A Polymorphism in the Glucocorticoid Receptor Gene is Associated with Refractory Hypotension in Premature Infants

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Abstract

Background: Glucocorticoids play an important role in endocrine control. The association of glucocorticoid receptor (GR) gene polymorphisms with altered sensitivity to glucocorticoid therapy has been reported in adults. However, there are few such reports in infants. The present study analyzed the prevalence of four GR polymorphisms in preterm infants born before 30 weeks of gestation and determined the associations between these polymorphisms and clinical outcomes in the infants.

Methods: Totally, 41 preterm infants born at two hospitals in Fukushima were retrospectively screened for the presence of four GR gene polymorphisms, using a TaqMan single-nucleotide polymorphism genotyping assay. The effect of GR gene polymorphisms on clinical outcomes during hospitalization was evaluated. The following primary clinical outcomes were assessed: refractory hypotension in the acute phase and/or severe bronchopulmonary dysplasia, maximum dopamine and dobutamine doses administered, and total hydrocortisone dose administered in the first 48 h of life. Multivariate analysis with logistic regression was used to assess the association between clinical factors and refractory hypotension.

Results: Of the four GR polymorphisms, only the *Bcl*I polymorphism was detected. The genotype distribution was as follows: C/C, 33; C/G, 8; and G/G, 0 infants. Significant differences were observed between the C/C and C/G genotypes with respect to the following variables: refractory hypotension (6% *vs.* 50%), dopamine dose [3.0 (2.0–4.0) *vs.* 4.8 (4.0–7.5) μg/kg/min], dobutamine dose [2.4 (0.0–3.6) *vs*. 4.0 (0–10.0) μg/kg/min], and total hydrocortisone dose administered in the first 48 h of life [2.0 (0–10.0) *vs.* 6.0 (0–12.0) mg/kg]. Multivariate analysis showed that the *Bcl*I genotype (C/C) was significantly less associated with refractory hypotension in the acute phase (odds ratio, 0.008; 95% confidence interval, 0.000–0.371; p = 0.013).

Conclusion: The incidence of refractory hypotension in infants with the C/C genotype was

initially expected to be higher than that in infants with the C/G genotype. However, the results of this study were rather different from what we originally expected. The suppressive effect of antenatal steroid use on the HPA axis of the preterm infants with the *Bcl*I variant may be associated with refractory hypotension in the acute phase.

Key words: *Bcl*I polymorphism; refractory hypotension; single-nucleotide polymorphisms

Introduction

Glucocorticoids (GCs) play an important role in endocrine control with regard to homeostasis, immune function, and cell growth and differentiation.[1] They are used to treat hypotension, bronchopulmonary dysplasia (BPD), and sepsis in premature infants. At the cellular level, the action of GCs is mediated by the intracellular glucocorticoid receptor (GR).[2] The GR, a member of the steroid hormone receptor family and the ligand-regulated nuclear receptor superfamily, is involved in the positive or negative regulation of the expression of GC-responsive genes.

The sensitivity of GRs to GCs can vary significantly among individuals,[3] and variability in the sensitivity to hydrocortisone therapy has been linked to several GR gene polymorphisms.[4] The following polymorphisms have been detected in the human GR-encoding gene: *N363S*, *Bcl*I, *R23K*, and *GR-9β*. The *N363S* polymorphism is associated with a high sensitivity to GCs *in vivo*, as demonstrated by the increased cortisol suppression observed using a 0.25-mg dexamethasone suppression test.[5] *Bcl*I polymorphism is associated with an increased sensitivity to GCs.[6] Conversely, *R23K* and *GR-9β* polymorphisms are associated with a relative resistance to GCs. Notably, *R23K* harbors two linked single-nucleotide polymorphisms (SNPs) in codons 22 and 23 of exon 2 in the human GR gene.

Several reports have described the effects of GR polymorphisms on clinical outcomes in adults; however, there are few such reports in infants.[7-9] We hypothesized that GR polymorphisms could affect clinical outcomes in preterm infants.

The present study analyzed the prevalence of four GR polymorphisms in preterm infants born before 30 weeks of gestation and determined the associations between these polymorphisms and clinical outcomes.

Methods

Study subjects

We enrolled infants born at 23–29 weeks of gestation to Japanese parents, between October 2010 and July 2013. Infants with major congenital defects were excluded from the study. The Ethics Committees of Fukushima Medical University School of Medicine and National Hospital Organization Fukushima National Hospital approved the study. All mothers provided written informed consent for the inclusion of their infants.

Definitions

Refractory hypotension in the acute phase was defined as a mean arterial pressure (mmHg) that was lower than the numerical value of the gestational age (weeks) of an infant[10] despite optimal management, including volume expansion, inotrope administration, and the administration of a single dose of hydrocortisone (2 mg/kg),[11, 12] in the first 48 h of life. Until the resolution of hypotension, infants were sequentially treated with the following therapies: (1) volume replacement (10 mL/kg of 0.9% saline), (2) dopamine administration (dose increased by 2–3 μg/kg/min every 30 min up to a maximum dose of 5–10 μg/kg/min), (3) dobutamine administration when the infant did not achieve the target blood pressure with only dopamine use, and (4) administration of a single dose of hydrocortisone (2 mg/kg).

BPD was diagnosed if oxygen therapy was administered for ≥28 days.[13] Severe BPD was diagnosed if an infant required positive airway pressure ventilation at 36 weeks' postmenstrual age or at discharge, whichever was earlier. A physiological test developed by Walsh *et al* was used to confirm oxygen requirement at the time of assessment.[14]

Analysis of gene polymorphisms

The following previously investigated GR gene polymorphisms were selected because of their reported association with an altered sensitivity to GCs: *N363S* (rs56149945), *Bcl*I

(rs41423247), *R23K* (rs6189), and *GR-9β* (rs6198). Blood samples were drawn immediately after birth from either the umbilical cord, a vein, or an artery. Genomic DNA was extracted from blood samples using the QIAamp® DNA Blood kit (Qiagen, Courtaboeuf, France). Genotypes were determined using a Custom TaqMan® SNP assay (Applied Biosystems, Foster City, CA, USA), which allowed custom allelic discrimination and included specific primers and corresponding probes for each SNP. Assay design was performed using the Custom TaqMan® SNP assay design tool (Applied Biosystems). *N363S*, *Bcl*I, and *R23K* genotyping was performed using the resulting Custom TaqMan® SNP genotyping assay (Applied Biosystems). *GR-9β* was assayed using the standard TaqMan® SNP genotyping assay [assay ID (SNP ID), hCV8951023]. The sequences of the primers and probes used for evaluating the GR gene polymorphisms (*N363S*, *Bcl*I, *R23K*, and *GR-9β*) are shown in Table 1. The polymerase chain reaction conditions and cycling parameters were set according to protocols described by the manufacturer. Allelic discrimination with endpoint measurements was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems). The results were analyzed using Sequence Detection Software, version 1.4 (Applied Biosystems).

Data collection

Both maternal and infant data, including demographic, birth history, laboratory, clinical, and postnatal complication-related data, were retrospectively collected from medical records. The Clinical Risk Index for Babies II score, a risk adjustment instrument, was used to evaluate preterm infants.[15] The postnatal complications included refractory hypotension, administration of maximum dopamine and dobutamine doses, hydrocortisone use, respiratory distress syndrome, severe BPD, duration of mechanical ventilation, duration of supplemental oxygen, intestinal perforation, necrotizing enterocolitis, hyperglycemia, intraventricular

hemorrhage (IVH), cystic periventricular leukomalacia (PVL), sepsis, and death. IVH was graded according to the criteria defined by Papile *et al*.[16]

Statistical analysis

Descriptive statistics are expressed as median (interquartile range) or frequency (percentage). The C/C, C/G, and G/G genotypes of *Bcl*I were assessed. Comparisons of the clinical characteristics and outcomes between the C/C and C/G genotypes were performed using the Mann–Whitney *U* test, Student's *t* test, or Fisher's exact probability test, as appropriate. The Mann–Whitney *U* test and *t* test were used to assess the between-group differences with respect to continuous variables. The Mann–Whitney *U* test was selected in case of non-normal distributions in the Shapiro–Wilk test. Fisher's exact test was used for categorical variables. Multivariate analysis with logistic regression was used to assess the association between clinical factors and refractory hypotension in the acute phase; $p < 0.05$ was considered to indicate a significant difference. All statistical analyses were performed using Dr. SPSS II (SPSS Inc., Chicago, IL, USA).

Results

43 infants were born during the study period and met the inclusion criteria. However, we excluded two infants with twin-twin transfusion syndrome. The remaining 41 preterm infants were included in the analyses. DNA samples from all the 41 infants were successfully subjected to *N363S*, *Bcl*I, *R23K*, and *GR-9β* SNP genotyping. No SNPs of *N363S*, *R23K*, and *GR-9β* were detected. The genotype distribution of the *Bcl*I polymorphism was as follows: C/C in 33 infants; C/G, 8; and G/G, 0; this result is similar to that reported in Japanese adults (Figure 1).[17]

Table 2 shows the clinical characteristics of the infants in our study. Notably, no differences were observed between infants with the C/C genotype and those with the C/G genotype. The clinical outcomes are detailed in Table 3. Refractory hypotension was significantly less common among infants with the C/C genotype than among those with the C/G genotype (6% *vs*. 50%, p = 0.009). In addition, the maximum dopamine dose was significantly lower among infants with the C/C genotype than among those with the C/G genotype [3.0 (2.0–4.0) μg/kg/min *vs.* 4.8 (4.0–7.5) μg/kg/min, p = 0.001], and similar results were observed for the maximum dobutamine dose [2.4 (0–3.6) μg/kg/min *vs.* 5.0 (2.5–7.5) μg/kg/min, p = 0.003]. Moreover, the total hydrocortisone dose administered in the first 48 h of life was significantly lower among infants with the C/C genotype than among those with the C/G genotype [2.0 (0–10.0) *vs.* 6.0 (0–12.0) mg/kg]. However, the *Bcl*I genotype did not affect the duration of mechanical ventilation, prevalence of respiratory distress, development of severe BPD, duration of supplemental oxygen, or occurrence of sepsis, severe IVH, PVL, hyperglycemia, intestinal perforation, necrotizing enterocolitis, and death.

Table 4 shows the clinical characteristics and outcomes in relation to refractory hypotension in the acute phase. The *Bcl*I genotype (C/C) was significantly associated with refractory hypotension (refractory hypotension *vs*. no refractory hypotension: 33% *vs*. 89%, p = 0.009).

Factors with $p < 0.15$ were *Bcl*I genotype (C/C) and birth weight ($p = 0.13$). Multivariate analysis with logistic regression showed that the *Bcl*I genotype (C/C) was significantly associated with refractory hypotension in the acute phase among preterm infants (odds ratio, 0.008; 95% confidence interval, 0.00–0.371; $p = 0.013$) (Table 5).

Discussion

Among the evaluated polymorphisms, only the C/C and C/G genotypes of the *Bcl*I polymorphism were detected in this study. Notably, the incidence of refractory hypotension, maximum dopamine and dobutamine doses used, and total hydrocortisone dose administered in the first 48 h of life were all significantly lower among infants with the C/C genotype than among those with the C/G genotype of *Bcl*I. Furthermore, multivariate analysis showed that the C/C genotype was significantly less associated with refractory hypotension in the acute phase. To our knowledge, this is the first study to demonstrate an association between *Bcl*I polymorphism in the GR gene and refractory hypotension in the acute phase among preterm infants. The G allele of *Bcl*I is associated with an increased sensitivity to GCs.[6] Hence, the incidence of refractory hypotension in infants with the C/C genotype was initially expected to be higher than that in infants with the C/G genotype. However, the results of this study were different from what we originally expected.

GCs are administered to very-low-birth-weight infants with hypotension, BPD, or hypoglycemia during postnatal periods. In some clinical cases, low hydrocortisone doses are needed to achieve adequate blood pressure, whereas other cases require higher total hydrocortisone doses. Notably, several SNPs in the GR gene may contribute to these differences in GC sensitivity. Therefore, we hypothesized that differences in GR polymorphisms would affect the sensitivity to GCs and consequently the clinical course of preterm infants. A previous study reported that GC sensitivity for the G allele was greater than that for the C allele of *Bcl*I,[6] which is consistent with our findings regarding this SNP.

In preterm infants, adrenal insufficiency was shown to be partially responsible for hypotension in the immediate postnatal period.[18, 19] For example, Ng *et al* reported that a stress hydrocortisone dose (1 mg/kg every 8 h for 5 days) is an effective rescue treatment for refractory hypotension.[19] Notably, some infants in this study required larger amounts of

hydrocortisone or vasopressin.

The relevant mutation at this polymorphic site (i.e., C to G substitution at intron 2) does not involve a coding, regulatory, or splicing part of the GR gene. However, Manenschijn *et al* suggested that this polymorphism could be related to the promoter region or to other functionally important polymorphisms.[20] The C>G polymorphism of *Bcl*I was shown to be associated with an increased sensitivity to and a higher level of cortisol, which resulted in the excessive retention of sodium ions and water in blood vessels, thus causing hypertension.[21] Srivastava *et al* reported that blood pressure levels were significantly higher in adult G allele carriers of *Bcl*I than in non-carriers.[22] Furthermore, this *Bcl*I *g*ene polymorphism inhibits prostaglandin and nitric oxide vasodilator production in endothelial cells, enhances smooth muscle contraction, and ultimately causes hypertension.[23] Although refractory hypotension was originally expected to be more common among infants with the C/C genotype than among those with the C/G genotype, the results of this study were different. The maximum dopamine and dobutamine doses were significantly lower among infants with the C/C genotype than among those with the C/G genotype. Schreiner also reported that in a large multicenter cohort of very-low-birth-weight preterm infants, the use of inotropes tended to be lower among infants who were carriers of the *Bcl*I variant (G/G, C/G) than among those with the C/C genotype ($p = 0.052$).[9]

Three of four refractory hypotension infants with the C/G genotype received antenatal

steroids in our study. The use of antenatal steroids induces the suppression of fetal hypothalamo–pituitary–adrenal (HPA) axis activity and reduces the cortisol levels at birth.[24] Furthermore, the G allele of *Bcl*I is associated with a high sensitivity to GCs, as demonstrated by the increased cortisol suppression after a 1-mg dexamethasone suppression test. According to these reports, the suppressive effect of antenatal steroids on the HPA axis of infants with the C/G genotype may induce refractory hypotension in the acute phase.

This study has several limitations. First, the sample size was too small to allow for an evaluation of the differences in GR SNPs. The incidences of BPD, PVL, IVH, and death did not significantly differ between the genotype groups. Notably, one of four infants with refractory hypotension in the C/G group died because we were unable to maintain adequate blood pressure, despite the administration of several doses of hydrocortisone and vasopressin. A study with a larger sample size will provide more accurate evidence. Therefore, we have continued to prospectively enroll more subjects to confirm whether the GR polymorphism status affects refractory hypotension in the acute phase. We believe that cohort studies are needed to investigate this topic further. The present study showed that the incidence of acute refractory hypotension in the C/G group was higher than that in the C/C group. There are reports that metabolic syndrome and hypertension were more in C/G and G/G genotype than C/C genotype in studies for adults.[4,6] In other words, premature infants with G carriers could have a tendency to be hypotensive in the neonatal period, but on the contrary, adults could

have a tendency to be hypertensive. We think that it would be meaningful to examine the influence of the SNP of *Bcl*I on the body in a cohort study from the neonatal period to adolescents. Second, we did not perform a detailed analysis of steroid hormones, including cortisol. However, we believe that the measurement of serum steroid hormone levels is necessary to elucidate the pathology of refractory hypotension and its association with GR polymorphisms. Third, there was also great variations in the observation period. The observation period of this study was duration of hospitalization at NICU. The gestational age of patients were from 23 to 29 weeks. A patient born in 29 weeks was hospitalized for the shortest duration of 63 days, while other patient born in 23 weeks was hospitalized for the longest duration of 296 days. If the neonate is more premature, the prematurity of the organ at birth is generally severer. The dose of the medicine would be higher and duration of ventilation and hospitalization would become longer. It is important to consider for the variation of gestational age in subsequent studies.

We noted that the total amount of administered hydrocortisone did not significantly differ between the C/C and C/G groups, although hydrocortisone use for BPD increased in the C/C group after the first week. However, the total hydrocortisone dose was not significantly different between the two groups. According to a Cochrane database systematic review, late (after the first week) postnatal corticosteroid therapy reduced the incidence of chronic lung disease at the postmenstrual ages of 28 days and 36 weeks.[25] Although this systematic review

included 21 randomized trials involving dexamethasone, only one trial investigated hydrocortisone.[26] The review revealed no significant short-term beneficial effects of hydrocortisone after the age of 1 week in extremely-low-birth-weight infants. Given that the causes of BPD are multifocal and characterized by interference with alveolar and vascular development,[12] mechanical ventilation, oxygen supplementation, inflammation, and genetic polymorphisms could all increase the risk of BPD development; therefore, the effects of GR SNPs may not be significant.

In conclusion, we detected only *Bcl*I polymorphism (C/C and C/G genotypes) of the GR gene in this study. Although the frequencies of the C/C and C/G genotypes varied with respect to ethnicity, the incidence of refractory hypotension was expected to remain significantly lower among infants with the C/C genotype than among those with the C/G genotype, as observed in this study. We believe that an association exists between the *Bcl*I polymorphism and the incidence of refractory hypotension in preterm infants born before 30 weeks of gestation. The suppressive effect of antenatal steroid use on the HPA axis of the preterm infants with the *Bcl*I variant may be associated with refractory hypotension in the acute phase.

Conflicts of Interest Statement

The authors declare no conflicts of interest associated with this manuscript.

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Figure legends

Figure 1. 43 infants were born during the study period and met the inclusion criteria. However, we excluded two infants with twin-twin transfusion syndrome. The remaining 41 preterm infants were included in this study. These 41 infants were divided into two groups according to the *Bcl*I genotype [C/C group (n = 33) and C/G group (n = 8)]. The genotype distribution of the *Bcl*I polymorphism was as follows: C/C in 33 infants; C/G, 8; and G/G, 0; this result is similar to the distribution reported in Japanese adults.

Fig. 1

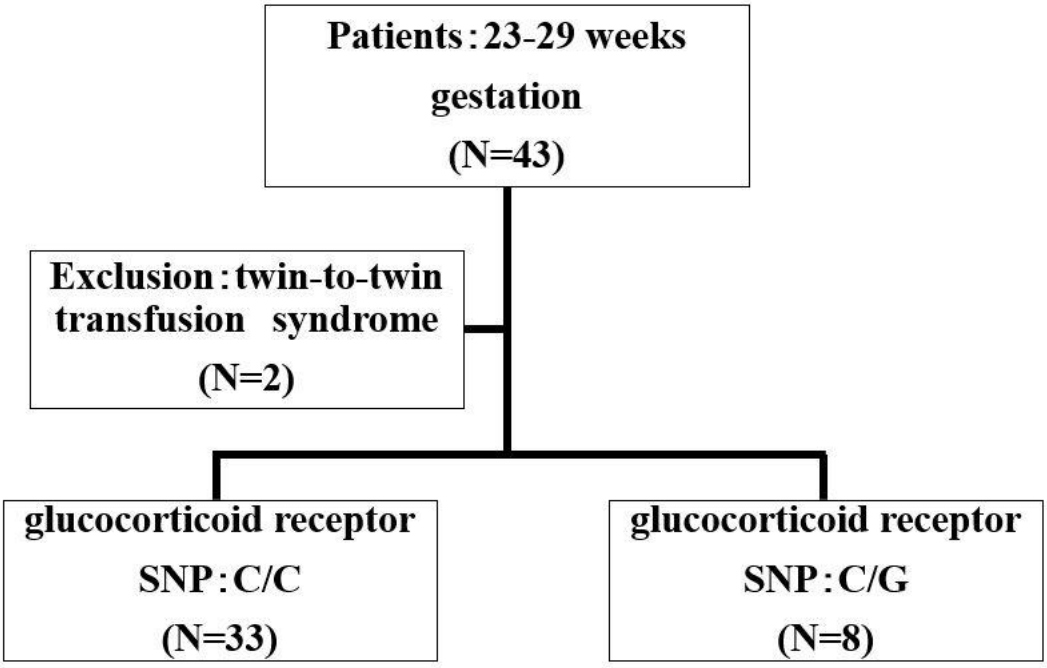
Patients : 23-29 weeks gestation (N=43)
Exclusion : twin-to-twin transfusion syndrome (N=2)
glucocorticoid receptor SNP : C/C (N=33)
glucocorticoid receptor SNP : C/G (N=8)

Table 1. Genotyping approch for polymorphism analysis of the GR gene.

Genotype	Reference SNP number	Primer/Probe	Sequence(5'-3')
N363S	rs56149945	Forword Primer	ATCTCCAGATCCTTGGCACCTA
		Reverse Primer	TGAATACAGCATCCCTTTCTCAACAG
		VIC-MGB	TGGTTCCGAAAGTTGGA
		FAM-MGB	TGGTTCCGAAAATTGGA
BCII	rs41423247	Forword Primer	CCATGTTGACACCAATTCCTCTCTT
		Reverse Primer	GGTCTTGCTCACAGGGTTCTTG
		VIC-MGB	TGCTGATCAATCTCT
		FAM-MGB	TGCTGATGAATCTCT
R23K	rs6189	Forword Primer	CAGTAGCTCCTCCTCTTAGGGTTT
		Reverse Primer	AGAAGAAAACCCCAGCAGTGT
		VIC-MGB	CCCCTTTCCTGAGAGCAA
		FAM-MGB	CCCCTCTCCTGAGAGCAA
GR-9β	rs6198	Forword Primer	*
		Reverse Primer	*
		VIC-MGB	GTAATACCAGAACAGCAAATTTAAACGAAAAAATAAAAGTTAAACATTTCC
		FAM-MGB	GTAATACCAGAACAGCAAATTTAAATGAAAAAATAAAAGTTAAACATTTCC

*The primer sequences were not supplied by Applied Biosystem.

Table 2 Clinical characteristics of the patients vs BclI genotype.

	C/C genotype (N=33)	C/G genotype (N=8)	p
Gestational age (weeks)	26.1 (23.0-29.9)	27.0 (23.9-28.9)	0.86**
Birth weight (g)	872 (686-1014)	1024(758-1046)	0.51**
Male	13(39)	5(62)	0.22†
C-section	30(91)	8(100)	0.51†
Apgar 1 minute	3(2-4)	4(1-4)	0.63*
Apgar 5 minutes	6(4-7)	7(4-7)	0.45†
Umbilical arterial cord blood pH	7.36(7.30-7.41)	7.30(7.26-7.32)	0.16*
CRIB II	10 (8－12)	9.5 (7－12.5)	0.74**
Maternal factors			
Pregnancy-induced hypertension	5(15)	1(13)	0.67†
Antenatal steroid	16(48)	5(63)	0.38†
Chorioamnionitis	15(71)	5(63)	>0.99†

Results are median (interquartile range) or n(%). All of the clinical characteristics described in the table were not significantly different between the two groups.

The record for chorioamnionitis of 12 patients in G/G genotype were unknown.

* Mann-Whitney U test.

** t-test.

† Fisher's exact probability test.

Table 3 Clinical outcomes vs BclI genotype.

	C/C genotype (N=33)	C/G genotype (N=8)	p
Refractory hypotension in acute phase	2(6%)	4(50%)	<0.001[a†]
The maximam dose of DOA (μg/kg/min)	3.0(2.0-4.0)	4.8(4.0-7.5)	0.001[a]*
The maximam dose of DOB (μg/kg/min)	2.4(0-3.6)	5.0(2.5-7.5)	0.01[a]**
The total amount of HDC in the first 48 hours (mg/kg)	2.0(0-10.0)	6.0(0-12.0)	0.02[a]*
The total amount of HDC in the hospital (mg/kg)	74.5(2.2-101.8)	70.6(38.3-78.8)	0.69*
RDS	32(97%)	8(100%)	0.81†
Severe BPD	22(67%)	3(38%)	0.13†
Duration of ventilation (days)	46(36-66)	34(21-49)	0.18*
Duration of supplemental oxygen (days)	73(57-90)	59(23-82)	0.22*
Sepsis	1(3%)	1(13%)	0.36†
IVH≧grade III	1(3%)	1(13%)	0.36†
PVL	1(3%)	0(0%)	0.81†
Intestinal perforation	2(6%)	0(0%)	0.64†
Necrotizing entrocllitis	0(0%)	0(0%)	—†
Hyperglycemia (≧180mg/dl)	13(39%)	3(38%)	0.63†
Death	0(0%)	1(13%)	0.21†

Results are median (interquartile range) or n (%).

[a] Statistically significant results.

* Mann-Whitney U test.

** t-test.

† Fisher's exact probability test.

Table 4 Clinical characteristics and outcomes of the patients in relation to refractory hypotension in acute phase.

	Refractory hypotension in acute phase (N=6)	No refractory hypotension in acute phase (N=35)	p
Gestational age (weeks)	25.0(23.8-26.9)	26.6(24.1-28.4)	0.17*
Birth weight (g)	707 (632-1005)	922(746-1104)	0.13**
Male	4(67)	14(40)	0.22†
C-section	6(100)	32(91)	0.61†
Apgar 1 minute	2.5(2-4)	3(2-4)	0.87*
Apgar 5 minutes	6(4.5-7)	6(4-7)	0.86**
Umbilical arterial cord blood pH	7.31(7.30-7.38)	7.40(7.29-7.42)	0.48*
CRIB II	11.5（10.3－13.5）	9.0（7－12）	0.17**
Maternal factors			
Pregnancy-induced hypertension	0(0)	6(17)	0.36†
Antenatal steroid	4(67)	17(49)	0.36†
Chorioamnionitis	5(83)	15(65)	0.38†
BclI genotype (C/C)	2(33)	31(89)	0.009[a]†
RDS	6(100)	34(97)	0.85†
Sepsis in the first 48 hours	0(0)	1(3)	0.85†

Results are median (interquartile range) or n(%).

The record for chorioamnionitis of 12 patiens in No refractory hypotensionwere in acute phase were unknown.

[a] Statistically significant results.

* Mann-Whitney U test.

** t-test.

† Fisher's exact probability test.

Table 5 Factors asociated with refractory hypotension in acute phase in preterm infants.

	OR (95% CI)	p
Birth weight (g)	0.991 (0.981-1.000)	0.054
BclI genotype (C/C)	0.008 (0.000-0.371)	0.013

OR= odds ratio; CI= confidence interval