Two Transforming *C-RAF* Germ-Line Mutations Identified in Patients with Therapy-Related Acute Myeloid Leukemia

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Abstract

Mutations leading to activation of the RAF-mitogen-activated protein kinase/extracellular signal-regulated (ERK) kinase (MEK)-ERK pathway are key events in the pathogenesis of human malignancies. In a screen of 82 acute myeloid leukemia (AML) samples, 45 (55%) showed activated ERK and thus were further analyzed for mutations in *B-RAF* and *C-RAF*. Two *C-RAF* germ-line mutations, S427G and I448V, were identified in patients with therapy-related AML in the absence of alterations in *RAS* and *FLT3*. Both exchanges were located within the kinase domain of *C-RAF*. *In vitro* and *in vivo* kinase assays revealed significantly increased activity for ^{S427G}C-RAF but not for ^{I448V}C-RAF. The involvement of the S427G C-RAF mutation in constitutive activation of ERK was further confirmed through demonstration of activating phosphorylations on C-RAF, MEK, and ERK in neoplastic cells, but not in nonneoplastic cells. Transformation and survival assays showed oncogenic and antiapoptotic properties for both mutations. Screening healthy individuals revealed a <1/400 frequency of these mutations and, in the case of I448V, inheritance was observed over three generations with another mutation carrier suffering from cancer. Taken together, these data are the first to relate *C-RAF* mutations to human malignancies. As both mutations are of germ-line origin, they might constitute a novel tumor-predisposing factor. (Cancer Res 2006; 66(7): 3401-8)

Introduction

The RAF-mitogen-activated protein kinase/extracellular signal-regulated (ERK) kinase (MEK)-ERK signaling cascade mediates mitogenic and antiapoptotic signals from the cell surface to intracellular effector molecules. It is preferentially activated through binding of growth factors to receptor tyrosine kinases (RTK) that associate with the 21-kDa small G-protein RAS (1). The serine-threonine kinases A-RAF, B-RAF, and C-RAF require RAS binding for activation and are additionally regulated through phosphorylation and binding to lipids and other protein regulators (2). Activated RAF phosphorylates MEK1/2, which in turn phosphorylate and thus activate ERK1/2. Constitutive activation of this pathway as a result of mutations is considered to be a key event in the development of several human malignancies including acute myeloid leukemia (AML; ref. 3). RTKs like *FLT3* are frequently mutated in AML, leading to ligand-independent pathway activation (4, 5). Mutations have also been described in *RAS*, which is constitutively active in ~20% of all human cancers (1) and in up to 25% of AML cases (5). Recently, activating mutations of *B-RAF* have been detected in 66% of melanomas and at lower frequency in a wide range of human solid cancers. All mutations were located within the kinase domain of *B-RAF* with a single substitution (V600E, formerly V599E) accounting for 80% of them (6). Until now, more than 60 different mutations have been identified in this *RAF* isoform, most of them exhibiting elevated kinase activity and the ability to transform NIH 3T3 cells. Some of these *B-RAF* mutants, however, have impaired kinase activity but nevertheless activate ERK by stimulating C-RAF (7).

In the study presented here, we have investigated the *B-RAF* and *C-RAF* genes in AML as constitutive activation of the RAF-MEK-ERK pathway frequently occurs in this disease. In a screen of 82 patient samples, we identified the first ever described transforming *C-RAF* mutations associated with human malignancies.

Materials and Methods

Patients and samples. Diagnostic blood and bone marrow samples with >80% blast cells of 82 AML patients were processed as previously described (8). AML was classified according to the WHO guidelines: AML with recurrent cytogenetic aberrations, $n = 5$; AML with multilineage dysplasia, $n = 8$; therapy-related AML (t-AML), $n = 12$; AML not otherwise categorized, $n = 49$; chronic myeloid leukemia-myeloid blast crisis, $n = 8$. The myeloid leukemia cell lines HL-60, KG1, and ML-2 were obtained from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany). Normal $CD34^+$ stem and progenitor cells were isolated from leukapheresis harvests of patients with nonmyeloid malignancies. Control samples of healthy individuals for denaturing high-pressure liquid chromatography were obtained from an anonymized DNA bank at the Medical University of Graz. For the pedigree analysis of the two patients with *C-RAF* mutations, peripheral blood was obtained from their relatives. The study was approved by the local ethics board and informed consent was obtained from all individuals.

Immunoblot analysis. Preparation of lysates and Western blotting procedures have previously been described (9). Membranes were incubated for 12 hours at 4°C with the following antibodies: anti–phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-ERK1/2, anti–phospho-MEK1/2 (Ser^{217}/Ser^{221}), anti-MEK1/2 (all from Cell Signaling Technology, Beverly, MA), anti–phospho-Raf-1 (Ser^{338}) (Upstate, Waltham, MA), and anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were reprobed with an anti-β-actin

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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antibody (Sigma-Aldrich, St. Louis, MO) to adjust for differences in protein loading of individual samples. Immune complexes were visualized by a horseradish peroxidase–conjugated secondary antibody using the enhanced chemiluminescence (ECL) reagents ECL or ECL plus (Amersham Biosciences, Piscataway, NJ). Signal intensities of the autoradiogram were quantified by densitometric scanning.

DNA/RNA extraction and reverse transcription. DNA and RNA isolation of peripheral blood, bone marrow, and paraffin-embedded samples was done as previously described (8, 10). All samples were diluted to a final concentration of 25 ng/μL DNA and 200 ng/μL total RNA. Reverse transcription of RNA was done with 2 μg of RNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA; manufactured by Roche, Branchburg, NJ) according to the protocol of the manufacturer and cDNA was stored at −20°C until further use.

Sequence analysis and denaturing high-pressure liquid chromatography. cDNA was amplified using overlapping primer pairs placed at the junction between two exons of *B-RAF* and *C-RAF*. For amplification of *C-RAF* exon 12, *FLT3* exons 11 and 12, and *N-RAS, K-RAS*, and *H-RAS* exons 1 and 2 from genomic DNA, we used intronic primers flanking the corresponding exons. Primer sequences, annealing temperatures, and amplicon sizes are listed in Supplementary Table S1. PCR, gel electrophoresis, and sequence analysis were done as previously described (8). For denaturing high-pressure liquid chromatography, we used genomic DNA from the two leukemia samples with the *C-RAF* mutations as well as genomic DNA extracted from 200 healthy individuals. PCR of *C-RAF* exon 12 was carried out in a volume of 20 μL using 25 ng of DNA, 1.5 units of AmpliTaq Gold DNA Polymerase, 1× buffer containing $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphate mix, and 4 pmol of each primer over 35 cycles. PCR conditions were initial denaturation at 95°C for 12 minutes, 35 cycles of amplification (45 seconds at 95°C, 30 seconds at 57°C, and 45 seconds at 72°C) with a final additional extension step at 72°C for 10 minutes. A 30-minute temperature ramp from 95°C to 65°C was added at the end of the PCR program. Denaturing high-pressure liquid chromatography was run with an injection volume of 5 μL and a pressure of 35 to 40 bar at 61°C melting temperature. The mobile phase consisted of buffer A [100 mmol/L triethylamine acetate (pH 7.0) and 0.1 nmol/L EDTA] and buffer B [100 mmol/L triethylamine acetate (pH 7.0), 0.1 mmol/L EDTA, and 25% (v/v) acetonitrile]. At a flow rate of 0.45 mL/min, samples were separated in 8 minutes and 50 seconds through the column.

Real-time expression analysis. Expression analysis was done on an ABI Prism 7000 Sequence Detection System using the TaqMan method (Applied Biosystems). *RAS* cDNA expressions were evaluated using the comparative $\Delta\Delta C_T$ method (ABI Prism 7000 Sequence Detection System User Bulletin #2). The HL-60 AML cell line served as calibrator and *c-ABL* as control gene as recommended for quantitative reverse transcription-PCR of leukemia-associated transcripts (11, 12). Primers and probes for *N-RAS, K-RAS*, and *H-RAS* were designed in cooperation with Applied Biosystems by the inventory assay system. Primers for *c-ABL* were designed using the Primer Express version 1.0 software (Applied Biosystems) based on published GenBank sequences. Primer and probe nucleotide sequences as well as reporter and quencher dyes are listed in Supplementary Table S1. Amplifications were done at ABI Prism 7000 standard conditions as previously described (13). Concentrations used at *c-ABL* were 150 nmol/L for the TaqMan probe and 300 nmol/L for forward and reverse primers. For *N-RAS, K-RAS,* and *H-RAS*, primers and probes were already premixed and added at 1× concentration.

Plasmids and generation of *C-RAF* expression constructs. A *Bam*HI/*Xba*I fragment derived from the pEFmRaf plasmid expressing full-length Myc-tagged *C-RAF* (14) was recloned into the corresponding sites of pBluescript KS− (Stratagene, La Jolla, CA) and site-directed mutagenesis was done using the QuikChange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The mutant primers 5′-GGTGCGAGGGCGGCAGCCTCTACAAAC-3′ and 5′-TCCAGCTAATTGACGTTGCCCGGCAGACG-3′ were used to generate amino acid exchanges S427G and I448V, respectively. Presence of the mutations was confirmed by direct sequencing and the mutated insert was transferred back into pEF. Expression constructs for His-tagged *MEK1* and HA-tagged *ERK1* have been described (15). Expression constructs for wild-type (wt) Myc-tagged *B-RAF* have been provided by Richard Marais (The Institute of Cancer Research, Signal Transduction Team, Cancer Research UK Centre of Cell and Molecular Biology, London, United Kingdom).

Transfection. NIH 3T3 and Cos-7 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (PAA, Pasching, Austria), penicillin/streptomycin (100 units/mL, Invitrogen), and 2 mmol/L L-glutamine (Invitrogen). NIH 3T3 fibroblasts and Cos-7 cells were seeded in six-well plates and transfected at 70% confluency by a high-efficiency liposome-mediated transfection method (Lipofectamine, Invitrogen). For kinase assays, routinely 1.5 μg of plasmid DNA were used. In cotransfection experiments, 0.7 μg of the *C-RAF* expression construct was transfected together with 0.3 μg of *MEK1* or *ERK1*. In another series of experiments, 0.4 μg of *B-RAF* and 0.4 μg of *C-RAF* were cotransfected together with 0.2 μg of His-tagged *MEK1* in Cos-7 cells.

Soft agar cloning. Three milliliters of a 0.5% Seaplaque GTG agarose (Cambrex, East Rutherford, NJ) solution in complete tissue culture medium were plated per well of a six-well tissue culture plate. Cells, 10^4 and 10^5, were resuspended in 2 mL of 0.3% agarose solution and layered over the bottom agar. Plates were maintained at 37°C under humid conditions and scored for colony formation after 28 days.

Cell lysis, immunoblotting, and kinase assays. Before lysis, cells were starved overnight in low-serum medium (0.3% for Cos-7, 0.05% for NIH 3T3), washed twice with room-temperature PBS, and lysed in a buffer containing 25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 10 mmol/L $Na_4P_2O_7$, 25 mmol/L $C_3H_7Na_2O_6P \times H_2O$, 10% glycerol, 0.75% NP40, 25 mmol/L NaF, and protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem, Darmstadt, Germany). SDS-PAGE and Western blot analysis were carried out as previously described (15). *C-RAF* was immunoprecipitated using the Myc-tag antibody 9B11 (Cell Signaling) and kinase activity was measured following an established protocol (16). For kinase assays of endogenous B-RAF and C-RAF, patient samples stored in liquid nitrogen after addition of DMSO were thawed in RPMI 1640 (PAA) supplemented with 10% heat-inactivated FCS (PAA), penicillin/streptomycin (100 units/mL, Invitrogen), and 2 mmol/L L-glutamine (Invitrogen), and maintained in this medium for 2 hours. Thereafter they were analyzed under the following conditions: (*a*) continued growth in full medium for further 6 hours (only for C-RAF); (*b*) deprivation of serum through three washes with serum-free RPMI 1640 followed by starvation for 6 hours in 0.05% serum before harvesting them for kinase assays; (*c*) restimulation with 10% serum for 15 minutes after starvation as mentioned in (*b*). Staining of cells with trypan blue before lysis confirmed that cells had remained sufficiently viable during these treatments. Endogenous C-RAF and B-RAF were immunoprecipitated with antibodies specific for C-RAF and B-RAF, respectively (Santa Cruz Biotechnology) and kinase assays were done as described above.

Cell survival assays. Cells (10^5) were seeded in a single well of a six-well plate and grown to confluency. Staurosporine (Sigma) treatment was carried out in normal growth medium. Apoptosis was analyzed using the Annexin V-FITC kit (BioCat, Heidelberg, Germany) according to the instructions of the manufacturer. Briefly, cells were trypsinized and pelleted by centrifugation at 250 × *g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 500 μL of binding buffer (PBS containing 2 mmol/L $CaCl_2$), followed by another centrifugation at 250 × *g* for 5 minutes. The cell pellet was resuspended in 100 μL of binding buffer containing Annexin V-FITC and incubated for 15 minutes at room temperature in the dark. Stained cells were analyzed in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. Percentage of apoptotic cells was calculated from Annexin V-FITC–positive cells using the CellQuest data analysis software.

Immunohistochemistry. Immunohistochemistry was done on paraffin-embedded biopsy specimens using the antibodies described for immunoblotting. The primary antibodies were diluted in antibody diluent (Dako, Glostrup, Denmark) and all phospho-antibodies in primary antibody diluent Monet blue (Biocarta, Carlsbad, CA). The alkaline phosphatase anti–alkaline phosphatase method was used for detection (17). Specimens were counterstained with hematoxylin.

Statistical analysis. Statistical analysis was done using SPSS 11.5 (SPSS, Inc., Chicago, IL). Distribution of ERK phosphorylated and unphosphorylated samples in the different AML subgroups was tested using Pearson's χ^2 test. All tests were two sided and $P < 0.05$ was considered statistically significant. As *RAS* expression values were not normally distributed, the median and the interpercentile range (20-80%) of 25 AML samples were determined and subsequently related to the two samples of interest (UPN 2681 and 3425).

Results

Constitutive ERK phosphorylation occurs frequently in AML patient samples. We initially identified those AML samples exhibiting constitutive activation of the RAF-MEK-ERK pathway. Eighty-two AML specimens and the AML cell lines HL-60, KG1, and ML-2 were analyzed for their phosphorylation status of ERK1/2 (Fig. 1*A*). For quantification of phosphorylation, we chose the pERK2 band as pERK1 was not detectable in some patient samples of lower quality. The ratio between total ERK2 and pERK2 determined densitometrically was taken as a measure for constitutive ERK phosphorylation. Samples were arbitrarily scored positive when this value exceeded 10% of the positive control (invasive ductal carcinoma of the breast; Fig. 1*B*). Forty five of 82 (55%) AML samples but none of the AML cell lines showed phosphorylation of ERK. No statistically significant differences in ERK phosphorylation between different WHO subgroups could be identified as shown in Fig. 1*C* ($P = 0.562$). We also tested the phosphorylation status of ERK1/2 of normal hematopoietic stem and progenitor cells. None of the five $CD34^+$ cell preparations showed phosphorylated ERK1/2.

Two novel *C-RAF* mutations in patients with t-AML. To screen for activating *RAF* mutations, we subjected the entire coding region of *B-RAF* and *C-RAF* of those 45 samples showing constitutive ERK1/2 phosphorylation to cDNA sequence analysis (exon 1 of both genes could not be amplified by PCR despite the use of different primer sets due to a very high GC content). Two silent *B-RAF* polymorphisms (A1225G and G1927A), as previously described by Davies et al. (6), but no single *B-RAF* mutation, could be detected. In addition to one already known silent *C-RAF* polymorphism (G807C; dbSNP:1063664), two novel heterozygous single-base substitutions of the *C-RAF* proto-oncogene were identified in 2 of 12 (17%) patients with t-AML. The presence of both mutations was confirmed using genomic DNA of the same samples. Both mutations are located in exon 12 within the highly conserved protein kinase domain (Fig. 2*A*). A1279G (serine replaced by glycine at position 427—S427G) was observed in the 43-year-old patient UPN 3425, and A1342G (isoleucine replaced by valine at position 448—I448V) in the 72-year-old patient UPN 2681 (Fig. 2*B*). Identical mutations were also detected in DNA from buccal epithelial cells and a skin biopsy of patient UPN 2681 as well as from buccal epithelial cells and an axillary lymph node of patient UPN 3425, respectively. They were therefore considered germ-line events. Primary malignancies of both patients (embryonal carcinoma of the testis in patient UPN 3425 and colorectal cancer in patient UPN 2681, respectively) exhibited the mutation as well.

To evaluate the frequency of these mutations in the general population, we did denaturing high-pressure liquid chromatography of 200 healthy individuals corresponding to 400 alleles. Both mutations could clearly be distinguished from the wt allele (data not shown). They could not be detected in any of these individuals.

Alterations in *RAS* and *FLT3* are absent in patients carrying mutant *C-RAF*. We further analyzed *RAS* and *FLT3*, which are potential upstream signaling components that may also have caused activation of the RAF-MEK-ERK pathway (1, 18) in these tumors. Sequence analyses of codons 12, 13, and 61 of *N-RAS, K-RAS*, and *H-RAS*, which contain the vast majority of *RAS* mutations in AML (5, 19), and of exons 11 to 12 of *FLT3*, to screen for *FLT3* internal tandem duplications, were done. In both patients, genomic DNAs obtained from the t-AML, primary malignancy, and buccal epithelial cells were used. With the exception of the colon cancer sample of patient UPN 2681, where inadequate material precluded the analysis, wt sequence could be shown in all tested codons and exons.

As cellular transformation can also be caused by *RAS* overexpression (20), we proceeded in testing the cDNA expression levels of *N-RAS, K-RAS*, and *H-RAS* in both t-AML samples and 25 additional AML samples, which served as control group. *RAS* expression levels of both samples were within the interpercentile range (data not shown).

S427G results in the constitutive activation of C-RAF. To address a possible role of mutant C-RAF proteins in the constitutive phosphorylation of ERK1/2, kinase assays were done with Myc-tagged C-RAF proteins immunoprecipitated from transiently transfected Cos-7 cells. Strong constitutive kinase activity was shown for ^{S427G}C-RAF whereas ^{I448V}C-RAF did not significantly differ from wt C-RAF (Fig. 3*A*). The previously published constitutively active C-RAF YY340/341DD protein served

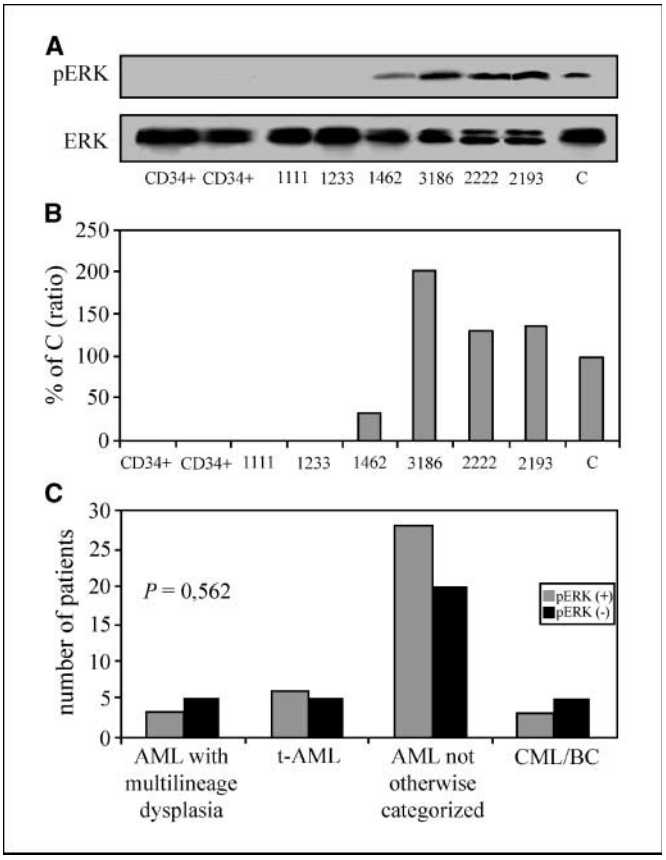

Figure 1. pERK status in AML patient samples. *A,* representative picture of the immunoblot analysis showing ERK phosphorylation in patient samples UPN 1462, 3186, 2222, and 2193 and absence in UPN 1111, UPN 1233, and $CD34^+$ samples. Lysates obtained from an invasive ductal carcinoma of the breast served as positive control (*C*). *B,* densitometric quantification of ERK phosphorylation. Samples were scored positive when (pERK2/ERK2) × 100 (in %) exceeded 10% of the positive control (set to 100%). *C,* the number of ERK phosphorylated samples did not differ among the different AML subgroups.

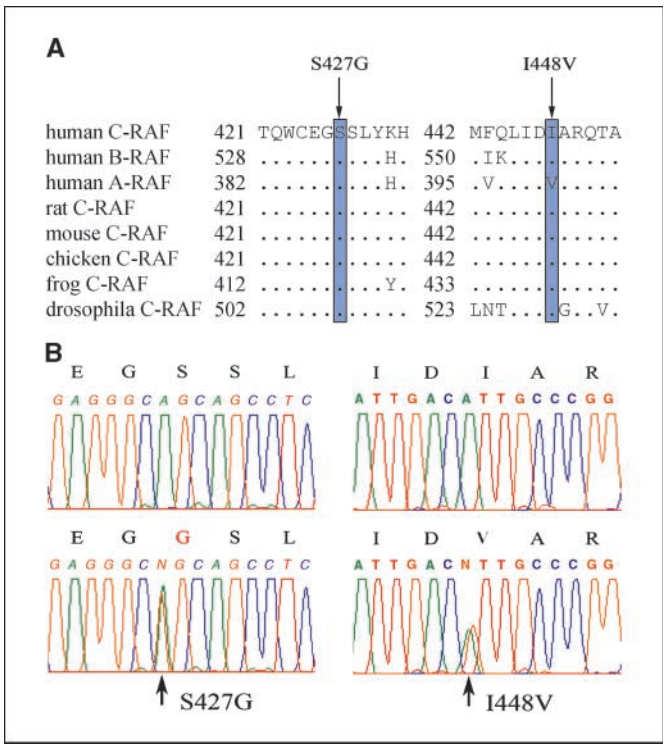

Figure 2. The S427G and the I448V *C-RAF* mutations. *A,* sequence alignment of the kinase activation segment containing both *C-RAF* single base substitutions. *Arrows,* mutations. *B,* electropherograms showing the heterozygous *C-RAF* mutations and their consequences on the amino acid sequence.

as a positive control in these experiments (21). We also tested the cooperation of the I4448V mutant with oncogenic H-RAS(V12) in the activation of MEK in Cos-7 cells. In these experiments, mutant RAF failed to enhance RAS induced MEK activation and also did not interfere with signaling downstream of RAS (data not shown). To further corroborate these observations, we analyzed the activity of endogenous C-RAF in our AML patient samples. However, due to the restricted availability of primary material, only a limited analysis of the kinase activity of mutant C-RAF was possible. The extent of phosphorylation detected in these reactions was weaker compared with the experiments carried out in NIH 3T3 and Cos-7 cells, which can be explained by the lower amount of protein, but was consistent with our *in vitro* assays after transfection of mutated cDNA. Again, S427G C-RAF stimulated the kinase activity towards MEK and ERK whereas 1448V C-RAF failed to do so (data not shown). In the case of S427G C-RAF, kinase activity was still detectable in cells, which have been starved for 6 hours by lowering the serum concentration in the medium to 0.05%, confirming the constitutive nature of this activation (data not shown). These findings were supported by data obtained with NIH 3T3 cells transiently transfected with C-RAF expression constructs together with either His-tagged wt MEK or HA-tagged ERK. The phosphorylation status of MEK and ERK was analyzed through the use of phosphorylation-specific antibodies. ^{S427G}C-RAF also strongly enhanced the activity of cotransfected MEK/ERK (Fig. 3*B*). Identical data on MEK and ERK phosphorylation were obtained when endogenous MEK and ERK were analyzed (Fig. 3*C*). Taken together, these experiments show that the S427G mutation by itself is sufficient to cause activation of MEK and ERK. However, they fail to provide an explanation for ERK phosphorylation detected in I448V mutant cells.

Activation of ERK in I448V mutant cells is not mediated via B-RAF. Some previously reported B-RAF mutants (6) lack elevated B-RAF kinase activity but still induce constitutive activation of ERK. Wan et al. (7) showed that these B-RAF mutants activate endogenous wt C-RAF, possibly via an allosteric or transphosphorylation mechanism, thereby causing constitutive ERK phosphorylation. To test for a similar interaction of mutant I448V with

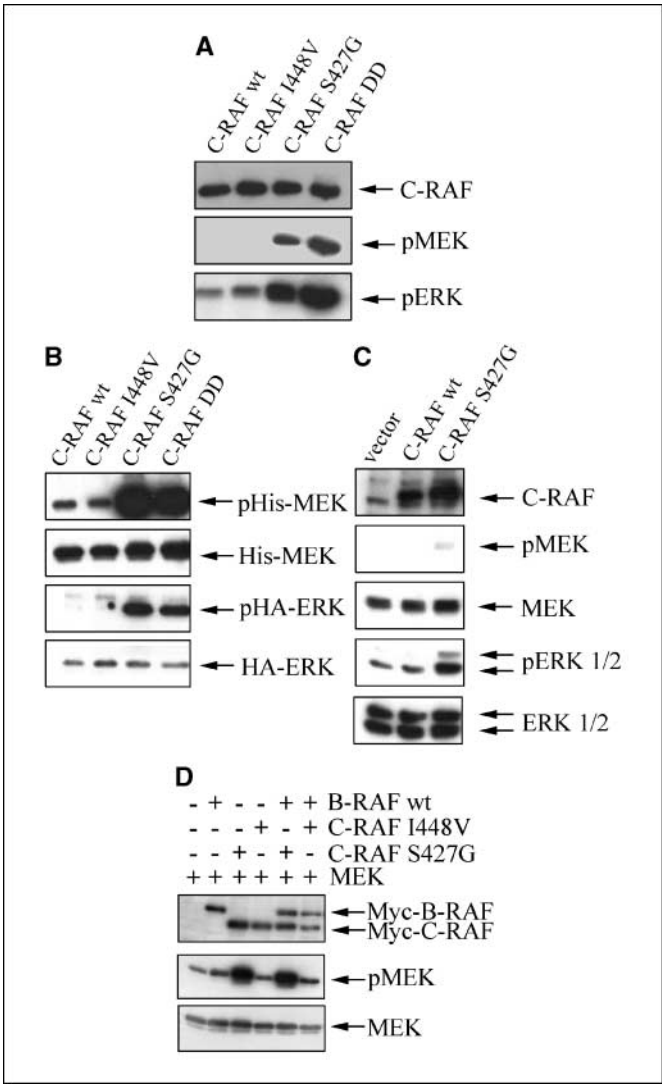

Figure 3. Analysis of the *in vitro* and *in vivo* kinase activities of mutant C-RAF proteins. *A,* immune-complex kinase assays after expression of mutant cDNAs in NIH 3T3 cells show elevated kinase activity for the ^{S427G}C-RAF mutant. The indicated Myc-tagged RAF proteins were immunoprecipitated and incubated with recombinant MEK and ERK proteins. Substrate phosphorylation was monitored by phosphorylation-specific antibodies. *B,* wt and mutant *C-RAF* constructs were cotransfected with either MEK or ERK in NIH 3T3 cells and the activation status of MEK and ERK was analyzed using phosphorylation-specific antibodies. Strong kinase activity was shown for ^{S427G}C-RAF, comparable to the previously published highly active *C-RAF* DD mutant (21), but not for ^{I448V}C-RAF. *C,* identical results were obtained when endogenous MEK and ERK in NIH 3T3 cells were analyzed. *D,* cotransfection experiments of the C-RAF mutants and wt B-RAF together with His-tagged MEK into Cos-7 cells. Analysis of MEK activation using phosphorylation-specific antibodies failed to detect MEK activation in the case of ^{I448V}C-RAF, irrespective of whether B-RAF was present or not. ^{S427G}C-RAF served as positive control. +, presence of a transfected construct; −, absence of a transfected construct.

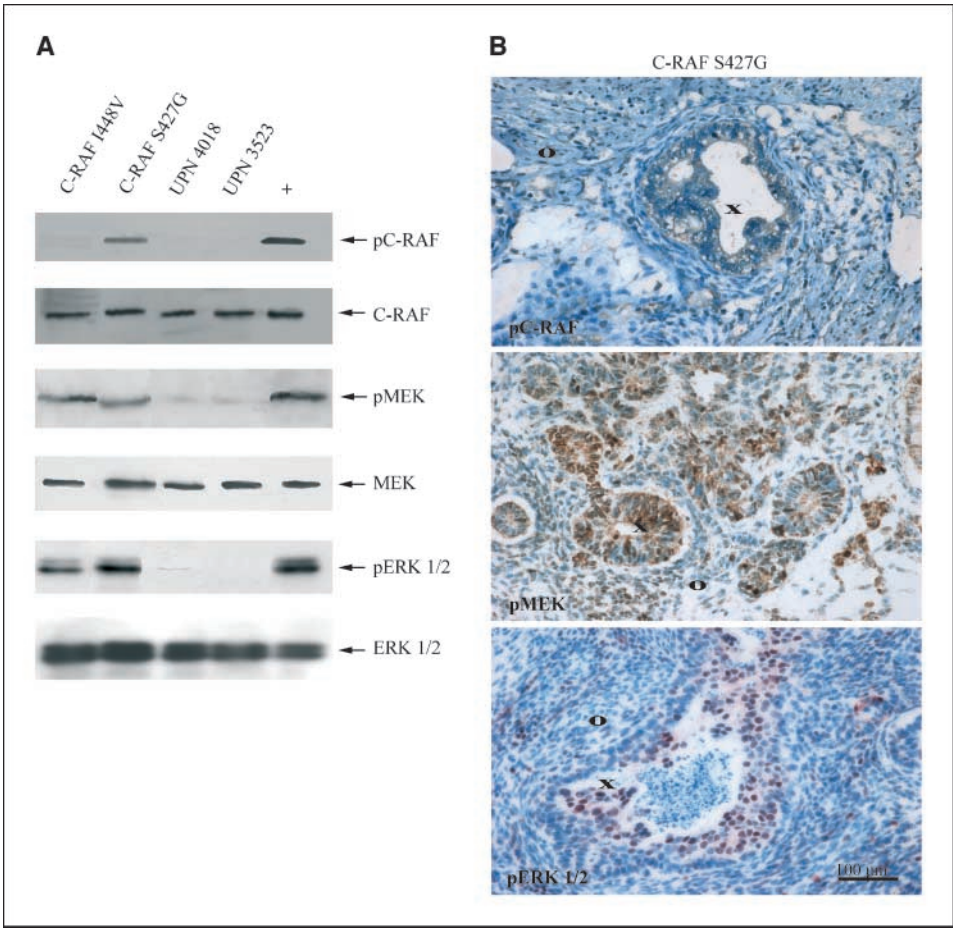

Figure 4. Activation of RAF-MEK-ERK pathway components in neoplastic tissues. *A,* lysates of two phospho-ERK–positive acute leukemia samples carrying I448V and S427G C-RAF mutations and of two phospho-ERK–negative leukemia samples were subjected to immunoblot analysis using phosphospecific ERK1/2, phosphospecific MEK, or phosphospecific C-RAF antibodies or total ERK1/2, MEK, or C-RAF antibodies. Fibroblasts that were serum stimulated for 5 minutes served as positive control. *B,* immunohistochemistry of the embryonal carcinoma with S427G mutation shows positive signals for indicated phosphospecific antibodies in tumor cells (**x**) but not in the surrounding stromal tissue (**o**).

wt B-RAF, we first did a transfection of ^{I448V}C-RAF with wt B-RAF together with His-tagged MEK into Cos-7 cells. ^{S427G}C-RAF was included in these experiments as positive control. Analysis of MEK activation using phosphorylation-specific antibodies failed to detect MEK activation in the case of ^{I448V}C-RAF, irrespective of whether B-RAF was present or not, thus making a possible activation of ERK via wt B-RAF implausible (Fig. 3*D*). To further prove these observations, we did kinase assays with endogenous B-RAF, immunoprecipitated from AML patient samples. The problems with this assay were similar to the ones described above for the analysis of C-RAF in tumor samples; however, the results obtained showed expression of B-RAF in primary tumor material but did not provide any hint for constitutive B-RAF activation. Hence, constitutive phosphorylation of ERK in I448V tumor samples as a consequence of B-RAF activation is an unlikely possibility.

Activated RAF-MEK-ERK pathway components in neoplastic tissues of S427G. Having shown the activating nature of the S427G C-RAF mutation, we further analyzed whether activation of ERK is accompanied by activation of the other components of the RAF-MEK-ERK pathway *in vivo*. For this, we used a phospho-specific MEK antibody and an antibody specific for phosphorylated S427G C-RAF at S338 (Fig. 4*A*). Phosphorylation of this site has been shown following activation of C-RAF by different stimuli (22). Activation of MEK and ERK was detected in acute leukemia samples carrying the I448V and S427G mutations, respectively. Other samples that did not show ERK activation, such as UPN 4018 and UPN 3523, were also negative for MEK phosphorylation. S338-phosphorylated C-RAF was only detected in the sample with the S427G mutation. Immunohistochemistry done on the primary tumor of the patient with the S427G mutation, an embryonal carcinoma of the testis, revealed, in concordance with the immunoblotting results of the leukemia sample, activation of all pathway components in tumor cells but not in the surrounding tissue (Fig. 4*B*).

C-RAF mutants display weak transforming potential. Transfection of NIH 3T3 cells with v-Raf and other highly transforming oncogenes results in the formation of foci composed of spindle shaped refractile cells (23). Similar attempts carried out with C-RAF mutants S427G and I448V failed to cause any gross morphologic abnormalities in these cells (data not shown). In a second set of experiments, NIH 3T3 cells were transiently transfected with pEF expression constructs for the mutant C-RAF proteins. Forty-eight hours later, cells were trypsinized and seeded in soft agar and monitored for colony growth. The retroviral v-Raf expression plasmid EHneo (24) served as positive control in these tests. Focus formation was consistently observed with both mutants. However, C-RAF mutant proteins caused the formation of much smaller cell aggregates (Fig. 5*A*) as compared with v-Raf. Identical data were obtained when pools of NIH 3T3 cells, stably transfected with the retroviral expression vector pBabe puro (25) carrying cDNAs for the C-RAF mutants, were subjected to soft agar cloning (data not shown).

Mutant C-RAF proteins protect against apoptotic cell death. One hallmark of the oncogenic potential of C-RAF is its ability to

delay apoptotic cell death (26). We thus tested the survival function of the mutant C-RAF proteins in NIH 3T3 cells stably expressing C-RAF mutants S427G or I448V. Cells transduced with the empty expression vector served as control. Subsequently, cells were treated with staurosporine, a condition under which survival activity for C-RAF had been shown before. Apoptosis was assessed by Annexin V staining. As shown in Fig. 5*B*, both C-RAF mutants provided protection under these conditions, suggesting that this may be one way through which the mutations contribute to tumor formation.

I448V is inherited in an Austrian family. As both mutations are of germ-line origin and thus may be hereditary, we did a pedigree analysis of both patients' families. Whereas the S427G substitution was shown to be a *de novo* mutation, inheritance over three generations could be shown for the I448V exchange. In this family, an additional mutation carrier (the patient's 74-year-old brother—case FII/6) suffers from prostate cancer. In generation FIII, ages 36 to 60 years, three of seven tested individuals and, in generation FIV, ages 5 to 31 years, one of two tested individuals showed the I448V mutation (Fig. 6). With the exception of case FIII/6, who died at the age of 3 months in 1954 (the cause of death could not be determined) and thus could not be analyzed, all tested and not tested individuals in generations FIII and FIV were asymptomatic at the moment this study was conducted. We also did immunohistochemistry using antibodies against phosphorylated ERK1/2 of the prostate carcinoma of case FII/6. ERK1/2 was phosphorylated in tumor tissue but unphosphorylated in the surrounding nonneoplastic tissue (data not shown).

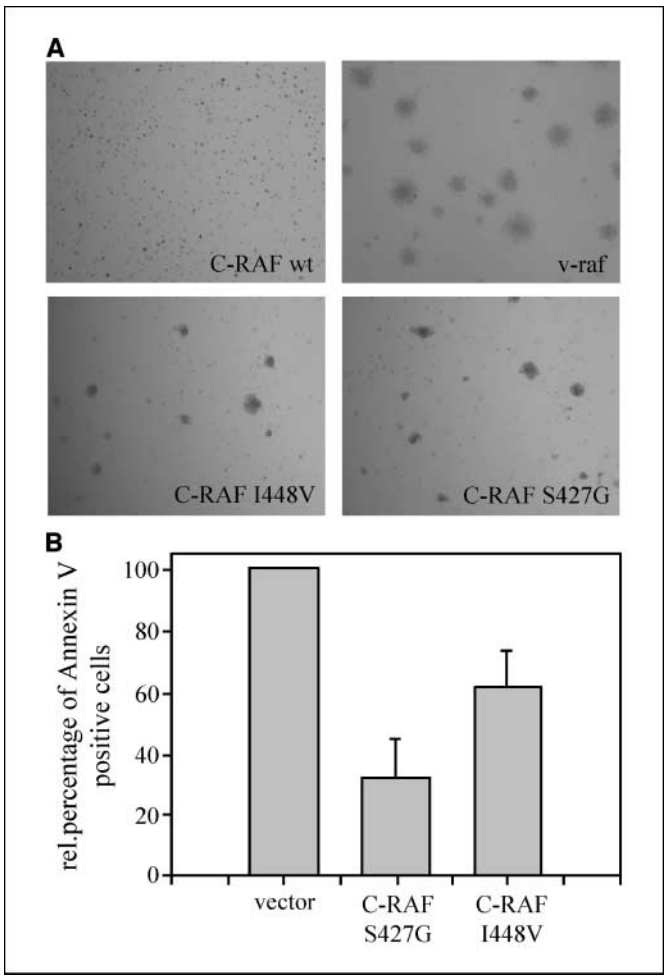

Figure 5. NIH 3T3 transforming and survival assays. *A,* NIH 3T3 cells were transiently transfected with expression constructs for wt and mutant C-RAF and 100,000 cells were plated in soft agar 48 hours later and monitored for growth. v-Raf-transformed cells served as positive control. Pictures were taken at day 28. *B,* NIH 3T3 cells containing either the mutant C-RAF proteins or an empty vector were treated with staurosporine, a common cytotoxic agent. Cell viability was assessed 8 hours later using Annexin V staining with apoptotic cells showing Annexin V positivity.

Discussion

Mutations in genes leading to activation of the RAF-MEK-ERK signaling cascade are critical in the development of malignant diseases. In 1983, the first of these mutations was discovered in *c-H-RAS*, the human homologue of the Harvey sarcoma virus oncogene, in a human bladder cancer cell line (27). Further studies also showed corresponding alterations in *K-RAS* and *N-RAS* (1); most of them result in pathway activation and cellular transformation (28, 29). In the case of *RAF* genes, *B-RAF* mutations, most of them transforming, can be observed in ~8% of human cancers (30, 31) whereas similar *C-RAF* mutations have not been reported yet. Nevertheless, the widely expressed C-RAF molecule is recognized to hold a central role in signal transduction, which is also supported by findings obtained in C-RAF deficient mice (32). Imbalance of RAF signaling by mutational events has recently been shown by Emuss et al. (30) who described four C-RAF mutations in human cancer cell lines, one of them (E478K) resulting in deregulated C-RAF kinase activity. In the study presented here, we analyzed the *B-RAF* and *C-RAF* genes in primary AML patient samples where constitutive activation of the RAF-MEK-ERK pathway is observed in the majority of cases (3).

We initially screened 82 diagnostic AML patient samples for their phosphorylation status of ERK. In accordance with previous studies (3), 55% exhibited constitutive phosphorylation of the ERK protein. No differences in ERK phosphorylation were observed between the different WHO subgroups, indicating that activation of the RAF-MEK-ERK pathway may be of equal importance for the development of all AML subtypes. Sequence analysis of the entire coding region of *B-RAF* and *C-RAF* of all ERK phosphorylated samples revealed two novel heterozygous single-base germ-line substitutions of the *C-RAF* proto-oncogene, S427G and I448V. Both are located within the highly conserved protein kinase domain of C-RAF and do not coincide with any of the previously described B-RAF mutations (33).

To gain insight into the effects of C-RAF mutations on RAF function, *in vitro* and *in vivo* kinase assays were done, which showed strong kinase activity towards MEK and ERK for ^{S427G}C-RAF whereas no significantly elevated activity could be shown for ^{I448V}C-RAF. These results prove that S427G C-RAF results in direct activation of the RAF-MEK-ERK pathway as a result of an elevated C-RAF kinase activity. They further suggest this position to be a yet unknown negative regulatory site of the C-RAF protein. However, the mechanisms underlying the ERK activation in neoplastic cells harboring I448V C-RAF currently remain enigmatic. Because we also failed to detect activation of B-RAF in cells expressing the I448V mutant of C-RAF, constitutive ERK phosphorylation in these malignancies could rather result from RAF-independent activation of MEK-ERK (15, 34, 35) or deregulation of phosphatases which normally counteract ERK activation (36). The former possibility is

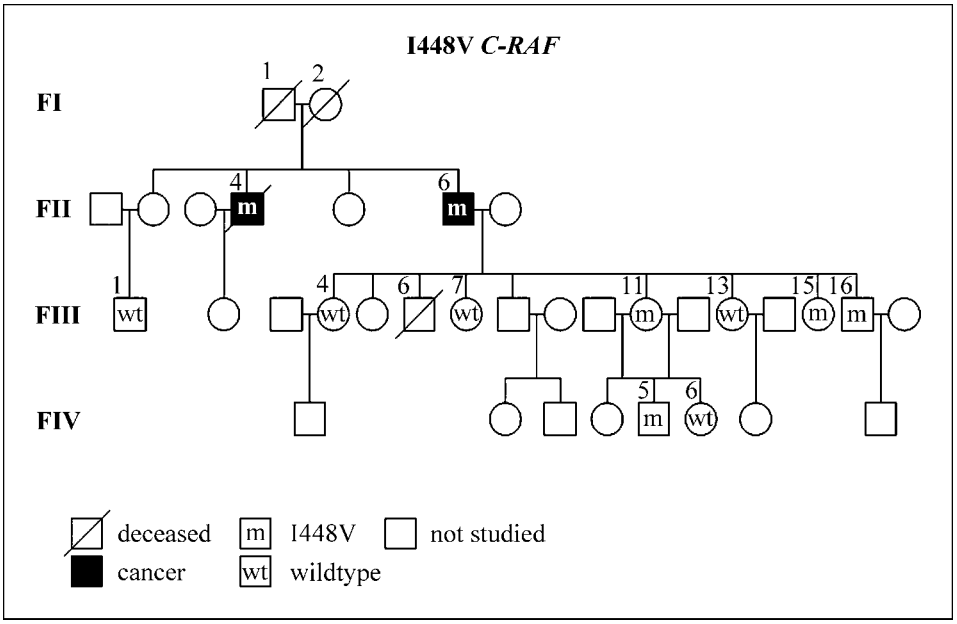

Figure 6. Pedigree of the Austrian family carrying the I448V *C-RAF* mutation. *Circles,* females; *squares,* males. The pedigree shows inheritance of this substitution over three generations with two mutation carriers suffering from cancer (cases FII/4 and FII/6).

supported by the presence of MEK, but absence of C-RAF, phosphorylation in the I448V positive primary tumors (Fig. 4).

To investigate the oncogenic potential of the C-RAF mutants, we did NIH 3T3 transforming assays. Contrary to the activating mutations in B-RAF (37), the mutations in C-RAF described here failed to induce morphologic alterations in NIH 3T3 cells. However, they were sufficient to sustain growth in soft agar, although much less efficiently than full-fledged oncogenes like v-Raf or v-Ras. This difference may also explain why these germ-line mutations can persist without affecting normal development or causing early-onset tumor development. However, they may render the carrier susceptible to further perturbations for full transformation.

Another hallmark of cancer genes is their ability to suppress apoptotic cell death. Using NIH 3T3 cells stably expressing mutant C-RAF proteins, we tested their antiapoptotic potential during staurosporine treatment. Both mutations significantly prolonged survival indicating an antiapoptotic potential. Taken together, these experiments show that the expression of either mutant, despite their difference with respect to MEK and ERK activation, results in weak oncogenic transformation as well as inhibition of apoptosis. These findings are striking given the previously established MEK requirement in transformation by C-RAF, as shown through the use of kinase-negative MEK mutants and MEK inhibitors (38, 39). Nevertheless, they further support more recent notions of MEK-independent C-RAF functions in cell survival or differentiation, processes also fundamental for cancer development (40, 41).

Finally, we focused on clinical aspects of these mutations. Both were detected in patients with t-AML (2 of 12; 17%), which occurs after chemotherapy and/or radiotherapy for a primary, and most often malignant, disease (42). This entity is characterized by nonrandom mutations in the *RAS, FLT3*, or *TP53* gene and typical cytogenetic abnormalities like monosomy 7/del(7q) and/or monosomy 5/del(5q) (43, 44). Especially *RAS* mutations have been shown to correlate with monosomy 7/del(7q) and therefore are thought to represent a subtle cytogenetic/molecular genetic pathway relevant to the pathogenesis of t-AML/therapy–related myelodysplastic syndrome (t-MDS; ref. 45). However, in these patients exhibiting a *C-RAF* mutation, we were unable to detect any *RAS* or *FLT3* alteration or chromosome 7 aberration. Nevertheless, as C-RAF is a direct effector of the *RAS* genes, assignment to this specific genetic pathway might be justified.

Of particular interest is the germ-line origin of these C-RAF mutations. By screening 200 healthy individuals, we were able to exclude S427G and I448V being common polymorphisms. By screening the patients' families, we were able to show the S427G substitution to be a *de novo* mutation whereas inheritance over three generations could be shown for the S427G mutation. In this family, an additional mutation carrier suffers from prostate cancer but all other tested and not tested individuals were asymptomatic when this study was conducted. Both affected mutation carriers in this family were of ages ~70 years when they were diagnosed with cancer. The advanced age of these cancer patients at disease manifestation also suggests that additional somatic mutations in other genes are necessary for the development of the malignant phenotype. The fact that C-RAF mutants do not act as full-fledged oncogenes has already been observed before (30, 46) and attributed to a lower basal kinase activity of C-RAF as compared with B-RAF (30). Hence, it has been suggested that C-RAF mutations may rather constitute predisposing factors to certain forms of neoplastic diseases (30). Our observation that only neoplastic tissue, but not the surrounding normal tissue, exhibits constitutive activation of the RAF-MEK-ERK pathway further supports this hypothesis (Fig. 4*B*). Another aspect of a potential predisposing role of both C-RAF mutants may be their occurrence in patients with t-AML. There is increasing evidence that the development of t-MDS/t-AML is due to a genetic predisposition rather than a drug-dosage effect (47). Children with neurofibromatosis type 1 are at increased risk for therapy-related neoplasms, including myeloid leukemias (48), and neurofibromatosis type 1 mutant mice treated with cyclophosphamide and radiation developed significantly more neoplasms than the wt group (49, 50). Interestingly, biochemical investigation of cell lines developed from these malignancies revealed deregulated Ras signaling (50).

In conclusion, these data relate mutations of the *C-RAF* proto-oncogene to human neoplasias. Considering their germ-line origin and biological properties, the S427G and I448V C-RAF mutations might constitute a novel tumor-predisposing factor.

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