

Letters to the Editor

Evidence for an Association between JC Virus and Colorectal Neoplasia

To the Editor: Newcomb et al. (1) have attempted to confirm the presence of JC virus (JCV) DNA in colorectal cancers and have failed to do so. They reached their negative conclusion, although other groups have been able to show JCV in colonic epithelium and colorectal neoplasia (2-4). Whether this polyomavirus, which encodes a potent oncogene (T antigen), causes colon cancer remains an open question. However, its presence in colonic tissues and colorectal neoplasms in particular is not a question. This has been reported convincingly from two laboratories.

Our group has repeatedly confirmed this observation on different specimens of colorectal cancer (published and unpublished), each time using multiple controls to ensure the absence of PCR contamination. The assay is somewhat technically difficult and requires a focused effort by a dedicated laboratory to perfect. We began work on this project in 1994, and although the initial experiments were positive, we took several months to optimize the PCR conditions. JCV is a small circular DNA virus and may be present in relatively small amounts in colorectal neoplasms. Newcomb et al. used novel primers and an entirely different method for their assays. Specifically, all of their assays were done on DNA extracted from formalin-fixed, paraffin-embedded specimens. We initially had some difficulty performing JCV PCR from paraffin. The formalin fixation breaks DNA and may make it more difficult to recover sufficient quantities of intact JCV DNA for amplification. Newcomb et al. may have had difficulty with PCR amplification of JCV out of paraffin as well. In fact, they reported no positive controls in which they were able to get this PCR to work from paraffin tissues. They readily obtained PCR products from a plasmid preparation or urine samples, much the same way we have readily obtained PCR evidence for JCV from frozen specimens that have never been fixed in formalin. The only positive PCR result obtained from the 233 fixed tissues by Newcomb et al. was not confirmed either by Southern blot or DNA sequencing; even this may have been a nonspecific result.

Essentially, Newcomb et al. have attempted to use an assay that had not undergone appropriate, relevant validation and speculated that their failure to obtain PCR products refutes multiple lines of evidence from other laboratories. A BLAST analysis indicates that the T-antigen primers in this study form multiple primer dimers, which would almost certainly limit the PCR efficiency. Moreover, other primers were found to have complete sequence matches with BK virus as well as JCV, suggesting that the primer design was not optimal.

It is unlikely that the results from our laboratory represent assay contamination because of the DNA sequence variations we have found in the T antigen and in the transcription control region. Contamination is likely to

provide a uniform product. Moreover, it is extremely unlikely (and would be unprecedented) that the rearrangements in the transcription control region reported by Ricciardiello et al. (5) represent PCR artifacts, as these sequence alterations are additions or deletions of a 98-bp cassette; similar rearrangements are generated during viral replication in nature and have been found in human samples by others (6, 7). The authors' suggestion that our PCR amplification of JCV represents nonspecificity due to the use of degenerate primers makes little sense, as our PCR products were validated both by Southern blot and by DNA sequencing. In addition, Enam et al. (4) have confirmed our findings, and extended these, by demonstrating protein expression of the T antigen and agnoprotein in colon cancers, but not normal colon, using immunohistochemistry. This important confirmation was dismissed without consideration by Newcomb et al. Considering the work of two independent laboratories, it is difficult to accept that a novel, unvalidated assay is sufficient to conclude that the prior investigators have been inaccurate.

It is reasonable for Newcomb et al. to conclude that they are unable to document the presence of JCV using *their* approach on paraffin-embedded tissues. The assay developed by our group and Khalili's group took considerable effort to validate. The best explanation for Newcomb et al.'s findings is that they have not made the assay sufficiently sensitive to find JCV DNA sequences that are actually present.

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In Response: Dr. C. Richard Boland raises concerns about the ability of our study to detect an association between JC virus and colorectal cancer. Our data do suggest that it can be present in colon, albeit at much lower frequency than his group (1, 2) and Enam et al. (3) report. However, in our opinion, neither their data nor ours support a role of JC virus as a cause of colon cancer (4). Ricciardiello et al. (2) report a high prevalence of JC virus in gastric and colonic tissue from patients without gastrointestinal neoplasia (>70% of participants). In our study, the prevalence of JC virus was <0.005% in normal tissue from colorectal cancer patients.

The cause of the large difference in JC virus prevalence probably is due to technical issues. Blast searches done at the time we designed the primers as well as Blast searches done now did not indicate homologies between our PCR primers and BKV sequences, as suggested by Dr. Boland. Our primers and probes were designed using PrimerExpress (Applied Biosystems, Foster City, CA), which eliminates primers pairs that are likely to form dimers.

In our assay design, we did take into account the lower quality of DNA from paraffin-embedded tissue we were testing by designing shorter amplicons (100-150 bp), which amplify well even from degraded DNA. As we explained in the Discussion section of our article, we had no problems amplifying 10 markers of similar size in single-copy genes from these DNAs and have no reason to believe that the DNA contained contaminants that inhibited amplification. We also screened DNA from normal, fresh frozen colon biopsies from 80 individuals, none of which turned out to be JC virus positive. Furthermore, their report that Mad-1 is the exclusive JC virus strain found in the human colon (5) is not consistent with our finding of a colonic isolate that did *not* have a rearranged regulatory region. This fact was not stated clearly enough in our article and we are grateful to Dr. Boland for an opportunity to make up for this omission.

Our criteria for a “JC virus positive” call were more stringent than those of Laghi et al. (1). Samples were classified positive only if we could obtain sequence for at least two of the three fragments tested, whereas in Laghi et al. (1) one positive amplification of an average of four amplifications per sample led to a positive call. This difference alone would have an effect on the proportion of positive calls.

Dr. Boland argues that the presence in their study of different JC virus sequences within an individual speaks against contamination with their positive control. Although it is well known that Taq polymerase is error prone (6), this is not usually a problem when direct sequencing PCR products but will become noticeable when cloning PCR products and then sequencing individual clones, especially when starting with a small copy number of template (6). In such a case, it is essential to sequence numerous clones to obtain a

consensus sequence. Thus, the sequence differences in clones derived from the same patient as reported by Dr. Boland’s group (1, 2, 5) are much more likely due to errors introduced during PCR amplification than intra-individual variation. Unfortunately, direct sequencing of some of their PCR fragments does not seem possible because of other fragments that are coamplified in their PCRs (see figures in refs. 1, 2, 7). Our amplifications, however, were optimized to allow direct sequencing, which is preferable to Southern hybridization as a tool for sequence verification. We agree that the 98-bp deletion in their clones is not likely due to PCR artifacts. However, because this region is prone to rearrangements, it is very conceivable that such rearrangements occur while growing up the clones. A comparison of direct sequencing of the PCR fragment before cloning and sequencing individual clones afterward could address this question.

In summary, the reports from Dr. Boland’s group had some important limitations. The intraindividual sequence heterogeneity on the one hand and the finding of the same strain in different individuals on the other (5) are not consistent with other published work: (a) longitudinal studies that show shedding of the same JC virus strain (8), (b) extensive work on the geographic distribution of different JC virus strains (e.g., ref. 9), and (c) uniqueness of rearrangements of neurotropic JC virus isolates from different individuals (e.g., ref. 10).

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