Letter to the Editor Re: How to Estimate Allele Frequencies and Make Statistical Comparisons in Case-Control Studies of Polymorphic X-Linked Loci

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Keywords

Allele frequency, Hardy-Weinberg equilibrium, polymorphisms, X-linked

Dear Editor,

It was with great interest that I read the article by Yaylacıoğlu Tuncay et al.[1] entitled "The role of *FOXP3* polymorphisms in Graves' disease with or without ophthalmopathy in a Turkish population" recently published in the *Turkish Journal of Ophthalmology.* The authors compared three *FOXP3* polymorphisms (rs3761549, rs3761548 and rs3761547) between Graves' disease patients with or without ophthalmopathy and between Graves' disease patients and controls.

As mentioned by the authors, the forkhead box P3 (*FOXP3*, MIM: 300292) gene has been mapped to human chromosome Xp11.23. Considering that males and females have one and two X chromosomes, respectively, the genotypic patterns are quite different between the two sexes. Females have three genotypes (including two homozygotes and one heterozygote) and males have only two hemizygous genotypes. Therefore, it is impossible to pool the genotypes of the sex groups. Unfortunately, the authors made a fatal mistake by pooling the genotypes of both sexes. I have already discussed this point in a debate.[2] However, I think it is necessary to explain the following points: 1) how we should estimate allele frequencies, and 2) how we should make statistical comparisons in case-control studies for such loci.

According to the STrengthening the REporting of Genetic Association studies statement, in genetic association studies, researchers should compare the observed and expected genotypic values based on Hardy-Weinberg equilibrium (HWE).[3]

Suppose we have two alleles A_1 and A_2, at an X-linked polymorphic locus. If the frequencies of A_1A_1, A_1A_2, and A_2A_2 genotypes in females are "a", "b" and "c", respectively, and the frequencies of A_1 and A_2 hemizygous genotypes in males are "d" and "e", then the allele frequency can be calculated by the counting method. The number of females and males is equal to n_1 (=a+b+c) and n_2 (=d+e), respectively.

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The allele frequencies of A_1 and A_2 are denoted by p and q, respectively, and are estimated using the following formulas:

$$p=(2a+b+d)/(2n_1+n_2)$$
$$q=(b+2c+e)/(2n_1+n_2)$$

Then, the expected values for the A_1A_1, A_1A_2, and A_2A_2 genotypes in the female samples, calculated using the estimated p and q, are equal to p2, 2pq, and q2, respectively. Finally, the observed and expected values for the genotypes should be compared using the chi-squared test. The degree of freedom is 1.

Because the number of X chromosomes differs between the sexes of the participants, to compare cases with controls (in case-control studies), participants should be stratified by sex. However, when allelic frequency is estimated in the manner described above, it is possible to compare allelic frequencies (and not genotypic frequencies) between all cases and controls, regardless of their sex.

Considering that Yaylacıoğlu Tuncay et al.[1] included both sexes among their participants, the reported results should be interpreted with caution. It is recommended that the authors present their data on the genotypes of each polymorphism according to the sex of the participants and reanalyze their data to address the major issues mentioned above. As emphasized elsewhere,[3,4,5] the researchers should also show that the frequencies of the observed genotypes are not significantly different from their expected frequencies based on HWE. I wish the esteemed authors all the best in their research.

Ethics

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Reply

First of all, we thank the author for evaluating our article.[1] As the author and we pointed out in our article, the Forkhead box P3 (*FOXP3*) gene is on the X chromosome. As far as we know, ten different reports in the literature have investigated the association between *FOXP3* polymorphisms and the development of Graves' disease (GD).[2,3,4,5,6,7,8,9,10,11] Seven of those reports presented the genotype and allele frequencies by pooling both sexes,[2,3,4,5,6,7,8] and the rest stratified the participants by sex and reported the genotype and allele frequencies separately in females and males.[9,10,11] In our article, we aimed to evaluate the frequency of *FOXP3* polymorphisms in GD with or without ophthalmopathy in a Turkish population.[1] Since the number of participants in each group was limited in our study and there were no corrections for the previously published articles,[2,3,4,5,6,7,8] we did not stratify the groups by sex and used the results of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) directly to analyze genotype and allele frequencies.[1] In the PCR-RFLP method, the results were shown by band patterns and those patterns do not indicate whether the sample had one or two alleles of the gene of interest. Additionally, using the same analysis method, we could compare our results with those seven reports that did not stratify the groups by sex.

However, as mentioned by the author, as males have one and females have two X chromosomes, the genotypic patterns differ between the two sexes. There are three genotypes (two homozygotes and one heterozygote) in females but only two hemizygous genotypes in males. Therefore, it will be better to stratify the groups by sex and define the genotype and allele frequencies separately in females and males for X-linked genes. Moreover, the author emphasized the importance of comparing the observed and expected genotypic values based on Hardy-Weinberg equilibrium (HWE) in his letter and explained the formula for an X-linked polymorphic locus. According to the concerns reported by the author, in this reply, we reported our additional analysis by stratifying the groups by sex as shown in Tables 1, 2, 3, and 4.

Firstly, we did the HWE analysis in both the controls and study groups, as suggested by the author. The frequencies of the observed genotypes were not significantly different from their expected frequencies based on HWE for all single nucleotide polymorphisms (SNPs) in female control groups (rs3761547, p=0.926; rs3761548, p=0.881; and rs3761549, p=0.926).