Polymorphisms of the Factor VII Gene Associated with the Low Activities of Vitamin K-Dependent Coagulation Factors in One-Month-Old Infants

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ITO, K., GOTO, K., SUGIURA, T., MURAMATSU, K., ANDO, T., MANIWA, H., YOKOYAMA, T., SUGIYAMA, K. and TOGARI, H. Polymorphisms of the Factor VII Gene Associated with the Low Activities of Vitamin K-Dependent Coagulation Factors in One-Month-Old Infants. Tohoku J. Exp. Med., 2007, 211 (1), 1-8 —— Despite administration of vitamin K (VK), some infants show lower activity of VK-dependent coagulation factors and they could develop intracranial hemorrhage. For preventing VK deficiency bleeding (VKDB) in infants, oral administration of VK and a screening test for VK deficiency are carried out in Japan. For the screening, the total activity of VK-dependent coagulation factors is measured using a commercial product, Normotest®. This study was undertaken to clarify the importance of the following genetic and environmental factors on the coagulation status in one-month-old infants: two polymorphisms in the factor VII gene, -323P0/10 (a 10-bp insertion in the promoter region at position -323) and R353Q (the replacement of arginine [R] with glutamine [Q] at residue 353) and sex, age, gestational age, birth weight, and feeding regimen. Two hundred Japanese infants (34.6 ± 4.0 days old) were screened for VK-dependent coagulation activity with Normotest and were genotyped for the two polymorphisms. Among the subjects screened, 18 infants (9%) carried the P10 allele and 26 (13%) carried the R353Q allele. Multiple regression analysis showed that the 10-bp inserted (P10) allele or the Q allele was associated with the lower coagulation activities. The coagulation activities for the R/Q genotype were significantly lower than those for the R/R genotype and those for the P0/P10 genotype were significantly lower than those for the P0/P0 genotype. Therefore, infants who carry the P10 allele or the Q allele show lower activity of VK-dependent coagulation factors. These infants may have a higher risk of VKDB manifestation. —— Normotest; factor VII polymorphisms; vitamin K; cycling probe; infants

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For the prevention of vitamin K deficiency bleeding (VKDB) in Japan, a protocol of three oral administrations of vitamin K (VK), 2 mg each, to all full-term newborns has been recommended; these are given on the day of birth, upon discharge from the maternity hospital, and at one month of age. The third administration of VK may be omitted if the deficiency is not detected at one month by the test of the total activity of VK-dependent coagulation factors II, VII, and X, using a commercial product called Normotest® (in a hepaplastin test) (Hanawa et al. 1988, 1990). Normotest is a combined prothrombin time reagent, which is lyophilized and contains rabbit brain thromboplastin and adsorbed bovine plasma. The administration at one month of age was set because the peak incidence of VKDB occurs at this age and because a routine health check at this age is widely performed in Japan. Some studies showed that the prophylactic effect of VK administered orally at birth cannot be maintained until one month of age, which supports the protocol of three oral administrations of VK (Motohara et al. 1987; Takahashi 1987).

Recently, several insertion-deletion and single-point mutations in the factor VII gene have been reported in the promoter region (e.g., -323P0/10 [a 10-bp insertion in the promoter region at position -323]) and within the protein-coding region (e.g., R353Q point mutation [the replacement of arginine with glutamine at residue 353]); those polymorphisms are known to affect either the function of the protein product or its level of expression (Green et al. 1991; Marchetti et al. 1993). In addition, the risk of spontaneous intracranial hemorrhage has been suggested to be linked to the factor VII polymorphism -323P0/10 in adults (Corral et al. 2001).

Although intracranial hemorrhage in newborns and infants may also be related to the factor VII polymorphisms, this relationship has not been well documented. Therefore, for further understanding of the causes of hemorrhagic disease in infants, we planned this study to determine the influence of the following genetic and environmental factors on coagulation status in one-month-old infants: two polymorphisms in the gene of factor VII, -323P0/10 and R353Q; sex; age; gestational age; birth weight; and feeding regimen.

**Materials and Methods**

**Subjects**

Study subjects were 200 Japanese infants (103 boys and 97 girls) aged 27 to 53 days (34.6 ± 4.0 days, mean ± s.d.) who visited Nishio Municipal Hospital from September 2003 to September 2005 for one-month examinations. All of them had been given 2 mg of vitamin K2 syrup (Kay Two Syrup®, Eisai Co., Ltd., Tokyo) orally at both birth and four days of age in the hospital. Infants with underlying diseases that could cause secondary VKDB, such as congenital biliary atresia or other apparent biliary tract diseases, etiologically proved hepatitis, severe diarrhea, or long-term antibiotic therapy, were excluded from the study. Normotest was carried out with capillary tubes. A 2-mg oral dose of vitamin K2 syrup was given to infants whose test values were below 80%, which has been defined empirically as the cut-off point. One week after the VK administration, Normotest was repeated. Normotest and therapeutic administration of 2 mg of VK were repeated at weekly intervals until the test value rose above 80%.

The experimental procedures were explained to all subjects’ parents and their written informed consent was obtained. The Ethics Committees of Nagoya City University and Nishio Municipal Hospital approved the study.

**Definition of feeding regimens**

The parents of the one-month old infants were asked: “What kind of milk did your child drink during the first month of life?” The infant was categorized as “breast-fed” if parents answered “Almost always human milk”. The infant was categorized as “formula-fed” if parents answered “Almost always formula”. Otherwise, the infant was categorized as “mixed-fed”.

**Blood sampling and Normotest**

Blood samples were taken from the dorsum of the hands by venopuncture, drawn into capillary tubes for Normotest, and the remnants were collected into ethylenediaminetetraacetic acid tubes for extraction of genomic DNA. All of the samples (about 1 ml) were collected at the same time to minimize the pain to the study subjects. Normotest was carried out soon after sampling by using Fukugo-Innshi H® (Kokusai-shiyaku,
After the blood samples were mixed with the reagent, the clotting time was measured. Reference correlation curves had been prepared by dilutions of standard plasma and the values of Normotest (%) were obtained.

Detection of the -323P0/10 polymorphism

DNA was purified from whole blood using a QIAamp DNA Blood Mini Kit® (QIAGEN, Tokyo). As described earlier (de Maat et al. 1997), genotyping for the -323P0/10 polymorphism was performed using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) with the forward primer 5′-GGCTGGTGCTGGACGCTCTCTC-3′ and the reverse primer 5′-GACGCGACGTTTTGTTGCCA GCG-3′. Amplification was carried out for 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The PCR products were digested with the restriction enzyme Sty I (TaKaRa Bio Inc., Shiga) and separated by electrophoresis on a 2% agarose gel. The allele with the restriction site and the noncleavable allele were designated P10 (10-bp inserted) and P0 (wild), respectively.

Detection of R353Q polymorphism

Genotyping for the R353Q polymorphism was performed by Cycling Probe Technology (CycleavePCR® Core Kit, TaKaRa Bio Inc.). Primers and cycling probes were designed to detect the R353Q. The probe for the detection of the R allele (5′-CCGGGAGCACG-3′) was 5′-labeled with reporter dye 6-carboxyfluorescein (FAM) and 3′-labeled with a quencher. The probe for the detection of the Q allele (5′-CAGGGACGCTCTC-3′) was 5′-labeled with reporter dye 6-carboxyrhodamine (ROX) and 3′-labeled with a quencher. The underlined nucleotides indicate the position of the RNA. The forward primer 5′-GGCCTGGTGCTGGACGCTCTC-3′ and the reverse primer 5′-GACGCGACGTTTTGTTGCCA GCG-3′ were used for amplification. All reagents for real-time PCR were obtained from a CycleavePCR® Core Kit. The amplification was carried out in a total volume of 25 μl. The reaction mixture contained 1 × CycleavePCR Buffer, 3 mM Mg²⁺, 78 μM of each dNTP, 0.3 μM of each primer, 0.2 μM of each probe, 100 U of Tli RNaseH II, and 1.25 U of TaKaRa Ex Taq HS. Cycling conditions were as follows: after holding at 95°C for 10 sec, 60 cycles of denaturation at 95°C for 5 sec, primer annealing at 65°C for 15 sec, and elongation at 72°C for 20 sec. Fluorescence was measured at 72°C.

![Fig. 1. FAM and ROX signals detected during the PCR reaction.](image)

The x-axis indicates the PCR cycle number; the y-axis indicates the fluorescence intensity. The simple curve represents fluorescence of FAM. The dotted curve represents fluorescence of ROX.

A: Results with R/R genotype. R/R genotype released FAM signal, and no ROX signal was observed. B: Results with Q/Q genotype. Q/Q genotype released ROX signal, and no FAM signal was observed. C: Results with R/Q genotype. R/Q genotype released both FAM and ROX signals.

Amplification by PCR, endpoint reading of fluorescence, and analysis of the data were performed with the Smart Cycler System (Cepheid, Sunnyvale, CA, USA) (Fig. 1).

**Statistical analysis**

Multiple regression analysis was used to investigate the influence of the independent variables of sex, body
weight, birth weight, gestational age, feeding regimen, and two factor VII alleles Q and P10 on the dependent variable, Normotest value. Normotest values were compared among the different genotypes of the -323P0/10 and R353Q polymorphisms, and among different feeding regimen groups by the Mann-Whitney’s U-test. Differences in qualitative variables between different genotype groups were assessed for statistical significance with the \( t \)-test. Qualitative data were analyzed with the \( \chi^2 \) test. All statistical analyses were performed with SPSS statistical software (version 13.0J, SPSS, Chicago, IL, USA).

**RESULTS**

**Characteristics of the subjects**

Subjects consisted of 200 Japanese infants (days 27-53). Backgrounds of the subjects are shown in Table 1. The mean and standard deviation of the alanine aminotransferase (ALT) levels of subjects were 18.5 ± 6.3 Karmen Units; thus, no obvious manifestation of hepatic dysfunctions was observed among them.

**Multiple regression analysis**

Of the genetic and environmental factors subjected to multivariate regression models using the Normotest value as the dependent variable, only P10 (\( p < 0.001 \)) or R353Q (\( p < 0.01 \)) was significantly associated with the Normotest value (Table 2).

**Frequency of Q and P10 alleles, and the different combinations of the alleles, and the lowering effect on Normotest values**

The frequency of the Q allele was 0.07, and the mean and s.d. of Normotest values for R/R, R/Q, and Q/Q genotypes were 91.2 ± 14.9%, 81.0 ± 14.1%, and 76%, respectively. The Normotest values for the R/Q genotype were significantly lower than that for the R/R genotype (\( p < 0.01 \)).

The frequency of the P10 allele was 0.05, and the Normotest values for P0/P0, P0/P10, and P10/P10 genotypes were 91.2 ± 14.7%, 75.9 ± 12.5%, and 76%, respectively. The Normotest value for the P0/P10 genotype was significantly lower than that for the P0/P0 genotype (\( p < 0.001 \)). There was only one case of the P10/P10 genotype, and it was the same case as the Q/Q genotype. No significant differences were observed in background factors (sex, body weight, birth weight, gestational age, feeding regimen, and ALT) between R/R and R/Q genotypes, and between P0/P0 and P0/P10 genotypes.

The frequencies of combinations of R353Q and P0/P10 genotypes were found to be as follows: R/R & P0/P0 (86.0%), R/R & P0/P10 (1.0%), R/Q & P0/P0 (5.0%), R/Q & P0/P10 (7.5%), Q/Q & P10/P10 (0.5%), R/R & P10/P10 (0.0%), R/Q & P10/P10 (0.0%), Q/Q & P0/P0 (0.0%), and Q/Q & P0/P10 (0.0%). The correlation between Q allele and P10 allele was signifi-

| Table 1. Clinical and biochemical data of one-month-old Japanese infants. |
|-----------------------|-------------------|---------------------|
| Number                | 200               |
| Sex (Male/Female)     | 103/97            |
| Age (days)\(^a\)      | 34.6 ± 4.0        |
| BW (g)\(^a\)         | 4,311.5 ± 467.0   |
| Birth weight (g)\(^a\)| 3,085.5 ± 341.6   |
| Gestational Age (weeks)\(^a\) | 39.5 ± 1.3      |
| Feeding regimen       |                   |
| Breast-fed (n)        | 126               |
| Mixed-fed (n)         | 63                |
| Formula-fed (n)       | 11                |
| ALT (Karmen unit)\(^a\) | 18.5 ± 6.3       |

\(^a\)This value was presented as the mean ± s.d.; Ranges were shown in parentheses. BW, body weight; ALT, alanine aminotransferase.
Polymorphisms of the Factor VII Gene in Infants

**Table 2. Multivariate regression models showing the association between Normotest value and the P10 allele and the R353Q allele.**

<table>
<thead>
<tr>
<th></th>
<th>P10 model</th>
<th>R353Q model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression coefficient</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (days)</td>
<td>0.392</td>
<td>−0.123 to 0.906</td>
</tr>
<tr>
<td>Gestational Age (weeks)</td>
<td>0.719</td>
<td>−1.122 to 2.560</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>−0.002</td>
<td>−0.009 to 0.005</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>0.702</td>
<td>−3.452 to 4.856</td>
</tr>
<tr>
<td>Breast-fed</td>
<td>−0.214</td>
<td>−6.417 to 2.128</td>
</tr>
<tr>
<td>P10</td>
<td>−14.413</td>
<td>−21.580 to −7.247</td>
</tr>
<tr>
<td>R353Q</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*The P10 allele and the R353Q allele cannot be put into the same model because of multi-collinearity problem. The correlation between the 2 parameters is r = 0.674, p < 0.01.

**Table 3. The Normotest values and frequencies of two polymorphisms of factor VII gene in 200 one-month-old Japanese infants.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>P0/P0</th>
<th>P0/P10</th>
<th>P10/P10</th>
</tr>
</thead>
<tbody>
<tr>
<td>R/R</td>
<td>172</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Normotest (%)</td>
<td>91.4 ± 14.7</td>
<td>68.5 ± 10.6 a</td>
<td>91.2 ± 14.9</td>
</tr>
<tr>
<td>R/Q</td>
<td>10</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Normotest (%)</td>
<td>87.2 ± 13.7</td>
<td>76.9 ± 12.7 b</td>
<td>81.0 ± 14.1 c</td>
</tr>
<tr>
<td>Q/Q</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Normotest (%)</td>
<td>-</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>Number (n)</td>
<td>182</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Normotest (%)</td>
<td>91.2 ± 14.7</td>
<td>75.9 ± 12.5 d</td>
<td>89.8 ± 15.1</td>
</tr>
</tbody>
</table>

Normotest values were presented as the mean ± S.D.

* p < 0.01. ** p < 0.001.

aTest for difference against the R/R&P0/P0 type.
bTest for difference against the R/R&P0/P10 type.
cTest for difference against the R/R type.
dTest for difference against the P0/P0 type.
Normotest values and feeding regimens

The Normotest values for the breast-fed group \((n = 126)\), formula-fed group \((n = 11)\), and mixed-fed group \((n = 63)\) were 88.7 ± 14.8%, 90.5 ± 18.1%, and 92.0 ± 15.3%, respectively. There was no significant difference in the Normotest values among the groups.

**DISCUSSION**

Normotest values reflect variation in the coagulation activities of coagulation factors II, VII, and X among VK-dependent coagulation factors II, VII, IX, and X, which are synthesized in hepatic cells. Thus, Normotest is useful for evaluating VK-dependent coagulation status. However, its values are affected not only by the status of VK deficiency, but also possibly by various genetic and environmental factors, especially factor VII polymorphisms.

Factor VII functions in the first process of the coagulation reaction. There are two common genetic polymorphisms of coagulation factor VII, both of which are known to lower the plasma factor VII activity: one is the replacement of arginine at 353 with glutamine in the catalytic region on Exon 8 (R353Q) (Green et al. 1991) and the other is a 10-base pair (CCTATATCCT) insertion in the promoter region at position -323 (-323P0/10) (Marchetti et al. 1993). It has been assumed that the plasma concentration of factor VII is lowered due to an impaired secretion level of factor VII in the R353Q variant (Hunault et al. 1997). Because a significant positive correlation between plasma factor VII activity and Normotest values has been suggested, it is considered useful to adopt Normotest for the screening of coagulation factor VII activity abnormalities (Dalaker et al. 1987).

In actual clinical cases, coagulation factor VII deficiencies and abnormalities were discovered in a follow-up investigation of abnormally low Normotest values in screening tests conducted as a part of health examinations for one-month-old infants (Takamiya et al. 1988; Fujita et al. 1991). It has been assumed that, in addition to malabsorption and malutilization of VK due to diarrhea, subclinical hepatic dysfunction and subclinical cholestasis also lower the coagulation ability (Matsuda et al. 1989; Tazawa et al. 1990). Other risk factors for lowering the coagulation ability in infancy have not been well documented. The present study investigated several environmental factors that could affect Normotest values of one-month-old infants, and then focused on two genetic polymorphisms of coagulation factor VII: R353Q and -323P0/10.

Among the factors examined, including sex, birth weight, gestational age, feeding regimen, the P10 allele, and the Q allele, the P10 allele or the Q allele was negatively associated with Normotest values in this study. The Normotest values in the P0/P10 group were significantly lower than those in the P0/P0 group \((p < 0.001)\). The Normotest values in the R/Q group were significantly lower than those in the R/R group \((p < 0.01)\). These data demonstrated that infants who carry the P10 allele or the Q allele show lower activity of VK-dependent coagulation factors. On the other hand, there was no significant difference in Normotest values among the different feeding regimen groups, which was consistent with some previous reports (Jimenez et al. 1982; Wago et al. 1998). It was assumed that feeding regimens were less influential, due to the administration of VK syrup after birth.

In the present study, we adopted cycling probe technology for detection of coagulation factor VII genetic polymorphisms. Cycling probe technology is a highly sensitive detection method utilizing a chimeric probe that consists of RNA and DNA, and RNaseH. The 5′ and 3′ ends of the probe are labeled with a fluorescent compound and a quencher compound, respectively. In an intact probe, no strong fluorescence is emitted due to quenching. After the sequence forms a hybrid with a complementary amplified product, and the RNA portion is digested by RNaseH, and the probe emits strong fluorescence. Based on this principle, when cycling probes and thermostable RNaseH are added in PCR reactions, the fluorescent strength is enhanced as the level of target amplified product is increased, thus allowing real-time detection of the product. Furthermore, when there is a mismatch near the RNA of a cycling probe, RNaseH does not work; thus, the fluores-


cent strength does not change. Because cycling probe technology is a detection method with extremely high sequence specificity, we evaluated it as a rapid and easy method for SNP typing.

The frequencies of the Q and P10 alleles presented in this study were 0.07 and 0.05, respectively. These frequencies were generally consistent with those reported in the Japanese population, 0.07 and 0.08 (Takamiya 1998). Next, to clarify the effects of each polymorphism on the total activities of VK-dependent coagulation factors, the frequencies of combinations of the genetic polymorphisms and the corresponding Normotest values were investigated. It was suggested that there was a strong correlation between the Q and P10 alleles \( r = 0.674, p < 0.01 \). The mean Normotest value in the R/R & P0/P10 group were significantly lower than those in the R/R & P0/P0 group by 25% \( p < 0.01 \). On the other hand, however, there was no significant difference between the test values in the R/Q & P0/P0 group and the R/R & P0/P0 group \( p = 0.279 \). Thus, it was suggested that the P10 allele may be a factor that strongly lowers the total activities of VK-dependent coagulation factors.

The coagulation ability of neonates is lower than that of adults due to various reasons, such as premature liver function, low concentration of VK in breast milk, low VK production ability of intestinal bacterial flora, and malabsorption of VK in intestinal mucosa; thus, a slight decrease of coagulation ability may affect manifestation of VKDB. There are three types of VKDB. The first type is the idiopathic type, in which VK deficiency is confirmed by coagulation studies, with no apparent cause being found. The second type is secondary VK deficiency, in which hepatobiliary tract disorders, severe diarrhea, or long-term antibiotic therapy are linked to VK deficiency. The third type is the so-called “near-miss” type. The hemorrhagic tendency of this group is discovered by chance. Apparently healthy infants having a Noromotest value less than 20% are considered to be in a latent hemorrhagic state, and are categorized as near-miss cases. Despite the prophylactic administration of VK, some cases of idiopathic type VKDB have been observed, suggesting that this disease has heterogeneity (Hanawa et al. 1990). As for the hemorrhage site, 82.7% of the cases of VKDB in infants showed intracranial hemorrhage (Hanawa et al. 1988). Regarding adult idiopathic intracranial hemorrhage, it is suggested that P10 allele carriers have significantly lower factor VII, giving a risk that is 1.5 times higher; thus, this polymorphism may be involved in the manifestation of idiopathic intracranial hemorrhage (Corral et al. 2001). However, no investigation has been conducted on the relationship between VKDB in infancy and the factor VII polymorphisms.

The present study indicates that the P10 allele carriers have a lower coagulation ability than non-carriers in the neonatal and infant periods by approximately 25%. This observation was assumed to be significant when considering the susceptibility of newborns to VKDB. In other words, being a carrier of the P10 or the Q allele alone may not cause a severe decrease of coagulation ability resulting in hemorrhagic symptoms, but in combination with other risk factors, such as bile efflux disorder, administration of antibiotics, prolonged diarrhea, and hepatic dysfunctions, being a carrier may increase the risk of VKDB manifestation.

Further studies for detecting factor VII polymorphisms in infants who bleed although VK is administered are needed to clarify the relationship between VKDB in infancy and factor VII polymorphisms. Further investigation focusing on the genetic polymorphisms of other coagulation factors is also required.

References


