Re: Biologic and Biochemical Analyses of p16\textsuperscript{INK4a} Mutations From Primary Tumors

In the September 15, 1999, issue of the Journal, Yarbrough et al. (1) report biologic and biochemical analyses of p16\textsuperscript{INK4a} mutations detected in pri-
primary tumors. One of the mutants described, a substitution of leucine for the wild-type proline amino acid residue in codon 48 (P48L), has been identified in human tumor cell lines, including two human melanoma cell lines (2,3), as stated in the report. However, in addition, we have previously reported that this alteration occurs as a germ-line mutation in affected individuals of Swedish kindred with hereditary cutaneous melanoma (4). This kindred also has a high incidence of other malignancies.

In their report, Yarbrough et al. state that the P48L mutant leads to a p16 protein that binds to cyclin-dependent kinases cdk4 and cdk6, but it has a defect in cdk4/6-cyclin D kinase inhibition and cell cycle arrest. This result is surprising because binding of p16 to cdk4/6 normally abolishes kinase activity. Moreover, this result is in contrast to our previously published findings (5) and those of others (6,7). These three reports all show that binding of the P48L mutant p16 protein to cdk4/6 is abolished or severely reduced. With respect to cdk6 binding, the results of these investigations differ somewhat. Although we were unable to detect binding of the P48L mutant protein to cdk6, Castellano et al. (6) and Ruas et al. (7) showed different levels of residual cdk6 binding. Ruas et al. also demonstrated loss of growth arrest of human fibroblasts after transfection of a vector containing a complimentary DNA encoding the P48L mutant differ from report (ii) and those of others (6,7). These three reports all show that binding of the P48L mutant p16 protein to cdk4/6 in vitro is abolished or severely reduced. With respect to cdk6 binding, the results of these investigations differ somewhat. Although we were unable to detect binding of the P48L mutant protein to cdk6, Castellano et al. (6) and Ruas et al. (7) showed different levels of residual cdk6 binding. Ruas et al. also demonstrated loss of growth arrest of human fibroblasts after transfection of a vector containing a complimentary DNA encoding the P48L mutant p16 protein (7). As further support for the significance of the CDKN2A-P48L germ-line mutation for the development of hereditary cutaneous melanoma, we have demonstrated loss of the wild-type allele in a primary melanoma and a metastatic tumor in a mutation carrier belonging to the Swedish melanoma kindred (5).

The discrepancy between the results published by Yarbrough et al. and those previously presented cannot be explained at present but may relate to methodologic differences. This observation supports the need for a common standard for functional testing of CDKN2A mutations.

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REFERENCES


NOTES

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RESPONSE

In the September 15, 1999, issue of the Journal, we published our findings of the biologic and biochemical analyses of tumor-associated p16^INK4a mutants (1). Dr. Hansson correctly notes that functional analyses of the proline-48 leucine (P48L) mutant differ from report to report. Previously published analyses of p16 mutants are confusing due to conflicting results, different methodologies, and incomplete testing for all known functions of p16—namely cyclin-dependent kinase (CDK) binding, inhibition of CDK activity, and ability to cause a cell cycle arrest.

Dr. Hansson suggests that data from three reports contradict our finding that the P48L mutant maintains CDK bind-

ing. Castellano et al. (2) show that immunoprecipitated p16, from a cell line known to contain the P48L mutant, is associated with endogenous CDK6 but not CDK4. Reports by Ruas et al. (3) and Hashemi et al. (4) test binding of CDK4 and CDK6 by mixing in vitro-translated (IVT) CDKs and p16 mutants followed by immunoprecipitation with antibodies specific to p16. With the use of the IVT binding assay, Ruas et al. (3) report that the P48L mutant has greatly reduced, but detectable binding to CDK4 and CDK6, whereas Hashemi et al. (4) suggest that CDK binding is lost.

Regarding the ability of the P48L mutant to bind CDKs, these reports are not consistent with one another or with our report. The differences in binding data are likely explained by the use of different methodologies. Our binding assay used affinity precipitation of bacterially produced glutathione S-transferase (GST)-p16 that had been mixed with ^35S-labeled in vitro-translated CDKs. Our assay is similar to that described by Ruas et al. (3) and Hashemi et al. (4), but there are some differences that may be critical. First, bacterial production of GST fusion proteins may allow for stabilization of protein structure relative to production of native proteins in a cell-free system. Second, production of proteins by IVT can result in decreased binding, even if the proteins are known to associate in vivo (i.e., CDK4 and cyclin D), an effect that could be enhanced by mutational alteration of protein structure. Third, bacterial production of GST-p16 mutants allows the addition of greater quantities of the p16 mutant in the binding assay relative to the IVT CDKs. Increasing the amount of mutant p16 relative to the CDKs may allow detection of CDK binding by mutants with decreased binding affinity. Our data are internally consistent in that the P48L GST-p16 fusion protein maintained some CDK6 inhibitory activity, a function that relies on CDK binding. Despite CDK binding and inhibitory activity, our data suggest that the P48L mutant has no cell cycle inhibitory properties, even when greatly overexpressed, and, as such, mutations resulting in this amino acid substitution should be considered inactivating (1).

Dr. Hansson’s letter supports our contention that multiple assays are necessary to determine the functional activity of p16 mutants and that slight varia-
tions in methodology may alter conclusions regarding the activity of individual p16 mutants. Comparison of our assays of p16 activity suggests that the in vivo cell cycle arrest assay is most sensitive and allows detection of p16 mutants with partial loss of function.

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