p53 Expression after Treatment with Zebularine Is Not Due to Demethylation

To the Editor:

In their recent article, Hodge et al. suggested that the expression of the p53 gene in the two myeloma cell lines KAS6/1 and IM-9 is regulated by methylation of CpG and CpA dinucleotides in the promoter region (1). This hypothesis is based on the observation that the expression of p53 increases after treatment with the DNMT inhibitor zebularine. Bisulfite sequencing is used to support this hypothesis. However, the primers listed in this article for amplification before sequencing are flawed in two ways. First, the primers are specific for nonconverted DNA. Treatment with sodium bisulfite converts unmethylated cytosine residues into uracils (2). Primer design for PCR reactions used for bisulfite sequencing must reflect this conversion (3). The primers used in this article would only amplify nonconverted DNA, with retention of CpG and CpA sequences interpreted as methylated sites, when in fact, these could be nonconverted sites. Second, all of the primers listed are “sense” primers that are complementary to the antisense strand. It is unclear whether this represents an omission of an actual antisense primer sequence used.

To verify the reported results, we did bisulfite sequencing on the KAS6/1 cell line according to standard conditions (4). Primer sequences were (sense) 5'-TGTttAGtTTTGTGttAGGAGttT and (antisense) 5'-CAaaAaCTTACCCAATCCAaaaAAaC (lowercase letters represent bisulfite-modified bases). PCR amplification preceded cloning into the pCR2.1-TOPO vector. Five clones were sequenced, and the results are shown in Fig. 1. No CpG or CpA methylation was observed. The increased expression of p53 after treatment with zebularine is therefore likely due to mechanisms other than decreases in promoter region methylation. Increased p53 expression in response to DNA damage has been reported in A549 NSCLC cells after treatment with the DNMT inhibitor decitabine (5).

Johann C. Brandes
James G. Herman
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
Baltimore, Maryland

References


Figure 1. The primer positions for PCR reactions that were used for bisulfite sequencing are displayed in relation to the transcription start site. Sequencing results are summarized in graphical form. Open circles display nonmethylated CpG's. No cytosine residues, either CpG or CpA, were present in the sequenced clones.
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In Response:

In their recent Letter to the Editor, Brandes et al. raised several issues regarding our research article entitled, “Interleukin 6 Supports the Maintenance of p53 Tumor Suppressor Gene Promoter Methylation” (1). First, they suggest that primers used in the amplification of bisulfite-converted DNA must reflect the cytosine-to-uracil/thymidine conversion that occurs with bisulfite treatment. We included the unconverted sequences for the primers used to amplify bisulfite-treated DNA, assuming that individuals familiar with the field would know that unmethylated cytosines are converted to uracils and appear as thymidines in the sequencing data. We also choose to include both the forward and reverse primers as they would appear in the p53 promoter sequence and to designate them as 'sense' and 'antisense' to indicate the directionality required for PCR. Showing the actual sequence allows the reader to quickly pinpoint the exact position of the primers on the promoter sequence. We are happy to include this information and will gladly submit this as a supplemental erratum at the Editor's request for inclusion in a future journal issue. The primers for the first round of PCR amplification are 5'-TGTTTTTATGTTTGTAGTTTT-3' (sense) and 5'-TAACATCAAAAACTCATCAAATTCAAT-3' (antisense); and for the second round of PCR amplification, the primers are 5'-GTTAATTTTTAAAGGTTTTTTAGAATTT-3' (sense) and 5'-CAAAATTTCAAAAACTTACCAAATCCAA-3' (antisense).

Next, Brandes et al. used primers of their own design to perform bisulfite sequencing on KAS6/1 cell line DNA and failed to detect any methylated cytosine residues. It should be noted that the primer sequences used in their analysis were (sense) 5'-TGTATTTCTGTTAGAATTTTT-3' and (antisense) 5'-CAAAATTTCAAAAACTTACCAAATCCAA-3' (lowercase letters represent bisulfite modified bases). These primers exclude nearly half of the promoter region we reported to contain methylated cytosines, and their sense primer overlapped two potential sites of methylation, excluding binding of this primer if either of these two cytosines were methylated (unconverted). There were no results presented, indicating that a combination of PCR primers was used to take into account the potentially variable methylation status of this primer-binding site. Using a primer that only recognizes the converted (unprotected or unmethylated form of cytosine) in a primer-binding site. Using a primer that only recognizes the converted (unprotected or unmethylated form of cytosine) in a PCR reaction would tend, in our opinion, to skew the results towards PCR amplification are 5'-CAAAATTTCAAAAACTTACCAAATCCAA-3'.

In support of this notion, we should mention that during our extensive genomic bisulfite sequencing (GBS) analysis of this region, we observed potential sites of methylation that were protected in 100% of the clones sequenced. However, we also observed sites where only 1 in 10 clones showed a protected cytosine residue, and some clones that showed no protection at all. As explained in our article, we reported the presence of methylation at a particular region of the p53 promoter and offered supporting data that indicated that the loss of methylation increased p53 gene expression. We have since repeated GBS analysis of the p53 promoter region in several other myeloma cell lines. The results were similar in nature to our previous findings, in that within a single cell line, there were clones with regions of high methylation, clones with regions of low to sporadic methylation, and finally, some clones that showed no methylation. Frankly, we are not surprised by this absence of methylation, even in the same cell line, because there is a possibility that these cell lines are actually heterogeneous in nature.

Furthermore, it is not clear if the DNA used in the Brandes et al. analysis was obtained from KAS 6/1 cells that were carried for many months in interleukin-6 as ours were. We believe the transient and pleomorphic nature of cytosine methylation that we have observed, even among isolates of the same cell line, could also be affected by other factors, such as time between passages, different sources of serum used for media supplementation, and simply different adaptations of isolates of the same cell line. Another possibility is that these differences in methylation could reflect the inherent heterogeneity between cells in culture, or fluctuations in methylation status of DNA taken at different points in the cell cycle (these cells were never synchronized).

Brandes et al. also suggests that “...the increased expression of p53 after treatment with Zebularine is therefore likely due to other mechanisms than decreases in promoter region methylation. Increased p53 expression in response to DNA damage has been reported in A549 NSCLC cells after treatment with the DNMT inhibitor decitabine.” That decitabine (5-aza-deoxycytidine) induces DNA damage and p53 expression in these cells is the primary reason we decided to use zebularine instead of this highly toxic DNMT inhibitor. Zebularine is more stable, has a much broader range of efficacy, is incorporated into DNA at far lower levels than decitabine, and has been given long term without similar off-target effects as observed with Decitabine (2). Furthermore, experiments conducted in our laboratory have demonstrated that zebularine, in contrast to the known DNA damage-inducing drug etoposide, does not increase phosphorylation of H2AX, which is an indicator of DNA damage in multiple myeloma cell lines. Therefore, we believe that the induction of p53 occurs through demethylation and not induction in response to DNA damage.

If this argument shows anything, it is that the analysis of even the same cell line requires great care to faithfully duplicate every condition and prior circumstance leading up to the time of analysis. The ambiguities in the field of epigenetic study are monumental, as we have only recently begun to unravel this complex system. Thus, it is premature and unwarranted to make unsupported conclusions until such time as all the pieces of the “puzzle” have been assembled. Having said this, we thank our colleagues for pointing out to us an opportunity to clarify our work, and we also thank them for their excellent reports in the past, many of which we have read with great interest.

David R. Hodge
William L. Farrar
National Cancer Institute at Frederick, Frederick, Maryland

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