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Letters to the Editor

Subsite-Specific Colorectal Cancer in Diabetic and Nondiabetic Patients

To the Editor: We read with interest the report by Limburg et al. (1), a large prospective study of postmenopausal women which noted the association between type II diabetes mellitus and incident colorectal cancer to be subsite specific. Specifically, they reported a statistically increased risk of proximal colon cancer (relative risk, 1.9; 95% confidence interval, 1.3-2.6). An alternate way of exploring the question of diabetes and subsite-specific bowel cancer risk is to start with a population of patients with colorectal cancer, looking for differences in the distribution of tumors in the diabetic versus nondiabetic populations. This also removes some of the potential biases in the report by Limburg et al., as they included only female patients, of a relatively tight age range (55-69 years at registration), and relied on a patient questionnaire (returned by 42% of women).

Here we report from our prospective colorectal cancer database of 1,139 patients entered over 14 years (from 1990 to 2004), at two Australian Hospitals. Prospective information on patient comorbidities has been collected on all patients, including diabetes mellitus. Using the same definitions as Limburg et al. (1), there were 308 proximal colon cancers, 456 distal colon cancers, and 365 rectal cancers. Ten patients with multiple primary tumors were excluded from the analysis. Of these colorectal cancer patients, 188 (16.5%) also had diabetes.

The subsite distribution of colorectal carcinoma was not statistically different in diabetic and nondiabetic patients; however, there was a trend for more proximal cancers in the diabetic patients, 35.1% having proximal tumors versus 26.8% in the control group. The odds ratio of having proximal tumors compared with other sites in diabetic patients was 1.50 (95% confidence interval, 1.08-2.09); this was consistently adjusted for age and sex. Further breakdown of this distribution according to sex revealed similar findings (42.3% versus 33.3% proximal tumors for females and 30.0% versus 21.1% proximal tumors for males). For completeness, the percentage figures for distal colon (36.7% versus 40.1%) and rectal cancers (28.1% versus 32%) were similar for diabetic and nondiabetic patients.

Our findings are consistent with the hypothesis that diabetic patients are more likely to develop proximal colon cancers, as reported by Limburg et al. (1) and previously in the Nurses Health Study, which reported a relative risk of 1.64 for proximal cancers (95% confidence interval, 1.04-2.60; ref. 2). Interestingly, both of these studies included only women, whereas our study included both male and female patients. In the only other study with significant numbers of patients with colorectal cancer (3), the authors also reported a slightly higher risk of proximal colon cancers. Based on all of these reports, we agree with Limburg et al. (1) that screening of diabetic patients with sigmoidoscopy alone may be of less value than in the nondiabetic population.

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Robustness of Case-Control Studies to Population Stratification

To the Editors: Using computer simulations, Khlat et al. (1) quantified type I error increase caused by population stratification. They argued that under ''realistic scenarios'' (where subpopulations account for ≤10% of the study population and allelic frequency differences are ≤0.2), the inflation of type I error is of limited concern.

Results from both computer simulations (2) and theoretical analyses (3) suggest a more nuanced and complex view of population stratification. Our results are consistent with some of those by Khlat et al. (1). First, a large inflation in type I error can occur when the two subpopulations are equal sized but have moderate marker allele and disease frequency differences. Second, the confounding risk ratio provides a poor measure of the increase in type I error rate under population stratification, as we showed in ref. (3). However, our computer simulations also show that having a mixture of unequal-sized subpopulations (e.g., 10% versus 90%) does not necessarily lead to a reduction in the inflated

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type I error rate; neither does an increase in subpopulation number, in contrast to Wacholder et al. (4). This is particularly true when marker allele frequency, disease prevalence, and population size are not independent (3). For example, founder effects and bottlenecks can create dependence between subpopulation size and disease prevalence and/or marker allele frequency. This may lead to subpopulations with substantially elevated marker allele frequency and disease prevalence rates relative to the majority population (5).

Consequently, a marker difference of 0.2 is not the maximum difference that one can expect, as suggested by Khlat et al. Indeed, Khlat et al. showed that when the marker difference is only 0.2, the type I error can reach 19% (i.e., on average, one of every five positive findings is false), which we argue is a substantial increase in the type I error rate over the nominal rate of 5%. This would be even higher with larger marker differences. Our results indicate that when the population variables are not independent, the type I error rate does not approach the nominal type I error rate of 0.05 even with a large number of subpopulations (3). Moreover, even when the population variables are independent, type I error may not converge to 0.05 until a very large number of subpopulations are reached.

Thus, the issue of population stratification is more complex than has been believed and is of more than ''limited'' concern. Allele frequency differences and possible interdependence of population variables suggest that population stratification cannot be dismissed out of hand.

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In Response: In our study of the robustness of case-control studies to population stratification, we conclude that the bias and type I error resulting from population stratification are likely to be ''limited in methodologically sound case-control studies of moderate size, except in quite unrealistic scenarios.'' Heiman et al. argue that their own analyses (1, 2) lead to a more nuanced view of the stratification bias in case-control studies. We fully agree with their assertion that ''relatively small CRR values can actually represent highly inflated type I error,'' and this is exactly the reason why we have focused on type I error in relation to the population variables and sample size. We feel that the conclusions of our study do complement and strengthen those of Wacholder et al. (3), and also that our findings are very much in accord with the findings of Heiman et al. The main point of divergence concerns the degree of variability of allelic frequencies across population subgroups, as Heiman et al. pinpoint the special situations of very small subpopulations exhibiting subtantially elevated marker allele frequency and disease rates relative to the majority population. More generally, they argue that, in populations containing a mixture of subpopulations, the disease prevalence can depend on the size of those subpopulations, and that the contention that ''the greater the number of distinct population subgroups, the smaller the bias'' does not hold in that case. This is indeed a very interesting and important point, and we do agree that, due to founder effects and genetic drift related to inbreeding, the allelic frequencies in some small subpopulations may shift far away from that of the majority population. And yet, as already pointed out by Wacholder et al. (3) ''only ethnic groups that maintain their individual identities are likely to remain endogamous and retain any important differences in genotype frequencies.'' It can therefore be argued that, whenever such a situation arises, it does concern distinguishable ethnic groups, and can be handled by matching or statistical adjustment. In that case, the allelic differences which have to be considered for the purposes of investigating the stratification bias are those which remain after accounting for ethnicity, and those ''residual'' differences are likely to be moderate. Whereas we find that the point made by Heiman et al. is very relevant, we believe that it does not question our conclusions, and maintain that, in carefully matched, moderate size studies, the type I error associated with population stratification remains very limited in most realistic scenarios.

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Accuracy and Reproducibility of Short Tandem Repeat Assays

To the Editor: By investigating the accuracy and reproducibility of short tandem repeat assays, Tran et al. (1) address an important issue in epidemiologic research. In their analyses, sequencing and sizing analysis yielded an exact match on repeat number for fewer than 10% of samples. Their conclusion that ''... laboratory analysis of dinucleotide STR may not be as reliable as originally thought'' is, therefore, very generous. For their sample size, one would normally expect 100% concordance between two assays. This kind of result should have prompted the authors to investigate the reason for the discrepancies.

However, there are several explanations for the high discrepancy between the two assays that the authors apparently did not address:

1. Because of the stutter bands in sizing assays, the data interpretation can be difficult (2). However, the second chromatogram in Fig. 2 shows a clear example of a 187/189 heterozygote and not a 187 homozygote.
2. Direct sequencing of an amplified PCR fragment is a poor choice when what is needed is an allele-sizing assay. As Fig. 2 shows, there is substantial—and unavoidable—stutter in the PCR reaction. Sequencing this mix of fragments will inevitably lead to an apparent heterozygous call.
3. The stutter bands are usually shorter than the original template. By using the number of repeats with unambiguous sequence calls as the allele size, the authors misclassified alleles as shorter than they really were; for example, if the true repeat length is 20, then the most prominent stutter band has 19 repeats. Sequencing will produce unambiguous calls only for 19 repeats. Furthermore, to allow proper comparisons, the authors would have been best advised to use the same method for scoring short and long alleles.
4. On any gel, DNA fragments usually run within ±10% of their true size. In addition to size, the migration is affected by secondary structure and charge. This applies to the fragment of interest as well as the size standards. Therefore, before launching into a genotyping project, it is essential to establish the correlation between apparent fragment length in a sizing assay and the number of repeats for each system individually. This is best done by cloning individual alleles and sequencing the plasmids without prior PCR amplification. Stutter during sequencing may still be an issue but is less of a problem than adding stutter in sequencing to stutter in PCR.
5. Because a population is never exclusively heterozygous for polymorphisms such as the insulin-like growth factor-I CA repeat, the authors might reasonably have been expected to question their sequencing results.

A reassessment of the data that takes into account the source of errors inherent in these assays may result in more meaningful conclusions regarding associations between insulin-like growth factor-I genotype and breast cancer risk.

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In Response: The comments made by Jeannette Bigler are valuable and helpful as one of them reiterates the point that we intend to emphasize in our report, which is that the determination of dinucleotide short tandem repeats using DNA sizing analysis is somehow subjective when the differences between homozygous and heterozygous alleles are not obviously shown on the chromatogram. The difference between the calls made by Jeannette Bigler and one of our investigators who did the sizing analysis without any information on the specimen is a perfect example that shows the inconsistency in determination of dinucleotide short tandem repeat genotype with the use of sizing analysis. Our comparison of sequencing results with those of sizing analysis also indicates that it is possible that some of the heterozygous samples may be misclassified as homozygous genotypes by the sizing analysis. Another interesting finding of our comparison is that sequencing results match well to the sizing results when concerning the size of short alleles; the discrepancies mainly come from the comparison of the long alleles. Thus, the explanations provided in the letter are not likely to change our findings even if we reassess our laboratory data accordingly.

The second point that we would like to convey in our article is that when using DNA sizing analysis to determine short tandem repeat genotype, one should be aware of the limit of the method in terms of resolution (i.e., the minimal size of nucleotide difference that the method can detect). Although sizing analysis is a perfect method for large short tandem repeats (trinucleotide or larger), the method may not have adequate resolution for a single dinucleotide difference. Under well-controlled laboratory conditions and careful performance of the method by experienced staff, we believe sizing analysis is capable of distinguishing a signal nucleotide difference. However, in large-scale epidemiologic studies, many stringent laboratory conditions

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are compromised and DNA samples are highly heterogeneous in terms of their quality due to variations in specimen collection, storage, processing, and handling. Under these situations, whether the method still maintains the same resolution is uncertain and needs reassessment.

Although sequencing analysis is not a method of choice for short tandem repeat genotyping, comparison of the results between these methods does reveal several interesting findings. As a result, questions are raised as to whether sizing analysis is sensitive enough to identify a single dinucleotide difference, and if not, whether misclassification generated by the method will affect study results and in which direction.

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