Functional Analysis of Mutations within the Kinase Activation Segment of B-Raf in Human Colorectal Tumors

Tsuneo Ikenoue,1,2 Yokho Hikiba,1 Fumihiko Kanai,2 Yasuo Tanaka,2 Jun Imamura,2 Takaaki Imamura,2 Miki Ohta,2 Hideaki Ijichi,2 Keisuke Tateishi,2 Takayuki Kawakami,2 Jun Aragaki,1 Masayuki Matsumura,1,2 Takao Kawabe,2 and Masao Omata2

1 Division of Gastroenterology, The Institute for Adult Diseases, Asahi Life Foundation, and 2 Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Abstract

Mutations in the B-Raf gene have been reported in a number of human cancers, including colorectal carcinoma. More than 80% of the B-Raf mutations were V599E. Although other mutations have been reported, their functional consequences were unclear. Here, we examined the effect of colon tumor-associated B-Raf mutations within the kinase activation segment, including V599E, on extracellular signal-regulated kinase (Erk) and nuclear factor-kB (NFkB) signaling, and on the transformation of NIH3T3 fibroblasts. Among the six mutations examined, only the B-Raf V599E and K600E mutations greatly increased Erk and NFkB signaling, and the transformation of NIH3T3 cells. The B-Raf F594L mutation moderately elevated Erk signaling and NIH3T3 transformation, but did not significantly increase NFkB signaling. Although the basal kinase activity of the B-Raf T598I mutant was comparable with that of wild-type, its oncogenic Ras-induced kinase activity was decreased to 60% of wild-type activity. The B-Raf D593V and G595R mutants showed severely reduced kinase activity and affected neither NFkB signaling nor NIH3T3 transforming activity. These results suggest that the B-Raf activation segment mutations other than V599E reported in colorectal tumors do not necessarily contribute to carcinogenesis by increasing kinase and transforming activities.

Introduction

The Raf serine/threonine-specific kinases serve as a key signal transducer in the Erk cascade, which regulates diverse physiological processes including cell growth, differentiation, and apoptosis (1–4). Activation of the small GTPase protein Ras recruits Raf to the plasma membrane, where it becomes activated. Activated Raf proteins directly phosphorylate and activate the downstream kinase MEK, which, in turn, phosphorylates Erk and thereby activates Erk. Activated Erk phosphorylates many cytoplasmic target proteins, such as Rsk, and several nuclear transcription factors, including Elk-1.

Raf-1, one of the Raf family of proteins, has been shown to activate NFkB transcription factor. NFkB plays an important role in distinct cellular functions, including the immune response, apoptosis, cell proliferation, and inflammation. NFkB is regulated by inhibitor proteins, IxBs, which reside in the cytoplasm. The signal-induced phosphorylation of the IxBs by IKK-α and -β, and their subsequent ubiquitination-dependent degradation are prerequisite for NFkB activation (5–7). Activation of NFkB has been shown to be critical for Raf-induced transformation (8). Raf-1 reportedly activates NFkB by the induction of an autocrine loop in some cell types (9–11), and it has been shown to induce NFkB more directly in an MEK-independent, but MEK kinase-dependent manner (8).

A common mechanism for protein kinase regulation is the phosphorylation of one or more residues in the activation segment that is part of the catalytic site of many protein kinases. T598 and S601 are the major phosphorylation sites of B-Raf in response to oncogenic Ras, and phosphorylation of these two residues is required for full activation of B-Raf (12). These two residues, which are located within the kinase activation segment between kinase subdomains VII and VIII of B-Raf, are conserved in RAF-1 (13). Furthermore, phosphorylation of T598 and S601 is important for B-Raf induction of Erk activation, Elk-1-dependent transcription, NIH 3T3 transformation and PC12 differentiation (12). The substitution of alamines for these residues reduces Ras-induced B-Raf activation; the replacement of these two sites by acidic amino acids results in the constitutive activation of kinase activity (12).

Mutations in the B-Raf gene have been reported in a number of human cancers, including malignant melanoma, thyroid cancer, and colorectal carcinoma (13–22). B-Raf mutations occur in two regions of the B-Raf kinase domain, the glycine-rich loop and the activation segment. More than 80% of the mutations were T to A transversions at nucleotide 1796 (T1796A), leading to a substitution of glutamic acid for valine at amino acid 599 (V599E) in the activation segment. The V599E mutation is thought to mimic phosphorylation by inserting a negatively charged residue adjacent to the phosphorylation site at T598, rendering B-Raf constitutively active. The other three cancer-associated B-Raf mutants studied previously, i.e., another activation segment mutant (L596V) and two glycine-rich loop mutants (G463V and G468A), also had elevated kinase activity and NIH3T3-transforming activity as compared with those of wild-type B-Raf (13).

Although several other B-Raf mutations were reported in previous studies (13–15), the biological effects of these mutations were not fully understood. In addition, the fact that the substitution of alanine for T598 or D593 reduced or abolished kinase and transforming activities prompted us to examine the possibility that some reported mutations in colorectal tumor such as D593V and T598I had no significant effect on tumorigenesis (12, 13, 15). However, the observed clustering of mutations within the activation segment, many at locations within one nucleotide of each other, would not appear to be the result of chance passenger mutations.

In the present study, we demonstrate that colon cancer-associated B-Raf mutations within the kinase activation segment are not necessarily associated with an increase in Erk or NFkB signaling activity, or in NIH3T3-transforming ability, suggesting that some reported
mutations are involved in carcinogenesis by other mechanisms than up-regulation of kinase and transforming activities.

**Materials and Methods**

**Plasmids.** A cDNA construct containing FLAG-tagged human B-Raf (pH8-FLAG-B-Raf) was generously provided by Dr. Toshihiko Kataoka (University of Kobe, Kobe, Japan). Constitutively active H-Ras (pCS2-H-Ras V12) was generously provided by Dr. Anne Wojtek (University of Michigan, Ann Arbor, MI). The FLAG-tagged dominant-negative mutant of IκBα (pDNA3-1xIκBα SS32/36AA), wild-type IκKα and IκKβ (pRK5-FLAG-IκKα and -IκKβ), and Smad4 (pRK5-FLAG-Smad4) were described previously (23, 24). Mutant constructs of B-Raf reported in colorectal tumors (D593V, F594L, G595R, T598I, V599E, and K600E) and dominant-negative mutants of IκKα and IκKβ (IKKα K44M and IKKβ K44M) were created by oligonucleotide-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were verified by DNA sequencing. The NFE2B-inducible reporter plasmid pNF-kB-Luc was purchased from Stratagene. The renilla luciferase control vector (pRL-TK) was purchased from Promega (Madison, WI).

**Reagents and Antibodies.** Anti-FLAG M2 monoclonal antibody and anti-FLAG M2 affinity gel were purchased from Sigma (St. Louis, MO). Anti-phospho-p44/42 MAPK, anti-p44/42 MAPK, and anti-MEK1/2 polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-IκBα-14-3-3 polyclonal (K-19) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hsp90 polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY). The MEK1 inhibitor PD98059 was purchased from Wako Pure Chemicals Industries (Osaka, Japan).

**Cell Culture and Transfection.** COS7 cells were cultured in DMEM containing 10% fetal bovine serum. NIH 3T3 cells were grown in DMEM containing 10% calf serum. Cells were transfected using the FuGENE6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions.

**Immunoprecipitation and Immunoblot.** COS7 cells were grown in 3.5-cm dishes and transfected with 1 μg of FLAG-tagged wild-type or each mutant B-Raf. Twenty-four h after transfection, cells were washed twice with ice-cold PBS and lysed in RIPA buffer [50 mm Tris-HCl (pH 7.4), 1% NP40, 150 mm NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonfluoride, 1 mm sodium fluoride, and 1 mm sodium vanadate, containing 1 μg/ml each of aprotinin, leupeptin, and pepstatin]. FLAG-B-Raf was immunoprecipitated by anti-FLAG M2 affinity gel, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked with anti-IκBα-14-3-3 and anti-hsp90 antibodies overnight at 4°C and then with peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the manufacturer’s instructions.

**In Vitro Kinase Assay.** For B-Raf activity assays, COS7 cells in six-well plates were transfected with FLAG-B-Raf constructs (20 ng each) in the presence or absence of H-Ras V12 (100 ng). Three h after transfection, cells were transfected to medium containing 0.1% serum and additionally cultured for 24 h. Cells were lysed in RIPA buffer. FLAG-B-Raf was immunoprecipitated by anti-FLAG M2 affinity gel. The immunoprecipitated FLAG-B-Raf was assayed using a B-Raf kinase cascade assay according to the manufacturer’s protocols (Upstate Biotechnology, Lake Placid, NY). Briefly, 0.4 μg of GST-MEK1 and 1 μg of GST-ERK2 were incubated with the precipitated FLAG-B-Raf for 30 min at 30°C. Then 4 μl of activated ERK2 was added to the mixture containing 20 μg of MBP substrate (Upstate Biotechnology) and 10 μCi of [γ-32P]ATP, and the mixture was incubated for 10 min at 30°C. The samples were spotted onto ϕ81 phosphocellulose filters. The filters were washed three times with 0.75% phosphoric acid and once with acetone. Bound radioactivity was measured as Cerenkov radiation.

**Erk Activation Assay.** COS7 cells in six-well plates were maintained in DMEM containing 0.1% serum and transfected with 1 μg of FLAG-B-Raf constructs, H-Ras V12, or empty vector. Cells were additionally cultured for 24 h and lysed in RIPA buffer. To examine the effect of the MEK1 inhibitor PD98059 on B-Raf-induced Erk activation, 25 or 50 μM of the MEK1 inhibitor PD98059 was added to the medium 2 h after transfection. Cell lysates were analyzed by SDS-PAGE and immunoblotting using anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies.

**Reporter Assay.** NIH3T3 cells grown in 12-well plates were cotransfected with 300 ng of pNFkB Luc, 100 ng of pRL-TK, and 200 ng of wild-type, mutant FLAG-B-Raf constructs or empty vector. To examine the effects of the dominant-negative mutants IκBα (IκBα SS32/36AA), IKKα (IKKα K44M), or IKKβ (IKKβ K44M) on B-Raf-induced NFκB-dependent transcription, NIH3T3 cells grown in 12-well plates were cotransfected with 300 ng of pNFkB Luc, 100 ng of pRL-TK, 200 ng of B-Raf mutants, and the indicated amounts of each dominant-negative vector. Cells were starved in 0.1% fetal bovine serum for 24 h. Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocols.

**Focus Formation Assay.** Focus formation assay was performed as described previously (12). NIH3T3 cells were cotransfected with pRL-TK and FLAG-B-Raf constructs or H-Ras V12 (positive control). Twenty-four h after transfection, cells were trypsinized, and 1 of 10 of the cells were taken to check the transfection efficiency among culture plates using the renilla luciferase assay. The remaining cells were plated onto 10-cm dishes and maintained in 5% calf serum medium with medium change every 3 days. After 14 days, cells were stained with crystal violet, and the number of transformed foci was counted.

**Results**

**Kinase Activity of B-Raf Mutants in Vitro.** We analyzed the biological effects of B-Raf mutations within the kinase activation segment that have been reported in colorectal cancer (Fig. 1). The K-Ras gene status of tumors with the B-Raf mutations examined in this study is shown in Table 1. Wild-type B-Raf reportedly had higher basal kinase activity than did Raf-1 due to the constitutive phosphorylation of S445 and the presence of the acidic residue D448, and the activity was stimulated by oncogenic Ras (26).

We first examined in vitro kinase activity of each B-Raf mutant. COS7 cells were transfected with each B-Raf mutant in the presence or absence of oncogenic H-Ras (Fig. 2). The kinase activities of immunoprecipitated B-Raf proteins were examined using the in vitro coupled Raf kinase assay. The B-Raf V599E mutant, which has been reported to be constitutively active, also showed high basal kinase activity in our assay (11.2-fold that of wild-type; Ref. 13). B-Raf K600E also had high basal kinase activity (9.0-fold that of wild-type), whereas the B-Raf D593V and G595R mutants had basal kinase activities that were significantly lower than wild-type B-Raf activity (0.67- and 0.23-fold that of wild-type, respectively). The B-Raf T598I mutant showed only slightly decreased basal kinase activity compared with that of wild-type B-Raf (0.84-fold that of wild-type). The B-Raf F94L mutant had moderately high basal activity (3.6-fold that of wild-type).

We also assessed whether oncogenic H-Ras stimulated all of the mutants. Oncogenic H-Ras increased the kinase activity of wild-type B-Raf by 11.5-fold. All of the B-Raf mutants were stimulated to different degrees by oncogenic H-Ras, although the fold activation of each mutant was lower than that of the wild-type (Fig. 2).

**Human B-Raf**

| Human B-Raf | TVK1GDFGLATVKGSRWSGHSQPOELSLGSLTM | Human Raf-1 | Q.V.P.T.V...... |
| Human A-Raf | T....AQP.L.P.D.V....A. | Human A-Raf | Q.V.P.T.V...... |

Fig. 1. Alignment of the kinase activation segment sequences of human B-Raf, Raf-1, and A-Raf. The B-Raf mutations examined in this study are indicated by arrows. The activation segment is boxed.
a previous observation of Raf-1 (29), which indicated that the reduced association of oncogenic Ras-activated Raf-1 with MEK reflected a faster turnover, and, hence, enhanced activation of MEK. The results of this study suggest that the differences in the activation of Erk signaling by B-Raf mutants are not due to the strengths of protein-protein interactions.

NFκB-Dependent Transcription of B-Raf Mutants. Raf-induced activation of NFκB signaling is reportedly critical for transformation by Raf (8–11). To examine whether colon cancer-associated B-Raf mutants activate the NFκB pathway, NFκB-dependent transcriptional activity was measured using a luciferase reporter assay. The NFκB-inducible reporter was slightly activated by transfection with wild-type B-Raf (1.2-fold that of empty vector; Fig. 5A). The B-Raf V599E and K600E mutants increased NFκB reporter activity more than did the wild-type (3.8- and 2.5-fold that of wild type B-Raf, respectively). The B-Raf F594L mutant only slightly activated NFκB reporter activity (1.3-fold that of wild-type B-Raf). However, the B-Raf D593V, G595R, and T598I mutants did not induce NFκB reporter activity (1.0-, 0.9-, and 1.0-fold that of wild-type B-Raf,

Table 1 B-Raf mutations and their functional consequences analyzed in this study and K-Ras status of the tumors with these B-Raf mutations

<table>
<thead>
<tr>
<th>B-Raf</th>
<th>B-Raf kinase cascade activitya</th>
<th>NFκB-dependent transcriptiona</th>
<th>NIH3T3 foci formationb</th>
<th>K-Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>D593V</td>
<td>0.06</td>
<td>1.0</td>
<td>0</td>
<td>G12D</td>
</tr>
<tr>
<td>F594L</td>
<td>3.6</td>
<td>1.3</td>
<td>4.0</td>
<td>Wild-type</td>
</tr>
<tr>
<td>G595R</td>
<td>0.23</td>
<td>0.9</td>
<td>0</td>
<td>Wild-type</td>
</tr>
<tr>
<td>T598I</td>
<td>0.84</td>
<td>1.0</td>
<td>1.0</td>
<td>G13D</td>
</tr>
<tr>
<td>V599E</td>
<td>11.2</td>
<td>3.8</td>
<td>18.3</td>
<td>Wild-type</td>
</tr>
<tr>
<td>K600E</td>
<td>9.0</td>
<td>2.5</td>
<td>13.0</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>

a Fold increase over wild-type B-Raf.

In Vivo Activation of Erk by B-Raf Mutants. We assessed the deregulation of the kinase activity of the B-Raf mutants in vivo by assaying Erk activity using a phosphospecific Erk antibody. Consistent with the in vitro kinase activity, the Erk activity was elevated significantly in COS7 cells expressing the B-Raf F594L, V599E, and K600E mutants, to a level comparable with that induced by oncogenic H-Ras (Fig. 3A). The Erk activity was not significantly stimulated by the B-Raf D593V, G595R, or T598I mutants as compared with wild-type B-Raf (Fig. 3A). The MEK1 inhibitor PD98059 blocked the Erk activation induced by the B-Raf F594L, V599E, and K600E mutants, indicating that these mutants activate Erk primarily through MEK1 (Fig. 3B).

Combining the results of the in vitro kinase cascade assay and the in vivo Erk activation assay, the B-Raf F594L, V599E, and K600E mutants activated the Raf/MEK/Erk signaling pathway, whereas the B-Raf D593V, G595R, and T598I mutants did not activate this pathway, as compared with the activation by wild-type B-Raf.

Association of B-Raf Mutants with MEK, 14-3-3, or Hsp90. Raf kinase activity is affected by interaction with other signaling components including 14-3-3 and Hsp90 (27, 28). B-Raf also directly binds to and activates MEK. To elucidate the possibility that the activation of Erk signaling by B-Raf mutants is due to the strength of interaction with signaling components, we performed coimmunoprecipitation analyses of B-Raf proteins overexpressed with endogenous MEK, 14-3-3, or Hsp90 (27, 28). B-Raf also directly binds 14-3-3, or Hsp90. As shown in Fig. 4, both wild-type and mutant B-Raf were able to bind to MEK, 14-3-3, and Hsp90. The B-Raf V599E and K600E mutants, which highly activate Erk signaling, showed a slight decrease rather than increase in MEK association compared with that of wild-type B-Raf (Fig. 4). Furthermore, cotransfection of H-Ras V12 slightly decreased the association of wild-type B-Raf with MEK (data not shown). These results were consistent with
NIH3T3 Transforming Activity in B-Raf Mutants. To quantify the transforming ability of each B-Raf mutant we performed a focus formation assay. Wild-type B-Raf generated only a few foci in the subline of NIH3T3 cells used for this assay (0.006 foci/ng DNA). Oncogenic H-Ras generated ~300-fold greater number of foci than wild-type B-Raf (2.0 foci/ng DNA). Consistent with a previous report (13), the B-Raf V599E mutant had a greater ability to form foci (0.11 foci/ng DNA; Fig. 6). Among the other five mutants, K600E and F594L induced greater foci formation than did the wild-type (0.078 and 0.024 foci/ng DNA, respectively). In contrast, the D593V and G595R mutants did not induce focus formation. The T598I mutant induced a few foci comparable with that of the wild-type (0.0063 foci/ng DNA).

Discussion

In the present study, we examined the biological effect of colon cancer-associated mutations within the kinase activation segment of B-Raf. The V599E mutation is thought to mimic phosphorylation by inserting a negative charged residue adjacent to the phosphorylation site at T598, rendering B-Raf constitutively active. Consistent with a previous report (13), the B-Raf V599E mutant showed greatly increased activity in the Raf/MEK/Erk pathway, both in vitro and in vivo. In this study, the V599E mutation was also shown to activate NFκB-dependent signaling. Constitutively active Raf-1 reportedly induces NFκB signaling via both MEK-dependent and MEK-independent pathways (8–11, 30). One of these previous studies revealed that IKKβ, and not IKKα, is involved in NFκB signaling induced by constitutively active Raf-1 (8). In these previous studies, the catalytic domain of Raf-1 from which the NH2-terminal regulatory domain had been deleted was used as constitutively active Raf-1. In this study, we showed that full-length B-Raf mutants found in human cancer can also induce NFκB signaling through IKKβ.

Another B-Raf mutant, K600E, also activated the Raf/MEK/Erk pathway to an extent comparable with that of the V599E mutant. In
addition, the B-Raf K600E mutant stimulated NFκB signaling and NIH3T3 transforming activity. In this mutant, negative charged glutamic acid residue was inserted adjacent to the phosphorylation site at S601, mimicking phosphorylation of S601. Thus, the B-Raf K600E mutant was thought to act like the B-Raf V599E mutant.

The B-Raf T598I mutant had basal kinase activity comparable with that of wild-type B-Raf, whereas its kinase activity stimulated by oncogenic Ras was decreased. In addition, the T598I mutation induced no significant NFκB-dependent reporter activity or NIH3T3 transforming activity as compared with the activity of wild-type B-Raf. The substitution of T598, one of the Ras-induced phosphorylation sites of B-Raf, with alanine (T598A) was reported to decrease the B-Raf kinase activity stimulated by oncogenic Ras (12). Thus, the B-Raf T598I mutant, in which T598 was replaced by the nonpolar amino acid isoleucine, was thought to act like T598A.

Most protein kinases contain the almost invariant Asp-Pho-Gly (DFG) motif at the beginning of the activation segment. The Asp in this motif is thought to bind to the Mg$^{2+}$ that bridges the $\beta$- and $\gamma$-phosphates of ATP. The sequence of this highly conserved motif changes in the B-Raf D593V, F594L, and G595R mutants. The B-Raf D593V and G595R mutants were unable to activate Erk and NFκB signaling or NIH3T3 transformation. The substitution of alanine for D593 reportedly abolishes the kinase and transforming activities of B-Raf (13). Thus, the B-Raf D593V, in which D593 is replaced by nonpolar amino acid valine, has been assumed to act like D593A. The fact that the kinase activity of the B-Raf G595R mutant was reduced suggests that the glycine in the conserved DFG motif is critical for B-Raf kinase activity. On the other hand, the B-Raf F594L mutant, moderately increased Raf/MEK/Erk signaling and NIH3T3 transforming activity, and very slightly stimulated NFκB signaling. In the B-Raf F594L mutant, the conserved DFG motif is replaced by the tripeptide sequence Asp-Leu-Gly (DLG) that is a variation of the DFG motif unique to G protein-coupled receptor kinases (31, 32), suggesting that this mutation maintains the kinase activity, and has different effects from those of the D593V and G595R mutations.

Coincident K-Ras and B-Raf mutations have been reported in previous studies (13, 15). Tumors with the B-Raf V599E mutations reportedly do not have K-Ras mutations, whereas the other mutations are often accompanied by K-Ras mutations (13–15). In addition, K-Ras and B-Raf seem to have some similar biological effects on colorectal tumorigenesis because both mutations occur at a similar stage of the adenoma-carcinoma sequence, both are associated with villous morphology, and both are less frequent in adenomas of patients with familial adenomatous polyposis (14, 15). Of the colon tumor-associated B-Raf mutations examined in this study, D593V and T598I were coincident with K-Ras mutations, whereas F594L, G595R, V599E, and K600E were not (Table 1; Refs. 14, 15). Because the D593V and T598I mutants showed no increased kinase activity in this study, it is likely that the coincident K-Ras, and not these B-Raf mutations, primarily contribute to tumorigenesis. In contrast, the F594L, V599E, and K600E mutants, of which the kinase activities were up-regulated, appear to have significant effects on tumor development. The fact that the G595R mutant did not have elevated kinase activity and that the tumor with the G595R mutation had no Ras mutation suggests the possibility that components of the Ras/Raf/MEK/Erk pathway other than Ras or B-Raf are activated in some colon tumors.

In conclusion, the reported colon tumor-associated B-Raf mutations did not necessarily stimulate Raf-mediated signaling cascades, such as Erk and NFκB, and did not necessarily increase the transforming activity of NIH3T3. Furthermore, the B-Raf mutations that were shown to be unable to activate the signalings in this study were found predominantly in the tumors with activating K-Ras mutations. However, the observed clustering of the B-Raf mutations would unlikely be the result of chance passenger mutations. Thus, the results of this study suggest that some of the non-V599E B-Raf mutations found in colorectal tumors have functionally different properties from increases in kinase and transforming activities. Additional studies are required to elucidate the functional significance among them.

Acknowledgments

We thank Drs. Tohru Kataoka and Anne Vojtek for providing the plasmids.

References


# Functional Analysis of Mutations within the Kinase Activation Segment of B-Raf in Human Colorectal Tumors

Tsuneo Ikenoue, Yohko Hikiba, Fumihiko Kanai, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/63/23/8132">http://cancerres.aacrjournals.org/content/63/23/8132</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 30 articles, 17 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/63/23/8132.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/63/23/8132.full.html#ref-list-1</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Citing articles</th>
<th>This article has been cited by 34 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/63/23/8132.full.html#related-urls">http://cancerres.aacrjournals.org/content/63/23/8132.full.html#related-urls</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>