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Exploring Environmental Causes of Altered *ras* Effects: Fragmentation Plus Integration?

Miquel Porta,[1,2]* Daniel Ayude,[1,2] Juan Alguacil,[1,3] and Manuel Jariod[1]

[1]*Institut Municipal d'Investigació Médica, Barcelona, Spain*
[2]*Universitat Autónoma de Barcelona, Catalonia, Spain*
[3]*Occupational Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland*

Mutations in *ras* genes are the most common abnormality of oncogenes in human cancer and a major example of activation by point mutation. Experimental and epidemiological studies support the notion that Ki-*ras* activation and expression may be chemically related. We discuss the potential role of several environmental compounds in the induction or promotion of *ras* mutations in humans, with a focus on exocrine pancreatic cancer, the human tumor with the highest prevalence at diagnosis of Ki-*ras* mutations. Organochlorine compounds, organic solvents, and coffee compounds may play an indirect role in causing Ki-*ras* mutations, rather than as direct inducers of the mutations. Although for some organochlorine compounds the induction of point mutations in *ras* oncogenes cannot be excluded, it seems more likely that the effects of these compounds are mediated through nongenomic or indirectly genotoxic mechanisms of action. Organic solvents also may act via enzymatic induction of *ras* mutagens or by providing a proliferation advantage to *ras*-mutated cell clones. In exocrine pancreatic cancer, caffeine, other coffee compounds, or other factors with which coffee drinking is associated could modulate Ki-*ras* activation by interfering with DNA repair, cell-cycle checkpoints, and apoptosis. Asbestos, cigarette smoking, and some dietary factors also may be involved in the initiation or the promotion of Ki-*ras* mutations in lung and colon cancers. Further development of the mechanistic scenarios proposed here could contribute to a meaningful integration of biological, clinical, and environmental knowledge on the causes of altered *ras* effects. © 2003 Wiley-Liss, Inc.

Key words: pancreas; neoplasms; *ras* genes; mutation spectra; organic chemicals; environment/chemistry

INTRODUCTION

The understanding of ras biology and effects has improved immensely during the past decade. Knowledge of ras signaling and function in human cancer has been analyzed in several reviews [1–10]. One feature common to several of these reviews is particularly evident in the article by Shields et al. [10]. Far from a mere description of the current state of knowledge, the paper tackles the conceptual, methodological, and technical reasons that influence this state. This analysis includes the fact that the "elegant complexity" of research approaches to *ras* is often "coupled with hopeless confusion on concepts that once seemed on the verge of comprehension" [10]. While suggesting that "those more removed from the field might have a better perspective," the paper ends with a call "to put into place all the pieces of the puzzle" [10], that is, to integrate the fragments of current knowledge. We believe that such a call is most pertinent and explore possible answers based on our work in pancreatic cancer.

ENVIRONMENTAL CORRELATES OF Ki-*ras* MUTATIONS IN EXOCRINE PANCREATIC CANCER

Mutations in *ras* genes are the single most common abnormality of oncogenes in human cancer and a major example of activation by point mutation [11–13]. Exocrine pancreatic cancer (EPC) is the human tumor with the highest prevalence at diagnosis of Ki-*ras* mutations (ranging from 75% to 85%) [11,14]. Molecular pathological and epidemiological studies suggest that wild-type Ki-*ras* carcinomas of the pancreas arise through a genetic pathway distinct from carcinomas that harbor a Ki-*ras* mutation [14–18]. In EPC, all *ras* mutations are limited to the Ki-*ras* gene; they are an early event, and practically all occur in codon 12. It is still unknown whether their occurrence and persistence in human neoplasms are associated with clinical, environmental, or other factors (e.g., tissue-specific processes) [10,14–21]. A

*Correspondence to: Institut Municipal d'Investigació Médica, Universitat Autónoma de Barcelona, Carrer del Dr. Aiguader 80, E-08003 Barcelona, Spain.

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Abbreviations: EPC, exocrine pancreatic cancer; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; PCBs, polychlorinated biphenyls; OC, organochlorine compound; CYP, cytochrome P450; TCDD, tetrachlorodibenzo-*p*-dioxin; NF-κB, nuclear factor kappa B.

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great deal of our research effort has been aimed at unveiling biological, clinical, and environmental correlates of the frequency and spectra of Ki-*ras* mutations in pancreatic cancer. Several of our results support the notion that Ki-*ras* activation and expression may be chemically related. Because they might help to ''connect pieces'' of current knowledge on *ras* biology, there are three results that are particularly relevant.

First, we found that in EPC cases with a Ki-*ras* mutation there were significantly higher serum concentrations of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), and some polychlorinated biphenyls (PCBs) than in EPC cases with wild-type Ki-*ras* [22,23]. DDE is the main metabolite of the pesticide DDT. DDE and PCBs are highly persistent and lipophilic organochlorine compounds (OCs), which accumulate in the trophic chain and in fat tissues of many human populations [22–26]. Levels of DDE and DDT were particularly high among cases with a Ki-*ras* GGT→GTT transversion (Gly → Val) [22]. These findings provided the first link between *ras* mutations and OCs among humans living under normal conditions of life [22,27,28]. The association was not indiscriminate with all types of OCs detected in EPC patients; for instance, levels of the OCs hexachlorobenzene and β-hexachlorocyclohexane were also high among the study subjects, and yet they were not associated with Ki-*ras* mutational status [22]. At first, this result might seem to support the role of DDE and DDT as initiators of Ki-*ras* mutations; however, as explained later, we think that it is more likely that DDT and DDE act as promoters of either spontaneous or induced mutations.

Second, we observed that in cases of Ki-*ras*–mutated tumors, there was a higher probability that the patients had been occupationally exposed to organic solvents than in cases with wild-type Ki-*ras* [29]. A significantly higher proportion of patients with a Ki-*ras* GGT → GTT transversion (Gly → Val) or a GGT → GAT transition (Gly → Asp) had been exposed to a hydrocarbon solvent. This finding raised the possibility that hydrocarbon solvents might be involved in the pathogenesis of EPC through indirect modulation of Ki-*ras* activation.

Finally, we noted that pancreatic tumors of regular coffee drinkers were significantly more likely to harbor a Ki-*ras* mutation than those of patients who were not regular coffee drinkers, with a dose-response relationship [30–34]. This finding is compatible with the hypothesis that caffeine, other coffee compounds, or other factors with which coffee drinking is associated (perhaps dietary factors) might modulate Ki-*ras* activation or the persistence of Ki-*ras* mutations.

The above-mentioned reviews [1–10] propose a diversity of mechanisms and signaling pathways by which *ras* and *ras*-related molecules effect their biological actions (maybe with some kind of species and tissue specificity); the reviews thus highlight the complexity of pathways leading to activation of the cell-surface receptors ultimately responsible for the activation of *ras*. Might connections exist between the environmental exposures that we found to be associated with Ki-*ras* mutations and some of the agents and processes controlling *ras* status and function? Our previous papers [22,23,29–31] sketch several mechanistic scenarios that could help address this question, offering ideas about possible factors that might play direct and indirect roles in Ki-*ras* activation, in preventing repair of such mutations, or in providing a growth advantage to *ras*-mutated cells. A summary of such mechanistic scenarios follows.

ORGANOCHLORINE COMPOUNDS AND *ras* EFFECTS

Several studies suggest that OCs may increase the risk of EPC [22,23,35–39]. Some OCs can act as tumor promoters and are suspected human carcinogens. The International Agency for Research on Cancer classifies DDT as ''possibly carcinogenic to humans'' [40] and PCBs as ''probably carcinogenic to humans'' [41]. Other OCs have been shown to be carcinogenic in animal models [22–25,40–43]. Doses of some OCs (including PCBs) far below those traditionally deemed ''safe'' may cause long-term biological effects [44,45].

Basic and clinical research also indicate that DDT and some PCB congeners may have weak estrogenic and anti-androgenic effects, whereas other OCs have antiestrogenic activity [24,46–48]. The low hormonal activity of these compounds may be counterbalanced by their long half-lives (about 8–10 yr for DDE and up to 30 yr or more for some PCBs) and by their concentrations in target tissues (levels are 100-fold to 350-fold higher in adipose tissue than in blood) [24,25]. Polypeptide and steroid hormones can both promote and inhibit carcinogenesis in the pancreas [49–51]. Knowledge is emerging on molecular mechanisms of hormonal carcinogenesis [52] and on the pathways through which hormones and nutritional substrates influence the expression of genes [39,53]. A recent study suggested that activated *ras*-dependent pathways may be required for the activation of the transforming growth factor β3 promoter by estradiol [54].

About half of EPC cases with a codon 12 Ki-*ras* mutation have a GGT → GAT transition (Gly → Asp), and about another half have a GTT transversion (Gly → Val). In EPC there are also a few less frequent mutations, for example, → CGT (→ Arg), → TGT (→ Cys), → AGT (→ Ser). About 10–15% cases have double mutations [14,16,19]. This diversity of mutations is thought to be suggestive of the involvement of several carcinogens, of other environmental and ethnic factors, or of errors in DNA repair [12,17].

While *ras* genes are critical DNA targets for chemical carcinogens [12,13], it is largely unknown whether links exist between environmental compounds stored in pancreatic tissue and the molecular signals that induce activation of *ras* proteins. Besides, the *ras* mutation spectrum is just beginning to be linked to compounds present in the human environment [12,13,21,49,55]. G-to-A transitions are thought to be the manner in which some aromatic amines, *N*-nitrosamines, and alkylating agents activate *ras* [21,56,57]. Patients with EPC whose tumors harbor Ki-*ras* mutations were found to have higher levels of aromatic DNA adducts than EPC patients with Ki-*ras* wild-type tumors; compared with the latter patients, those patients with G-to-A substitutions have higher adduct levels, and these levels are even higher among cases with G-to-T mutations [57].

Although it has not been ruled out that OCs, organic solvents, and coffee compounds play a direct role in causing Ki-*ras* mutations, it seems more likely that their effects, if real, are mediated through nongenomic or indirectly genotoxic mechanisms of action. For instance, PCB mixtures may increase c-*raf* and c-*erb* mRNA levels in human liver cells; that is, they may act as tumor promoters through activation of cell-proliferation pathways [58,59]. Free radicals and oxidative DNA damage are produced during oxidation of lower-chlorinated PCBs [42,60]. The pesticides paraquat and endosulfan also generate reactive oxygen species [61,62]. Thus, while it is unclear whether the more estrogenic OCs are directly mutagenic in humans, there is substantial evidence of their role as ''tumor promoters'' [40,41,63,64].

Certainly, many OCs are mutagenic in experimental systems [40], and pancreatic neoplasms have been induced in experimental animals by a variety of chemical carcinogens [49,50]. Of particular interest are ductlike pancreatic adenocarcinomas induced in Syrian hamsters by nitrosamines and dimethylbenzanthracene, because they harbor GGT → GAT point mutations in codon 12 of Ki-*ras* [21,50], which also could be the initiating event in the hamster model [49]. Heptachlor, a chlorinated hydrocarbon insecticide, can affect *ras* expression in human myeloblastic leukemia cells [65]. Pyperonyl butoxide (a pesticide synergist and food additive) and bisphenol A (an environmental estrogenic chemical used in the production of plastic products), induced Ki-*ras* codon 12 mutations in human cultured RSa cells [66,67]. Many studies have shown that after treatment of mice with various genotoxic or non-genotoxic compounds (including OCs), Ha-*ras* mutational patterns exhibited by hepatocellular tumors vary specifically with the chemical and that the intrinsic resistance to tumor formation is influenced by the OC used as promoter [68]. In dimethylbenzanthracene-initiated mouse skin tumors, for instance, a high number of tumors promoted by mirex (an organochlorine pesticide) contain a characteristic A → T transversion in codon 61 of Ha-*ras* [69].

The association that we observed of DDE and DDT with the GGT → GTT transversion [22,23] could point to a molecular ''fingerprint'' of the DDT family [70], or, more likely, it could reflect the action of some carcinogen promoted by DDE or DDT, such as benz[*a*]pyrene, which has been associated with G → T transversions [12,17]. In our study PCBs were not associated with any specific mutation, which again might reflect a wider promoting effect upon a variety of carcinogens. The expression of several members of the cytochrome P450 (CYP) superfamily (including CYP1A1/A2, CYP1B1, CYP2B1/B2, and CYP3A) is induced by DDE and PCBs (either by the nonplanar, di-*ortho*-chlorinated PCBs highly prevalent in human cells rich in lipids or by coplanar, dioxin-like PCBs, aryl hydrocarbon receptor agonists present at much lower concentrations in humans but which are highly carcinogenic) [24,46,71–73]. CYP1A1 and CYP1B1 are examples of enzymes involved in the activation of environmental carcinogens. In EPC, CYP1A1 polymorphisms have been found to be associated with levels of aromatic DNA adducts [57].

Inhibition of apoptosis is one possible mechanism of tumor promotion [74]. A change in the balance of effector functions can determine whether *ras* causes pro- or anti-apoptotic responses [10,75,76]. Phosphoinositide 3-kinase and, under some circumstances, raf, may act as ras effectors, which are thought to be involved in anti-apoptotic stimuli [76,77]. Addition of heptachlor to human lymphocytic cells causes a marked increase in the expression of activated extracellular signal–regulated kinase 1 and extracellular signal–regulated kinase 2 [78], which are ras effectors through the raf cascade. Another OC, tetrachlorodibenzo-*p*-dioxin (TCDD), has been found to be nongenotoxic in short-term in vitro tests but strongly carcinogenic in two-stage models of hepatocellular carcinogenesis in female rats. TCDD prevents apoptosis in the human cell line MCF-10A by stimulating the autocrine production of *ras* inducers [79]. TCDD also may promote tumors by contributing to downregulation of Ki-*ras* and stimulation of raf-1 [80]. Because Ki-ras p21 functions in transmembrane signaling, it is interesting that the membrane/cytosol Ki-ras p21 ratio may be influenced by TCDD and PCBs [81].

Inhibition of reactive oxygen species production [82] or nuclear factor kappa B (NF-κB) activation [83] has been shown to block oncogenic *ras* transformation. Furthermore, because NF-κB inhibition causes *ras*-transformed NIH3T3 cells to undergo apoptosis [75], the role of NF-κB activation in *ras,* and possibly *rac*, transformation may be to cause upregulation of as-yet-unidentified anti-apoptotic genes. The anti-apoptotic role of NF-κB has been described for other extracellular stimuli [84].

ORGANIC SOLVENTS AND *ras* EFFECTS

Organic solvents have been shown to be associated with *ras* gene activation in human hepatocarcinoma, myeloid leukemia, and EPC [29,85,86]. In animal models, 1,3-butadiene and vinyl chloride, a degradation product of chloroethylene solvents (perchloroethylene and trichloroethylene), induces *ras*-mutated tumors [87–91]. The carcinogenicity of tricholoroethylene may be related to downregulation of apoptosis [92]. Vinyl chloride also is linked to *ras* alterations in humans [55,85,93]. Although they were based on low numbers of subjects, other studies did not find a correlation between exposure to organic solvents (mainly benzene) and altered expression of *ras* genes in healthy human populations [94–96]. While benzene can induce GC-to-AT transitions and GC-to-TA transversions [97], it seems to be weakly effective in inducing point mutations. Another aromatic solvent, styrene, produces high levels of O^6-styrene guanine adducts and elevated rates of mutant hypoxantine guanine phosphoribosyl transferase [98]. O^6-guanine adducts are considered promutagenic lesions that may induce GC-to-AT transitions [99]. The chlorinated solvent methylene chloride also has been reported to promote growth of cells with spontaneous *ras* lesions in the liver and in the lung [100]. Finally, some organic solvents are the substrate of several polymorphic enzymes [101,102] that have been found to be associated with Ki-*ras* mutations [103]. Therefore, the available evidence suggests that organic solvents may act via enzymatic induction of *ras* mutagens or by providing a proliferation advantage to *ras*-mutated cell clones [29].

COFFEE DRINKING AND *ras* EFFECTS

In agreement with our findings [30–34], a study conducted in California [35] showed that EPC patients with Ki-*ras*–mutated tumors were almost three times more likely to be in the upper category of coffee consumption than patients without a mutation (odds ratio = 2.78). Because this study probably suffered from a small bias [104], the association was probably stronger, with a true odds ratio closer to the figure of 3.65 that we noted [30]. Thus, both studies suggest that caffeine, other coffee compounds, or other factors with which coffee drinking is associated could modulate Ki-*ras* activation in EPC. Additional confirmation of the interaction between coffee and Ki-*ras* could yield new insights into such processes as cell-cycle checkpoints, signal transduction, genomic stress, DNA repair, apoptosis, and the role of metabolic polymorphisms [30–33] in *ras* biology.

The most accepted role of caffeine in carcinogenesis is interference with DNA repair [30–33,105]. Caffeine has been shown to inhibit repair of ultraviolet-induced DNA damage in several cell-culture models [106–109]. Caffeine inhibits both the ataxia telangiectasia gene and ataxia telangiectasia and Rad-3-related kinases at millimolar doses [110,111]. When the protein kinases ataxia telangiectasia mutated gene and ataxia telangiectasia and Rad-3-related detect double-strand breaks, they phosporylate p53 and produce its dissociation from the negative regulator MDM2. Then, p53 undergoes further modification and activates transcription of genes responsible for cell-cycle arrest. Under certain circumstances, p53 also activates transcription of genes responsible for apoptosis. The dysfunction of this cascade of events is oncogenic, with the inactivation of ataxia telangiectasia mutated gene kinase being an alternative to *p53* mutation [112]. As a result of inactivation of p53 functions responsible for control of the G_1 checkpoint and/or DNA repair, chromosomal breaks may occur in dividing cells [113]. Caffeine also has been shown to modulate apoptosis [114], which might help Ki-*ras* mutations prosper, overriding apoptosis. The lack of specific Ki-*ras* mutations that we found among coffee drinkers [30] also supports the hypothesis of the indirect role of caffeine in *ras* carcinogenesis (e.g., obstruction of *ras* repair). Finally, caffeine and its metabolites interact with enzymes like CYP1A2, CYP2A6 [115], CYP2E1 [116], and xanthine oxidase [117], which are involved in the metabolism of *ras* carcinogens.

THE FUTURE: PUTTING INTO PLACE MORE PIECES OF THE PUZZLE

Knowledge is continuing to emerge on the potential influence of environmental agents upon *ras* effects. For instance, cigarette smoking and occupational exposure to asbestos have been associated with Ki-*ras* mutations in patients with lung adenocarcinoma [118,119]. In animal models, nitrosamine compounds produce *ras* mutations, allowing for the proliferation of pancreatic, lung, and liver carcinomas [120–122]. Associations between dietary habits and specific Ki-*ras* mutations also are observed in patients with colorectal cancer [123]. Exposure to nitroso compounds has been associated with G-to-A transitions in codons 12 and 13 of Ki-*ras* in colorectal cancer patients with high meat consumption and in tumors induced in rodents [122,124].

Dietary fat is reported to modulate *ras* functions during the promotion and progression of colon cancer [125,126]. Experiments in mice show that corn oil (a widely used fat in the American diet) enhances the expression of farnesyl protein transferase and that fish oil (rich in omega-3 polyunsaturated fatty acids) reduces its levels of expression [126]. This enzyme catalyzes a critical step (farnesylation of ras precursors) in the modification of ras oncoproteins, allowing their anchorage to the plasma membrane. On the other hand, high levels of folate, carotene, and vitamins may have a protective effect against mutations in oncogenes and suppressor genes,

perhaps through the correct maintenance of mechanisms of DNA synthesis and repair. Thus, in chemically induced lung carcinogenesis in mice, a diet supplemented with vitamin E reduces the frequency of Ki-*ras* mutations [127].

To conclude, we openly admit that the proposed interpretative scenarios may still be too broad, unspecific, or even speculative. Many fragments of the puzzle [10] may still be missing for a meaningful integration of knowledge on the potential environmental causes of altered *ras* effects. Yet we believe that the magnitude of the task should not deter us from trying. We hope others will build further upon our models, support or contradict them, and thus provide new avenues for acquiring knowledge.

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