Acquisition of Anoikis Resistance Promotes the Emergence of Oncogenic K-ras Mutations in Colorectal Cancer Cells and Stimulates Their Tumorigenicity In Vivo\(^1\)

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Abstract
Detachment from the extracellular matrix causes apoptosis of normal epithelial cells—a phenomenon called anoikis. K-ras oncogene, an established anoikis inhibitor, often occurs in colorectal carcinoma (CRC). In addition to blocking anoikis-inducing mechanisms, oncogenic K-ras can cause anoikis-unrelated changes in CRC cells, such as induction of events promoting their deregulated mitogenesis, ability to trigger angiogenesis, and so on. Thus, whether ras-induced anoikis resistance of CRC cells is essential for their ability to form tumors in vivo or represents a mere epiphenomenon is unclear. We found that when poorly tumorigenic, oncogenic, K-ras-negative, anoikis-susceptible human CRC cells were cultured under anoikis-inducing conditions in vitro, they spontaneously gave rise to an anoikis-resistant cell population harboring de novo oncogenic K-ras mutations and manifesting dramatically increased tumorigenicity. We further observed that a variant of the same oncogenic K-ras-negative anoikis-susceptible cells selected for increased tumorigenicity acquired de novo oncogenic K-ras mutations and manifested increased anoikis resistance. Unlike the case with anoikis, oncogenic K-ras did not rescue CRC cells from death caused by hypoxia or anticancer agents. Taken collectively, our results support the notion that ras-induced anoikis resistance of CRC cells is essential for their ability to form tumors in vivo and thus represents a potential therapeutic target.

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Introduction
Colorectal carcinoma (CRC) originates from within the orderly monolayer of the normal intestinal epithelium. Normal epithelial cells that compose this epithelium are known to be continuously attached to a form of the extracellular matrix (ECM) called basement membrane (BM). Detachment of such cells from the ECM triggers a form of apoptotic cell death \(^1\) often referred to as anoikis \(^3\). Unlike normal epithelia, advanced colorectal tumors represent largely disorganized three-dimensional multicellular masses in which cell–ECM contacts are significantly altered or even disrupted \(^4\)–\(^6\). It is known in this regard that carcinoma cells typically grow as multilayers, and at least some of these cells are detached from the BM. In addition, cancer cells often secrete BM-degrading enzymes, which allows tumors to invade adjacent tissues. Furthermore, in the process of metastatic progression (a series of events that result in the dissemination and colonization of malignant cells at ectopic sites), these cells completely detach from the primary tumor. However, despite the fact that carcinoma cells are deprived of normal contacts with the BM during tumor progression—conditions that can be expected to induce a rapid demise (anoikis) of their normal epithelial counterparts, a significant fraction of these malignant cells likely remains viable \(^5\)–\(^6\). Although this ability to survive in the absence of adhesion to the ECM is readily demonstrable in vitro (in suspension culture), the role of this viability in carcinoma progression still remains largely inferential, and direct evidence supporting the critical role of the anoikis resistance of cancer cells in their ability to form tumors in vivo is lacking.

One line of evidence that is thought to support the involvement of the anoikis resistance of CRC cells in tumor progression is the fact that the death of intestinal epithelial cells in suspension culture can be attenuated or aborted by the...
expression of oncogenic ras [7–10], a lesion commonly found in human CRC [11]. However, it is possible that ras mutations emerge in this disease for reasons unrelated to anois resistance, such as the need of the respective tumor cells to be able to proliferate in an uncontrolled manner, promotion of tumor angiogenesis, or the dependence of these cells on one of many other changes that are known to occur in response to Ras activation [12–14]. Establishing whether ras-induced anois resistance contributes to CRC progression could have important therapeutic implications. If this resistance does indeed contribute to the progression of the disease, then targeting the mediators of ras-dependent antianois effect would be expected to create a situation in which ras-transformed cells can no longer survive in the absence of adhesion to the ECM and die by apoptosis. This, in turn, would result not only in the inhibition of the ability of CRC to survive without being attached to the ECM but also in the elimination of cells carrying mutant ras (and hence displaying numerous ras-dependent properties that drive CRC progression) [12–14] from the CRC cell population.

Here, we show that culturing K-ras–negative, poorly tumorigenic, anois-susceptible CRC cells in the absence of adhesion to the ECM (i.e., under conditions designed to selectively induce anois) leads to a spontaneous generation of a variant of these cells that is anois-resistant, harbors de novo K-ras mutations, and possesses strongly enhanced tumorigenicity in vivo. We observed that the presence of mutant K-ras in cancer cells resulted in their increased resistance to anois, but not to other forms of cell death, including that induced by hypoxia or anticancer agents. These data support the notion that K-ras–dependent inhibition of anois plays an important casual role in CRC progression and, thereby, may represent a valuable target for cancer therapy and prevention.

Materials and Methods

Cell Culture

ras knockout derivatives of human CRC-derived cells (HCT-116, Hkh-2, and Hke-3 cells) have been previously described [15]. Hkh-2T cells (referred to as Hkh-TUM2 in Yu et al. [14]) have been previously described [14]. The p53 knockout variant 379.2 of HCT-116 cells was kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) [16]. To generate Hkh-2AR cells, 1000 Hkh-2 cells were cultured in suspension for 72 hours; cells that survived this treatment were replated in monolayer, allowed to grow for 7 days, and then used for subsequent studies. All cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For suspension cultures, cells were plated above a layer of 1% sea plaque agarose polymerized in Dulbecco's modified Eagle's medium.

Soft Agar Colony Formation Assay

A total of 10³ cells was suspended in 2 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum containing 0.3% melted Bacto agar. The resulting suspension was added to a 60-mm plate covered with a 2-ml layer of solidified 0.5% Bacto agar in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cell colonies were allowed to form for 7 to 10 days and counted.

Cell Death Enzyme-Linked Immunosorbent Assay (ELISA)

Cells growing in monolayer or suspension culture were removed from the plates and assayed for the presence of nucleosomal fragments in the cytoplasm by the Cell Death Detection ELISA kit (Roche Applied Science, Laval, QC, Canada), according to the manufacturer’s instructions.

K-ras Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis

Activating mutations in codon 13 of the K-ras gene were detected by PCR-RFLP assay, as previously described [14]. Chromosomal DNA was extracted by using a DNeasy tissue kit (Qiagen, Valencia, CA). PCR was subsequently performed in a reaction volume of 50 µl containing 250 ng of DNA, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.2 µM of each primer, and 2.5 U of Taq polymerase (Invitrogen, Carlsbad, CA). The primers used in the reaction were RAS A (sense) 5'-ACTGAAATATCTGTTGGCCATGAGC-3' and RAS B (antisense) 5'-TTATCTGTATCAAAGATTGCTGGCACC-3'. Amplification reaction consisted of 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. Two rounds of PCR were performed to obtain a clean 166-bp product. Then, a 10-µl aliquot of a PCR reaction mixture was treated with 10 U of XcmI (New England Biolabs, Beverly, MA) for 20 hours at 37°C, in a total volume of 40 µl. Digestion products were subsequently detected by electrophoresis in a 3.5% agarose gel. PCR-RFLP analysis of mutations at the third position of codon 61 of the K-ras gene was performed as described [17].

Tumorigenicity Assay

A total of 10 × 10⁶ cells was suspended in 0.2 ml of phosphate-buffered saline and injected subcutaneously into the flanks of 8- to 12-week-old female nude athymic BALB/C mice. The resulting tumors were measured by a vernier caliper, and tumor volumes were then calculated by using the formula: \( V = \frac{a \times b \times l}{2} \), where \( a \) is width and \( b \) is the length of an ellipsoid tumor perimeter.

Hypoxia Assay

Cells were cultured in a hypoxic chamber (Coy Laboratory Products, Grass Lake, MI) at < 0.1% oxygen.

Drug-Induced Apoptosis Assay

A total of 10⁵ cells was plated in 60-ml dishes and subjected to treatment with various concentrations of drugs for 24 hours. A culture medium was subsequently replaced with a fresh medium. Cells were then allowed to form colonies for 7 days, colonies were stained by crystal violet and counted.
Results and Discussion

**Oncogenic K-ras Is Required for the Anoikis Resistance of Human CRC Cells**

To address the role of oncogenic ras-dependent anoikis resistance in the ability of human CRC cells to form tumors in vivo, we chose to study a human CRC cell line (HCT-116) that carries oncogenic mutation in codon 13 of one of two K-ras alleles and is highly tumorigenic [15]. In addition, we used two different isogenic derivatives of HCT-116 cells (Hkh-2 and Hke-3) [15] in which oncogenic K-ras allele was disrupted by homologous recombination. Hkh-2 and Hke-3 cells are poorly tumorigenic [15] and, according to our studies, display a significantly reduced ability to grow as colonies in soft agar compared to HCT-116 cells (Figure 1, A and B). We established that this reduction does not represent merely a consequence of K-ras–dependent changes in cellular proliferation because the rate of cell death (anoikis) in suspension culture, as assayed directly by measuring chromosomal DNA fragmentation (a characteristic feature of apoptosis), was significantly increased in the two oncogenic K-ras–deprived cell lines relative to parental HCT-116 cells (Figure 1, C and D). It is noteworthy that HCT-116 cells and their mutant K-ras knockout variants are known to contain an array of additional (and only partially characterized) genetic alterations [18,19]. It is therefore remarkable that the expression of oncogenic K-ras appears to be particularly critical for the maintenance of the anoikis-resistant phenotype of these CRC cells irrespectively of coexisting genetic lesions.

**Oncogenic K-ras Is Acquired by Oncogenic K-ras–Negative CRC Cells Selected for Increased Resistance to Anoikis**

Whether the tumorigenic capacity of CRC cells depends on their anoikis resistance and whether this resistance is, in turn, necessarily associated with the presence of oncogenic K-ras mutations in these cells (of all possible mutations that

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**Figure 1.** Loss of the oncogenic K-ras allele by human colorectal cancer HCT-116 cells renders them anoikis-susceptible. (A and B) CRC-derived HCT-116 cells and oncogenic K-ras knockout variants of these cells (A, Hkh-2; B, Hke-3) were plated in soft agar or monolayer culture, and colonies formed by the cells were counted 7 to 10 days later. Results are expressed as a percentage of the number of cells plated in the monolayer. (C and D) HCT-116 and Hkh-2 (C) or Hke-3 (D) cells were cultured while attached to or detached from the ECM for 24 hours and assayed for apoptosis by cell death ELISA, which detects the presence of oligonucleosomes in the cytoplasm of apoptotic cells. The levels of apoptosis observed in attached cells were subtracted from the respective numbers obtained in case of detached cells as background. Results are expressed as a ratio between the apoptotic signal observed for Hkh-2 (C) or Hke-3 (D) cells and that detected in case of HCT-116 cells. Results represent the average of duplicates (A, B, and D) or triplicates (C) plus the standard deviation. All experiments were repeated twice, with similar results.
can, in principle, be acquired by these cells during their natural history [19]) are not known. To address this question, anoikis-susceptible Hkh-2 cells were maintained in suspension culture to enforce the selection for cellular changes required for the acquisition of anoikis resistance by these cells and to exclude all other potential influences that could affect the emergence of oncogenic mutations in CRC cells in vivo (e.g., inflammation, limited access to oxygen and nutrients, and so on). We observed that 21.5% of Hkh-2 cells originally plated in suspension culture (SD = 0.5%; \( n = 2 \)) remained clonogenic when replated in monolayer after being cultured in suspension for 72 hours. We thus established these anoikis-resistant cells as a cell line (called Hkh-2AR) in monolayer culture.

We found that Hkh-2AR cells exhibited a stable anoikis-resistant phenotype on secondary detachment from the ECM (Figure 2, A and B). We reasoned that these cells likely harbor permanent de novo–acquired genetic alterations that could trigger the effects that have been previously observed due to the presence of mutant K-ras oncogene (Figure 1). In this regard, we considered two important circumstances. First, Hkh-2 cells are known to possess a deficient DNA repair system and, therefore, if necessary, are likely to undergo secondary mutations [19]. Second, such mutations could activate proteins that are known to be able to block anoikis, including K-Ras [9,10] encoded by the wild-type K-ras allele that is still present in Hkh-2 cells after the disruption of the oncogenic K-ras allele [15]. Therefore, we carried out an RFLP analysis of the remaining K-ras allele in Hkh-2AR cells to test whether this allele had acquired an oncogenic mutation [14]. To this end, a fragment of the K-ras gene containing codon 13 was amplified by PCR. PCR primers were designed in such a way that the cleavage site for the restriction enzyme XcmI would have been created if codon 13 was wild-type, but not if this codon was mutant [14]. Remarkably, a significant fraction of anoikis-resistant Hkh-2AR cells exhibited the presence of the mutant K-ras allele (Figure 2C). By contrast, RFLP analysis revealed that neither HCT-116 nor Hkh-2 or Hkh-2AR cells displayed an A-to-C or A-to-T mutation of the third base of codon 61 of K-ras (not shown; the latter mutation was observed by others in a fraction of colorectal cancers) [20].

To eliminate the possibility that the emergence of the codon 13 mutation of the K-ras gene in Hkh-2AR cells was the consequence of the carryover of a subpopulation of parental HCT-116 cells to the population of Hkh-2AR cells that might have occurred at some point during the natural history of the latter cells, we took advantage of the fact that HCT-116 cells are neomycin-susceptible whereas Hkh-2 cells—and consequently Hkh-2AR cells (derived from Hkh-2 cells)—are neomycin-resistant. This is due to the fact Hkh-2 cells were generated from HCT-116 cells by the disruption of the mutant K-ras allele by a cassette containing a neomycin resistance gene [15]. We thus used RFLP analysis to test Hkh-2AR cells

**Figure 2.** The population of oncogenic K-ras allele–deprived CRC cells selected for increased anoikis resistance is enriched with cells carrying an oncogenic mutation of the second K-ras allele. Hkh-2 cells and a variant of these cells (Hkh-2AR) that was selected for increased anoikis resistance were assayed for the ability to form colonies in soft agar (A) and to undergo anoikis (B), as in Figure 1. Results in (A) and (B) represent the average of the duplicates plus the standard deviation. Experiments in (A) and (B) were repeated twice, with similar results. (C) Genomic DNA was extracted from Hkh-2 and Hkh-2AR cells, and two rounds of PCR were performed to obtain a 166-bp product containing the sequence corresponding to codon 13 of K-ras. PCR was treated with XcmI. When codon 13 is wild type, the PCR product contains a restriction site for XcmI, and digestion yields bands of 138 and 28 bp. If a mutation is present in either of the first two bases of codon 13, the mutant PCR fragment is not digested by XcmI and remains at its original size of 166 bp. Bands were visualized by electrophoresis in agarose gel. This experiment was repeated three times with three independently obtained preparations of genomic DNA, with similar results. (D) Hkh-2AR cells were analyzed as in (C) for the presence of oncogenic mutation in codon 13 of K-ras before (–) and after (+) treatment with 1.3 mg/ml neomycin for 5 to 7 days. This experiment was repeated three times with three independently obtained preparations of genomic DNA, with similar results.
for the presence of K-ras mutation in codon 13 after neomycin treatment. We reasoned that any HCT-116 cell that might be present in the population of Hkh-2AR cells should die in response to neomycin treatment. In this case, the signal corresponding to mutant K-ras should no longer be detectable in neomycin-treated Hkh-2AR cells. We have not observed any obvious changes in the viability and growth of Hkh-2AR cells during or after exposure to neomycin, whereas all HCT-116 cells died as a result of a similar treatment in parallel control experiments. As shown in Figure 2D, exposure to neomycin did not result in any significant change in the ratio between the signal corresponding to mutant K-ras and that corresponding to the wild-type K-ras observed in case of Hkh-2AR cells. Therefore, the emergence of the codon 13 mutation of K-ras gene in Hkh-2AR cells was not the consequence of the carryover of a subpopulation of parental HCT-116 cells to the population of Hkh-2AR cells.

Based on the results presented above, we concluded that the acquisition of anoikis resistance by parental Hkh-2 cells was accompanied by a significant enrichment of a population of these cells with those that have acquired a secondary oncogenic mutation in codon 13 of the K-ras proto-oncogene.

**CRC Cells Selected for Increased Resistance to Anoikis**

Whether CRC cells that have acquired increased anoikis resistance can necessarily be expected to possess an increased tumorigenic potential is not known. In this regard, we observed that Hkh-2AR cells, but not their parental Hkh-2 counterparts, formed aggressive tumors in immunodeficient mice with 100% efficiency and within < 2 months of subcutaneous tumor cell injection (Figure 3A). We also observed that the tumorigenicity of Hkh-2AR cells was lower than that of HCT-116 cells (Figure 3, A and B). This was likely due to the fact that, unlike HCT-116 cells, all of which harbor mutant K-ras, only a fraction of Hkh-2AR cells (Figure 2C) carries oncogenic K-ras mutation.

The observations presented above (Figures 2 and 3A) lead to at least two important conclusions. First, acquisition of anoikis resistance by CRC cells is, at least under some circumstances, sufficient to trigger the attainment of an overt tumorigenic phenotype by these cells. Second, CRC cells likely became anoikis-resistant and tumorigenic, at least in part, due to the acquisition of oncogenic mutations of the K-ras proto-oncogene gene by these cells.

**CRC Cells Selected for Increased In Vivo Tumorigenicity**

**Display the Presence of De Novo—Acquired Oncogenic Mutations of K-ras and Increased Resistance to Anoikis**

We reasoned that if anoikis resistance is required for the acquisition of an aggressive tumorigenic phenotype by CRC cells in vivo, then the CRC cells selected for elevated in vivo tumorigenicity should display increased anoikis resistance. To test whether this is the case, we took advantage of our recent observations indicating that even though Hkh-2 cells are poorly tumorigenic in vivo, small tumors do arise from these cells in mice eventually after a long latency period [14]. We thus established a cell line (called Hkh-2T) from one of these tumors and demonstrated that the population of tumor-derived Hkh-2 cells is significantly enriched with cells carrying the oncogenic mutation of K-ras in codon 13 of the remaining K-ras allele compared to parental Hkh-2 cells [14]. We have now compared the anoikis resistance of Hkh-2 cells to that of their tumorigenic derivative Hkh-2T. As shown in Figure 4, A and B, Hkh-2T cells were significantly more capable of growing anchorage-independently and were significantly less susceptible to detachment-induced apoptosis than their parental Hkh-2 counterparts. Collectively, our observations enforce the notion that anoikis resistance and the tumorigenicity of CRC cells are tightly linked (and interdependent) not only at the functional level but also at the molecular level. Namely, attainment of both of these properties by CRC cells (at least in our experimental conditions) is invariably paralleled by (and likely represents the consequence of) the acquisition of oncogenic K-ras mutations by these cells. It, however, remains to be established whether the indicated effects of this oncogene pertain to its general ability to protect CRC cells from cell death, regardless of the nature of the death-promoting stimulus or whether these...
effects are, at least to a degree, unique to death induced by cell detachment from the ECM.

Oncogenic K-ras Does Not Confer Survival Advantage to CRC Cells Exposed to Anoikis-Unrelated Proapoptotic Stimuli

To test whether the effect of oncogenic K-ras on the survival of CRC cells is relatively unique to anoikis, we chose to expose HCT-116 cells or their K-ras–disrupted counterparts to apoptosis-inducing effects of hypoxia (Figure 5) or chemotherapeutic agents (Figure 6), both of which are highly relevant to CRC progression and treatment [21–23]. In particular, tumor hypoxia can arise as a natural consequence of a disorganized and dysfunctional tumor vasculature [21] or else result from treatment with antiangiogenic agents, some of which (bevacizumab) have recently become a front-line therapy in CRC [24]. These agents restrict tumor perfusion and oxygen supply and thus cause the apoptotic death of cancer cells [24,25]. HCT-116 cells appear to represent an adequate model for studying this therapeutic paradigm in that exposure to hypoxia in vitro results in a gradual loss of their clonogenicity (Figure 5). This loss of clonogenicity, however, was not enhanced by loss of oncogenic K-ras. We found, in this regard, that the two K-ras–deficient variants of HCT-116 cells (Hkh-2 and Hke-3) exhibited susceptibility to hypoxia-induced death similar to that of parental HCT-116 cells (Figure 5). Thus, expression of mutant K-ras appears to have little effect on the survival of these CRC cells under hypoxic conditions. Therefore, the status of K-ras cannot be expected to have a significant impact on the growth of colorectal tumors under hypoxia and, more importantly, would not be predictive of the responses of CRC patients to antiangiogenic therapy, a notion supported by recent clinical evidence [26].

Another relevant inducer of the apoptotic death of CRC cells is cytotoxic chemotherapy. To this end, we examined the role of ras in the protection of HCT-116 cells from death-promoting stimuli, such as those induced by a topoisomerase I inhibitor irinotecan (Camptosar; Pfizer Inc., New York, NY) and an antimetabolite 5-fluorouracil (5-FU). Both of these drugs are presently being used in clinics for CRC treatment [22,23]. In addition, we used a cyclooxygenase-2 inhibitor sulindac, which is now considered to represent a prototypic anti-CRC agent [27]. All three chemotherapeutic drugs are well known to be able to trigger apoptosis [27–29]. As a control for these studies, we used another isogenic

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**Figure 4.** A variant of oncogenic K-ras allele–deprived CRC cells selected for increased in vivo tumorigenicity (and previously reported to carry an oncogenic mutation of the second K-ras allele) displays increased anoikis resistance. Hkh-2 cells and a variant of these cells (Hkh-2T) that was selected for increased in vivo tumorigenicity were assayed for the ability to form colonies in soft agar (A) and undergo anoikis (B), as in Figure 1. Results in (A) and (B) represent the average of the duplicates plus the standard deviation. Experiments in (A) and (B) were repeated twice, with similar results.

**Figure 5.** Oncogenic K-ras does not protect colorectal cancer cells from hypoxia-induced death. HCT-116, Hkh-2, and Hke-3 cells were cultured under hypoxia for indicated times. Cells were subsequently cultured under normoxic conditions, and colonies formed by cells that survived after being cultured under hypoxia were stained with crystal violet 7 to 10 days later and counted. Results are expressed as a percentage of the number of colonies obtained after culturing cells under normoxia and represent the average of the duplicates plus the standard deviation. This experiment was repeated twice, with similar results.
variant of HCT-116 cells, called 379.2, in which disruption of both p53 alleles resulted in resistance to cytotoxic agents, such as 5-FU [16,30].

As may have been expected based on what has already been published [30], the survival of p53 knockout cells in the presence of 5-FU, irinotecan hydrochloride, or sulindac markedly exceeded that of parental HCT-116 cells (Figure 6). Unlike the case with p53, the status of mutant K-ras did not predict the responses of CRC cells to treatment with the aforementioned anticancer agents. For instance, the

Figure 6. Oncogenic K-ras does not protect colorectal cancer cells from death induced by chemotherapeutic drugs. HCT-116, Hkh-2, and 379.2 cells were treated with indicated doses of irinotecan hydrochloride (A), 5-FU (B), and sulindac (C) for 24 hours. Cell colonies were then allowed to form for 7 to 10 days, stained with crystal violet, and counted. Results in (A) to (C) are expressed as a percentage of the number of colonies derived from vehicle-treated cells and represent the average of the duplicates plus the standard deviation. All experiments were repeated twice, with similar results.
survival of Hkh-2 cells (deprived of oncogenic K-ras and carrying intact p53) in the presence of irinotecan or sulindac was significantly greater than that of parental HCT-116 cells (Figure 6, A and C). However, Hkh-2 cells displayed sensitivity to 5-FU treatment similar to that of parental HCT-116 cells (Figure 6B). Although these analyses are not intended to establish the predictive value for the efficiency of CRC therapy as a function of the presence of K-ras or p53 mutations in a wide context of CRC, they do indicate that, in the same cellular background, the expression of mutant K-ras can have dramatically different effects on CRC cell survival, depending on whether these cells are exposed to conditions related or unrelated to the induction of anoikis.

Loss of p53 Does Not Enhance the Anoikis Resistance of Human CRC Cells

In the course of CRC progression, acquisition of activating mutations of K-ras by malignant cells is often followed by loss of the p53 tumor-suppressor protein [31]. Given that p53 is well known to be able to stimulate apoptosis [32], we decided to test whether loss of p53 can further enhance the anoikis resistance of HCT-116 cells. As shown in Figure 7, A and B, the p53 knockout variant of HCT-116 cells, 379.2, displayed anoikis resistance similar to that of parental HCT-116 cells. Thus, our data indicate that once CRC cells have acquired oncogenic K-ras and K-ras–dependent anoikis resistance, loss of p53 does not enhance this resistance further.

Our data indicating that the anoikis resistance of CRC cells tends to depend on the status of K-ras, rather than on that of p53, are consistent with our previous observations [9] demonstrating that DLD-1 cells, another CRC-derived cell line that carries oncogenic K-ras [15], failed to remain anoikis-resistant after the ablation of the oncogenic K-ras allele by homologous recombination, even though these cells do not express functional p53 [9,33]. Furthermore, despite the absence of functional p53, the resulting oncogenic K-ras–deprived variants of DLD-1 cells were found to be incapable of growing in the absence of adhesion to the ECM as colonies in soft agar (unlike parental DLD-1 cells that are capable of such growth) [15]. Thus, based on these data and on our results presented above (Figure 7), it appears that it is the acquisition of oncogenic ras, rather than loss of p53, that renders CRC cells anoikis-resistant.

Our study offers several novel observations relevant to the role of the anoikis resistance of CRC cells in the progression of the disease. We have demonstrated that enforced survival of nontumorigenic intestinal epithelial cells under anoikis-inducing conditions can lead to the emergence of de novo K-ras mutations in these cells and renders them overtly tumorigenic. This is consistent with the notion that anoikis resistance is a prerequisite of tumor growth and not just an epiphenomenon. Importantly, we were able to induce the anoikis resistance of CRC cells in two independent ways, namely, by forcing these cells to survive in suspension culture and by forcing them to form tumors in vivo. In both cases, one genetic lesion that consistently emerged in resulting cells was the oncogenic mutation of K-ras. This suggests that, in the context of CRC, this lesion is particularly efficient in rendering cancer cells anoikis-resistant. Indeed, K-ras mutations occur with high frequency, mainly during the early stages of CRC progression [31] and in tumors that tend to display a three-dimensional, rather than a flat, growth pattern [34] (i.e., under circumstances where overcoming anoikis could be especially important).

Our data indicate that anoikis resistance is required for the ability of CRC cells to form subcutaneous three-dimensional tumors in mice. Thus, anoikis resistance of carcinoma cells may be important not only for their metastatic potential, as has been previously suggested by others [5,6], but also for the ability of primary tumors to grow as disorganized three-dimensional masses in which a significant proportion of malignant cells is deprived of proper contacts with the BM. This possibility is consistent with our earlier findings. We demonstrated in this regard that ras oncogene blocks the anoikis of rat and human intestinal epithelial cells by downregulating a proapoptotic protein Bak [7] and by upregulating an antiapoptotic protein Bcl-XL [8]. We also observed that the reversal of the effects of ras

![Figure 7](https://example.com/figure7.png)  
**Figure 7.** Loss of p53 does not enhance the anoikis resistance of human CRC cells. (A) HCT-116 and a p53 knockout variant of these cells (379.2) were assayed for the ability to form colonies in soft agar (A) and to undergo anoikis (B), as in Figure 1. Results in (A) and (B) represent the average of the duplicates plus the standard deviation. Experiments in (A) and (B) were repeated twice, with similar results.
on Bak and Bcl-XL suppressed the anoikis resistance of ras-transformed cells and significantly blocked their ability to form subcutaneous tumors on injection into mice [7,8]. In view of these data, as well as of our findings reported in the present study, the fact that the frequency of oncogenic mutations of ras is known to be significantly higher in three-dimensionally growing colorectal tumors compared to those growing in a flat manner [34] does not appear to be coincidental.

What molecular mechanisms could have resulted in secondary oncogenic K-ras mutations in the tumorigenic Hkk-2T and anoikis-resistant Hkh-2AR cells that we observed in this study, as well as in a previous study [14]? It seems possible that these mutations represent the consequence of the documented DNA mismatch repair defects of HCT-116 cells and the subsequent increased rate of spontaneously occurring mutations that have been reported in these cells by others [19]. According to one likely scenario, a small and initially undetectable fraction of oncogenic K-ras knockout Hkk-2 cells could have spontaneously acquired an oncogenic mutation of the second K-ras allele during their natural history. The population of Hkh-2 cells containing this oncogenic mutation could have received a selective growth/survival advantage under the pressure existing during tumor growth in mice and under anoikis-inducing conditions in the absence of adhesion to the ECM. In addition, it cannot, in principle, be excluded that the frequency of K-ras mutations could be increased by the influence of the restrictive conditions themselves. In this regard, hypoxia and hypoglycemia (not uncommon in the tumor microenvironment) were demonstrated to lead to the downregulation of a key mismatch repair protein MSH2 in Hkk-2 cells and to the increased rate of spontaneously occurring mutations in these cells, including the acquisition of de novo oncogenic mutations of K-ras [35]. Thus, it is conceivable that conditions existing during cell growth both in vivo and in the absence of cell adhesion to the ECM could have triggered mechanisms resulting in the acquisition of mutant K-ras by the respective variants of CRC cells.

Our studies indicate that the role of mutant K-ras in the ability of CRC cells to resist apoptosis depends on the nature of a proapoptotic stimulus. This is important for at least two reasons. First, understanding pathways resulting in the anoikis resistance of CRC cells (in which K-ras is protective) may offer the opportunity to restore the functionality of these pathways (downstream of K-Ras) and thus suppress tumor growth. Second, our data indicate that the susceptibility of CRC cells to anoikis-promoting treatment may not be abrogated by prior exposure to chemotherapy because the responsiveness of CRC cells to the latter appears to, at least in our hands, be driven by mechanisms/pathways (oncogenic K-ras—dependent) different from those involved in anoikis resistance (oncogenic K-ras—dependent).

In summary, our results support the notion that rendering malignant intestinal epithelial cells anoikis-resistant represents one of the essential mechanisms by which oncogenic ras promotes the growth of tumors formed by these cells in vivo.

References


