mutation (+) and those without mutation (-) in 98 BCP-ALL cell lines. In panels (e), (f), (i) and (j), the IC50 values of Dex (e and i) and Pred (f and j) were compared between the cell lines with mutation (+) and those without mutation (−) in the 84 NR3C1 unmutated cell lines. In each panel, vertical axis indicates log-scaled IC50 value of Dex or Pred and p-value in Mann-Whitney U test is indicated on the top.](image-url)
Li J et al. focused on this issue using the PDX model [56]. Consistent with the findings in ALL cell lines, Dex-treatment failed to promote survival of the mice inoculated with WHSC1 E1099 K mutated ALL samples, while it significantly promoted survival of the mice inoculated with ALL sample with wild type WHSC1. They also demonstrated that introduction of WHSC1 E1099 K mutation by genome editing into ALL cell line downregulated basal NR3C1 gene expression level, which was associated with the accumulation of H3K27me3-binding at the NR3C1 gene promoter. Consistently, in the present study, the NR3C1 gene expression levels in the 10 cell lines with the NR3C1 mutations (p = 0.081, Supplement Fig. 4). These results strongly suggested that WHSC1 is an upstream regulatory factor of the NR3C1 gene and that the WHSC1 mutation subsequently induces GC-resistance by downregulating NR3C1 gene expression level.

In contrast to the WHSC1 mutations, even when focused on the NR3C1 unmutated cell lines, we could not confirm the association of the mutation status of the CREBBP gene with the GC-sensitivities and the NR3C1 gene expression levels. Association of CREBBP mutations with GC-resistance was originally suggested by the findings that all of the five T-ALL cell lines with CREBBP mutations were resistant to Dex [29]. However, in this original report, three of four T-ALL cell lines without CREBBP mutation were also resistant to Dex. Consistently, in the present study, 13 out of 14 CREBBP mutated T-ALL cell lines were resistant to Dex, whereas 6 out of 8 CREBBP unmuted T-ALL cell lines were resistant to Dex. Thus, most of T-ALL cell lines were resistant to Dex regardless of their mutational status of the CREBBP gene. Accordingly, one background aspect for difference in the association of CREBBP mutations with GC sensitivity between previous observation [23] and present study might be difference between T-ALL cell lines and BCP-ALL cell lines.

Finally, we tried to evaluate the significance of the NR3C1 mutation in the GC-sensitivity by disrupting ligand binding and DNA binding domains of the NR3C1 gene using the CRISPR/Cas9 genome editing system in GC-sensitive cell line. We targeted the PAM site adjacent to codons 473–474 of the NR3C1 gene, and selected the GC-resistant sublines in the presence of Pred at a 25 times higher concentration than IC50. The obtained subline was confirmed to be various acquired in 1 cell line in the presence of Pred at a 25 times higher concentration than IC50. The obtained subline was confirmed to be various acquired in 1 cell line. We performed non-homologous end-joining, no common functional features were observed in terms of electrical charge or water solubility. These mutations were located at the C-terminal end of the zinc finger domain for dimerization [57] and the adjacent R477 residue plays a critical role in the GC transactivation activity [58]. Thus, these mutations may abrogate transactivation activity of the GC-GR complex by disrupting dimerization in the obtained GC-resistant subline.

In conclusion, our analyses provided direct evidence showing that the loss-of-functional mutations of the intrinsic NR3C1 gene induce GC resistance in BCP-ALL cell lines. Of clinical importance, the NR3C1 mutations were more commonly observed in the cell lines established at relapse than at diagnosis. These observations in BCP-ALL cell lines suggest that the acquisition of the loss-of-functional mutation of the
NR3C1 gene may render the GC-resistant phenotype and, subsequently, induce expansion of the mutated clone during chemotherapy (including GC therapy), as observed in our CRISPR-Cas9 mediated NR3C1 gene disruption model. Although further evaluations are required, our observations also suggest that the WHSC1 mutation may provide some growth advantages as well as the GC-resistant phenotype of BCP-ALL cells. In addition, these large series of BCP-ALL cell lines are useful tools to evaluate the involvement of somatic gene mutations in the drug-resistant phenotype of poor prognostic leukemia.

Author contribution

Minori Tamai: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, and Writing – original draft. Shin Kasai, Thao Nguyen Thu, Atsushi Watanabe, Koshi Akahane, and Kumiko Goi: Investigation. Keiko Kagami, Chiaki Komatsu, and Masako Abe: Formal analysis and Investigation. Kunio Miyake: Data curation and Methodology. Toshiya Inaba, Junko Takita, Hiroaki Goto, Masayoshi Minegishi, Shotaro Iwamoto, and Kanji Sugita: Providing leukemia cell lines. Takeshi Inukai: Conceptualization, Data curation, Funding acquisition, Project administration, and Writing – review and editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbm.2022.106068.

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