Re: HRAS1 Rare Minisatellite Alleles and Breast Cancer in Australian Women Under Age Forty Years

The report by Firgaira et al. (1) makes the potentially important observation that breast cancer in women under 40 years of age may not demonstrate association with rare alleles of the HRAS1 minisatellite. Breast cancer in younger women may differ genetically and biologically from late-onset disease and, therefore, the lack of association with genetic modifiers of modest effect would be an interesting, but not unexpected, outcome.

Although the authors mention potential biologic differences between cancers in patients of different age groups as one possible basis for the observed difference in rare allele association, they
clearly favor a more prosaic explanation: that “new methods” with greater resolving power challenge the original observations and meta-analysis. Under this construction, the previously observed association is merely an artifact of the inability of previous methods to resolve more rare alleles among larger sized, common (low-risk) alleles.

I believe this study has two flaws, one technical and one conceptual, that undermine its conclusions. On the technical side, the newer methods using fluorescence-based genotyping on automated sequencing platforms do resolve alleles better. But a critical issue is the reliability of polymerase chain reaction (PCR) amplification in detecting the larger alleles that sequencers are capable of resolving. The PCR-based method described in the report by Firgaira et al. is not satisfactory in this regard. We know from our own experience that PCR strategies with straightforward cycling protocols can preferentially amplify lower molecular weight alleles at the expense of larger ones (“allele steal”) or they can create artifactual rare alleles from larger rare and common alleles. Our own protocol combines additives (dimethyl sulfoxide and betaine [N,N,N-trimethylglycine]), proofreading enzymes, and a multistage cycling protocol to minimize artifactual loss of larger alleles (2,3).

It seems likely that genotyping artifacts introduced by a technique that is not robust may have affected the outcome of their study in a major way. For example, the frequency of their largest common allele, a4, is only slightly more than half of that reported in other studies. This loss is not compensated by the appearance of newly resolved alleles a4−3, 4−2, 4−1, 4+1, 4+2, and 4+3. In contrast to the statements in the “Discussion” section that provide a larger number, I compute a total allele frequency in this group from Table 1 (1) of 0.05, compared with the meta-analysis value of 0.09. Therefore, nearly half the high-molecular-weight, low-risk alleles are unaccounted for in their cohort. Since the authors maintain that the population they are studying is ethnically comparable to that of other studies in the literature (an assertion with which I concur), then there must be a technical flaw in the genotyping. Of potential relevance is the fact that only 63% of available blood samples are reported for case patients and 80% of available samples are reported for control subjects. It would be important to know if technical failures account for any of this discrepancy. Although the differences discussed above in low-risk allele frequencies are small, these differences can have a very large effect on the case–control distribution of rare alleles.

The authors state that their analysis reveals no deviation from Hardy–Weinberg equilibrium, which, if true, would argue against substantial technical artifact. However, I strongly question whether, given the sample size, such analysis is sensitive enough to detect critical differences among so many sparsely populated allele classes. Therefore, I believe that any conclusions drawn from these data must be set aside until the technical question is resolved. A blind exchange of samples for repeat genotyping between my laboratory and that of the Australian group would settle this issue.

On the conceptual side, one would predict that, if lack of resolution of more alleles in larger, low-risk allele classes were the basis for the previously noted association, then simply regrouping newly resolved alleles should reproduce the old association. In other words, pretending that a2−2, a2−1, a2, a2+1, and a2+2 are all a2 (and likewise for a3 and a4) should recreate the case–control differences. No rebinning of a1-related alleles is required, since they were resolved by earlier methods. Ignoring the issue of genotyping accuracy for the moment, rebinning the alleles in this report still reveals no difference in the frequency of rare alleles between case patients and control subjects. Therefore, the conclusion concerning the effect of technical resolution of alleles on cancer association is questionable.

Until the issues raised herein are addressed, the possibility of age-related differences in the association of rare HRAS1 alleles with breast cancer remains an interesting, but unproved, outcome.

THEODORE G. KRONTIRIS

REFERENCES


Note

Correspondence to: Theodore G. Krontiris, M.D., Ph.D., City of Hope National Medical Center, Beckman Research Center, Division of Molecular Medicine, 1500 East Duarte Rd., Duarte, CA 91010 (e-mail: tkrontir@coh.org).

Response

We welcome Dr. Krontiris’ offer to exchange samples for repeat genotyping between our laboratories to help resolve the issues raised by our report and his letter.

Dr. Krontiris considers our study to have two flaws. On the technical side, we do not believe that our polymerase chain reaction (PCR)-based genotyping system is failing to detect large HRAS1 variable number of tandem repeats (VNTR) alleles due to “allele steal” (dropout). We were well aware of this phenomenon when we commenced this work and undertook considerable PCR optimization to reduce or eliminate it (1). We used a proofreading enzyme system. Trials with dimethyl sulfoxide and multistage cycling protocols did not provide improvements. (Betaine was not tested.) Our technique allowed us to amplify HRAS1 allele heterozygote combinations with large size differences; i.e., a1, a4 + 3, and a1, a4 + 8 (see Table 1 from our study) and a1, a4 + 15 (unpublished results). We did not experience allele dropout.

Furthermore, although our finding of a combined case and control a4 allele frequency of 0.03 (95% confidence interval [CI] = 0.02–0.04) clearly differs from the value of 0.09 (95% CI = 0.07–0.11) quoted by Dr. Krontiris based on older studies using Southern blot analysis protocols (2), it is not inconsistent with more recent work reporting values in control subjects from 0.015 to 0.045 (3–5) or with the estimate derived from controls by Dr. Krontiris’ laboratory (6) of 0.06 (95% CI = 0.03–0.09) (P = .15; two-sided test).

An important finding of our study was that, in control subjects, the rare a-
malle frequency using the newer method (0.17; 95% CI = 0.14–0.20) was substantially different from that found by older Southern blot analysis (0.06; 95% CI = 0.05–0.07) (P < .001; two-sided test). Even in recent work from Dr. Krontiris’ laboratory (6), the allele frequency in control subjects is more than twice as large (0.13; 95% CI = 0.09–0.18) as that found in his previous work using Southern blot analysis (P < .001), yet it is no different from our estimate, once sample sizes are taken into consideration (P = .25; two-sided test).

On the conceptual side, the argument proposed by Dr. Krontiris is predicated on the assumption that there is a real association of breast cancer with the so-called “rare alleles,” as measured by Southern blot analysis. We do not presume to know why there appears to be a difference between the previous apparent association with a risk factor measured with poor resolution and the lack of association in our study using a higher resolution technique. We are not at odds with Dr. Krontiris’ summation that, with or without rebinning of HRAS1 alleles, our data from women under the age of 40 years reveal no difference in frequency of rare alleles between case and control subjects.

Despite the now apparent large misclassification error of the Southern blot analysis methodology for assigning rare alleles, Dr. Krontiris seems to continue to believe that the results of the meta-analysis remain valid when the newer high-resolution technologies are applied, referring to the common alleles as “low-risk” alleles. The possible explanation for the discrepancy between our null result and that of the meta-analysis—that the putative genetic effect “may be confined to or may be stronger in cancers occurring at a later age”—was only raised by us in response to a reviewer’s opinion and is not one that we would favor without having studied older onset case patients using the newer methods. In this regard, we would invite Dr. Krontiris to help resolve the issue of a possible age-related difference in association by studying with us a large number of population-based case and control subjects with later onset disease, whom we have recently collected as part of the Cooperative Family Registry for Breast Cancer Studies (CFRBCS) (7), funded by the National Institutes of Health.

REFERENCES


NOTES

Affiliations of authors: J. L. Hopper, G. S. Dite, The University of Melbourne, Centre for Genetic Epidemiology, Carlton, Victoria, Australia; F. A. Firgaira, R. Seshadri, C. R. E. McEvoy, Department of Haematology and Genetic Pathology, Flinders University, and Flinders Medical Centre, Bedford Park, South Australia; G. G. Giles, Cancer Epidemiology Centre, Anti-Cancer Council of Victoria, Carlton, Australia; M. R. E. McCredie, Cancer and Epidemiology Research Unit, New South Wales Cancer Council, Kings Cross, Australia, and Department of Preventative and Social Medicine, University of Otago, New Zealand; M. C. Southey, D. J. Venter, Department of Pathology and Research, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia, and Department of Pathology, The University of Melbourne, Parkville, Victoria, Australia.

Correspondence to: John L. Hopper, Ph.D., The University of Melbourne, Centre for Genetic Epidemiology, 200 Berkeley St., Carlton, Victoria 3053, Australia (e-mail: j.hopper@gpph.unimelb.edu.au).