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Article

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Oncogenic *AKAP9-BRAF* fusion is a novel mechanism of MAPK pathway activation in thyroid cancer

Raffaele Ciampi,¹ Jeffrey A. Knauf,² Roswitha Kerler,³ Manoj Gandhi,¹ Zhaowen Zhu,¹ Marina N. Nikiforova,¹ Hartmut M. Rabes,³ James A. Fagin,² and Yuri E. Nikiforov¹

¹Department of Pathology and Laboratory Medicine and ²Division of Endocrinology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA.

³Institute of Pathology, University of Munich, Munich, Germany.

Genes crucial for cancer development can be mutated via various mechanisms, which may reflect the nature of the mutagen. In thyroid papillary carcinomas, mutations of genes coding for effectors along the MAPK pathway are central for transformation. *BRAF* point mutation is most common in sporadic tumors. By contrast, radiation-induced tumors are associated with paracentric inversions activating the receptor tyrosine kinases *RET* and *NTRK1*. We report here a rearrangement of *BRAF* via paracentric inversion of chromosome 7q resulting in an in-frame fusion between exons 1–8 of the *AKAP9* gene and exons 9–18 of *BRAF*. The fusion protein contains the protein kinase domain and lacks the autoinhibitory N-terminal portion of *BRAF*. It has elevated kinase activity and transforms NIH3T3 cells, which provides evidence, for the first time to our knowledge, of in vivo activation of an intracellular effector along the MAPK pathway by recombination. The *AKAP9-BRAF* fusion was preferentially found in radiation-induced papillary carcinomas developing after a short latency, whereas *BRAF* point mutations were absent in this group. These data indicate that in thyroid cancer, radiation activates components of the MAPK pathway primarily through chromosomal paracentric inversions, whereas in sporadic forms of the disease, effectors along the same pathway are activated predominantly by point mutations.

Introduction

Thyroid papillary carcinoma is the most common type of endocrine malignancy. In this tumor, mutations of genes coding for effectors along the MAPK pathway are central for transformation. Indeed, activating mutations of *BRAF*, *RET*, or *RAS* are found in 70% of all cases and rarely overlap in the same tumor (1–3).

The *RET* gene codes for a cell membrane receptor tyrosine kinase (4, 5). In papillary carcinomas, it is activated via chromosomal rearrangement, which results in fusion of the 3' portion of the gene coding for the tyrosine kinase domain to the 5' portion of various genes, creating chimeric oncogenes named *RET/PTC* (6, 7). The 2 most common rearrangement types, *RET/PTC1* and *RET/PTC3*, are paracentric inversions, since both *RET* and its respective fusion partner, *H4* or *NCOA4* (*ELE1*), reside on the long arm of chromosome 10 (8, 9). The fusion leads to ligand-independent activation of the truncated *RET* receptor. Binding of activated *RET* to *SHC* via Y1062 has been shown to be critical to *RET*-mediated transformation and dedifferentiation, primarily through the *RAS-RAF-MAPK* pathway (10, 11). *RAS*, in turn, can be independently activated by point mutations, primarily in the follicular variant of papillary thyroid cancer (12).

Most recently, point mutations activating the *BRAF* serine/threonine kinase have surfaced as the most common genetic event in sporadic thyroid papillary carcinomas (1, 13). *RAF* proteins, including *ARAF*, *BRAF*, and *CRAF* or *RAF-1*, phosphorylate

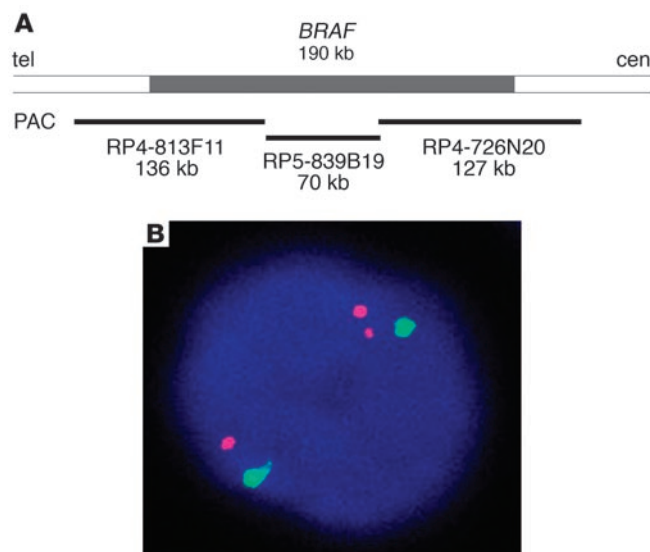
MAPK/ERK kinase (*MEK*) within the *MAPK* cascade upon stimulation by *RAS*. *BRAF* somatic mutations were first discovered in malignant melanomas and in a smaller subset of colorectal and ovarian cancers (14). The majority of mutations in those tumors and virtually all mutations in thyroid carcinomas result in a valine-to-glutamate substitution at residue 600 (V600E), formerly designated as V599E. This mutation is believed to produce a constitutively active kinase by disrupting hydrophobic interactions between residues in the activation loop and residues in the ATP binding site that maintain the inactive conformation, allowing development of new interactions that fold the kinase into a catalytically competent structure (15). Correspondingly, *BRAF*^{V600E} exhibits elevated basal kinase activity and transforms NIH3T3 cells with high efficiency (14).

Conceivably, other alterations that would release the inhibitory constraints of the catalytic domain of *BRAF* could also result in kinase activation. Indeed, loss of the N-terminal regulatory domains as a result of fusion to different genes during in vitro transfection has been shown to activate *BRAF* kinase and transform NIH3T3 cells (16, 17). Despite those reports, which appeared more than a decade ago, this mechanism of *BRAF* activation has not been identified in human tumors. Here, we report the activation of *BRAF* by chromosomal rearrangement that results in its N-terminal truncation in several papillary thyroid carcinomas. We show that the fusion gene exhibits elevated basal kinase activity, stimulates *ERK* phosphorylation, and induces transformation of NIH3T3 cells, which is consistent with its functioning as an oncogene. This observation also provides insights into distinct mechanisms of activation of components of the *MAPK* pathway in radiation-induced cancers, which involve chromosomal inversions, in contrast to sporadic cancers, where it happens predominantly by point mutations.

Nonstandard abbreviations used: BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; MEK, MAPK/ERK kinase; ORF, open reading frame; PAC, P1 artificial chromosome; PKA, protein kinase A; RACE, rapid amplification of cDNA ends; RBD, RAS-GTP binding domain; RII, type II regulatory subunit.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Results

Identification of *BRAF* rearrangement. The possibility of *BRAF* involvement in chromosomal rearrangement was studied by fluorescence in situ hybridization (FISH). A contig of 3 P1 artificial chromosome (PAC) clones of approximately 330 kb, spanning the entire *BRAF* gene, was assembled to use as a probe (Figure 1A). Benign thyroid cells showed, as expected, 2 signals. Analysis of 32 papillary thyroid carcinomas revealed 1 tumor containing 3 signals, whereas subsequent hybridization with a chromosome 7 centromeric probe demonstrated the presence of 2 copies of the chromosome (Figure 1B). Since the split of one *BRAF* signal resulted in a pair of signals located at a relatively constant distance from each other, the likely mechanism for this event was intrachromosomal inversion.

Identification and characterization of the fusion partner. The identification of the fusion partner was achieved by 5' rapid amplification of cDNA ends (RACE) using tumor poly(A)⁺ RNA and a pool of primers designed along the 3' end of the *BRAF* gene. RACE products were sequenced, and several of them revealed a fusion between exon 9 of *BRAF* and exon 8 of the *A-kinase anchor protein 9* (*AKAP9*) gene (Figure 2A). Confirmation of the fusion was obtained by RT-PCR with primers located in exon 8 of *AKAP9* and exon 10 of *BRAF*, which yielded the expected product of 181 bp. Genomic DNA was used for PCR to identify the fusion at the

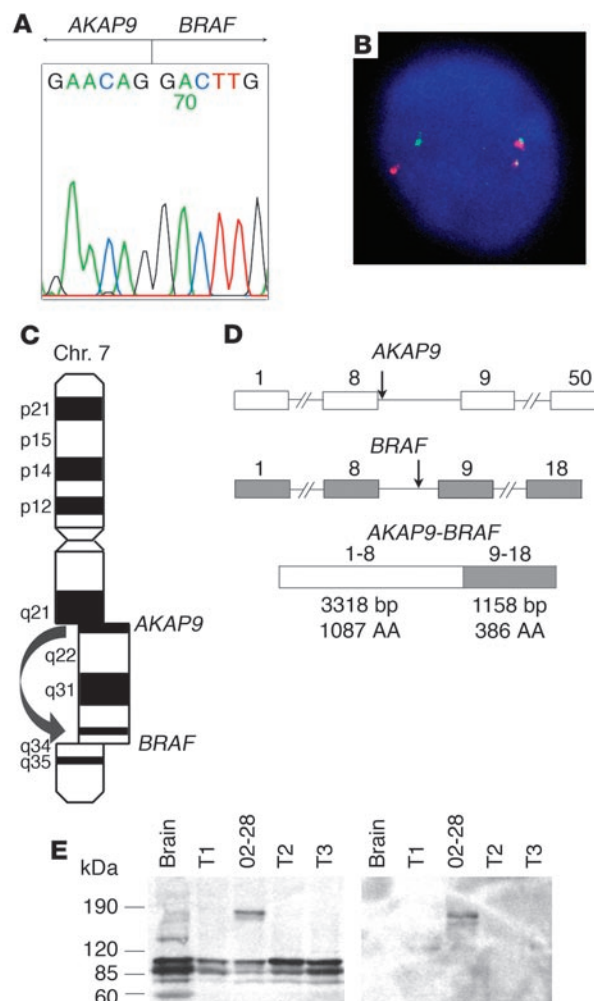
Figure 2

BRAF is recombined with the *AKAP9* gene and results in expression of a fusion protein. (A) Sequence of the 5' RACE product showing a fusion point between *AKAP9* and *BRAF*. (B) Confirmation of the reciprocal fusion by FISH with probes corresponding to the *BRAF* (red) and *AKAP9* (green) genes. (C) The fusion is a result of paracentric chromosomal inversion inv(7)(q21–q22q34). Chr., chromosome. (D) Genomic structure of the *BRAF* and *AKAP9* genes showing the location of breakpoints (arrows) and the organization of the chimeric cDNA. Exons are represented by boxes and introns by lines. Numbers above indicate exon numbers. (E) Western blot analysis using *BRAF* antibody (left) and *AKAP9* antibody (right), showing an approximately 170-kDa protein, corresponding to the predicted molecular weight of the fusion protein in the index case (number 02-28). Three other papillary carcinomas (T1–T3) are shown for comparison. Wild-type *AKAP9* is 453 kDa in size and was not detected in this Western blot.

Figure 1

Identification of the *BRAF* gene rearrangement. (A) Genomic region on 7q containing the *BRAF* gene and position of PAC clones used as a probe for FISH. (B) Interphase nucleus from the index tumor showing split of 1 *BRAF* signal (red) and preservation of 2 chromosome 7 centromeric signals (green), which indicates the rearrangement of the *BRAF* gene.

genomic level, which was found to reside between nucleotide 364 of *AKAP9* intron 8 and nucleotide 4,930 of *BRAF* intron 8. The reciprocal fusion gene product was subsequently identified by RT-PCR using tumor cDNA, indicating the reciprocal nature of the rearrangement. Further confirmation was obtained by double-color FISH with probes corresponding to the *BRAF* and *AKAP9* genes (Figure 2B). Since the *BRAF* gene is located on chromosome 7q34 (18) and *AKAP9* on 7q21–q22 (19–21), the *AKAP9-BRAF* fusion results from inv(7)(q21–q22q34) (Figure 2C). Using primers located within the 5' and 3' untranslated region of *AKAP9* and *BRAF*, respectively, the *AKAP9-BRAF* coding cDNA sequence was amplified from the tumor, cloned into the pCR-XL-TOPO vector, and sequenced (GenBank accession number AY803272). Analysis of the sequence (4,567 bp total size) revealed a 4,476-bp open reading frame (ORF) containing exons 1–8 of *AKAP9* fused in-frame with exons 9–18 of *BRAF* (Figure 2D). Based on the known



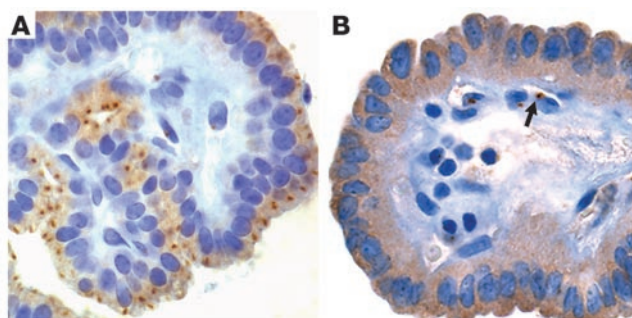


Figure 3

Subcellular localization of AKAP9 and AKAP9-BRAF detected by immunohistochemistry with an AKAP9 antibody. (A) In a papillary thyroid carcinoma negative for the fusion, the protein is predominantly targeted to centrosomes, seen as a single perinuclear dot. There is also weak diffuse cytoplasmic staining. (B) The tumor expressing AKAP9-BRAF reveals diffuse cytoplasmic distribution of the chimeric protein and the perinuclear dot is no longer visible. The shift in localization involves only tumor cells but not adjacent vascular endothelial cells, which preserve the dotted pattern of staining (arrow). Magnification, $\times 200$.

sequences of the untranslated regions of both genes, the predicted length of the complete *AKAP9-BRAF* cDNA is 4,849 bp. Four transcript variants of human *AKAP9* have been previously identified based on several coding region differences. The *AKAP9* sequence of the chimeric cDNA lacked 36 nucleotides at position 269–304 of transcript version 1 (GenBank accession number NM_147171), which indicates that it corresponded to either transcript version 2 or 3 of the gene. Multiple RT-PCR reactions revealed no splice variants of the fusion transcript in the tumor cDNA. Western blot analysis of the tumor using an antibody to the C-terminus of BRAF showed a band of approximately 170 kDa, corresponding

to the predicted molecular weight of 172 kDa for the fusion protein (Figure 2E). The same band was detected with an antibody against the N-terminus of AKAP9.

The wild-type *AKAP9* gene encodes a 453-kDa protein capable of binding the type II regulatory subunit (RII) of protein kinase A (PKA) as well as other signaling proteins, anchoring them to the centrosome and Golgi apparatus (19–21). Several isoforms arising through alternative splicing are selectively expressed in most human tissues at low abundance (19, 20). The AKAP9 protein localizes by immunofluorescence as a single perinuclear dot and some surrounding dispersed fluorescence, corresponding to the centrosome and Golgi compartments, respectively, in interphase HeLa and SaOS2 osteosarcoma cells (19, 20). Based on the sequence of the chimeric cDNA, the fusion protein is predicted to lack 2 putative RII binding regions and the centrosome localization domain of AKAP9 (20, 22, 23).

In papillary thyroid carcinomas negative for the fusion and in non-neoplastic thyroid tissue, *AKAP9* expression was readily detectable by RT-PCR (data not shown), and the protein was seen as a single perinuclear dot with faint diffuse cytoplasmic staining (Figure 3A). In the tumor expressing the AKAP9-BRAF protein, the perinuclear dot was no longer identifiable, whereas diffuse staining was observed in the cytoplasm (Figure 3B). However, the presence of intact centrosomes in the tumor cells was confirmed by immunohistochemistry with an antibody against the centrosomal protein γ -tubulin (data not shown). This is consistent with the absence of the centrosomal localization domain of AKAP9 in the fusion protein, which prevents its preferential targeting to the centrosomal compartment.

Functional characterization of the AKAP9-BRAF fusion. The wild-type human *BRAF* gene codes for an 84-kDa serine/threonine kinase in the MAPK pathway (16, 24). Structurally, BRAF, similar to other RAF kinases, contains 2 highly conserved N-terminal regulatory

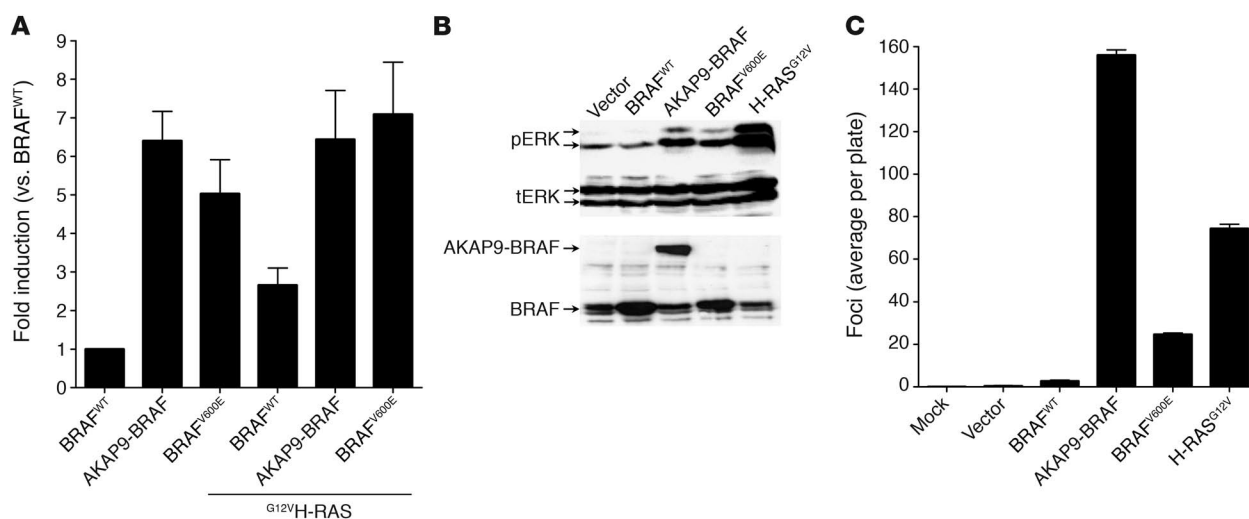


Figure 4

The AKAP9-BRAF chimeric protein has increased BRAF kinase activity and results in NIH3T3 cell transformation. (A) In vitro kinase activity of myc-tagged BRAF^{WT}, AKAP9-BRAF, and BRAF^{V600E} in the absence or presence of H-RAS^{G12V} in COS7 cells. BRAF kinase activity was measured in myc-IgG immunoprecipitates using MEK1 substrate phosphorylation as a read-out. Each sample was assayed in triplicate, and bars represent the standard deviations from the mean. Similar results were obtained in 2 independent transfections. (B) Western blots of lysate from cells stably transfected with pEFP, pEFP-BRAF^{WT}, pEFP-AKAP9-BRAF, pEFP-BRAF^{V600E}, or pBabe puro-H-RAS^{G12V} probed with antibodies to phospho-ERK1/2 (pERK), total ERK (tERK), or C-terminus of BRAF. Similar results were obtained in 3 additional independent experiments. (C) Foci formation of NIH3T3 cells transfected with pEFP-BRAF^{WT}, pEFP-AKAP9-BRAF, pEFP-BRAF^{V600E}, or pBabe puro-H-RAS^{G12V}. Bars represent the average number of foci per plate from 6 plates. Similar results were obtained in 2 independent experiments.



Table 1
Prevalence of *BRAF* alterations in papillary thyroid carcinomas

	<i>n</i>	Age at surgery	Age at exposure	AKAP9-BRAF	BRAF ^{V600E}
Early radiation-associated tumors (latency period 5–6 yr)	28	11.4 ± 3.6	5.0 ± 3.8	11% ^A	0 ^B
Late radiation-associated tumors (latency period 9–12 yr)	64	16.0 ± 5.0	5.4 ± 5.1	0	16%
Sporadic tumors	102	40.0 ± 17.7	–	1%	37%

^A*P* = 0.03 compared with sporadic tumors and late radiation-associated tumors. ^B*P* < 0.0001 compared with sporadic tumors; *P* = 0.03 compared with late radiation-associated tumors.

domains, CR1 and CR2, that mediate autoinhibition of BRAF and a C-terminal protein kinase domain (CR3). CR1 includes the RAS-GTP binding domain (RBD), which mediates RAS binding, triggering the recruitment of the protein to the cell membrane and kinase activation (25). Based on the cDNA sequence, the AKAP9-BRAF fusion protein is predicted to lack the BRAF N-terminal regulatory domains, including RBD, and to retain an intact kinase domain. Loss of the regulatory domains would be expected to lead to constitutive activation of BRAF kinase in a RAS-independent manner.

We characterized the kinase activity of the AKAP9-BRAF fusion protein by transient transfection of myc epitope-tagged versions of cDNAs for AKAP9-BRAF, BRAF^{V600E}, or wild-type BRAF into COS7 cells, using bacterially purified MEK1 as a substrate for phosphorylation (Figure 4A). Basal BRAF kinase activity of the fusion protein was 6-fold greater than BRAF^{WT} activity and not further increased by coexpression of activated RAS. Basal activity of BRAF^{V600E} was also increased, but to a lesser extent, and was modestly increased by H-RAS^{G12V}. By contrast, H-RAS^{G12V} increased BRAF^{WT} kinase activity by about 2.5-fold. The fusion protein stimulated endogenous ERK1/2 phosphorylation to a similar extent as BRAF^{V600E} in pooled stably transfected NIH3T3 cells, whereas overexpression of BRAF^{WT} had no discernible effect (Figure 4B).

The ability of AKAP9-BRAF fusion protein to induce transformation was studied in an NIH3T3 cell focus assay (Figure 4C). Vector and BRAF^{WT} transformed cells at very low efficiency. AKAP9-BRAF showed the highest transformation activity in this assay, followed by H-RAS^{G12V} and BRAF^{V600E}. In addition, NIH3T3 cells stably transfected with empty vector, BRAF^{WT}, AKAP9-BRAF, BRAF^{V600E}, and H-RAS^{G12V} were assayed for tumorigenicity in nude mice. Two weeks after injection of 10⁶ cells, tumors of at least 5 mm in diameter were formed with complete penetrance (6 out of 6 animals, repeated in 2 independent experiments) for AKAP9-BRAF, BRAF^{V600E}, and H-RAS^{G12V} constructs, whereas vector and BRAF^{WT} did not lead to the development of tumors (0 out of 6 animals). These results demonstrate that AKAP9-BRAF fusion leads to activation of BRAF kinase and transformation of NIH3T3 cells.

Prevalence of AKAP9-BRAF fusion and BRAF point mutation in thyroid cancer. To determine the prevalence of AKAP9-BRAF fusion in various populations, we used RT-PCR with primers flanking the fusion point to study a large series of papillary thyroid carcinomas (Table 1). In a group of 102 sporadic papillary tumors, no additional case of the fusion was found. We also examined papillary carcinomas from 2 cohorts of Belarussian children and adolescents exposed to radiation after the Chernobyl nuclear accident in 1986. Among 28 tumors that developed 5–6 years after exposure, 3 (11%) were positive for the fusion, whereas 64 tumors developing 9–12 years after the accident were all negative (Figure

5A). Paraffin blocks were available for immunohistochemical analysis with AKAP9 antibody in 2 out of 3 positive cases. Both cases showed a shift in subcellular localization of AKAP9 from the centrosomal pattern seen in adjacent non-neoplastic thyroid tissue to diffuse cytoplasmic distribution in tumor cells (Figure 5B).

We also tested these tumors for BRAF point mutation lead-

ing to the V600E substitution. The mutation was identified in 38 (37%) sporadic tumors, in 10 (16%) tumors developing 9–12 years after exposure, but in none of the tumors that developed 5–6 years after exposure (Figure 6). The differences in prevalence of the 2 types of BRAF activation were statistically significant between the early radiation group and both late radiation and sporadic groups (Table 1).

Discussion

The MAPK signaling pathway is a ubiquitously present regulator of cell growth, proliferation, apoptosis, and differentiation. Recently, BRAF, an essential component of the kinase cascade, has received renewed attention since mutations in the gene were identified in several types of human cancer. Indeed, activating point mutations of BRAF have been found in more than 60% of malignant melanomas, 10–14% of colorectal and ovarian cancer, and in very few cases of lung cancer, soft tissue sarcomas, and other cancer cell lines (14, 26, 27). Thyroid cancer was promptly added to the list with 36–69% prevalence of BRAF mutation (1, 2, 13, 28). However, neither BRAF nor other

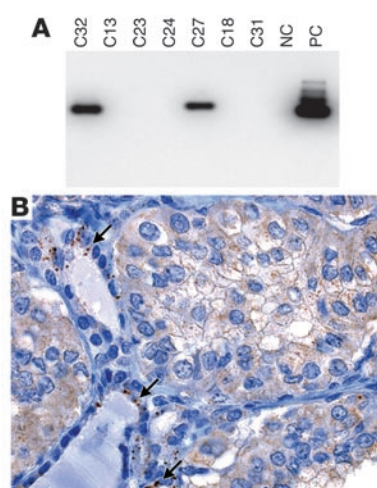
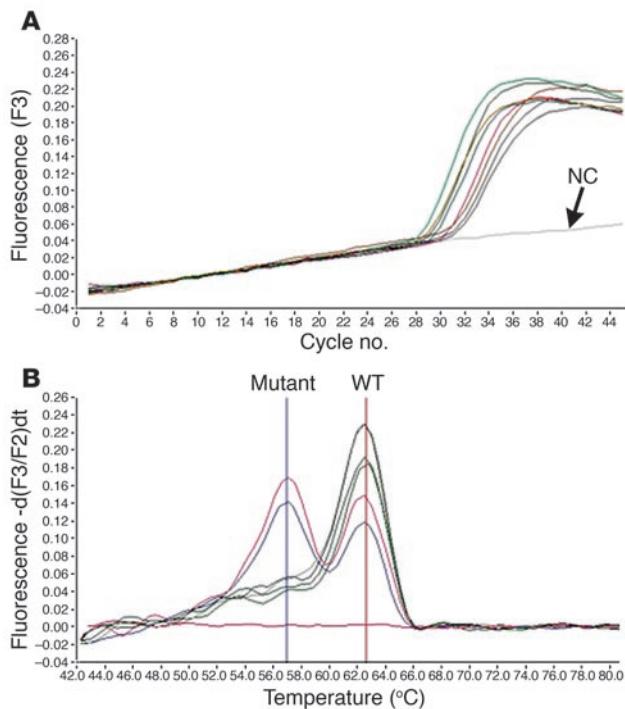


Figure 5
Analysis of post-Chernobyl tumors that developed 5–6 years after exposure. (A) Southern blot of RT-PCR products amplified with primers Ex8A and Ex10B and hybridized with probe PR showing 2 positive cases (C32 and C27). Other tumor samples are negative (C13, C23, C24, C18, and C31). NC, negative control; PC, positive control (index case). (B) Immunohistochemical analysis of tumor C32 with AKAP9 antibody showing a shift in subcellular localization of the protein from a single dot/centrosomal pattern observed in the entrapped non-neoplastic follicles (arrows) to diffuse cytoplasmic staining in tumor cells. Magnification, ×200.

**Figure 6**

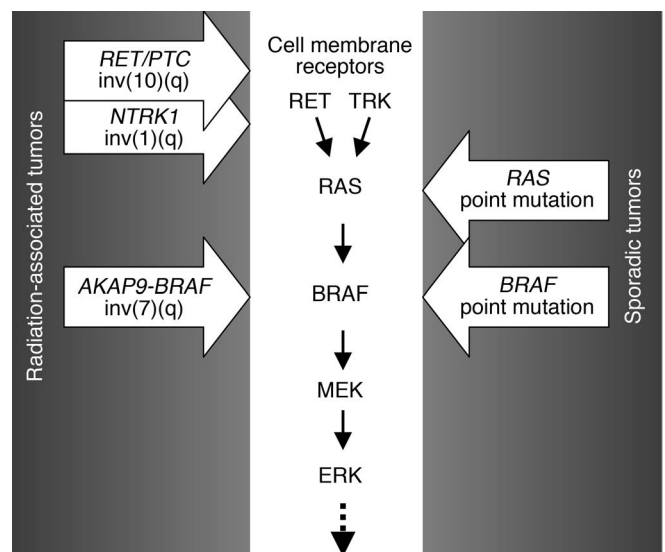
Detection of V600E *BRAF* mutation cDNA from post-Chernobyl tumors that developed 9–12 years after exposure using LightCycler real-time PCR followed by fluorescence melting curve analysis. **(A)** Real-time amplification of cDNA samples. **(B)** Detection of point mutations by the postamplification fluorescence melting curve analysis based on a distinct melting temperature of duplexes formed between the WT probe and either WT (62.8°C) or mutant (57.0°C) sequences. The mutations in both samples are heterozygous, since both mutant and WT peaks are detectable.

intracellular effectors along the MAPK pathway have been previously implicated in human carcinogenesis through mechanisms other than point mutation. In the present study, we demonstrate *BRAF* activation by chromosomal rearrangement that results in *BRAF* fusion to the *AKAP9* gene in thyroid papillary carcinomas. The fusion protein lacks the regulatory domains of *BRAF*, which results in constitutive kinase activation in a RAS-independent manner, and exhibits transforming activity similar to that of the most common *BRAF*^{V600E}.

The fusion partner of *BRAF*, the *AKAP9* gene, belongs to the group of A-kinase anchor proteins that have the common function of binding to the regulatory subunit of PKA and targeting it to discrete locations within the cell (29). *AKAP9*, in particular, has predominantly centrosomal and Golgi compartmentalization (19–21). The fusion protein lacks the C-terminal centrosomal domain and, as expected, loses its centrosomal localization in cancer cells. Moreover, immunohistochemical analysis with *AKAP9* antibody revealed no evidence of wild-type *AKAP9* within centrosomes of cancer cells in all cases positive for the fusion by RT-PCR. This may suggest the competitive inhibition of wild-type *AKAP9* by *AKAP9-BRAF*, although further studies are required to confirm this. *AKAP9* is an important regulator of c-AMP-dependent PKA and interacts with many other signaling proteins, including serine/threonine kinase protein kinase N, protein phosphatase 1, protein phosphatase 2A, and PKC- ϵ (20, 30). Thus, deregulation of its function in cells harboring *AKAP9-BRAF* fusion may affect a variety of physiological functions. On the other hand, neither *AKAP9* nor other *AKAP* genes have been implicated in carcinogenesis so far. Therefore, it remains unclear whether or not the *AKAP9* portion of the fusion gene contributes independently to its transforming potential, besides providing an active promoter driving the expression of truncated *BRAF*.

Over the last decade, it has become apparent that activation of components of the MAPK pathway in thyroid cancer can be

achieved through several distinct genetic events, i.e., *RET/PTC* (or, less frequently, *TRK*) rearrangements, *RAS* point mutations, or *BRAF* point mutations. However, the relative frequency of each event varies significantly in different populations. *BRAF* point mutation is the most common change in sporadic adult papillary carcinomas. In contrast, tumors associated with accidental or therapeutic irradiation have exceedingly high frequency of *RET/PTC* rearrangements and absence or low prevalence of *BRAF* point mutations (31–38). The 2 most common *RET/PTC* types found in up to 80% of radiation-associated tumors, *RET/PTC1* and *RET/PTC3*, are both paracentric inversions of chromosome 10q (39, 40). Another rearrangement, which contributes to about 7% of radiation-induced papillary carcinomas, involves the nerve growth factor gene *NTRK1* and results from paracentric inversion of chromosome 1q (41, 42). Here, we report the identification of another paracentric inversion, involving 7q, which is also more common in radiation-associated tumors, especially those appearing with a short latency. These tumors, however, revealed no *BRAF* point mutations. The sharp contrast in mechanisms of activation of the same gene in radiation-induced and sporadic tumors points to the association between environmental factors and distinct genetic mechanisms of activation of MAPK pathway components: through chromosomal rearrangement in patients exposed to radiation or through point mutations in patients with no radiation history (Figure 7).

**Figure 7**

Activation of MAPK pathway in papillary thyroid carcinogenesis involves predominantly point mutations in sporadic tumors or chromosomal rearrangements in radiation-associated tumors.



The vast majority of *RET/PTC* and all *TRK* and *AKAP9-BRAF* chromosomal rearrangements in radiation-induced tumors are paracentric inversions. In epithelial cancers as a whole, point mutations are more prevalent than chromosomal rearrangements, and the latter are usually translocations or interchromosomal exchanges. The reason for the striking predisposition of radiation-induced thyroid cancers to paracentric inversions is not entirely clear, although some evidence suggests that this may be related to the organization of chromosomal domains and arm domains and spatial interaction between pairs of various chromosomal regions. Indeed, there is a general consensus that free DNA ends produced in the nuclear volume after passage of radiation tracks have to be within a certain distance of each other in order to recombine (43, 44). Residing within the same chromosomal arm domain would limit their maximum separation and help to fulfill this requirement. Indeed, the yields of intra-arm chromosomal exchanges after both low- and high-linear energy transfer radiation has been shown to exceed the expected levels, calculated based on spatial randomness, by up to 10-fold (45). The conditions would be even more favorable if potentially recombinogenic foci within the arm domain were frequently juxtaposed to each other, as was demonstrated in human thyroid cells for *RET* and *H4*, the *RET/PTC1* partners (46).

In this study, we observed significant difference in mechanisms of BRAF activation not only between early radiation-associated tumors and sporadic cancers, but also between early and late post-radiation tumors. Such a difference is not without precedent. Similar variation has been previously observed with respect to *RET/PTC* rearrangements between post-Chernobyl tumors developing 10 years or less after exposure and those manifesting after a longer latency (34, 35). Indeed, the overall prevalence of *RET/PTC* was significantly higher in the former group, and *RET/PTC3* was the most common type. By contrast, tumors arising after a longer latency had lower prevalence of *RET/PTC*, and the predominant type was *RET/PTC1*, both of which are features more typical of sporadic tumors. The present study demonstrates that late post-radiation tumors also occupy an intermediate position with respect to the predominant mechanism of BRAF activation. This suggests that tumors arising early after exposure are likely to be initiated through direct radiation damage-related genetic events, such as chromosomal rearrangements occurring after double-stranded DNA breaks. As for the tumors developing after a longer latency, their pathogenesis may involve a combination of radiation-related and -unrelated mechanisms and rely on molecular alterations which develop after repair of radiation-induced DNA damage that are stimulated by genomic instability that persists in the progeny of the exposed cells (47, 48).

In summary, we report here a novel mechanism of *in vivo* BRAF activation via inversion of chromosome 7q, resulting in *BRAF* fusion to the *AKAP9* gene. *AKAP9-BRAF* functions as an oncogene and is more prevalent in thyroid carcinomas with recent history of radiation exposure. The significance of this study is 2-fold. It demonstrates a novel mechanism of BRAF activation in human tumors, showing for the first time that intracellular effectors along the MAPK pathway can be activated by recombination. In addition, it shows correlation between the nature of the mutagen and the mechanism of mutation and suggests that paracentric chromosomal inversions represent a common genetic mechanism of radiation-associated thyroid carcinogenesis.

Methods

Tumor samples. Frozen tissues from sporadic thyroid carcinomas were obtained from the Department of Pathology, University of Cincinnati, following an institutionally approved tissue procurement protocol and through the Cooperative Human Tissue Network. Post-Chernobyl tumors from Belarussian patients who underwent surgery in 1991–92 were available as archival paraffin-embedded tissue. Samples from Belarussian patients who underwent surgery in 1995–1998 were from the Experimental Division of the Institute of Pathology, University of Munich, and available as RNA extracted from frozen tissue; they were negative for *RET/PTC* and *TRK* rearrangements (35). The study was approved by the University of Cincinnati Institutional Review Board.

FISH analysis. Tumor touch preparations were made from snap-frozen tissues. PAC clones for *BRAF* (RP4-726N20, RP5-839B19, RP4-813F11) were obtained from BAC/PAC Resources, Children's Hospital Oakland, and bacterial artificial chromosome (BAC) clones for *AKAP9* (CTB-104F4, CTB-161K23) from Open Biosystems. The probes were labeled by nick translation using SpectrumOrange-dUTP and SpectrumGreen-dUTP (Vysis Inc.), respectively. An α -satellite DNA centromeric probe for chromosome 7 was obtained from Vysis Inc. Nuclei were counterstained with DAPI. Microscopy was performed with a Leica Microsystems TCS 4D confocal laser scanning fluorescence microscope with digital image capture. One hundred nuclei were scored for each preparation.

5' RACE. Fifty micrograms of poly(A)⁺ RNA from the index case were used for 5' RACE using the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen Corp.). The *BRAF* gene-specific primers were designed for cDNA synthesis (5'-GATGACTTCTGGTGCCATCC-3', 5'-AACTGCTGAGGTGTAGGTGCTGTC-3', and 5'-GTCTCGTTGCCCAAATTGAT-3') and for subsequent PCR reactions (5'-TTTCACTGCCACATCACCAT-3' for first PCR and 5'-TCACTCGAGTCCCGTCTACC-3' for nested PCR). The 5' RACE products were excised from the gel, purified, and sequenced using an automated ABI 377 sequencer (PerkinElmer).

Cloning and sequencing analysis. Total RNA was used to amplify the ORF of the chimeric cDNA using primers located in the 5'-untranslated region of *AKAP9* (5'-ACCACCCCTCAACCCCTGTTTT-3') and 3'-untranslated region of *BRAF* (5'-TTCCTTTTGTGCTACTCTCCTGA-3'). The PCR product was electrophoresed in an agarose gel, purified from the gel, and cloned into the pCR-XL-TOPO vector (Invitrogen Corp.). Several clones were fully sequenced. The genomic *AKAP9-BRAF* fusion was amplified with primers 5'-GCAACTCAACCAAGTGAATG-3' and 5'-GGTTGATCCTCATCACCAC-3', yielding a product of approximately 2.4 kb, and the reciprocal fusion product cDNA was amplified with primers 5'-ACCCGCCTCGGACTCTATT-3' and 5'-GTTGGGCTTCCATCTGTAGC-3', yielding a product of 242 bp.

Western blot and immunohistochemistry. Frozen tissues from papillary carcinomas were used for Western blotting, which was performed as previously described (49). We loaded 50 μ g of protein on a 5% polyacrylamide gel. After transfer, the membranes were incubated with anti-C-terminus BRAF polyclonal (Raf-B, C-19; Santa Cruz Biotechnology Inc.) or anti-N-terminus AKAP9 monoclonal (AKAP450; BD Biosciences) antibodies. The same AKAP9 antibody in 1:50 dilution was used for immunohistochemical analysis, which was performed on paraffin sections after overnight pretreatment in borate buffer using avidin-streptavidin immunoperoxidase detection. Rabbit anti- γ -tubulin polyclonal antibody was provided by K. Fukasawa (University of Cincinnati College of Medicine, Cincinnati, Ohio, USA) (50) and was used in 1:800 dilution on paraffin sections after pretreatment in borate buffer.

In vitro kinase assay. COS7 cells were transiently cotransfected with either pEFP vector or pEFP-containing myc-tagged BRAF^{WT}, AKAP9-BRAF, or BRAF^{V600E} and either pBabe puro or pBabe puro-HRAS^{G12V} using Lipo-



fectamine 2000 (Invitrogen Corp.). After 48 hours, cell extracts were prepared, immunoprecipitated with mouse anti-myc-IgG (Upstate Cell Signaling Solutions), and kinase activity determined as directed by the manufacturer using bacterially purified MEK1 as substrate, and Western blotting with pMEK1 antibody as a read-out (B-RAF Kinase Assay Kit; Upstate Cell Signaling Solutions). To determine the effects of the BRAF mutants on endogenous ERK1/2 phosphorylation, we incubated pools of stably transfected cells for 12 hours without serum prior to lysate preparation. Lysates were centrifuged, the supernatant collected, and 100 µg of extract subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and subject to Western blot analysis with anti-phospho-ERK1/2, anti-total ERK1/2, or anti-BRAF (Santa Cruz Biotechnology Inc.) antibodies.

Transformation assays. For focus assays, NIH3T3 cells were transfected with empty vector or the indicated BRAF and H-RAS expression plasmids using lipofectamine. After 2 days, cells were split into six 100-mm³ dishes and incubated with DMEM containing 5% calf serum for 21 days. Cells were stained with 0.4% crystal violet and foci counted. For the mouse xenograft experiments, NIH3T3 cells were cotransfected with pBabe puro and pEFP vector, pEFP-myc BRAF^{WT}, pEFP-AKAP9-BRAF, pEFP-myc BRAF^{V600E}, or pBabe puro-H-RAS^{G12V} and mass selected by incubation with DMEM containing 5% calf serum and 1 µg/ml puromycin for 3 weeks. A total of 10⁶ pooled cells was injected into nude mice. Animals were killed after 2 weeks, and nodules larger than 5 mm in diameter were scored as positive.

Mutation analysis. For the detection of AKAP9-BRAF fusion, cDNA amplification was achieved by PCR using primers located in exon 8 of AKAP9 (Ex8A, 5'-AGCAAGAACAGTTGATTTTGA-3') and exon 10 of BRAF (Ex10B, 5'-GCAGACAAACCTGTGGTTGA-3'), with the expected product of 181 bp. The PCR products were electrophoresed on agarose gels and bands visualized by staining with ethidium bromide (frozen tissues) or by transfer to a nylon filter and hybridization with an internal oligonucle-

otide probe (PR, 5'-AACTTCAGAAAGAACTCAATGTACTT-3') labeled with 50 µCi of [γ -³²P]-ATP (paraffin-embedded tissue). Detection of V600E BRAF mutation was performed from DNA using real-time PCR and fluorescence melting curve analysis, as previously reported (51), or from cDNA using real-time RT-PCR with primers 5'-CGACAGACTGCACAGG-3' and 5'-TGACTTCTGGTGCCAT-3' and the same probes.

Statistical analysis. The comparison between groups was performed using a 2-tailed Fisher exact test. Differences were considered significant when *P* was less than 0.05.

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Address correspondence to: Yuri Nikiforov, Department of Pathology, University of Cincinnati, 231 Albert Sabin Way, PO Box 670529, Cincinnati, Ohio 45267-0529, USA. Phone: (513) 558-5798; Fax: (513) 558-2289; E-mail: Yuri.Nikiforov@uc.edu.

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