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UDP-glucuronosyltransferase 2B17 genotyping in Japanese athletes and evaluation of the current sports drug testing for detecting testosterone misuse

Masato Okano,[a]* Toshihiko Ueda,[b] Yasunori Nishitani,[a] Hiroko Kano,[a] Ayako Ikekita[a] and Shinji Kageyama[a]

Ethnicity has been found to influence urinary testosterone glucuronide to epitestosterone glucuronide (T/E) ratios among athletes. Uridine diphospho-glucuronosyltransferase 2B17 (UGT2B17) is the most active enzyme in testosterone glucuronidation. UGT2B17 polymorphism analysis is rarely performed in Japanese athletes, and the influence of testosterone administration on steroid profiles and carbon isotope ratios, according to gene polymorphisms, in Asians remains unknown. The prevalence of UGT2B17 genotypes and urinary androgenic steroid profiles, classified according to UGT2B17 genotypes, was investigated in Japanese athletes (255 male and 256 female). Testosterone enanthate (100 mg) was administered intramuscularly to Japanese female volunteers (*del/del*: n = 6, *del/ins*: n = 3, *ins/ins*: n = 1). The distribution rates of the UGT2B17 *del/del* genotype in Japanese male and female athletes were 74.5% and 60.2%, respectively. The *ins/ins* genotype was detected in only three male (1.2%) and seven female (2.7%) athletes. The prevalence of the UGT2B17 deletion genotype was extremely high in Japanese athletes. The T/E ratio in the *del/del* group was significantly lower than that in the other groups. After testosterone was administered to female volunteers, the T/E ratios for the *del/del* individuals failed to reach the positivity criterion of 4. By contrast, in all of the *del/del* subjects, the gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS) analysis successfully fulfilled the positivity criterion. The overall result has demonstrated the limited effectiveness of population-based T/E ratios in screening tests for testosterone use. Subject-based steroid profiling with UGT2B17 genotyping will be an effective strategy for detecting testosterone misuse. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: UGT2B17; testosterone; doping; carbon isotope ratio; T/E ratio; steroid profile

Introduction

Exogenous testosterone administration is widely used for treating testosterone deficiency; however, its abuse by athletes has been a serious problem in sports for decades. For sports doping control, Donike *et al.* reported that urinary testosterone glucuronide to epitestosterone glucuronide (T/E) ratio as determined by gas chromatography/mass spectrometry (GC-MS) is increased by exogenous testosterone administration.[1] The International Olympic Committee (IOC) defined a T/E ratio above 6 as evidence of testosterone doping on the basis of statistical data in 1982; however, some individuals have been found to have naturally elevated T/E ratios.[2–5] Therefore, it is necessary to conduct further investigation to determine whether the elevated T/E ratio is a consequence of a physiological or pathological condition. In 1994, Becchi *et al.* developed an innovative technique employing gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS) for discriminating testosterone origin.[6] When GC-C-IRMS analysis reveals that a steroid has an exogenous origin, the result is considered as an adverse analytical finding (AAF). However, this technique is labour-intensive and costly, thereby making it impractical for analyzing multiple urine samples. In 2005, the reporting threshold value of the T/E ratio was redefined from 6 to 4 by the World Anti-Doping Agency (WADA) to improve the rates of detecting testosterone doping. Currently, WADA requires a follow-up investigation or GC-C-IRMS measurement for T/E ratios above 4.[7] In addition to the T/E approach, when the concentrations of urinary steroid glucuronides are above the threshold levels (e.g. testosterone and epitestosterone >200 ng/ml, androsterone (A) and etiocholanolone (Et) >10000 ng/ml, dehydroepiandrosterone (DHEA) >100 ng/ml), further GC-C-IRMS measurements are recommended.[7]

In contrast, Asians were found to have significantly lower levels of urinary testosterone glucuronide excretion than Caucasians, suggesting that urinary T/E ratios are influenced by ethnicity.[8–11]

In humans, androgens and their metabolites are excreted mainly as gluco-conjugates after metabolism by uridine diphospho (UDP)-glucuronosyltransferases.[12] The metabolic pathway of relevant steroids is shown in Figure 1. Testosterone is mainly conjugated by UDP-glucuronosyltransferase 2 family, polypeptide

* *Correspondence to: Masato Okano, Anti-Doping Laboratory, Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku Tokyo, 174–8555, Japan. E-mail: okano.masato@mk.medience.co.jp*

a *Anti-Doping Laboratory, Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku Tokyo, 174-8555, Japan*

b *Advanced Medical Science Research Center, Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku Tokyo, 174-8555, Japan*

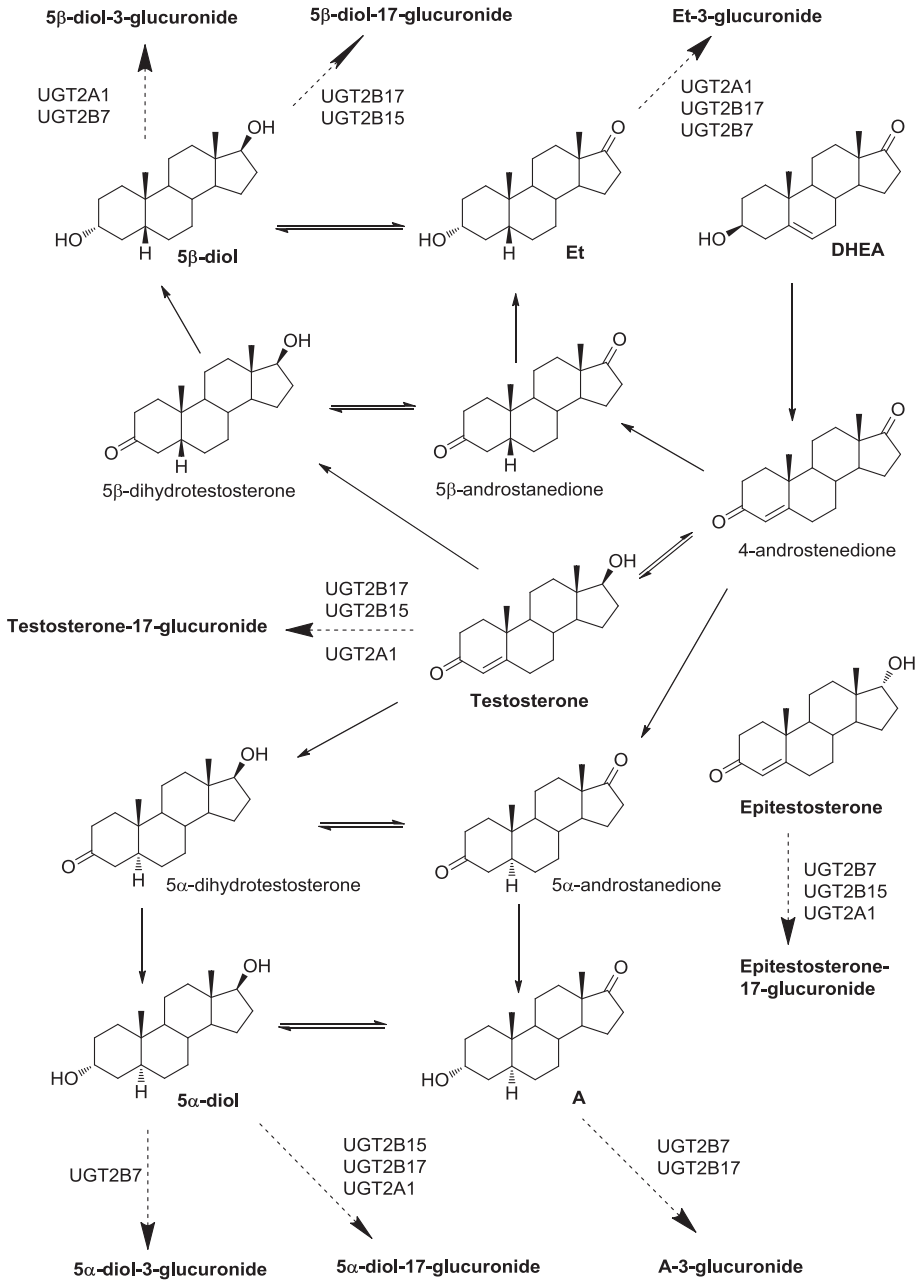

Figure 1. Metabolic pathway of the related steroids.[15,16,28] A solid line with an arrow and a broken line with an arrow represent phase I and phase II metabolism, respectively.

B17 (UGT2B17) and slightly by UDP-glucuronosyltransferase 2 family, polypeptide B15 (UGT2B15).[13] UDP-glucuronosyltransferase 2 family, polypeptide B7 (UGT2B7) has been demonstrated to have the capacity to conjugate epitestosterone.[14] UDP-glucuronosyltransferase 2 family, polypeptide A1, complex locus (UGT2A1), an extrahepatic enzyme, catalyzes the glucuronidation of both testosterone and epitestosterone.[15] Sten *et al*. reported the UDP-glucuronosyltransferase activities of 5α- and 5β-androstane steroids.[16] Wilson *et al*. characterized UGT2B17 gene deletion polymorphism, and they revealed that the frequencies of the homozygous insertion (*ins/ins*), heterozygous (*del/ins*) and homozygous deletion (*del/del*) alleles differ between African–Americans and Caucasians.[17] Jakobsson *et al*. also reported that the UGT2B17 *del/del* genotype is 7-fold more common in Korean males than in Swedish males.[18]

When 500 mg of testosterone enanthate was administered to Caucasian males, 40% of the *del/del* subjects failed to reach the T/E ratio of 4. By contrast, the mean T/E ratio increased to the WADA threshold of 4 for the *del/ins* and *ins/ins* subjects.[19] UGT2B17 genotyping in Japanese male and female athletes is hardly ever performed and the influence of testosterone administration in Asians on parameters such as steroid profiles together with carbon isotope ratios has to our knowledge not been reported. Given this background, the distribution of UGT2B17 genotypes together with urinary androgenic steroid profiles in Japanese athletes was investigated. The applicability

of steroid profiling and GC-C-IRMS analysis in Japanese female volunteers after a small intramuscular dose of testosterone enanthate is also discussed.

Experimental

Materials

All reagents and solvents were of analytical grade. β-Glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostics GmbH (Mannheim, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Marcherey-Nagel (Duren, Germany). NH_4I was purchased from Sigma (St Louis, MO, USA). Ethanethiol was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Acetic anhydride was obtained from Kanto Chemical Co., Ltd (Tokyo, Japan). Pyridine was purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q Ultra pure system (Millipore, Bedford, MA, USA). Bond Elut LRC-C18 (500 mg) was obtained from Varian Inc. (Lake Forest, CA, USA). QIAamp® DNA Mini Kit was purchased from QIAGEN K.K. (Tokyo, Japan). TaKaRa Ex Taq® Hot Start Version was purchased from Takara Bio Inc. (Shiga, Japan). DNA-2500 Reagent Kit was obtained from Shimadzu (Kyoto, Japan). pGEM® DNA Markers were obtained from Promega K.K. (Tokyo, Japan). Primers and the BigDye® Terminator v1.1 Cycle Sequencing Kit were purchased from life Technologies Japan Ltd. (Tokyo, Japan). The UGT2B15 specific primer master mix (Exon 1, marker G) consisted of forward (CCTGGAAGAGCTTGTTCAGA) and reverse (CTGCCAGAATGACAT-CAAAC) primers. The UGT2B17 specific primer master mix (5′, marker D) consisted of forward (TCACAAGTCAATCTCCCATCC) and reverse (CTGCAGAATATGTCAATAATTGGC) primers. The UGT2B17 deletion specific primer master mix (3′ of UGT2B15, marker J) consisted of forward (TGCACAGAGTTAAGAAATGGAGAGATGTG) and reverse (GATCATCCTATATCCTGACAGAATTCTTTTG) primers.

Epitestosterone, 17α-methyltestosterone (MT) and A were purchased from Sigma. Et and 5α-androstan-3β-ol were obtained from Steraloids Inc. (Newport, RI, USA). Testosterone, DHEA, 5α-androstan-3α,17β-diol (5α-diol), 5β-androstan-3α,17β-diol (5β-diol), [16,16,17-$^2H_3$]-testosterone (d_3-T), [16,16,17-$^2H_3$]-epitestosterone (d_3-E), [2,2,3,4,4-$^2H_5$]-Et (d_5-Et), [16,16,17-$^2H_3$]-5α-diol (d_3-5α-diol), [2,2,3,4,4-$^2H_5$]-5β-diol (d_5-5β-diol) and [2,2,4,4-$^2H_4$]-A-β-glucuronide (d_4-A-g) were obtained from National Measurement Institute (New South Wales Pymbel, Australia). Pregnanediol (Pdiol) was purchased from Merck. Testosterone propionate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). [2,2,3,4,4,6-$^2H_6$]-DHEA (d_6-DHEA) was obtained from Isotec (Miamisburg, OH, USA). The internal standard mixture was prepared in ethanol, and the concentrations of MT, d_3-T, d_3-E, d_5-Et, d_3-5α-diol, d_5-5β-diol, d_4-A-g and d_6-DHEA were 4.1, 7.2, 1.9, 9.2, 3.7, 3.6, 7.9 and 6.5 μg/ml, respectively. For the excretion study, an injectable solution of 100 mg testosterone enanthate (ENARMON DEPOT®) was obtained from ASKA Pharmaceutical Co., Ltd (Tokyo, Japan).

Analysis of urinary steroid profiles by GC-MS

Urine samples were prepared according to the common steroid analytical procedure validated for sports doping tests.[20,21] In brief, 3 ml human urine fortified with 50 μl internal standard mixture were incubated with 50 μl β-glucuronidase from *E. coli* K12 solution (pH 7.0, 50°C, 60 min). After incubation, target steroids were extracted with 5 ml of tert-butyl methyl ether (TBME) at pH 9.6. The extracts were derivatized to trimethylsilyl steroids using MSTFA, NH_4I, and ethanethiol (1000/2/6, *v/w/v*). The mixture (2 μl) was injected using the split mode (11:1) at 280°C into the GC-MS system. The GC-MS system was an Agilent 6890N GC/5975 inert XL mass selective detector equipped with an Ultra-1 capillary column (17 m length, 0.25 mm internal diameter and 0.11 μm film thickness; Agilent Technologies, Palo Alto, CA, USA). The oven temperature was set at 180°C (hold, 1 min) and increased by 3°C/min to 229°C and then by 40°C/min to 300°C (hold, 3 min). Helium (at 12 psi) was used as the carrier gas. The injector and interface temperatures were set at 280°C and 300°C, respectively. Electron ionization was accomplished at 70 eV, and the source temperature was set at 230°C. All data were acquired using the selected ion monitoring (SIM) mode, and the quantifier ions of steroid trimethylsilyl derivatives were set to *m/z* 432 (testosterone, epitestosterone and DHEA), *m/z* 434 (A and Et) and *m/z* 241 (5α-diol and 5β-diol). The SIM of the internal standards was used with the following ions: *m/z* 439 for d_5-Et, *m/z* 435 for d_3-T and d_3-E, *m/z* 244 for d_3-5α-diol, *m/z* 246 for d_5-5β-diol, *m/z* 438 for d_6-DHEA, *m/z* 438 for d_4-A and *m/z* 446 for MT. d_4-A-g was used as a marker of the hydrolysis step. Quantitative analysis was performed by isotope dilution mass spectrometry using one-point calibration with a mixture of steroids (i.e. 70 or 2.4 ng/ml for testosterone, 20 ng/ml for epitestosterone, 1330 ng/ml for A, 1330 ng/ml for Et, 110 ng/ml for 5α-diol, 334 ng/ml for 5β-diol and 104 ng/ml for DHEA). The deuterated internal standards were used for quantification of the corresponding non-deuterated standards (i.e. testosterone: d_3-T, epitestosterone: d_3-E, A and Et: d_5-Et, 5α-diol: d_3-5α-diol, 5β-diol: d_5-5β-diol and DHEA: d_6-DHEA). This study focused on subjects with very low urinary testosterone levels. Because the linearity ranging from 0.5 to 16 ng/ml (mean of inter-seven days, $R^2 = 0.9997$) and the assay accuracies ranging from 2.2 to 9.6% at low levels were validated, the calibrator with 2.4 ng/ml was used for quantifying low levels of testosterone (0.5–16 ng/ml). A single calibration point of 70 ng/ml was used to quantify samples with testosterone concentrations exceeding 16 ng/ml. Urine concentrations were calculated by the peak areas of the detected signals relative to the internal standards. The calculated concentrations of steroids were adjusted for specific gravity (S.G.) of 1.020 according to Equation (1)[7] as follows:

$$\text{Concentration}_{\text{corrected}}(\text{ng/ml}) = (1.020 - 1)/(\text{S.G.}_{\text{sample}} - 1) \times \text{Concentration}_{\text{measured}}. \quad (1)$$

Quality control samples for all of the target steroids were analysed simultaneously during all batch analyses.

Carbon isotope ratio analysis of urinary steroids by GC-C-IRMS

Urine samples (10–30 ml) were prepared according to a previously described sample preparation method [22–24] with minor modifications. The sample preparation consisted of solid-phase extraction (Bond Elut LRC-C18), removal of free steroids using TBME, enzymatic hydrolysis (β-glucuronidase from *E. coli*, 50°C, 60 min) and liquid–liquid extraction using *n*-pentane. The dried extracts were dissolved in 110 μl of 30% CH_3CN, and 50 μl of the mixture was injected into an Alliance high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) with a C8 analytical column (150 mm × 2.0 mm, 5 μm particle size; Shiseido, Tokyo, Japan). Fractionation was monitored by HPLC/UV at 191 nm using a Waters 2998 Photodiode Array Detector. The shift

of the retention time was monitored using testosterone propionate as an internal standard. The mobile phases were 30% CH_3CN in water (mobile phase A) and CH_3CN (mobile phase B). The column oven temperature was 40°C, and the flow rate was 0.5 ml/min. The conditions for gradient elution were as follows: 0% B, linear to 50% B in 20 min (hold 3 min) followed by a decrease to 0% B in 3 min. Finally, the column was equilibrated for 7 min. Five fractions (I: testosterone, II: DHEA, III: 5α-diol and 5β-diol, IV: A and Et, V: Pdiol) were collected, and 5α-androstan-3β-ol (3 μg) was added to each fraction as an internal standard to monitor isotopic fractionation during acetylation. The dried residue of each fraction was acetylated (acetic anhydride:pyridine, 1:2; *v/v*; 60°C; 60 min). Then, the dried analytes were dissolved in 30 μl cyclohexane, and 2 μl of the mixture were injected into the GC-C-IRMS system (splitless). For fractions I and III, if co-eluted substances are found by GC-MS, then additional HPLC cleanup is conducted. In this study, there was no need to conduct further purification by HPLC. The GC-C-IRMS system was an Agilent 7890A GC/Isoprime stable isotope ratio mass spectrometer (Isoprime Ltd., Cheadle, UK) equipped with a 50% phenylmethylsilicone DB-17 capillary column (30 m length, 0.25 mm internal diameter and 0.25 μm film thickness; Agilent Technologies). The oven temperature was set at 50°C (hold 1 min) and increased by 20°C/min to 250°C and then by 5°C/min to 300°C (hold 9 min). The carrier gas was helium at a flow rate of 1 ml/min. The injector temperature was set at 260°C. The interface temperature was set at 350°C. The combustion furnace glass tube packed with copper oxide pellets was heated at 850°C. The temperature of the cryotrap was set at −100°C to remove water. The $\delta^{13}C$ value of the reference CO_2 gas was calibrated against NBS-19 limestone. The ^{13}C to ^{12}C isotope ratio ($^{13}C/^{12}C$) was expressed as $\delta^{13}C$ values against an international standard (Vienna Pee Dee Belemnite), and the $\delta^{13}C$ value was calculated as described in Equation (2):

$$\delta^{13}C‰ = [(^{13}C/^{12}C)\text{sample} - (^{13}C/^{12}C)\text{standard}]/(^{13}C/^{12}C)\text{standard} \times 1000. \quad (2)$$

The $\delta^{13}C$ values obtained were corrected for the acetate moieties as described in Equation (3):[24]

$$n_{as}\delta^{13}C_{as}‰ = n_s\delta^{13}C_s + n_a\delta^{13}C_{acorr}, \quad (3)$$

where n = number of carbon atoms, as = acetylated steroid, s = underivatized steroid and a = acetyl group. The $\delta^{13}C_{acorr}$ was calculated by measurements of both the native and acetylated steroids.

The differences between the target steroid (i.e. A, Et, testosterone, 5α-diol and 5β-diol) and Pdiol as the endogenous reference compound (ERC) were expressed as $\Delta\delta^{13}C$ values based on Equation (4) as follows:

$$\Delta\delta^{13}C‰ = \delta^{13}C(\text{Pdiol}) - \delta^{13}C(\text{target steroid}). \quad (4)$$

WADA defined the positivity criterion for $\Delta\delta^{13}C$ as 3‰.[7]

The repeatability, reproducibility and precision of the method were validated using the linear mixing models for all target steroids in accordance with a procedure described by Piper *et al.*[24] As representative example of validation results using a linear mixing model, the linear equation of 5α-diol was $y = (10.9 \pm 0.18‰) \times x + (-31.1 \pm 0.64‰)$, whereas the values of the standard of 5α-diol and the endogenous 5α-diol were −31.2 ± 0.07 and −20.1 ± 0.49‰, respectively. Two types of quality control samples (negative pooled urine and the sample fortified with reference target steroids) were analysed simultaneously during 11 batch analyses. All samples were also analysed by GC-MS (6890N GC/5973 inert mass selective detector, Agilent Technologies) in the cyclic scan mode (*m/z* 50 to 550, 2.9/s) using the same gas chromatographic conditions as GC-C-IRMS to identify the peak purity and to ensure the absence of any interfering organic compounds in all fractions.

Genotyping of UGT2B17

Genotyping was performed in accordance with the literature.[17,18] In brief, genomic DNA was extracted from whole blood samples (0.2 ml) using a QIAamp® DNA Mini Kit. Polymerase chain reaction (PCR) amplifications were performed on genomic DNA samples (10 ng) containing primer master mix at 94°C for 3 min followed by 30 cycles of 94°C for 20 s, 60°C for 30 s and 72°C for 90 s in a GeneAmp® PCR System 9700 (Life Technologies Japan Ltd). PCR products were analysed using the MultiNA MCE®-202 microchip electrophoresis system (Shimadzu). pGEM® DNA Markers were used as the DNA ladder. To confirm the presence of genomic DNA, marker G (UGT2B15) was amplified by PCR. The sequence of PCR products was confirmed by an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies Japan Ltd). The presence or absence of the UGT2B17 gene was identified by the following scheme: homozygous *del/del* genotype: presence of the PCR product of UGT2B17 (marker J); homozygous *ins/ins* genotype: presence of the PCR product of UGT2B17 (marker D) and heterozygous *del/ins* genotype: presence of the PCR products of both markers J and D.

Statistical analysis

Statistical analysis was conducted using EXCEL Statistics 1.01 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan). All values were described as the mean ±standard deviation, if not mentioned otherwise. The normality of the distribution was verified by the Kolmogorov–Smirnov test. A nonparametric Mann–Whitney U-test was used for comparison between *del/del* subjects and the other UGT2B17 carriers (*del/ins* + *ins/ins*). To examine the deviation from the Hardy–Weinberg equilibrium, Pearson's χ^2 test (5% significance level at 3.84, df = 1) was used. The χ^2 test was used for a test of independence between gender and genotype. The Wilcoxon signed-rank test was used for comparisons prior to and after the injection of testosterone enanthate. *P* values <0.0001, <0.01 or <0.05 was considered statistically significant. The 95% confidence interval (95% CI, between the 2.5th and 97.5th percentiles) was defined as the reference range in this study.

Human subjects

The protocol was performed in accordance with the Helsinki Declaration and approved by the Ethical Review Board of Mitsubishi Chemical Medience Corporation. The details of the study were explained to all human subjects, and written consent was obtained from each subject. EDTA tubes were used to collect blood for UGT2B17 genotyping. After collecting urine and blood samples, the samples were immediately frozen at −20°C.

Japanese male athletes

A total of 255 samples were collected from male Japanese university athletes (age: 20.3 ±1.3 years, body mass index (BMI):

23.3 ± 2.1). The sport categories consisted of athletics, football, basketball, baseball, volleyball, and dance sports. The volunteers refrained from ingesting alcohol or any supplements/medicines 3 days before sample collection. Their diet intake was not controlled during the study. All samples were collected between 9:00 am and 12:30 pm.

Japanese female athletes

A total of 256 samples were collected from female Japanese university athletes (age: 19.8 ±0.9 years, BMI: 21.8 ±2.2). The sport categories consisted of athletics, football, basketball, cheerleading, badminton, baseball, volleyball, skiing, lifesaving, fencing, lacrosse, soft tennis, handball, kendo, golf, gymnastics, and dance sports. The volunteers refrained from ingesting alcohol or supplements/medicines 3 days before sample collection. Their diet intake was not controlled during the study. All samples were collected between 9:00 am and 12:30 pm.

Study on testosterone enanthate administration

Forty-two Japanese female nonathletes voluntarily participated in the study. UGT2B17 genotyping revealed that the volunteers included 28 homozygous *del/del* subjects, 13 heterozygous *del/ins* subjects and only 1 homozygous *ins/ins* subject. After quasi-randomized selection and medical examination, 10 healthy female volunteers (age: 21–38 years, BMI: 21.3 ±1.9) consisting of 6 *del/del* volunteers (subjects-02, -03, -04, -06, -08 and −10), 3 *del/ins* volunteers (subjects-01, -05 and −07) and 1 *ins/ins* volunteer (subject-09) participated in the administration study.

An injectable solution of 100 mg of testosterone enanthate (72 mg as free testosterone) was administered intramuscularly to 10 volunteers after fasting. The volunteers refrained from ingesting alcohol or medicines (e.g. contraceptive pill or finasteride) and did not perform any exercise prior to and during the studies. Urine samples were collected on Days 0 (pre-administration), 1, 2, 3, 4, 6, 8–9, 10–11, 12–13 and 15–16.

Results

UGT2B17 genotyping

Genotyping of the UGT2B17 deletion was conducted using blood samples collected from 511 Japanese student athletes (255 males and 256 females). As shown in Table 1, the distribution rates of the UGT2B17 homozygous *del/del* genotype in Japanese athletes were 74.5% for male athletes and 60.2% for female athletes. The distribution rates of the heterozygous *del/ins* genotype were 24.3% for male athletes and 37.1% for female athletes. Only three male (1.2%) and seven female (2.7%) athletes carried the homozygous *ins/ins* genotype. The genotype distributions of Japanese athletes did not deviate from the Hardy–Weinberg equilibrium (H_0 was not rejected, $\chi^2 = 0.7$ for males, 2.9 for females, df = 1). The prevalence of the *del/del* genotype in male athletes was significantly higher than that in female athletes (H_0 was rejected, $\chi^2 = 12.0$, df = 1, $P < 0.01$).

Steroid profiles in Japanese student athletes

The target steroid concentrations of the quality control samples were within the acceptance range. The intermediate precision of the testosterone concentration in the quality control samples ranged from 1.6% (70 ng/ml) to 10.1% (0.5 ng/ml). The results of comparative analysis of urinary androgenic steroid profiles and their ratios classified according to UGT2B17 genotypes in Japanese student athletes (254 males and 256 females) are shown in Tables 2 and 3 and Figure 2. One homozygous *del/del* male sample, which was positive for a prohibited substance was excluded from the statistics. In female athletes, the data for testosterone, 5α-diol and 5β-diol in two *del/del* subjects were excluded because of the presence of co-eluted substances at each retention time. All statistical analyses were performed by the non-parametric method because the parameters displayed a non-normal distribution as confirmed by the Kolmogorov–Smirnov test.

Japanese male athletes

The testosterone concentration of the combined *del/ins* (median: 33.9 ng/ml) and *ins/ins* (median: 45.8 ng/ml) group was significantly higher than that of the *del/del* (median: 4.4 ng/ml) group ($P < 0.0001$), whereas the epitestosterone concentration of the *del/del* (median: 26.7 ng/ml) group was not significantly different from that the combined *del/ins* (median: 31.7 ng/ml) and *ins/ins* (median: 13.9 ng/ml) group ($P > 0.05$). Consequently, the T/E ratio in the *del/del* (median: 0.16) group was significantly lower than that in the combined *del/ins* (median: 1.1) and *ins/ins* (median: 3.3) group ($P < 0.0001$). The T/E ratios of two male subjects with heterozygous *del/ins* genotypes were above the WADA threshold of 4 (4.5 and 5.0, respectively); however, the result of GC-C-IRMS analysis was negative, and physiological conditions were considered to be the cause for these elevated values (data not shown). No significant difference in the 5α-diol concentration was observed among the genotypes (median *del/del*: 49.3 ng/ml, *del/ins*: 50.0 ng/ml, *ins/ins*: 40.2 ng/ml) ($P > 0.05$). By contrast, the 5β-diol concentration of the combined *del/ins* (median: 63.3 ng/ml) and *ins/ins* (median: 41.2 ng/ml) group was significantly higher than that of the *del/del* (median: 30.1 ng/ml) group ($P < 0.0001$). Consequently, the 5α-diol-to-5β-diol (5α-/5β-diol) ratio of the *del/del* (median: 1.7) group was significantly higher than that of the

Table 1. Comparison of the UGT2B17 genotype distribution among ethnic groups

Origin	Gender	Number Rate%			Reference
		del/del	*del/ins*	*ins/ins*	
Asian (Japanese)	Male	190 74.5%	62 24.3%	3 1.2%	
Asian (Japanese)	Female	154 60.2%	95 37.1%	7 2.7%	
Asian (Koreans)	Male	44 66.7%	15 22.7%	7 10.6%	[18]
Caucasian (Swedes)	Male	8 9.3%	34 39.5%	44 51.2%	[18]
Caucasian (pubertal boys)	Male	10 8.6%	52 44.8%	54 46.6%	[25]

Table 2. Statistical analysis of urinary androgenic steroid concentrations and the ratios classified according to UGT2B17 genotypes in Japanese male student athletes

	Testosterone (ng/ml)	Epitestosterone (ng/ml)	A (ng/ml)	Et (ng/ml)	5α-diol (ng/ml)	5β-diol (ng/ml)	DHEA (ng/ml)
del/del Male							
n	189	189	189	189	189	189	189
Mean	4.8	31.2	2386	1364	55.1	33.2	34.4
Median	4.4	26.7	2240	1249	49.3	30.1	30.6
Maximum	32.6	107	7772	6168	235	154	143
Minimum	0.6	3.5	701	330	15	9	9
25th percentile	3.5	19.0	1746	956	37	21	24
75th percentile	5.4	38.9	2732	1565	66	40	41
95 % CI	2.1-9.1	7.4-75.1	1113-4765	566-2688	21.6-121	11.0-71.6	13.4-77.1
del/ins Male							
n	62	62	62	62	62	62	62
Mean	37.7	33.9	2532	1648	56.4	65.0	36.8
Median	33.9	31.7	2337	1508	50.0	63.3	30.3
Maximum	76.6	85.6	6548	6792	132	169	132
Minimum	4.3	7.4	763	385	20.6	19.0	11.9
25th percentile	26.2	20.6	1731	1161	36.8	42.7	23.2
75th percentile	48.3	42.7	3111	2027	70.7	84.8	46.3
95 % CI	6.6-72.1	9.9-70.0	955-4753	522-3027	21.9-121	24.8-118	12.3-74.9
ins/ins Male							
n	3	3	3	3	3	3	3
Mean	46.7	15.8	1957	1243	36.4	61.2	24.1
Median	45.8	13.9	1837	1061	40.2	41.2	24.9
Maximum	55.5	23.0	2412	1751	41.7	104	28.6
Minimum	38.7	10.4	1622	918	27.2	38.2	18.9
P value	<0.0001	NS	NS	<0.01	NS	<0.0001	NS

	T/E	A/Et	5α-/5β-diol	5α-diol/E	5β-diol/E	A/T
del/del Male						
n	189	189	189	189	189	189
Mean	0.19	1.9	1.8	2.2	1.3	548
Median	0.16	1.9	1.7	1.8	1.0	509
Maximum	0.94	4.6	4.4	10.1	6.2	2387
Minimum	0.03	0.6	0.4	0.5	0.2	68.6
25th percentile	0.12	1.4	1.4	1.3	0.7	399
75th percentile	0.22	2.2	2.1	2.6	1.5	635
95 % CI	0.07-0.49	0.8-3.6	0.9-3.1	0.6-6.2	0.4-4.5	260-978
del/ins Male						
n	62	62	62	62	62	62
Mean	1.4	1.6	0.9	2.0	2.3	87.8
Median	1.1	1.6	0.9	1.6	2.1	63.2
Maximum	5.0	3.3	2.2	4.7	6.6	749
Minimum	0.10	0.87	0.5	0.6	0.7	24.7
25th percentile	0.8	1.3	0.67	1.2	1.4	50.5
75th percentile	1.7	1.9	1.1	2.5	2.9	86.1
95 % CI	0.2-4.1	0.9-2.8	0.5-1.9	0.8-4.6	0.7-5.3	32.9-335
ins/ins Male						
n	3	3	3	3	3	3
Mean	3.2	1.7	0.7	2.5	3.7	42.5
Median	3.3	1.8	0.7	2.6	3.7	41.9
Maximum	3.7	2.3	1.0	3.3	4.3	52.6
Minimum	2.4	1.0	30.4	1.7	3.2	33.1
P value	<0.0001	<0.01	<0.0001	NS	<0.0001	<0.0001

P value: *del/del* vs. *del/ins* + *ins/ins*.
NS: not significant.

Table 3. Statistical analysis of urinary androgenic steroid concentrations and the ratios classified according to UGT2B17 genotypes in Japanese female student athletes

	Testosterone (ng/ml)	Epitestosterone (ng/ml)	A (ng/ml)	Et (ng/ml)	5α-diol (ng/ml)	5β-diol (ng/ml)	DHEA (ng/ml)
del/del Female							
n	153	154	154	154	153	153	154
Mean	1.5	11.5	2002	1955	20.4	21.0	41.9
Median	1.4	10.1	1819	1729	18.2	17.5	37.8
Maximum	4.7	48.6	5957	5515	85.1	93.9	148
Minimum	0.5	2.4	478	377	7.2	5.7	8.9
25th percentile	1.1	7.0	1239	1259	13.6	11.6	25.9
75th percentile	1.7	13.7	2473	2570	24.5	25.5	49.6
95 % CI	0.6-3.0	3.5-32.2	561-4259	693-4643	8.7-48.1	6.3-53.5	15.3-98.2
del/ins Female							
n	95	95	95	95	95	95	95
Mean	5.9	11.1	2064	2055	23.7	27.1	39.9
Median	5.2	9.2	1806	1800	19.5	22.7	35.1
Maximum	19.9	55.2	6853	5036	81.6	79.8	95.5
Minimum	0.6	1.7	553	727	5.8	6.5	12.1
25th percentile	1.4	6.0	1162	1287	14.2	15.0	25.4
75th percentile	8.8	14.2	2570	2617	28.7	34.4	53.1
95 % CI	0.8-16.3	2.9-33.0	667-5182	791-4570	7.4-60.8	8.8-72.5	15.0-81.1
ins/ins Female							
n	7	7	7	7	7	7	7
Mean	10.1	7.0	1654	1782	23.1	46.4	37.0
Median	9.5	5.8	1364	1549	18.5	35.6	39.9
Maximum	13.7	15.5	2967	3750	47.6	134	56.2
Minimum	6.0	3.2	1058	797	14.6	12.9	19.4
25th percentile	8.5	4.0	1157	1014	15.4	22.9	25.5
75th percentile	12.1	8.5	1935	2177	25.1	48.2	46.3
95 % CI	6.3-13.6	3.2-14.8	1064-2888	821-3568	14.7-44.6	13.2-122	19.9-55.1
P value	<0.0001	NS	NS	NS	NS	<0.01	NS

	T/E	A/Et	5α-/5β-diol	5α-diol/E	5β-diol/E	A/T
del/del Female						
n	153	154	152	153	153	153
Mean	0.16	1.1	1.2	2.1	2.3	1437
Median	0.14	1.0	1.1	1.9	1.8	1398
Maximum	0.8	3.1	6.9	12.4	16.0	3483
Minimum	0.03	0.3	0.2	0.4	0.4	284
25th percentile	0.09	0.8	0.8	1.3	1.1	949
75th percentile	0.2	1.4	1.4	2.8	2.8	1881
95 % CI	0.05-0.4	0.4-2.5	0.4-2.4	0.7-4.0	0.5-5.8	473-2807
del/ins Female						
n	95	95	95	95	95	95
Mean	0.6	1.1	1.0	2.8	3.1	713
Median	0.6	1.0	0.9	2.2	2.8	337
Maximum	2.6	3.2	2.8	20.3	10.7	3430
Minimum	0.05	0.4	0.3	0.5	0.6	109
25th percentile	0.2	0.7	0.7	1.6	1.8	218
75th percentile	1.0	1.4	1.1	3.5	3.8	1024
95 % CI	0.06-1.9	0.4-2.7	0.5-1.9	0.7-6.2	0.7-7.4	120-2434
ins/ins Female						
n	7	7	7	7	7	7
Mean	1.8	1.0	0.7	4.0	7.1	165
Median	1.7	1.0	0.5	3.3	8.7	143
Maximum	3.4	1.5	1.1	8.8	11.2	236
Minimum	0.8	0.6	0.4	1.3	1.2	95.0
25th percentile	1.2	0.8	0.4	3.1	5.4	134
75th percentile	2.1	1.2	0.9	4.2	9.0	206
95 % CI	0.8-3.2	0.6-1.5	0.4-1.1	1.6-8.2	1.5-10.9	100-236
P value	<0.0001	NS	<0.01	<0.01	<0.0001	<0.0001

The data for testosterone, 5α-diol and 5β-diol in two *del/del* subjects were excluded because of the presence of co-eluted substance at each retention time.
P value: *del/del* vs. *del/ins* + *ins/ins*.
NS: not significant.

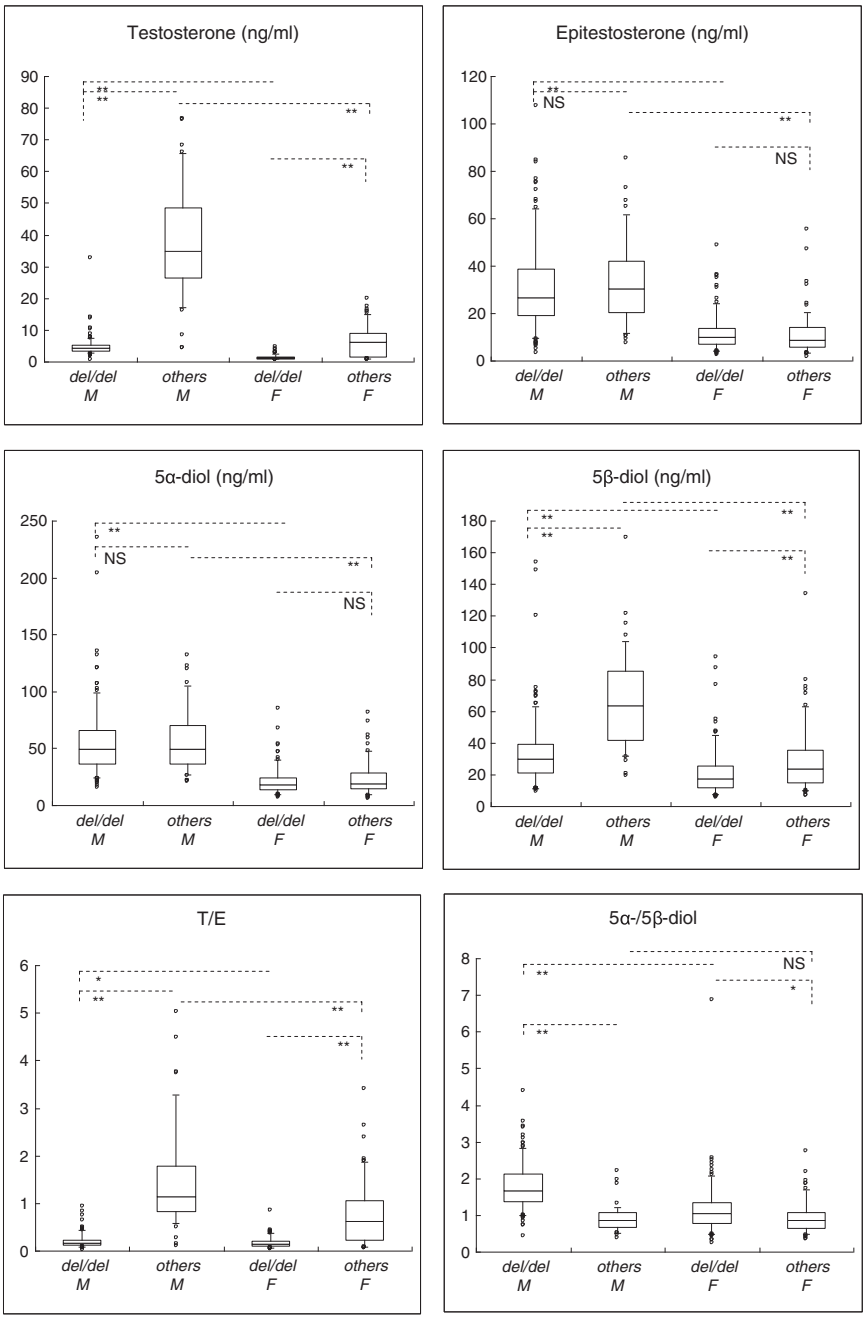

Figure 2. Box plots of urinary androgenic steroid concentrations and the ratios classified according to gender and UGT2B17 genotypes in Japanese male (M) and female (F) student athletes. The whiskers indicate the 5th and 95th percentiles. $^{**}P < 0.0001$, $^{*}P < 0.05$, NS: not significant, others: *del/ins* + *ins/ins*.

combined *del/ins* (median: 0.9) and *ins/ins* (median: 0.7) group ($P < 0.0001$). No significant difference was observed in the DHEA concentration among genotypes ($P > 0.05$). On the basis of the 95% CI, the genotype-based upper limits of the T/E ratio in the *del/del* individuals were set to 0.5 (*del/del*) in this study.

Japanese female athletes

The testosterone concentration of the combined *del/ins* (median: 5.2 ng/ml) and *ins/ins* (median: 9.5 ng/ml) group was significantly higher than that of the *del/del* (median: 1.4 ng/ml) group ($P < 0.0001$), whereas the epitestosterone concentration of the *del/del* (median: 10.1 ng/ml) group was not significantly different from that of the combined *del/ins* (median: 9.2 ng/ml) and *ins/ins* (median: 5.8 ng/ml) group ($P > 0.05$). Consequently, the T/E ratio in the *del/del* (median: 0.14) group was significantly lower than that in the combined *del/ins* (median: 0.6) and *ins/ins* (median: 1.7) group ($P < 0.0001$). The maximum T/E ratio was 3.4 in the homozygous *ins/ins* genotype group. No significant difference in the 5α-diol concentration was observed among the genotypes (median *del/del*: 18.2 ng/ml, *del/ins*: 19.5 ng/ml, *ins/ins*: 18.5 ng/ml) ($P > 0.05$). By contrast, the 5β-diol concentration of the combined *del/ins* (median: 22.7 ng/ml) and *ins/ins* (median: 35.6 ng/ml) group was significantly higher than that of the *del/del* (median: 17.5 ng/ml) group ($P < 0.01$). Consequently, the 5α-/5β-diol ratio of the *del/del* (median: 1.1) group was

significantly higher than that of the combined *del/ins* (median: 0.9) and *ins/ins* (median: 0.5) group ($P < 0.01$). No significant difference in the DHEA concentration was observed among the genotypes ($P > 0.05$). As expected, gender differences were observed in the values of testosterone, epitestosterone, 5α-diol, 5β-diol, the T/E ratio and the 5α-/5β-diol ratio (Figure 2). On the basis of the 95% CI, the genotype-based upper limits of the T/E ratio were set to 0.4 (*del/del*), 1.9 (*del/ins*) and 3.2 (*ins/ins*) in this study.

Administration of testosterone enanthate to Japanese females

Urinary steroid profiles

Testosterone enanthate (100 mg) was intramuscularly administered to six *del/del* individuals, three *del/ins* individuals and one *ins/ins* individual, and the results are shown in Figures 3, 4, and 5. The rate of AAF samples fulfilling the criteria based on the WADA threshold value and the genotype based on the cut-off limit set in this study are shown in Table 4. After injection, the maximum concentrations of testosterone in the six *del/del* individuals (11.9–29.2 ng/ml) were significantly higher than that prior to testosterone enanthate administration (0.5–2.6 ng/ml) ($P < 0.05$) and that in the *del/ins* and *ins/ins* subjects increased by 843–1607%. However, the testosterone concentration did not reach 200 ng/ml for any phenotype. As all female volunteers were not using oral contraceptives, the concentrations of epitestosterone increased and decreased in concert with the female menstrual cycle (Figure 3).[21]

The maximum T/E ratios for the six *del/del* individuals (0.6–1.3) were significantly higher than prior to administration (0.07–0.2) ($P < 0.05$); however, all of the *del/del* subjects failed to reach the WADA threshold value of 4. In the *ins/ins* subject (subject-09), the T/E ratio increased from 0.8 to 7.1 on Day 3. In the *del/ins* subjects, the basal T/E ratios of subjects-01, -05 and −07 were 1.0, 0.2 and 0.5, respectively, and the ratios reached 3.9, 1.0 and 5.6, respectively.

As shown in Figures 4 and 5, the concentrations of testosterone metabolites A, Et, 5α-diol and 5β-diol significantly increased ($P < 0.05$ at Day 3) in all phenotypes. Only two *del/ins* individuals (subjects-01 and −07) exhibited an A or Et concentration greater than 10000 ng/ml. Significant decreases of the 5α-/5β-diol ratios were observed after the injection of testosterone enanthate in all *del/del* subjects compared to the ratios before injection ($P < 0.05$ at Day 3).

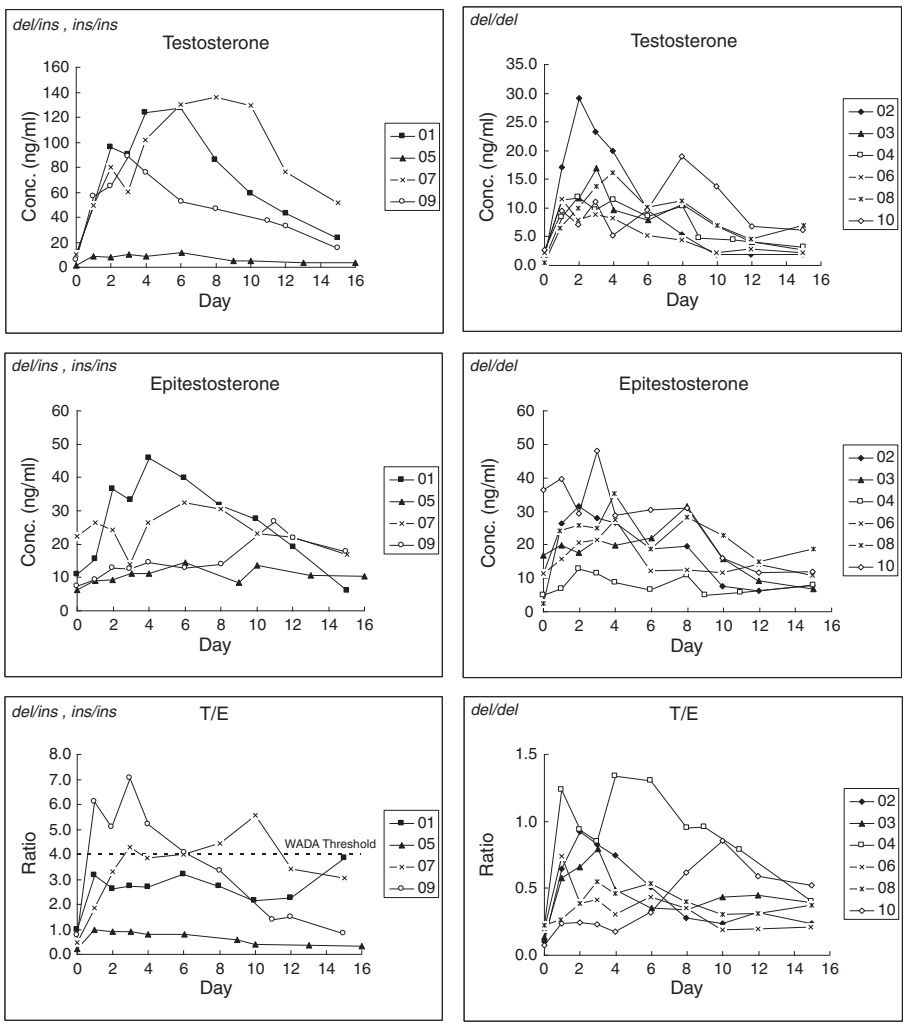

Figure 3. Change in the urinary concentrations of testosterone and epitestosterone and the ratios after the intramuscular administration of testosterone enanthate. *del/del*: subjects-02, -03, -04, -06, -08 and −10, *del/ins*: subjects-01, -05 and −07, *ins/ins*: subject-09.

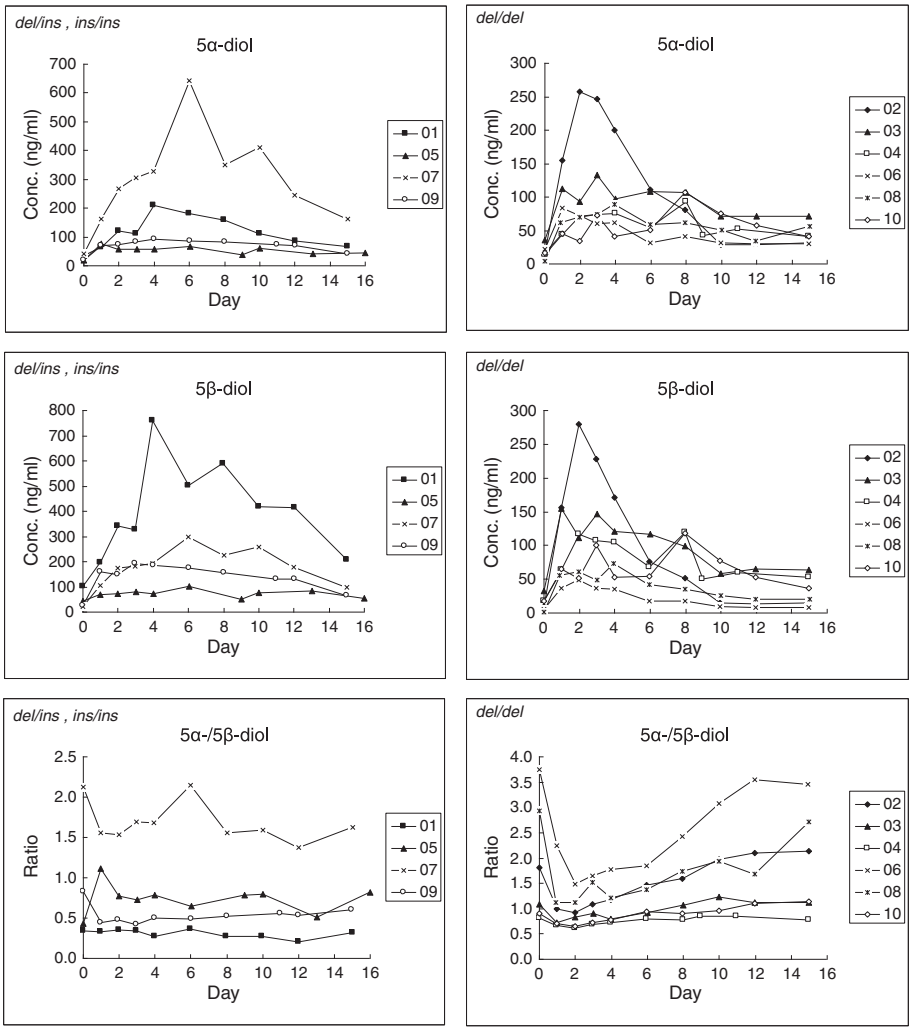

Figure 4. Change in the urinary concentrations of 5α-diol and 5β-diol and the ratios after the intramuscular administration of testosterone enanthate. *del/del*: subjects-02, -03, -04, -06, -08 and −10, *del/ins*: subjects-01, -05 and −07, *ins/ins*: subject-09.

Carbon isotope ratios

The standard deviation of the $\delta^{13}C$ value of the internal standard were 0.1–0.2‰ for intraday and 0.3–0.4‰ for interday (n = 11). No isotopic fractionation was observed in any sample. Standard deviations of the $\delta^{13}C$ and $\Delta\delta^{13}C$ values of the quality control samples were 0.4–0.8 and 0.3–0.6‰, respectively. Thus, no analytical deviation was observed in the $\delta^{13}C$ values during the day-to-day analysis. All fractions were analysed by GC-MS in scan analysis to identify the purity of each peak. Afterwards, two urine samples collected from a *del/del* individual (subject-04 at Days 3 and 12) were removed from the data analysis because invalid data with a poor peak intensity were obtained due to an insufficient urine volume. Unfortunately, the $\delta^{13}C$ values of testosterone in eight samples could not be correctly measured due to the influence of co-eluted substances and low concentrations.

The changes of $\delta^{13}C$ values and their difference values ($\Delta\delta^{13}C$) after testosterone enanthate administration in the typical eight subjects (*del/del*: subjects-02, -03, -04, -06 and −10, *del/ins*: subjects-05 and −07, *ins/ins*: subject-09) are shown in Figures 6 and 7. The rates of the AAF samples fulfilling the WADA criteria are shown in Table 4. The mean absolute $\delta^{13}C$ values of A, Et, testosterone, 5α-diol and 5β-diol in the *del/del* subjects at pre-administration were −20.5, −21.4, −21.2, −19.7 and −19.5‰, respectively. After the injection of testosterone enanthate, the respective $\delta^{13}C$ values of the aforementioned metabolites were found to be significantly depleted to minimum mean values of −25.7, −26.2, −27.0, −28.2 and −28.0‰, respectively ($P < 0.05$). Similarly, the $\delta^{13}C$ values were affected by the administration of testosterone enanthate in the *del/ins* and *ins/ins* subjects. No significant changes of those of Pdiol were observed in any phenotype ($P > 0.05$). The mean $\Delta\delta^{13}C$ values of A, Et, testosterone, 5α-diol and 5β-diol in the *del/del* subjects at pre-administration were 0.4, 1.3, 1.0, −0.4 and −0.6‰, respectively. After the injection of testosterone enanthate, the respective $\Delta\delta^{13}C$ values for these metabolites significantly increased to maximum mean values of 5.6, 6.2, 7.0, 8.1 and 7.9‰, respectively ($P < 0.05$). Thus, the $\Delta\delta^{13}C$ values were higher than the WADA threshold of 3‰ throughout the period of testosterone application for all of the phenotypes.

Discussion

As shown in Table 1, the distribution rate of the UGT2B17 homozygous *del/del* genotype in Japanese males was 74.5%, whereas those in Swedish men and Caucasian pubertal boys were

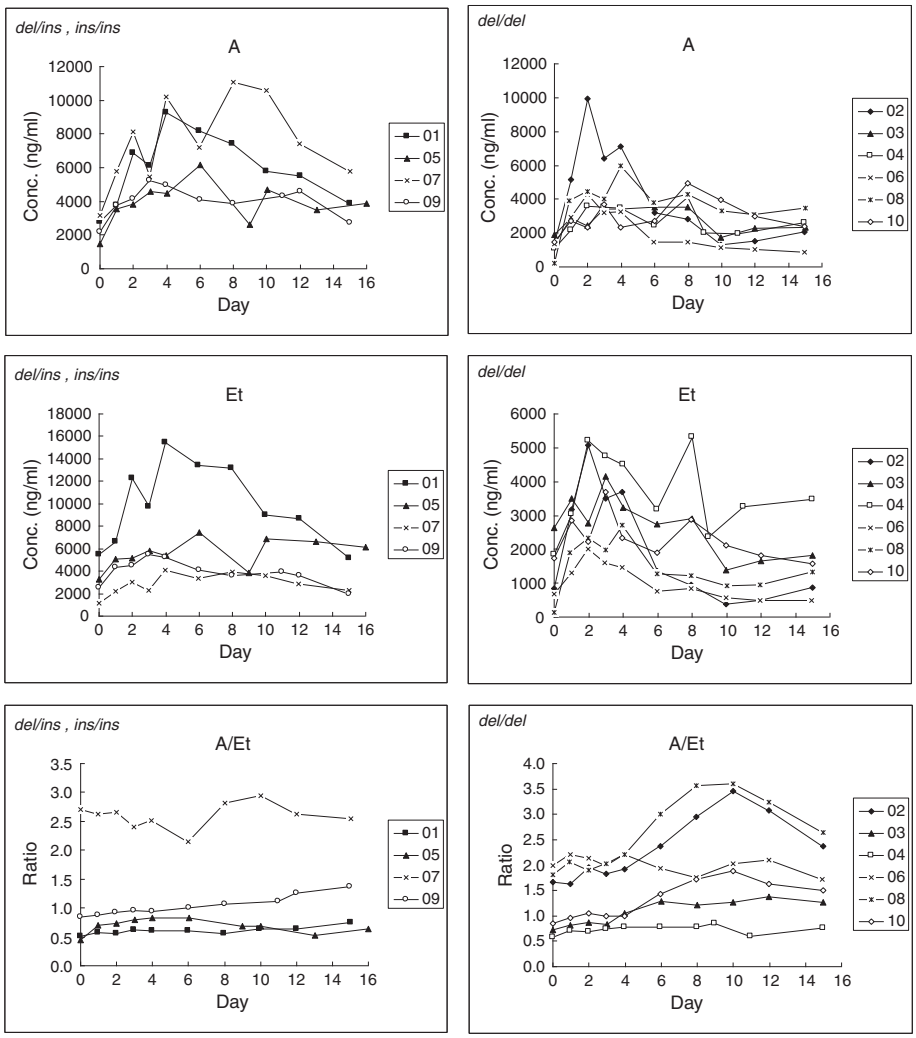

Figure 5. Change in the urinary concentrations of A and Et and the ratios after the intramuscular administration of testosterone enanthate. *del/del*: subjects-02, -03, -04, -06, -08 and −10, *del/ins*: subjects-01, -05 and −07, *ins/ins*: subject-09.

only 9.3 and 8.6%, respectively.[18,25] Although 60.2% Japanese females (14.3% lower than that for Japanese males) carried the *del/del* phenotype, the distribution of the UGT2B17 deletion was extremely high in both Japanese males and females. It is noteworthy that the distribution of the *ins/ins* genotype in Japanese men (1.0%) is less than one-tenth of that in Koreans (10.6%), who are also Asians, compared with the data from Jakobsson *et al.*[18]

One of the reasons for this may be closely related to Japan's geographic distance from Europe and its racially homogeneous island nature.

The T/E ratio of the *del/del* (median: 0.16) group for Japanese male athletes was significantly lower than that in the combined *del/ins* (median: 1.1) and *ins/ins* (median: 3.3) group as well as that reported in the data from Juul *et al.*, in which the mean T/E ratios of the *del/del*, *del/ins* and *ins/ins* groups in Caucasian pubertal boys were 0.29, 1.4 and 2.1, respectively.[25] Thus, the lower T/E ratio in Asians appears to be caused by the extremely low distribution of *ins/ins* genotypes in the Asian region, particularly in Japan, compared with their distribution in Europe.

The 5α-/5β-diol ratio is one of the most useful parameters for detecting 5α-dihydrotestosterone doping.[26] The 5α-/5β-diol ratio in the *del/del* group of Japanese student athletes was significantly higher than that of the combined *del/ins* and *ins/ins* group for both males and females. Strahm *et al.* reported that the 5α-/5β-diol ratio in Japanese soccer players (median: 0.9) was significantly higher than that in Caucasians (median: 0.4), and one of the reasons for this could be that the 5α-reductase activity in Asians tends to be higher than that in Caucasians.[10] However, Juul *et al.* demonstrated that the 5α-/5β-diol ratio in Caucasian pubertal boys carrying the *del/del* phenotype was also significantly higher than that of the *ins/ins* group.[25] This can best be explained by the valuable study regarding the UDP-glucuronosyltransferase activities of 5α- and 5β-androstane steroids by Sten *et al.*[16] As shown in Figure 1, UGT2B17 exhibits a strong preference for glucuronidation at the 17-hydroxy position in both 5α-diol and 5β-diol, whereas UGT2B15 catalyzes stereoselectively at the 17-hydroxy position in 5α-diol and slightly at that position in 5β-diol. In addition, UGT2B7 stereoselectively glucuronidates 5α-diol at the 3-hydroxy position, and its activity for 5α-diol is much higher than that for 5β-diol. Thus, UGT2B17 deletion influences glucuronidation at the 17-hydroxy position in 5β-diol. Hence, it might be concluded that the concentration of 5β-diol-17-glucuronide in the UGT2B17 *del/del* individuals is significantly lower than that in the *del/ins* and *ins/ins* groups, whereas no significant

Table 4. Rate of an AAF sample fulfilling the criteria based on the WADA Technical Document and the genotype-based on the cut-off limit set in this study

	Rate (%) of AAF per sample ( ): per individual									
	Day after administration of testosterone enatnthate									
	0	1	2	3	4	6	8–9	10–11	12–13	15–16
del/del subjects (n = 6)										
Testosterone (ng/ml) >200*	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
A (ng/ml)>10000*	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
Et (ng/ml)>10000*	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
T/E>4*	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
T/E>0.4**	0% (0/6)	67% (4/6)	67% (4/6)	83% (5/6)	67% (4/6)	50% (3/6)	50% (3/6)	50% (3/6)	50% (3/6)	33% (2/6)
$\Delta\delta^{13}$C(Pdiol—A) ‰>3*	0% (0/6)	100% (6/6)	83% (5/6)	100% (5/5)	100% (6/6)	83% (5/6)	83% (5/6)	67% (4/6)	60% (3/5)	33% (2/6)
$\Delta\delta^{13}$C(Pdiol—Et) ‰>3*	0% (0/6)	100% (6/6)	100% (6/6)	100% (5/5)	100% (6/6)	100% (6/6)	100% (6/6)	100% (6/6)	80% (4/5)	33% (2/6)
$\Delta\delta^{13}$C(Pdiol—5α-diol) ‰>3*	0% (0/6)	100% (6/6)	100% (6/6)	100% (5/5)	100% (6/6)	100% (6/6)	83% (5/6)	83% (5/6)	80% (4/5)	83% (5/6)
$\Delta\delta^{13}$C(Pdiol—5β-diol) ‰>3*	0% (0/6)	100% (6/6)	100% (6/6)	100% (5/5)	100% (6/6)	100% (6/6)	83% (5/6)	83% (5/6)	80% (4/5)	67% (4/6)
$\Delta\delta^{13}$C(Pdiol—Testosterone) ‰>3*	0% (0/5)	100% (5/5)	100% (6/6)	100% (4/4)	100% (6/6)	100% (5/5)	100% (4/4)	100% (5/5)	50% (2/4)	33% (2/6)
del/ins subjects (n = 3)										
Testosterone (ng/ml) >200*	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)
A (ng/ml)>10000*	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	33% (1/3)	0% (0/3)	33% (1/3)	33% (1/3)	0% (0/3)	0% (0/3)
Et (ng/ml)>10000*	0% (0/3)	0% (0/3)	33% (1/3)	0% (0/3)	33% (1/3)	33% (1/3)	33% (1/3)	0% (0/3)	0% (0/3)	0% (0/3)
T/E>4*	0% (0/3)	0% (0/3)	0% (0/3)	33% (1/3)	0% (0/3)	33% (1/3)	33% (1/3)	33% (1/3)	0% (0/3)	0% (0/3)
T/E>1.9**	0% (0/3)	33% (1/3)	67% (2/3)	67% (2/3)	67% (2/3)	67% (2/3)	67% (2/3)	67% (2/3)	67% (2/3)	67% (2/3)
$\Delta\delta^{13}$C(Pdiol—A) ‰>3*	0% (0/3)	100% (3/3)	67% (2/3)	100% (3/3)	67% (2/3)	67% (2/3)	67% (2/3)	33% (1/3)	0% (0/3)	0% (0/3)
$\Delta\delta^{13}$C(Pdiol—Et) ‰>3*	0% (0/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	67% (2/3)	33% (1/3)	33% (1/3)	0% (0/3)
$\Delta\delta^{13}$C(Pdiol—5α-diol) ‰>3*	0% (0/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	67% (2/3)
$\Delta\delta^{13}$C(Pdiol—5β-diol) ‰>3*	0% (0/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	67% (2/3)
$\Delta\delta^{13}$C(Pdiol—Testosterone) ‰>3*	0% (0/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	67% (2/3)
ins/ins subject (n=1)										
Testosterone (ng/ml) >200*	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)
A (ng/ml)>10000*	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)
Et (ng/ml)>10000*	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)
T/E>4*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)
T/E>3.2**	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)
$\Delta\delta^{13}$C(Pdiol—A) ‰>3*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)
$\Delta\delta^{13}$C(Pdiol—Et) ‰>3*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)
$\Delta\delta^{13}$C(Pdiol—5α-diol) ‰>3*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)
$\Delta\delta^{13}$C(Pdiol—5β-diol) ‰>3*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)
$\Delta\delta^{13}$C(Pdiol—Testosterone) ‰>3*	0% (0/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)

* WADA criteria[7]

** genotype-based cut-off limit

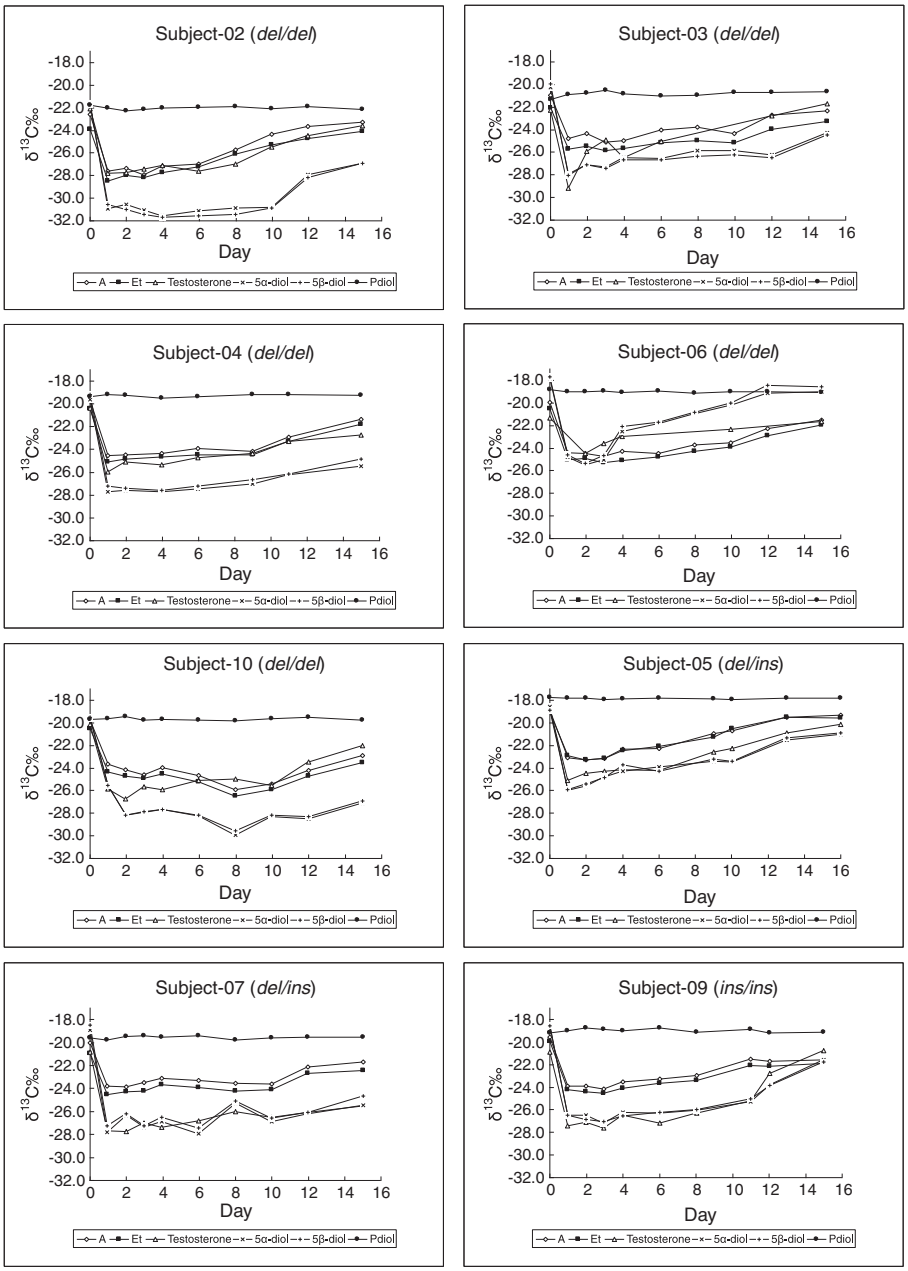

Figure 6. Change in the $\delta^{13}C$ values after the intramuscular administration of testosterone enanthate. *del/del*: subjects-02, -03, -04, -06 and −10, *del/ins*: subjects-05 and −07, *ins/ins*: subject-09.

difference in the 5α-diol concentration was observed. To draw definitive conclusions regarding these findings, the blood levels of free 5α-diol and 5β-diol as well as the 3- and 17-glucuronides of both 5α-diol and 5β-diol in urine should be subjected to further comparative investigations.

As shown in Table 4, the use of testosterone enanthate would have remained undetected, especially in the *del/del* cases, if the laboratories had limited their investigations to a T/E value exceeding 4. Of note, after the injection of testosterone enanthate, the urinary testosterone concentration and T/E ratio in subject-05 (*del/ins)*, who is a UGT2B17 gene carrier, failed to increase as expected (Figure 3). It should be mentioned here that the bioavailability of testosterone derived from testosterone enanthate has recently been demonstrated to be associated with a polymorphism in the PDE7B gene.[27] The PDE7B polymorphism had a significant effect on the rate at which testosterone was released in the system, which could be observed both in serum and in the urinary testosterone levels and the T/E ratio. Therefore, the PDE7B polymorphism might actually have an effect on some of the subjects in this administration study of testosterone enanthate.

Although the glucuronidation of 5β-diol is much less than 5α-diol in the *del/del* subjects,[16] the 5α-/5β-diol ratio tended to decrease after the injection of testosterone enanthate in all *del/del* subjects (Figure 4). With regard to the phase I metabolism of 3-oxo-4-ene-17β-hydroxy steroids, it is known that the production of 5β-metabolites is superior to that of 5α-metabolites.[28] It can be interpreted by the superior phase I metabolism of testosterone might affect the urinary 5α-/5β-diol ratio, which is the ratio of phase II metabolites. However, further investigation of 5α-diol and 5β-diol levels in blood as mentioned above should be conducted.

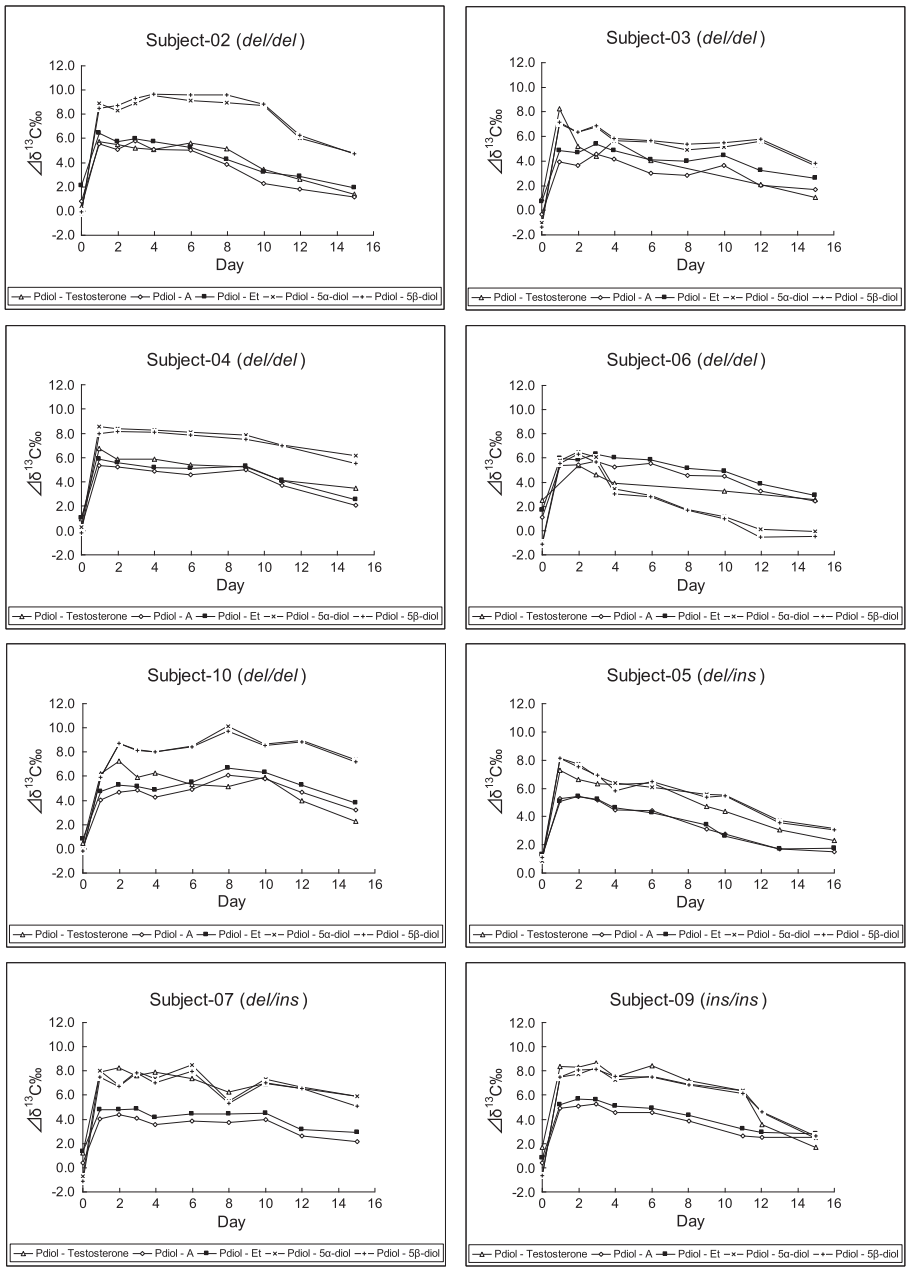

Figure 7. Change in the $\Delta\delta^{13}C$ values after the intramuscular administration of testosterone enanthate. *del/del*: subjects-02, -03, -04, -06 and −10, *del/ins*: subjects-05 and −07, *ins/ins*: subject-09.

Even using other GC-C-IRMS conducting criteria (e.g. A and Et >10000 ng/ml) defined by WADA[7] would have not resulted in detection of testosterone doping. Regarding the 5α-diol-to-E (5α-diol/E), 5β-diol-to-E (5β-diol/E) and A-to-testosterone (A/T) ratios of other steroids, the parameters increased after administration (data not shown). However, their populations have exhibited wide variation among Japanese athletes (Tables 2 and 3). Therefore, the 5α-diol/E, 5β-diol/E and A/T ratios might be suitable for subject-based detection but not for population-based detection. When the genotype-specific threshold level of 0.4 for the *del/del* females was applied (Table 4), testosterone use could be detected in 33.3–83.0% of subjects within the investigated period. In addition, the sensitivity was increased, and false negatives were eliminated. However, 3.3% of Japanese female athletes with the *del/del* phenotype exceeded the T/E ratio of 0.4. Moreover, 3.2% of male athletes exceeded the T/E ratio of 0.5. This suggests that GC-C-IRMS analysis, for which negative results are expected, might be required in routine doping control testing. In our WADA Tokyo laboratory, 19175 doping control samples were analysed between 2009 and 2011, and only 31 specimens (0.2%) displayed a T/E ratio > 4, two of which (6.5%) tested positive by GC-C-IRMS. By contrast, in a German laboratory, the frequency of finding samples with T/E >4 was much higher (2.3%), 5.5% of these samples were tested positive by GC-C-IRMS analysis.[29] This difference is considered to be related to the population of UGT2B17 deletion subjects among Asians and Caucasians.

The GC-C-IRMS analysis for detecting testosterone use was a particularly powerful tool even in the early and elimination phases after the application of testosterone enanthate. It is important to note that the GC-C-IRMS analyses of 5α-diol and 5β-diol are strongly impacted by testosterone administration, whereas large amounts of endogenous A and Et have greatly influence the $\delta^{13}C$ values. Contrary to the typical findings, the impact on the $\delta^{13}C$ values of 5α-diol and 5β-diol were less than those of the other steroids in the *del/del* subject-06, which might have been caused by the difference in the enzymatic activities of 3α-dehydrogenase and 17β-dehydrogenase. Pdiol was used as the ERC in this study; however, it has been reported that the $\delta^{13}C$ value depends on the ERC used.[24] It should be considered that our result would be different if other ERCs (e.g. 5α-androst-16-en-3β-ol, 11β-hydroxyandrosterone and 11-ketoetiocholanolone) were used. Furthermore, it was recently reported that several black market testosterone preparations had similar $\delta^{13}C$ values as endogenous steroids.[30] It should be noted that the use of these illicit products by athletes might lead to a decrease in positive findings by GC-C-IRMS.

Although the application of testosterone enanthate could be detected for long periods, clever athletes are known to use short-lasting preparations such as testosterone propionate, orally applicable testosterone undecanoate or transdermal testosterone preparations. The administration of these testosterone preparations in relation to the phenotype should be further investigated.

Conclusions

The prevalence of the UGT2B17 deletion genotype is extremely high in Japanese subjects. The overall result has demonstrated the limited effectiveness of a population-based T/E ratio for detecting testosterone abuse.

Asian laboratories must pay sufficient attention to individuals with low T/E values because of the high distribution of *del/del* individuals in the Asian region. Introducing genotype-specific cut-offs for the T/E ratio in screening tests for doping control will be a highly sensitive approach for detecting testosterone abuse. GC-C-IRMS analysis is a particularly powerful tool in detecting testosterone doping in athletes carrying the UGT2B17 deletion. These arguments point to a need to include UGT2B17 genotyping in subject-based profiling methods such as athlete biological passports for detecting testosterone doping, which will be an effective strategy for interpreting the results. It is important to proceed with the inter-laboratory standardization of more high-precision determination at low urinary testosterone levels for subject-based profiling, and the implementation of a highly sensitive determination such as gas chromatography-tandem mass spectrometry should be considered in future doping control programmes.

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