K-ras Asp12 mutant neither interacts with Raf, nor signals through Erk and is less tumorigenic than K-ras Val12

Introduction

The ras genes code for proteins that transduce signals through different effectors (e.g. Raf, PI3K, Raf-GDS, Ras-GAP), which regulate diverse cell functions (1). They become oncogenic by single point mutations, mainly at codons 12 or 13 (2), which lead to constitutive signaling and cell transformation associated with changes in morphology, increased proliferation and/or inhibition of apoptosis (3).

Using K-ras, the most clinically relevant ras gene, to transfec NIH3T3 cells, we have demonstrated previously the differences in predisposition to apoptosis and signaling through the Ras downstream antiapoptotic pathway PI3K/Akt between codon 12 and codon 13 mutants (4). We have also demonstrated that these differences were maintained when the transformants were implanted in vivo, producing sarcomas of different type and aggressiveness (5). These experiments were performed on the basis of previous reports describing different transformation capacities for ras genes bearing codon 12 or codon 13 mutations in vitro (6,7) and different aggressiveness of human tumors bearing K-Ras codon 12 or codon 13 mutations (8).

There is also evidence suggesting that the malignant potential of tumor cells may be influenced, not only by the mutated codon in the ras gene but by different alterations within single codons. Thus, distinct mutant amino acids in the Ras proteins lead to different capacities for foci formation in cultured cells: H-Ras Val12 being highly transforming, whereas K-Ras Asp12 (K12D) being only moderately transforming (7,9). K-Ras mutants also associate with different aggressiveness in human tumors. Thus, in aberrant crypt foci of human colon, K12D is as frequent as K-Ras Val12 (K12V), however, only the latter predominates in adenocarcinomas (10). Moreover, K12V is more prevalent in primary and metastatic deposits of Dukes’ C/D than in primary Dukes A/B colorectal carcinomas, whereas K12D does not associate with metastatic capacity (11,12). In addition, K12V increases the risk of tumor recurrence and death in colorectal carcinomas and is an independent predictor of decreased overall survival (13). In contrast, K12D is not associated with alteration of any of these parameters. Finally, in lung adenocarcinomas K12V confers a poorer prognosis than K12D (14).

Here, we tested, in vitro and in vivo, whether different human K-Ras oncoproteins mutated at codon 12 to Val, Asp or Cys would confer NIH3T3 fibroblasts distinct oncogenic phenotypes. We studied tumor histology and growth, apoptotic and mitotic rates, activation of signal transduction pathways downstream of Ras and regulation of the cell cycle and apoptotic proteins in tumors derived from the implanted transformants. We found that the K12V oncogene induces a more aggressive tumorigenic phenotype than the K12D oncogene, whereas K12C does not induce tumors in this model. Thus, K12V mutant tumors proliferate about seven times faster, and have higher cellularity and mitotic rates than the K12D mutant tumors. A molecular analysis of the induced tumors shows that the K12V mutant protein interacts with Raf-1 and transduces signals mainly through the Erk pathway. Unexpectedly, in tumors induced by the K12D oncogene, the K-Ras mutant protein does not interact with Raf-1 nor activates the Erk canonical pathway. Instead, it transduces signals through the PI3K/Akt, JNK, p38 and FAK pathways. Finally, the higher growth rate of the K12V tumors associates with changes in morphology, consistent with faster G1/S and G2/M phase transitions, without alteration of apoptotic regulation.

**Abbreviations:** Asp12, codon 12 mutation to aspartic acid; Val12, codon 12 mutation to valine; Cys12, codon 12 mutation to cysteine; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; IPTG, isopropyl-β-D-thiogalactopyranoside.
We found, in vitro, before their injection in mice, an increase in proliferation of single unattached cells for the K12V or Asp12 transfectants, as compared with K12C cells. In vivo, whereas K12C did not induce tumors, the K12V mutant yielded tumors with significantly higher growth and mitotic rates and higher cellularity than the K12D mutant. These functional differences associate with differences in the K-Ras interaction with its effectors and in downstream signaling. Thus, the K12V mutant interacts with Raf-1 and transduces signals mainly through the Erk pathway. In contrast, the K12D mutant, unexpectedly, does not interact with Raf-1 nor transduces signals through the canonical Erk pathway, but through the PI3K/Akt, JNK, p38 and FAK pathways, leading to less aggressive tumors. Moreover, K12V tumors show enhanced phosphorylation of the Rb protein and upregulation of PCNA and cyclin B.

Materials and methods

Reagents

NIH3T3 cells were obtained from the American Type Culture Collection. Dulbecco’s MEM (25 mM HEPES), FBS, glutamine, fungizone, penicillin/ streptomycin, ampicillin, acrylamide and bis-acrylamide were obtained from Life Technologies, Hoescht, Trizo–HCl, Triton X-100, SDS, glycero, benzamidone, phenylmethylsulfonyl fluoride, leupeptin, sucrose, sodium acetate, sodium chloride, EDTA, EGTA, bromphenol blue, DT, gllycine, sodium β-glycerophosphate, sodium fluoride and PP, were obtained from Sigma. Gentamicin sulfate was obtained from BioWhittaker and neomycin (geneticin) from Life Technologies. APS, TEMED and 2-β-mercaptoethanol were from Fluka. Sodium orthovanadate was from Panreac. Molecular weight markers were from Bio-Rad.

Generation of the transfectants

We used plasmids containing a K-ras minigene with a mutation to Val, Cys, Asp at the first position of codon 12. Plasmids were a gift from Dr Manuel Perucho of the Burham Institute at La Jolla, CA, and were derived from pBR and contain a neo-resistant selectable gene under the thymidine kinase gene promoter and an ampicillin resistance gene. The K-ras minigene contains all four coding exons (4B is the fourth exon) separated by intronic regions, under the promoter of K-ras, and a polyadenilation signal. Cells were stably transfected, using lipofectamine (Invitrogen) and selected and maintained as described previously (4).

Characterization of the transfectants in vitro

The different transfectants were analyzed for morphological appearance, characterizing shape, refringency and ability to grow in colonies, unattached to less aggressive tumors. Moreover, K12V tumors show enhanced phosphorylation of the Rb protein and upregulation of PCNA and cyclin B.

Evaluation of tumorigenicity in vivo

Animal experiments were performed according to the requirements of the Animal Committee of the Institut de Recerca de l’Hospital de Sant Pau, Four-week-old male Nu/Nu Swiss mice, weighing 18–20 g (Charles River), were used to evaluate the tumorigenicity of the transfectants. Mice were housed in a sterile environment; cages, water and bedding were autoclaved and food was γ-ray sterilized.

Nine animals per transfectant were injected intramuscularly in the posterior right back with 100 µl of DMEM containing 105 cells of each studied clone (constituting four different groups: empty vector 3F3wt, Asp12, Cys12 and Val12). Tumor volume was measured with a caliper using the following formula: volume = length × width2/2 every week over a period of 25 days. Animals that did not develop macroscopically apparent tumors were also killed and samples of the tissue at the injection site were taken for histological analysis. Mice were killed when tumors became too large, if animals lost more than 10% of their body weight, or if they showed signs of pain during the experiments. Tumor fragments were frozen in liquid nitrogen for molecular analysis or fixed on formalin for histopathological analysis.

Histological analysis and tumor growth

We evaluated cell morphology and histology of tumor sections of all generated tumors, after H&E staining of formalin-fixed tissue. Moreover, we evaluated tumor growth by measuring tumor volume twice a week, starting when tumors were visible and until the end of the fourth week after implantation. We also performed, at the end of the experiment, a mitotic count in 10 consecutive high-power fields, in tumor sections, to compare the mitotic index between the groups. The presence of apoptotic cells within the macroscopically viable tumor tissue was also assessed staining with Hoescht (Roche). We also counted the apoptotic figures in 10 consecutive high-power fields in five different tumor samples per group. The Hoescht and immunohistochemistry (IHC) analyses were performed on formalin-fixed paraffin-embedded tumor tissue. Tissue sections (5 µm) were dewaxed in xylene, dehydrated and rinsed three times with phosphate-buffered saline (PBS). To perform the nuclear staining with Hoescht, sections were permeabilized, incubated with 0.5% Triton X-100 in PBS pH 7.4 for 5 min at room temperature, then rinsed again twice in PBS. Finally, cells were stained with Hoescht (1 : 5000 in PBS) for 1h and washed with water. Sections were mounted and observed under a fluorescent microscope at 334 nm absorption and 365 nm emission light.

Immunohistochemical analysis of tumors

We studied molecular markers for mesenchymal or epithelial cells by IHC. To carry out the IHC analysis, we deparaffinized and hydrated tumor sections, immersed them in an aqueous solution, containing 10 mM citric acid/0.1 mM Na citrate pH 6.0 and heated them for 10 min to unmask antigenic epitopes. Sections were quenched for endogenous peroxidase, incubated with 3% hydrogen peroxide for 10 min, and subsequently incubated with primary antibodies anti-vimentin (Abcam), cytokeratins AE1/3 (DAKO), cyclin D1 (Lab vision) or p53 (Abcam) for 30 min. Samples were finally developed using the DAKO cytomation EnVision Kit (DAKO), according to the manufacturer and co-stained with hematoxylin. We also studied the possible oncogenic activation by upregulation of cyclin D1 or the possible inactivation of the tumor suppressor p53, leading to its overexpression, by western blot, as secondary hits during tumorigenesis.

Analysis of Ras interaction with effectors and downstream signaling

We studied the differential activation of the pathways downstream of two of the effectors of Ras, Raf/Erk and PI3K/Akt, in K12V and K12D tumors, determining the amount of active K-Ras bound to them and the activation of signal transduction pathways downstream of the K-Ras protein.

Ras pull-down analysis

We used GST-Ras binding domains (RBD) of Raf-1 and PI3K (GST-Raf-1 and GST-PI3K), to measure the active (GTP-bound) K-Ras protein that interacted with these effectors in tumor lysates. Plasmids containing pGEX-PI3K or pGEX-Raf-1 (Piero Crespo gift; CSIC, Spain) were transfected into strain and incubated with the beads for 2 h with gentle mixing. The beads were, then, washed five times with 400 µl of PBS. Cell pellets were lysed by sonication on ice for 6 × 15 s and separated by 60 s intervals. Cell debris was pelleted at 14 000 g of the purified GST-Raf-1 or GST-PI3K. The bead pellet and supernatant were resolved by SDS–PAGE gel and a BSA standard curve, and visualized by Coomassie brilliant blue R250 staining.

Following, we incubated ~20 µg of the purified GST-Raf-1 or GST-PI3K proteins, immobilized on glutathione-agarose beads, with the 0.1 ml of tumor extract supernatants at 4°C for 2 h with gentle mixing. The beads were, then, washed five times with 400 µl of lysis buffer and centrifuged (2000 r.p.m., 1 s at 4°C). The bead pellet and supernatant were resolved by SDS–PAGE. Equivalent amounts of the fusion protein or GST alone were used. Resolved proteins were electrotransferred onto nitrocellulose membranes. Membranes were blocked with TBS-T buffer and nonfat milk, and shaken at RT for 1.5 h, incubated with K-Ras antibody for 1 h and, then, with the corresponding secondary antibody (1 h) at 1 : 20 000 dilution. Protein bands were detected by chemiluminescence using Supersignal (Pierce).

Quantitation of K-Ras downstream protein expression or activation

Western blots for the assessment of protein expression of K-Ras, or Ras downstream protein expression or activation by upregulation of cyclin D1 or the possible inactivation of the tumor suppressor p53, leading to its overexpression, by western blot, as secondary hits during tumorigenesis.

2191
phosphatase and kinase inhibitors, as described previously (4). The amount of protein was quantitated by the Bradford method using the Bio-Rad protein assay dye. 7.5% or 15% PAGE was performed. Samples were denatured at 100°C for 3 min, and after loading 75 μg of total protein, electrophoresis were run at 30–40 mAmp in Laemml buffer with molecular weight markers.

After the electrophoresis, samples were transferred at 200 mAmp overnight to nitrocellulose membranes. To control for protein loading, membranes were incubated for 10 min in 2 g/l PonceauS (Sigma) in 3% acetic acid and rinsed with water. Afterwards, they were blocked in TBS-T buffer, containing 5 g/100 ml of nonfat milk, and shaken at room temp for 1.5 h. Membranes were then, incubated with the corresponding primary antibody at the indicated dilution (in TBS-T buffer with 1 g/liter BSA), shaken for 1 h at RT and then with the corresponding secondary antibody at the indicated dilution. Dilutions for primary antibodies were as follows: K-Ras (Calbiochem), 1 : 2000; phospho-Erk1 and Erk2 (Promega), 1 : 20 000; PI3K (Santa Cruz), 1 : 5000; phospho-p38 (Santa Cruz), 1 : 5000; PCNA (Transduction Laboratories), 1 : 6000; phospho-JNK1 and JNK2 (Promega), 1 : 10 000; phospho-Stat3 (Promega), 1 : 20 000; FAK (Santa Cruz), 1 : 400; p53 (Calbiochem), 1 : 7000; Caspase 3 (Pharmigen), 1 : 8000; Src (Biosource), p-Stat3 (Santa Cruz), cyclin D1 (Upstate Cell Signaling), cyclin E (Pharmin- gen); cyclin B1 (Santa Cruz), 1 : 7000 and c-Myc (Pharmin- gen), β-actin (Santa Cruz) at 1 : 2000; phospho-Erk1 and Erk2 (Promega), 1 : 20 000; PI3K (Santa Cruz), 1 : 5000; phospho-p38 (Santa Cruz), 1 : 5000; PCNA (Transduction Laboratories), 1 : 6000; phospho-JNK1 and JNK2 (Promega), 1 : 10 000; phospho-Stat3 (Promega), 1 : 20 000. Protein bands were detected by chemiluminescence using Supersignal (Pierce).

The chemiluminescent bands corresponding to expression or phosphorylated forms of the different proteins were acquired using a Kodak 440 Digital Science Image Station. Quantitation and analysis of the bands were performed using the Kodak ID Image Analysis Software.

Statistical methods
Differences in tumor volume and number of mitotic or apoptotic figures between groups (K12V and K12D) were analyzed using the Mann–Whitney test. The mean intensities of the chemiluminescent bands, after western blot, were also compared using the same test. Differences were considered significant at a P < 0.05.

Results
We tested the hypothesis that the molecular nature of K-Ras codon 12 mutations would confer tumors bearing them distinct transforming phenotypes. We used NIH3T3 fibroblasts as the recipient cell, because it is a reliable model to study transformation by ras and other oncogenes (15). We selected stable transfectants, expressing constructs with K12V, K12D or K12C mutations and performed an evaluation of some of the functional and molecular alterations associated with their transformation in vitro and tumorigenesis in vivo.

Differences in morphology and growth among K-Ras codon 12 transformants in culture
We first characterized in vitro the morphology and duplication time of all transformants. K12V and K12D mutant cells in culture behaved in a similar manner, and differently from Cys12. Thus, control NIH3T3 cells had a fusiform and flattened morphology and grew attached to the plate without colony formation. K12G cells grew in groups, with higher cell densities than control cells, and rapidly formed big and dense colonies, which spontaneously detached from the plate, forming spheroids. No single suspended cells were identified in these transfectants (Figure 1A). In contrast, K12V and K12D transformant cells showed a similar morphology; being more scattered, rounded and refringent than K12C cells (Figure 1A); moreover, a high proportion of them were able to grow as single cells without attachment. In addition, the doubling times for K12V (15.4 ± 0.1 h) or K12D (15.1 ± 0.2 h) transformants were significantly shorter than for K12C (20.6 ± 0.3 h) transformants (Figure 1B).

We did not find any difference among transformants in cell death or apoptosis. Thus, there were no signs of cell death, nuclear condensation or fragmentation, in the K12V, K12D or K12C transformants, when cultured under the same conditions (data not shown).

Tumorigenesis of K12V and K12D transfectants
First of all, we selected clones for each transfectant that expressed the exogenous mutant K-Ras at a similar level
before their injection in mice. This way, we ensured that the different tumor phenotypes were due to the effect of their particular mutation, rather than to a variable level of expression.

Only injection of K12V and K12D transfectants resulted in tumor development in nude mice. The generation of tumors, from the initial inoculus and until reaching a macroscopically detectable tumor size, was faster in K12V than in K12D transformants.

At the end of the first week, 6 out of 9 K12V implants had already generated visible tumors and by the end of the second week all 9 implants had generated tumors. In contrast, only 2 out of 9 K12D implants by the end of the first week and 4 out of 9 at the end of the second week generated tumors (Figure 2A). Thus, the appearance of K12D tumors always lagged behind K12V tumors. No tumors were generated in any of the mice injected with the NIH3T3 cells transfected with the empty vector or with the K12C mutant construct, after macroscopic or microscopic inspection of the implanted area at the end of the study period. Moreover, K12V tumors grew significantly faster, and had higher final tumor volumes, than K12D tumors.

Table I. Growth, mitotic and apoptotic rates in tumors derived from K12V and K12D transfectants

<table>
<thead>
<tr>
<th>Transfectant-derived tumors</th>
<th>K-Ras Val12 (n = 9)</th>
<th>K-Ras Asp12 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean growth rate (mm³/day)</td>
<td>30.4 ± 2.1 a⁺</td>
<td>4.3 ± 2.2 a⁺</td>
</tr>
<tr>
<td>Mitotic rateᵇ</td>
<td>30.3 ± 3.4 a⁺</td>
<td>11.3 ± 3.1 a⁺</td>
</tr>
<tr>
<td>Apoptotic rateᵇ</td>
<td>6.9 ± 1.3</td>
<td>5.2 ± 0.9</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE.

a⁺Statistically significant differences at a P < 0.01 (Mann–Whitney U test).

bAverage count of 10 independent 10⁰ fields per tumor.

In addition, K12V tumors had also a significantly higher (P < 0.001) mitotic rate (30.1 ± 3.4), per 10 consecutive high-power microscopic fields, than K12D tumors (11.3 ± 3.1) (Figure 3A and B, Table I). A low number of apoptotic figures per 10 consecutive high-power microscopic fields were recorded in K12V (6.9 ± 1.3) or K12D (5.2 ± 0.9) tumors; these differences were not statistically significant (Figure 3E and F, Table I). Thus, despite depicting the same histological appearance, K12V tumors appear to be significantly more aggressive than K12D tumors, mainly because of significantly higher proliferative and mitotic rates, which could lead to a higher cellularity.

IHC analysis for epithelial and mesenchymal markers, showed that virtually all tumor cells in K12V (Figure 4B) or K12D (Figure 4C) tumors showed a strong immunoreactivity to vimentin and a lack of cytokeratin AE1/AE3 expression in K12V (Figure 4E) or K12D (Figure 4F) tumors.

Regulation of the K-Ras mutant proteins and interaction with their effectors

We assessed the level of K-Ras protein expression, the interaction of active K-Ras with two of its effectors, and the activation of the signal transduction pathways downstream of K-Ras in the established KV12 and KD12 tumors. Secondary mutations in p53 or cyclin D1 were not found in tumors. Thus, cyclin D1 or p53 expression levels by western blot were similar in cultured K12V and K12D cells, and in tumors derived from them (Figure 5A), and there were no significant differences in the expression of these proteins between tumors of these two groups (Figure 6B and Table II). We also confirmed, by IHC, the lack of variation...
in the expression of cyclin D1 and the lack of immunoreactivity to p53 in both mutant tumors (data not shown).

Nevertheless, despite K-Ras expression among K12V and K12D transfecants, before their injection to the animals, was similar (Figure 5B), once tumors appeared K-Ras expression was significantly increased in K12D as compared with K12V tumors (Figure 5C and Table II); in the latter the K-Ras protein level was similar to that observed in vitro (Figure 5B). Thus, transfecant cells expressing the K12D mutant required an increase in their level of K-Ras protein expression to become established tumors.

Next, we performed Ras pull-down experiments to determine the levels of active K-Ras bound to effectors (Figure 5D). The Ras binding domain of PI3K (GST-PI3K) pulled down active K-Ras from K12V (V12)-derived tumors (Figure 5D). Thus, whereas the K12V mutant protein interacts with Raf-1 and PI3K, the K12D mutant protein interacts with PI3K, but it does not interact with Raf-1.

Analysis of the activation of the signaling pathways downstream of K-Ras

Next, we wanted to explore whether the differences in the interaction of K-Ras with its effectors were translated into a differential activation of the pathways that transduce signals from them. We considered the existence of differences in protein expression or activation between K12V and K12D-derived tumors only when the mean of the quantitated bands reached statistical significance (Table II). Thus, consistently with its interaction with Raf-1, tumors expressing the K12V mutant protein showed a high activation (phosphorylation) of the Erk1 and Erk2 proteins (Figure 6A and Table II). On the other hand, most of the K12D tumors lacked any activation of the Erk1 or Erk2 proteins (Figure 6A).

In contrast, and consistent with its interaction with PI3K and its lack of interaction with Raf-1, K12D strongly activated Akt and did not activate the Erk pathway. Moreover, the interaction of the K12V mutant with PI3K, which we observed, did not lead to the activation of Akt, as this protein was not phosphorylated in those tumors. In addition to the PI3K upregulation and Akt activation, the K12D mutant also activated the JNK, p38, Stat-3 and FAK pathways and upregulated Src and c-Myc (Figure 6A and Table II). Moreover, there was a strong correlation between the activation of the p38, JNK pathways and c-Myc upregulation in K12D tumors (Figure 6A). In contrast, none of these pathways or proteins were activated or upregulated in K12V tumors (Figure 6A). Therefore, our results indicate that K12V tumors transduce signals mainly through the canonical Erk pathway; whereas multiple pathways, alternative to the canonical Erk pathway, maintain tumorigenesis in K12D tumors.

Analysis of cell cycle and apoptotic regulatory proteins

The established connections among Ras downstream pathways and cell cycle regulatory proteins prompted us to evaluate whether the significantly different pathway activation associated with the two K-Ras mutant proteins was also translated into differences in cell cycle or apoptotic regulation. We observed that the K12V tumors, which show much higher growth and mitotic rates, had an enhanced phosphorylation of pRb, and an increase in PCNA and cyclin B1 expression (Figure 6B and Table II), which could explain their higher proliferative activity and faster G1/S and G2/M transitions. In contrast, cyclin E was significantly upregulated in K12D tumors, as compared with K12D tumors (Figure 6B and Table II). Finally, there were no differences in the levels of cyclin D1 expression between K12V and K12D tumors (Figure 6B). No changes in apoptotic regulators were observed either. Thus, there was a similarly low level of procaspase 3 or 9 proteolysis in the studied tumors from both groups (Figure 6C and Table II), consistent with the similarly low level of apoptosis observed with Hoescht staining (Figure 3E and F, Table I).
Fig. 4. High expression of vimentin, and the absence of cytokeratin expression, in K12V or K12D tumors by IHC. (A) Cytoplasmatic immunostaining for vimentin of muscular cells in the normal mouse bowel (positive control). Virtually all tumor cells in K12V (B) or K12D (C) tumors showed a strong immunoreactivity to vimentin. (D) Crypts of the mouse colonic epithelium showing cytoplasmatic immunoreactivity to cytokeratin AE1/AE3 (positive control). Lack of cytokeratin AE1/AE3 expression in K12V (E) or K12D (F) tumors.

Fig. 5. Comparison of cyclin D1, p53 and K-Ras expression between K12V or K12D cultured cells and established tumors, and K-Ras binding to effectors in tumors. (A) Cyclin D1 was not upregulated in tumors (1, 2, 10 and 11) as compared with K12V or K12D transformant cells; p53 levels were similarly low in cultured K12V or K12D cells and derived tumors (control HCT116 cells, mutant for p53, and show p53 overexpression). (B) K-Ras expression was similar in K12V and K12D cultured transformants before its injection. (C) Established tumors (samples 10–18) derived from K12D transformants expressed significantly higher levels of the K-Ras protein than K12V tumors (samples 1–9) (see Table II for quantitation). (C) In K12D tumors the active K-Ras protein is pulled down with Ras binding domain of PI3K (GST-PI3K), but is not with the Ras binding domain of Raf-1 (GST-Raf-1), whereas in K12V tumors both GST-PI3K and GST-Raf-1 pull down active K-Ras. V12 or D12 = pull-down extracts; Sp-V12 or Sp-D12 = supernatants obtained after pull-down; C-V12 or C-D12 = extracts that did not undergo pull down.
Discussion

We have demonstrated in an in vivo model that the K12V oncogene induces a more aggressive tumor phenotype than the K12D oncogene. We found differences in tumor growth, cellularity and mitotic rates, as well as in the interaction of the respective K-Ras mutant proteins with Ras effectors, activation of downstream pathways and deregulation of cell cycle regulatory proteins.

The K12V oncogene induces more aggressive tumors than K12D

The differences in in vivo tumorigenic capacity between K12V and K12D transformants were not obvious in vitro. Thus, K12V or K12D cultured transformants had a similar behavior, and appear to be more aggressive than K12C. K12V and K12D cells look alike, being more rounded and refringent, showing a higher level of cell dispersion, and having a higher proliferation rate than K12C transformants. In contrast, K12C transformants were able to grow in suspension, forming spheroids and maintaining the cell–cell contacts. Thus, it appears that K12C transformants are significantly more dependent on cell–cell contacts for growth than K12D or K12V.

In vivo, K12V and K12D tumors were mesenchymal malignancies, both expressing vimentin, but not cytokeratins; K12V tumors being more aggressive than K12D tumors. Thus, their latency for tumor appearance was significantly shorter and their mean growth rate was about seven times higher in
K12V than in K12D tumors. In addition, this enhanced growth was associated with a higher mitotic rate and a higher cellularity in K12V tumors, two markers for higher grade and poor prognosis in soft tissue sarcomas (16). Moreover, despite the fact that both, K12V and K12D, cultured transfectant cells displayed a similar level of K-Ras expression before its inoculation to mice, K12D established tumors showed significantly higher K-Ras expression than K12V tumors. This is consistent with the K12D mutant being less tumorigenic than the K12V mutant, similar to our previous observation that weak transformants (e.g. K-Ras codon 13 mutation) need to build up their level of expression before being capable of inducing tumorigenesis in vivo (5).

On the other hand, we could not study the aggressiveness of the K12C transfectants because they did not form tumors. Whereas we did not expect the 3T3 cells transfected with the empty vector to be tumorigenic, the lack of tumor formation by the K12C transfectants was unexpected. We attribute this finding to the influence of local growth factors and stroma cells in tumorigenicity, as here we injected K12C cells in the back skeletal muscle, whereas in a previous experiment injecting the same transfectant cells, subcutaneously, we were able to generate tumors (5).

**K12D does not interact with Raf-1 nor activates the Erk pathway, whereas K12V does**

In addition to the described differences in K-Ras expression levels, K12V and K12D tumors showed dramatic differences in the interaction of their respective K-Ras mutant proteins with their effectors and in the activation of signal transduction pathways downstream of K-Ras. Thus, whereas the K12V mutant interacts with the Ras binding domain of both Raf-1 (GST-Raf-1) and PI3K (GST-PI3K), the K12D mutant interacts with PI3K, but not with Raf-1. This is consistent with previous observations that mutant amino acids at codon 12 induce structural changes in the effector domain of the Ras protein (17), which, in turn, alter the affinity of the interaction of the Switch I for Raf-1 (18) or the Switches I and II for PI3K (19). Specifically, Ras Asp12 substantially differs in the arrangement of Gln-61, Tyr-32 and Pro-34, as compared with Ras Val12 or Ras wild-type, significantly changing its affinity for Raf-1 (20).

These altered interactions may lead to differential downstream signaling and regulation of K-Ras transforming functions. Thus, consistent with its interaction with Raf-1, the K12V mutant activates the Erk pathway; however, its interaction with PI3K does not lead to the activation of its effector Akt (see Figures 5 and 6). Nevertheless, we cannot exclude that other pathways downstream of PI3K may be active. In contrast, and consistent with its interaction with PI3K and its lack of interaction with Raf-1, K12D strongly activates Akt whereas it does not activate the Erk pathway. Moreover, in addition to the PI3K/Akt pathway, the K12D mutant activates additional proteins such as JNK, p38, Stat-3 and FAK.

Thus, the higher aggressiveness we observed in the K12V tumors may be due to the fact that the K-Ras Val12 mutant activates mainly the Erk pathway, which is capable on its own of inducing transformation (21). In contrast, K-Ras Asp12 tumors may be less aggressive because the pathways this mutant protein activates, PI3K/Akt, JNK or p38, are not able on their own of transforming 3T3 cells (22,23) but need their cooperative action (24). Thus, K12D transformation may be accomplished through the cooperation of pathways downstream of Ras, which do not involve Raf-1 or Erk (24,25). This conclusion is also in agreement with the findings that transgenic (26) or ‘knock in’ (27) mice, expressing the K12V mutant, activate the Erk, but not the Akt, pathway and yield lung and intestinal carcinomas, whereas K12D ‘knock in’ mice activate the Akt, but not the Erk, pathway and yield only lung and intestinal hyperplasias, rather than carcinomas (28).

The lack of Erk activation in K12D tumors was unexpected, being an exception to the requirement of the canonical Raf/Erk pathway activation for tumorigenesis by Ras (29,30). We think the conclusion of such a requirement was drawn using mainly the H-ras Val12 oncogene; however, our results suggest that it may not apply to other Ras oncogenes, such as the K12D mutant.

Therefore, K12V and K12D mutants appear to use two completely different pathway activation patterns for cell growth and tumor formation.
transformation. Thus, in K12D tumors the concomitant activation of the PI3K/Akt, JNK, p38 and FAK pathways, occurs in the absence of Erk activation. In contrast, K12V tumors activate the Erk pathway, but none of the other pathways evaluated. These findings are consistent with previous reports demonstrating that the activation of the Erk pathway, together with JNK and p38, in a single tumor is unlikely to occur, since they show crossed downregulation. Thus, p38 (31) or JNK (32) activation repress Erk activation; whereas, activated Erk downregulates the JNK and p38 pathways (33). Similarly, the Ras effector MEKK (34) is known to activate p38 and Jnk pathways, but not Erk (35,36). In addition, Src transformation requires activation of Jnk and p38 (37) and inhibits Erk (38). Moreover, the strong correlation we observed in the activation of the p38 and JNK in K12D tumors suggest that these pathways could be activated by the same effector of Ras in these tumors; nevertheless, this aspect requires its direct evaluation.

K12V and K12D mutants differentially activate cell cycle regulatory proteins

We analyzed the cleavage of caspase 3 and caspase 9 in tumor tissue. Consistently with the low level of chromatin condensation and fragmentation we reported with Hoescht staining, there was also a low level of procaspase 3 or 9 activation in all studied tumors from both groups. Thus, differences in proliferation rather than in apoptosis should account for the tumor growth differences between groups.

Consistent with the dramatic increases in growth and mitotic rate in K12V, as compared with K12D tumors, we expected that the differential patterns of pathway activation between the two K-Ras mutant tumors led to a distinct deregulation of some of the proteins controlling the $G_1/S$ and $G_2/M$ cell cycle phases.

Regarding the $G_1/S$ transition both mutants induced a similar expression of cyclin D1; the K12V may do this by signaling through the Erk pathway, whereas K12D may use at least the PI3K/Akt, JNK and p38 pathways to do so. Thus, the RAF/Erk pathway upregulates cyclin D1 (39). On the other hand, the PI3K/AKT pathway also upregulates cyclin D1 (40), and in Src transformed cells, JNK and p38 coactivation upregulates cyclin D1 and induces the $G_1/S$ transition (37).

Because the activation of cyclin D-dependent kinases is responsible for Rb phosphorylation and inducing the $G_1/S$ transition (37), the higher levels of Rb phosphorylation we observed in K12V, as compared with K12D, tumors suggest that Erk activation is more efficient than the coactivation of the PI3K/Akt, JNK, p38 pathways in inducing this transition. Moreover, the upregulation of PCNA (an S-phase induced protein), observed in K12V tumors is also consistent with their higher proliferative rates. On the other hand, the dramatic upregulation of cyclin E and c-Myc, observed only in K12D tumors, is consistent with Src transformation inducing c-Myc overexpression and activating the cyclin E–CDK2 complex (41). In contrast, and consistently with the low levels of cyclin E expression observed in K12V tumors, Ras Val12 is only a weak cyclin E activator (42).

Similarly, the pathway activation pattern induced by each K-Ras mutant may vary its efficiency in activating the cyclin B–Cdc2 complex and in inducing the $G_2/M$ transition. Thus, K12V may activate the cyclin B–Cdc2 complexes through Erk (43,44), whereas K12D could do it through the PI3K/Akt and JNK pathways. This is consistent with JNK phosphorylating Cdc2/cyclin B and controlling the $G_2$ phase in Src transformed cells (45,46). It is also in agreement with PI3K/Akt regulating cyclin B/Cdc2 and playing a role in the $G_2/M$ transition (47,48). In summary, the increased Rb phosphorylation and PCNA and cyclin B upregulation we observed in the K12V tumors suggest that the activation of the Erk pathway is more efficient in inducing faster $G_1/S$ and $G_2/M$ transitions, leading to significantly enhanced mitotic rate, growth and tumor cellularity, than the coactivation of the PI3K/Akt, JNK, p38 and FAK pathways observed in the K12D tumors.

Cyclin D1 or p53 mutations did not occur as secondary hits during tumorigenesis, as we found a similar level of cyclin D1 and p53 in all tumors from both groups, by both IHC and WB, which were also similar to the levels found in cultured transformants before their injection.

Conclusion and clinical implications

In summary, we found that the K12V mutant protein induces very aggressive tumors by interacting with its effector Raf-1 and activating the Erk pathway, which leads to an increase in Rb phosphorylation and upregulation of PCNA and cyclin B, which may, in turn, induce higher growth and mitotic rates. In contrast, the K-Ras mutant protein induces significantly less aggressive tumors by interacting with PI3K (but not with Raf), and activating the PI3K/Akt, JNK, p38 and FAK pathways which leads to a less intense upregulation of the same cell cycle regulatory proteins and to a slower tumor growth. Our results offer an explanation for the higher aggressiveness of the K12V, as compared with K12D, tumors observed in clinic. Thus, K12D is more prevalent in benign than in malignant human colorectal tumors (10), whereas K12V associates with more advanced and metastatic colorectal carcinomas (11,12), higher recurrence and decreased survival (13,49).

Our observations demonstrate that the mutant amino acid in the K-Ras protein influences its in vivo tumorigenic capacity. Therefore, despite that most prognostic studies attribute to all Ras mutants similar transforming properties, knowledge on the mutant amino acid may be critical in identifying subsets of patients with different tumor aggressiveness. Our results also suggest that the Raf-1 (50) or the MEK1 (51) inhibitors may not work in the subset of tumors bearing K12D mutations (and possibly in other Ras mutant tumors) because these tumors do not use the canonical Raf/MEK/Erk pathway to maintain their transforming state.

Acknowledgements

We would like to thank Judith Darrical (contract FIS 01A041) and QD for their technical assistance. This work was supported in part by Grants of the Spanish Ministerio de Educacion y Ciencia SAF03-07437 to RM and from the Ministerio de Sanidad, FIS 01/0853 to RM and FIS01/3085 to MP and from Fundación BBVA and SAF-05-01627 to MC. RM and MP were supported partially by FIS contracts 98/3197 and 01/3085, respectively. RM is a researcher of the Catalanon Public Health System. The Grup d Oncogenesi i Antitumorals is supported by a Grant (SGR 1050) by AGAUR Agency of the Generalitat de Catalunya. The research team belongs to the Network of Cooperative Research on Cancer (CO3/10), funded by the Instituto Carlos III, of the Spanish Ministerio de Sanidad.

Conflict of Interest Statement. None declared.
Nature of Ras mutation influences tumorigenesis and signaling

phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell, 89, 457–467.


*Received November 23, 2005; revised March 24, 2006; accepted April 15, 2006*