Determining valid zygosity is a basic requirement in a twin study, because misdiagnosing zygosity leads to biased results. The Healthy Twin Study has collected data from adult like-sex twins and their families since 2005. In the study, a questionnaire to determine zygosity was developed comprising four questions; one concerning the degree of resemblance, and three concerning the degree of confusion by the resemblance. Among 2,761 individuals (624 twin pairs) of twin and their families, 406 pairs of twins (mean age 38.3, 63.5% women) with both questionnaire and genotype information were selected to examine the validity of the zygosity questionnaire using 16 short tandem repeat markers. We first determined individual zygosity including undetermined category, and then decided the zygosity of a twin pair using a decision tree. Sensitivity of questionnaire diagnosis was 98.8% for monozygotic (MZ) and 88.9% for dizygotic (DZ) twins, and positive predictive value was 97.2% for MZ and 95.0% for DZ. When we compared correctly and wrongly diagnosed twin pairs, misdiagnosed DZ twins (nine pairs) showed striking similarity in stature or obesity even exceeding that of true MZ twins. Our finding suggests that a parsimonious questionnaire method of diagnosing the zygosity will be useful, and adding physical or physiological measurements to a questionnaire of zygosity diagnosis will either confirm the correct diagnosis or reduce the efficiency of the study compared with using questionnaire alone or with introducing genotyping.

Keywords: zygosity determination, twin study, Korea, questionnaire, genetic markers

Determining valid zygosity is a basic requirement in a twin study. Misdiagnosed zygosity will substantially distort estimated parameters from the study, such as heritabilities or logarithm of the odds ratio (LOD) scores in linkage analyses. Historically, various methods have been applied to decide zygosity; blood type or serological markers (Selvin, 1970; Wyslouchowa, 1970); physical characteristics such as facial traits or dermatoglyphics (Forget-Dubois et al., 2003; Gao et al., 2006; Rao & Greene, 1977); zygosity questionnaires administered by twins or their parents (Bonnelykke et al., 1989; Eisen et al., 1989; Price et al., 2000); or highly informative genetic markers (Hannelius, et al., 2007; Jackson et al., 2001; Yang et al., 2006). Among them, the zygosity questionnaire confers the advantage of both simplicity and relative accuracy (Jackson et al., 2001), and so is the method of choice in large-scale epidemiologic studies. A questionnaire of zygosity diagnosis (QOZD) commonly comprises two parts; degree of perceived resemblance and confusion by others (Rasmussen & Johansson-Kark, 2002).

Although QOZDs share similar questions across the studies, algorithms by which zygosity is determined are less standardized. Moreover, the accuracy of questionnaire-based algorithms differs between studies (Christiansen, et al., 2003; Ooki et al., 1990). For example, some studies have included undetermined
zygosity for diagnosis, others applied individual-wise decision trees, while others utilized pair-wise ones (Rasmussen & Johansson-Kark, 2002). Thus, it is a priority to formulate and examine appropriate QOZD and an algorithm to determine the zygosity for any studies incorporating twins.

The Healthy Twin Study was launched in 2005 as a nationwide twin-family study in South Korea, in which a QOZD was newly adapted and primarily administered as a means of determining zygosity (Sung et al., 2006; Sung et al., 2006). The project is still ongoing; all the twin participants have completed the QOZD, and two-thirds of them also have 16 short tandem repeat (STR) genetic marker information to validate their QOZD results.

Through the process of evaluating the QOZD, we became determined to add insight to the existing abundant knowledge of zygosity determination by taking advantage of our study settings. First, we compared genotype information not only with zygosity-predicted individuals but with undetermined ones, so that overall accuracy of the QOZD could be estimated. Additionally, as the number of twin pairs with both QOZD and genotype results are relatively large, and all the participants have taken a health examination and completed the full survey, we could compare the characteristics of the twin pairs between correctly and (rarer) incorrectly diagnosed twins by our QOZD.

We hope this article will serve as a reference to the Healthy Twin Study, as well as to other epidemiologic studies recruiting adult twins, particularly those performed in Asian countries.

Methods
Participants and Zygosity Questionnaire in the Healthy Twin Study
The Healthy Twin Study was first launched in 2005 as a part of government supported genome cohort project. The study has recruited like-sex twins over 30-years-of-age and their adult family members (the premise is that a family consists of an inclusion unit and not just an individual). A family unit should be a twin pair, or a twin pair plus more than two other first-degree family members to constitute a family unit. Participation of large families with twins has always been encouraged. The Healthy Twin program is advertised through media and health-related governmental agencies. Those who agreed to participate in this program take comprehensive health examination and complete a full-length questionnaire survey. The QOZD used in the Healthy Twin Study consists of four questions; one question concerns the degree of twin resemblance, and three questions concern the degree of appearance-related confusion in discriminating twins (Appendix A). Because Koreans do not use an expression that is similar to ‘two peas in a pod’, we translated the resemblance question as ‘mirror-like similarity in appearance’. We included three groups of people representing different levels of confusion: whether twins were confused by their parents or siblings, confusion by teachers or friends, and confusion by strangers not familiar with the twins. The responses to degree of confusion were categorized as ‘never’, ‘sometimes’, ‘often’, and ‘almost always’. One additional question asking if the twins had previously provided zygosity information (Appendix), was not included in formulating the decision tree. Other measurements including physical examination, blood tests, and lifestyle questionnaire were previously reported (Sung et al., 2006).

Genotype Information
Among 2,761 individuals (as of September 2009) collected, 1,700 persons (831 individuals of twin and 869 family members) were genotyped. We only analyzed twin pairs of which both of the co-twins were genotyped. Genotyping is ongoing in order to verify the identity of biological specimens as well as to validate the QOZD. The AmpFISTR Identifier Kit with 16 STR markers (15 autosomal STR markers + one sex determining marker) was used (Perkin Elmer, Waltham, MA, USA) as previously described (Cotton et al., 2000). Considering the possible genotyping errors, a twin pair was regarded as monozygotic if more than 14 markers matched between the cotwins.

Decision Tree for Zygosity Prediction
We formulated a decision tree to determine individual zygosity based on previous studies (Ooki & Asaka, 2004; Rasmussen & Johansson-Kark, 2002) and on discussions among the researchers (Figure 1). The above-mentioned four QOZD questions constituted an algorithm to classify the zygosity. In this decision tree algorithm, those who had ever been confused by their parents were classified as monozygotic (MZ). Similarly, those who had never been confused by strangers were considered to be dizygotic (DZ). Otherwise, other questions were combined to decide individual zygosity first. Since we added ‘undetermined zygosity (XZ)’ category for individual zygosity diagnosis, there were six possible pair-wise combinations. XZ was diagnosed only at individual level, and we assigned definite zygosity (either MZ or DZ) in the final pair-wise discrimination. That is, if there was a discordant diagnosis (A–D cells in Figure 1), we provided the pairs with more probable zygosity, based on genotype information. The decision tree was tested to choose different cut-off points of being confused, i.e., between ‘never’ and ‘sometimes’, between ‘sometimes’ and ‘often’, and so on.

Characterizing Inaccurately Diagnosed Twin Pairs
We compared the characteristics of four groups according to the correctness of zygosity diagnosis: true MZs, true DZs, misdiagnosed MZs, and misdiagnosed DZs. We selected several anthropometrics and biochemical tests proven to have higher heritabilities. Previous perception of zygosity (Appendix, question 3) was examined to ascertain whether the information source is associated with the propensity for correctness of the diagnosis. Pearson’s correlation coefficients
between cotwins were compared for the selected phenotypes among the four groups.

Results

Among 831 individuals of twins, a total of 406 twin pairs with both questionnaire and genotype information were available for the analysis. General characteristics of total participants and twins analyzed in this study are presented in Table 1. The mean ± standard deviation age of the twins was 38.9 ± 7.4 years for men (range 30–63 years) and 38.0 ± 7.2 years for women (range 30–73 years). The majority (63.5%) of the twins were women. There were 325 MZ and 81 DZ pairs by genotype (Table 2). Pair-wise comparisons revealed twin pairs with discordant individual zygosity. Setting a cut-off point between Never confused and Sometimes confused revealed a best sensitivity value for all three questions on the level of twin recognition confusion. There were 284 concordant MZ (MZ/MZ) and 63 concordant DZ (DZ/DZ) pairs, while 59 pairs were discordant for their zygosity; six, 33, seven, and 13 pairs were MZ/DZ, MZ/XZ, DZ/XZ, XZ/XZ combinations, respectively, corresponding to cells A–D in Figure 1. The zygosity of discordant cells was determined as the more probable one guided by genotype results. For example, since five of six discordant MZ/DZ combination pairs (cell A in Figure 1) turned out to be DZ, all twins in this category were regarded as six DZ pairs. The sensitivity of diagnosing MZ and DZ by this pair-wise algorithm was 98.8% and 88.9%, and the overall agreement between questionnaire and genotyping in terms of kappa value was 0.90. When we projected this pair-wise decision algorithm to the individual level, our QOZD had a positive-predictive value of 97.2% for MZ and 95.0% for DZ. When the individual diagnosis was XZ, it could be classified as MZ with 80.3% accuracy (Table 2).

When we further analyzed the characteristics of zygosity-misdiagnosed twin pairs, perceived zygosity before the survey tended to be less correct if it was simply derived from their resemblance (four MZ and 16 DZ individuals), which might have influenced their parents’ beliefs on their zygosity as well (one MZ and eight DZ twin individuals). Only a few had been informed of their zygosity by medical doctors or formal zygosity tests (Table 3). When we compared
twins whose zygosity was misdiagnosed with those correctly diagnosed, intra-class correlations between physical characteristics and biochemical tests exhibited different trends. For physical traits, misdiagnosed MZ twins showed less similarity in their height or weight than correctly diagnosed MZ twins, while misdiagnosed DZ twins showed strong similarity for those traits than correctly diagnosed DZ twins (Table 3). However, for biochemical markers with high heritabilities such as blood pressure or blood lipids, similarity of the misdiagnosed group did not demonstrate material difference with that in correctly diagnosed groups, partially due to limited sample size of misdiagnosed twin pairs reflected in wide confidence intervals.

**Discussion**

In most twin studies, genotyping of all the participants is usually inefficient or not feasible, even though the cost per genetic marker is getting lower. Considering the importance of correct zygosity diagnosis in twin research, it is crucial to have a good and efficient
method of diagnosis. A diagnosis based on QOZD is preferred because it can be reliably applied in a mailing survey as well as large-scale studies. The primary goal of this study was to estimate the validity of QOZD used in the Healthy Twin Study. By summarizing previously used methods, we attempted to develop a parsimonious method of zygosity diagnosis with acceptable accuracy. Additionally, we tried to examine the physical and biochemical characteristics of correctly and wrongly diagnosed pairs, to provide a rationale to add or not to add those characteristics to a diagnosis algorithm. Our zygosity determining method used a QOZD consisting of only four questions, and it turned out to have relatively high sensitivity (MZ: 98.8%, DZ: 88.9%), positive predictive value (MZ: 97.2%, DZ: 94.7%), and overall agreement rate with genotype information (simple overall agreement: 0.97, kappa value: 0.90), if information of both co-twins was available. When we estimated validity of diagnosing individual co-twin’s zygosity, those whose zygosity were decided as MZ (97.2%) or DZ (95.0%) showed a good positive predictive value as well. However, if individual zygosity was undetermined by the QOZD, about 80% turned out to be MZ. Considering that about 80% of the twins in this analysis were MZ, QOZD did not add information for the XZs if only one co-twin responded. The overall accuracy or predictive value is as high as previous studies which used QOZD methods with or without addition of physical characteristics to the questionnaire. (Chen et al., 1999; Gao et al., 2006; Ooki et al., 1990; Sarna et al., 1978) Most of the present MZ/XZ or XZ/XZ pairs turned out to be MZ twins, and most DZ/MZ and DZ/XZ twins were DZ twins. Considering the higher prior probability toward MZ, given from higher MZ proportions, it is remarkable that most of DZ/MZ or DZ/XZ pairs turned out to be DZ. However, considering the excess of MZ pairs, our findings indicating that 84.6% of XZ/XZ pairs were MZ would be less informative.

This study used relatively larger twin samples for validating zygosity. Thus, it was possible to explore the characteristics of the misdiagnosed twin pairs. The physical resemblance of misdiagnosed twin pairs differed from that of correctly diagnosed pairs for body mass index, weight, standing height, and sitting height. Misdiagnosed MZ pairs tended to have lesser degree of physical resemblance including height than correctly diagnosed MZs, although the reliability of this finding may be limited due to small size of this group (four pairs). However, misdiagnosed DZ pairs showed striking features in their physical resemblance. Not only the physical resemblance, in terms of correlation coefficient, of this misdiagnosed DZ group (nine pairs) exceeded that of DZ, it was even higher than that of true MZ pairs. As height and obesity influence the overall appearance of the body, it is logical to interpret the results as indicating that some DZ twins share many physical characteristics, which

<table>
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<th>Previously perceived zygosity (Q3 in the Appendix 1)</th>
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<th>Misdiagnosed</th>
<th>Correctly diagnosed</th>
<th>misdiagnosed</th>
</tr>
</thead>
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<tr>
<td>No (40 of 812 individuals)</td>
<td>34</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Yes (772 of 812 individuals (95.1%)</td>
<td>608</td>
<td>4</td>
<td>143</td>
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<td>From doctors</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>From Parents</td>
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<td>1</td>
<td>53</td>
<td>8</td>
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<tr>
<td>Based on resemblance</td>
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<th>intraclass correlation coefficients (p value)</th>
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<td>N = 4</td>
<td>N = 72</td>
</tr>
<tr>
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<td>0.93 (0.06)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Body mass index</td>
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</tr>
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<td>Sitting height</td>
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<tr>
<td></td>
<td>0.93 (&lt; .001)</td>
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<td>N = 72</td>
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<tr>
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<td></td>
<td>Not significant</td>
<td>Not significant</td>
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</table>

- Table 3: Characteristics of Misdiagnosed Twin Pairs for Selected Phenotypes
make them appear as MZ twins. Similarly, a smaller proportion of MZ twins have discordant height or obesity, which make twins believe they do not look alike, as do MZ twins. Given the unusual conditions in the misdiagnosed twin pairs, adding information on physical resemblance such as height or obesity will not improve the discrimination power. Conversely, our finding indicates that adding these general physical characteristics can even lower the discrimination power. Biochemical markers with higher heritabilities such as blood lipids or blood pressure did not show clear distinction between each group. Among physiologic traits, diastolic blood pressure tends to be more alike among true MZs than among DZs, regardless of the correctness of zygosity diagnosis. However, the correlation coefficients exhibited less consistent patterns, and marginal benefits of applying blood test results will be debatable. We did not test the power of discriminating misdiagnosed twin pairs for other frequently applied methods such as fingerprints, photographs, or various blood types. Considering the additional cost and efforts required for introducing these additional methods other than QOZD and ever lowering genotyping costs, the usefulness of those methods may be attenuated.

In our study, as in other Asian studies, almost all participants had dark eyes and dark hair color, which lessens the information value of these characteristics. We did not test models with the addition of various blood test results because, even if they could provide a small benefit, it was not economical to use those markers compared with using genotype information for discriminating zygosity.

Among the 16 STR markers, three MZ twin pairs showed two mismatched STR markers (i.e., only 14 identical markers). We considered them as MZ twins because other DZ twins had up to 11 identical STR markers (range 4–11 matches). For those DZ with participating parents, Mendelian inheritance patterns were all satisfied to validate that they were true DZ.

We projected the pair-wise decision algorithm to project the accuracy of determining individual zygosity. The predictive value and decision algorithm for individual zygosity will make a reference value for future larger scale mailing questionnaire surveys.

This study had several potential limitations. First, it was performed for adults over 30-years-of-age, and so is not directly applicable to any studies recruiting young adults or childhood twins. Second, the pairwise decision was guided by genotype results, and we did not introduce independent validation set. Because we attempted to examine the characteristics of misdiagnosed pairs, we did not divide twin samples for ‘formulating’ and ‘validating’ purposes. Finally, owing to abundance of MZ, true discrimination power of decision method for MZs could be inflated. However, overall validity of diagnosing MZs are much higher than proportion of MZ twins.

Although this study used simple decision tree-based zygosity discriminating algorithm based on QOZD with four questions, our findings suggest that the parsimonious method of diagnosing zygosity will be useful, especially for adult twins. Adding physical or physiological measurements to QOZD will either confound the correct diagnosis, as shown by height or obesity findings, or make the overall project less efficient if the measurements are not superior to genotyping in terms of cost-efficiency.

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Appendix A

Zygosity Questionnaire used in the Healthy Twin Study

Fraternal twins are not much different from siblings born together. But, identical twins very much resemble each other in body shape, skin color and appearance like 'looking reflection in the mirror' and therefore it is very difficult to distinguish. Please keep this in mind and answer the following questions.

Q1. In light of above mentioned facial and physical resemblance, my twin sister/brother and I are _______________twins.
   1. Identical   2. Fraternal   3. Don't know

Q2. When you were in your school age, how much you and your twin brother/sister was alike?
   1. When someone first saw us, they could not distinguish us.
      □ Never   □ Sometimes   □ Often   □ Almost Always
   2. Teachers and friends could not distinguish us.
      □ Never   □ Sometimes   □ Often   □ Almost Always
   3. Parents and other sister/brothers could not distinguish us.
      □ Never   □ Sometimes   □ Often   □ Almost Always

Q3. If you knew whether you are fraternal twin or identical twin, how did you know?
   □ I did not have any previous zygosity information
   □ I had information on my zygosity based on
      □ What Doctor told me
      □ What Parents told me
   □ resemblance, because we have too much points alike or different.
      (Explain in detail)______________________________________________
   □ After zygosity-diagnosing blood test
   □ Do not know