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Point Mutations of *ras* Genes in Human Adrenal Cortical Tumors: Absence in Adrenocortical Hyperplasia

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Abstract: Point mutations of *ras* genes (K-, H-, and N-*ras*) at codons 12, 13, and 61 and of the $G_i2\alpha$ gene at codons 179 and 205, were studied in 56 primary adrenal cortical tumors and 6 adrenal cortical hyperplasias. Of 56 tumors, 24 were carcinomas and 32 were benign. The 24 carcinomas and 20 of the benign tumors were from American patients; the 12 remaining adenomas were from Japanese patients. Of the benign tumors 12 were cortisol-producing adenomas, 15 were aldosterone-producing adenomas, 3 were nonfunctioning adenomas, and 2 were adenomas that produced a virilizing syndrome. Tumor DNA obtained from archival formalin-fixed, paraffin-embedded tissue or fresh frozen tissue was amplified by polymerase chain reaction; and point mutations were detected by sequence-specific oligonucleotide hybridization. Activating *ras* mutations were found in 7 of 56 (12.5%) of all tumors: 3 of 24 (12.5%) carcinomas and 4 of 32 (12.5%) adenomas. Of adenomas from an American population, 4 of 20 (20%) exhibited positive *ras* mutations, whereas none was present in the Japanese tumors. All mutations detected were adenine to guanine transitions at the second position of N-*ras* codon 61, resulting in a conversion from glutamine to arginine. No mutations were found in K-*ras* or H-*ras* genes. Furthermore, no mutations of the $G_i2\alpha$ gene were identified. These findings demonstrate that N-*ras* mutations at codon 61 may contribute to the genesis of both benign and malignant human adrenal cortical tumors. Finally, no mutations of the *ras* or $G_i2\alpha$ genes were identified in hyperplastic adrenocortical tissues.

Studies have demonstrated that genetic alterations—*activation* of proto-oncogenes by point mutations, rearrangements, or amplification or *inactivation* of tumor suppressor genes—are involved in the carcinogenesis of various human tumors. Among the genetic alterations identified, activation of K-, H-, and N-*ras* proto-oncogenes by single point mutations at codons 12, 13, or 61 are one of the most prevalent aberrations [1]. Mutated *ras* oncogenes have been recognized to be important in the development of human colon and exocrine pancreatic cancers [2–4], for example. The prevalence of *ras* mutations in adrenal cortical cancers, however, has not been thoroughly evaluated, possibly because of their relative rarity and the difficulty of accumulating a sufficient number of cases at one institution. At this time, only 15 adrenal cortical cancers have been studied for all possible *ras* mutations, and no *ras* mutations have been detected [5].

In addition, controversy exists concerning the importance of mutations of the $G_i2\alpha$ gene in the pathogenesis of adrenocortical tumors. Like the mutated *ras* proteins, a mutated $G_i2\alpha$ protein acts as a signal transducer that fails to "turn off," thereby stimulating growth autonomously [6–8]. Mutations at codon 179 of the $G_i2\alpha$ gene have been reported by Lyons et al. [9] to occur in 3 of 11 human adrenal cortical tumors (one cancer, two adenomas). However, a more recent study of 18 adrenal neoplasms and 2 human adrenocortical tumor cell lines by Reincke et al. [10] failed to demonstrate any mutations of the $G_i2\alpha$ gene. Mutations at codon 205, in addition to mutations at codon 179, also have been reported to activate the $G_i2\alpha$ gene into an oncogene referred to as gip2 [6–8]. Therefore we investigated the prevalence of *ras* mutations and of mutations of the $G_i2\alpha$ gene at codons 179 and 205 in both adrenocortical tumors and hyperplasia.

Materials and Methods

Patients and Tissues

Tissues from 62 patients with adrenal cortical tumors ($n = 56$) or adrenocortical hyperplasia ($n = 6$) who were treated between 1942 and 1991 were studied. Of these tumors, 24 were carcinomas and 32 others were benign. Of the latter group, 12 were cortisol-producing adenomas, 15 were aldosterone-producing adenomas, 3 were nonfunctioning adenomas, and 2 were adenomas with virilizing syndrome; tissue from six patients with adrenocortical hyperplasia due to pituitary corticotropin (ACTH)-producing adenomas was also studied. These specimens were largely selected from the files of the Department of Surgical Pathology and the Registry of Neoplastic Disease at the University of Chicago Medical Center, although, 12 adenomas resected at Tokyo Women's Medical College were also studied.

After reviewing the histology by light microscopy and con-

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Table 1. Primers used for PCR amplification of K-, H-, N-*ras* and $G_i2\alpha$ genes.

Gene/codon	Primers	Annealing temperature (°C)	PCR product (basepairs)
K-*ras*/12,13	5′-GACTGAATATAAACTTGTGG-3′ 5′-CTATTGTTGGATCATATTCG-3′	55	107
K-*ras*/61	5′-GCAAGTAGTAATTGATGGAG-3′ 5′-AGAAAGCCCTCCCCAGTCCT-3′	56	111
H-*ras*/12,13	5′-GACGGAATATAAGCTGGTGG-3′ 5′-TGGATGGTCAGCGCACTCTT-3′	55	63
H-*ras*/61	5′-AGACGTGCCTGTTGGACATC-3′ 5′-AACACACACAGGAAGCCCTC-3′	60	100
N-*ras*/12,13	5′-GACTGAGTACAAACTGGTGG-3′ 5′-CTCTATGGTGGGATCATATT-3′	55	109
N-*ras*/61	5′-GGTGAAACCTGTTTGTTGGA-3′ 5′-CTTGCTATTATTGATGGCAA-3′	52	123
$G_i2\alpha$/179	5′-GGAGCGTATTGCACAGAGTG-3′ 5′-AGGTCCTTGAAGGTGAAGTG-3′	58	108
$G_i2\alpha$/205	5′-TCTGCAGGATGTTTGATGTG-3′ 5′-CTCAAGGCTACGCAGAAGAT-3′	58	100

PCR: polymerase chain reaction; G: guanine; A: adenine; C: cytosine, T: thymine.

firming the original diagnosis, we selected 101 appropriate tissues, 89 of which were from formalin-fixed and paraffin-embedded tissue blocks obtained at the University of Chicago. Of these 89 specimens, 40 were from primary tumors, 14 were metastatic lesions (4 liver, 4 lymph nodes, 6 other sites), 12 were from 6 patients with hyperplastic lesions, and 23 were control tissues (e.g., adjacent uninvolved adrenal gland, spleen, or lymph nodes).

In all of these specimens, DNA was extracted from both tumor and control tissues as described by Goelz et al., [11] with slight modification [12]. A section of each paraffin block was stained with hematoxylin and eosin to confirm the presence or absence of tumor cells. In addition, fresh frozen tumor tissues from 12 adenomas obtained from Japan were investigated. DNA from these tumors was extracted from 100 to 200 mg of this tissue by proteinase K digestion and phenol-chloroform extraction as previously described [13].

Polymerase Chain Reaction

DNA amplification was performed by polymerase chain reaction (PCR) using a DNA Thermal Cycler 480 (Perkin-Elmer/Cetus Corporation, Norwalk, CT, USA). The primers were 20 base length oligonucleotides complementary to sequences flanking codons 12, 13, and 61 of K-, H-, and N-*ras* genes and codons 179 and 205 of the $G_i2\alpha$ gene (Table 1). Sequences were amplified to give products between 63 and 123 basepairs (bp). All oligonucleotides used were synthesized by a 380B synthesizer (Applied Biosystems, Inc., Foster City, CA, USA) with the exception of the MUTALYZER probe panels for the *ras* series (Clontech Laboratories, Palo Alto, CA, USA). PCR was performed as previously described [14]. PCR-amplified DNA products were verified by 4% NuSieve agarose gel (FMC Bioproducts, Rockland, MD, USA) electrophoresis and visualized by ethidium bromide staining. In a few cases when the initial PCR failed to amplify sufficient product for dot blot hybridization, a second PCR was employed: 5 μl of initial PCR product was used as the template DNA, and PCR was carried out under the same conditions.

Dot Blot Hybridization

Point mutations were analyzed using sequence-specific oligonucleotide hybridization (SSOH) as previously described [14]. Briefly, PCR products were applied and immobilized to 0.2 μm pore hybridization transfer membranes (MSI, Westborough, MA, USA). Twenty base oligonucleotide probes specific for the wild-type and mutant sequences at codons 179 (six mutant codons tested) and 205 (eight mutant codons tested) of the $G_i2\alpha$ gene were designed according to the published sequences of Itoh et al. [15]. As mentioned above, the probes for codons 12, 13, and 61 of K-, H-, and N-*ras* were commercially obtained. All probes were 5′-end-labeled using T4 polynucleotide kinase (Pharmacia, Piscataway, NJ, USA) and gamma-^{32}P-adenosine triphosphate (6000 Ci/mmol) (DuPont Company, Wilmington, DE, USA), and were purified using G-25 Sephadex columns (Boehringer Mannheim Corp., Indianapolis, IN, USA). The membranes were prehybridized, hybridized, washed, and autoradiographed as previously described [14]. The high stringency washing temperatures were increased to 59° to 61°C, and the exposure time was limited to 1 to 4 hours. For every point mutation identified, both the tumor and corresponding control (if available) tissue DNAs were amplified and hybridized at least once more to confirm the results.

DNA Sequencing

The sequence of DNA at codon 61 of the N-*ras* gene was studied in one adrenal cortical cancer and in splenic tissue from the same patient using the dsDNA Cycle Sequencing System (Life Technologies, Grand Island, NY, USA). The PCR-amplified N-*ras* codon 61 DNA products were purified in a Centricon 100 microconcentrator tube (W.R. Grace & Co., Beverly, MA, USA). A 5 μl aliquot of the product was electrophoresed on a 2% agarose gel, visualized by ethidium bromide staining, and quantified by comparing the relative intensity of the molecular weight marker Phi X 174 RF DNA/Hae III fragment (Life Technologies). N-*ras* codon 61 PRI-MATE™ 3′-amplimer 40 pmol (Clontech Laboratories) was end-labeled as described

above. The sequencing reaction was performed per the manufacturer's protocol with the following exceptions: 50 to 100 fmol of DNA was employed, and the cycling program was altered to 20 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension/termination at 70°C for 1 minute, followed by 10 cycles of denaturation at 94°C for 1 minute and extension/termination at 70°C for 1 minute. A 4 μl aliquot of the sample was electrophoresed through a 6% polyacrylamide/TBE-urea gel (Life Technologies) at 60 watts for 1.0 to 1.5 hours. The gel was transferred and exposed to film for 2 to 14 hours.

Results

Activating *ras* mutations were found in 7 of 56 (12.5%) of the adrenocortical tumors: 3 of 24 (12.5%) carcinomas and 4 of 32 (12.5%) adenomas. All seven tumor samples with a *ras* mutation were from American patients. Thus the prevalence of *ras* mutations in the 44 tumors collected from American patients is 16% overall: 12.5% in carcinomas and 20% in adenomas. None of the tissues from 12 benign adenomas obtained from Japanese patients contained a *ras* mutation. The reasons for these differences remain unknown.

All mutations detected were adenine (A) to guanine (G) transitions at the second position of N-*ras* codon 61, resulting in a conversion from the amino acid glutamine to arginine (Fig. 1). DNA sequencing autoradiographs from the one adrenocortical cancer that was studied (patient M.K., Table 2) and its splenic control tissue are shown in Figure 2.

Among the three N-*ras* mutations in the malignant lesions, one was identified in a nonfunctioning carcinoma from a 46-year-old male patient (M.K.) who died of this malignancy 8 months after adrenalectomy (Table 2). The second was detected in a functioning carcinoma that resulted in Cushing syndrome and virilization in a 41-year-old woman (M.C.K.) who died of cancer 2 months after a biopsy of her adrenal tumor. The final mutation was identified in a large carcinoma (3065 g: 28.0 × 19.0 × 8.5 cm) associated with a virilizing syndrome in a 29-year-old woman (B.S.) who died of massive bleeding from the inferior vena cava during the early postoperative period in 1964.

Among the four benign tumors that exhibited *ras* mutations, two were aldosterone-producing, the third was detected in a nonfunctioning adenoma, and the fourth in an adenoma that resulted in virilization. No K-*ras* or H-*ras* mutations were found in any of the 56 adrenal cortical tumors analyzed.

No mutations at codons 179 or 205 of the $G_i2\alpha$ gene were identified in any of the 56 benign or malignant adrenal cortical tumors analyzed, although amplification of tumor DNA was successful, as evidenced by the intensity of those samples probed for the wild-type gene (Fig. 3). Finally, no *ras* mutations or mutations of $G_i2\alpha$ were identified in tissue from six patients with adrenocortical hyperplasia.

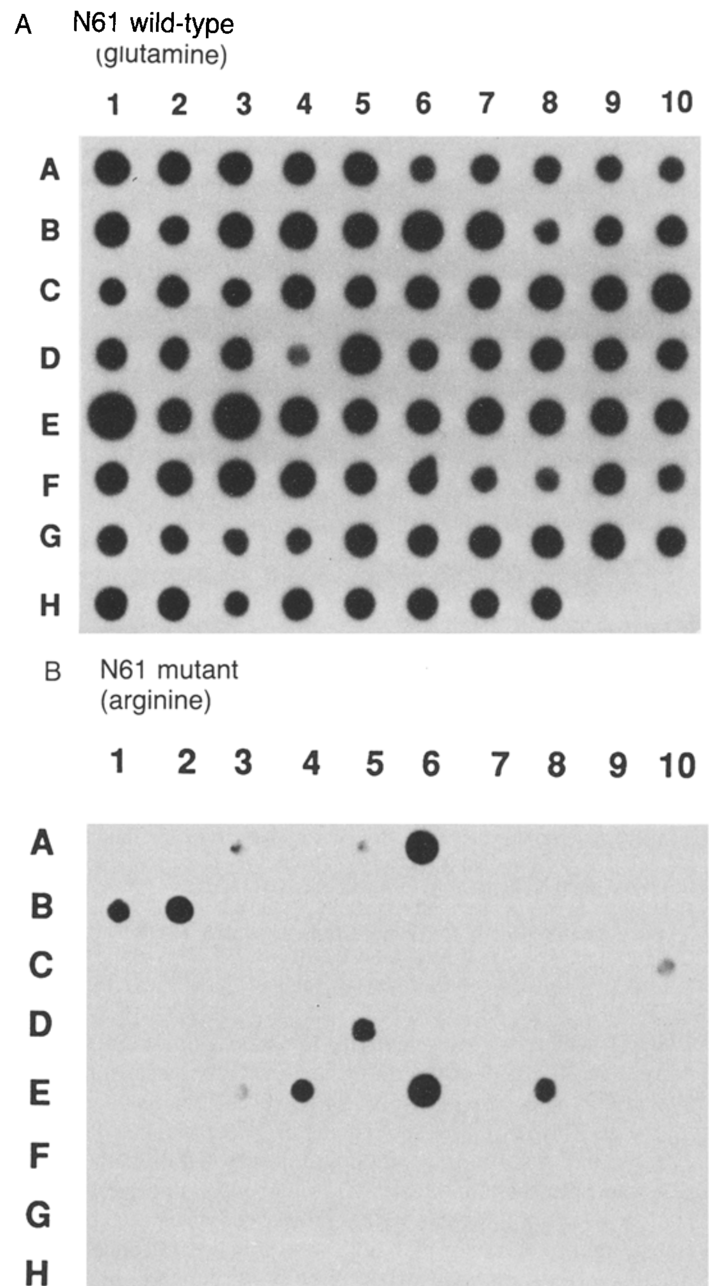

Fig. 1. Autoradiography of N-*ras* 61 hybridization. Two blots, each containing the *same* PCR product at the same position, which have been subsequently hybridized with sequence specific radiolabeled oligonucleotide probes. **A.** Wild type N-*ras* codon 61 (CAA: glutamine). **B.** Mutant N-*ras* codon 61 (CGA: arginine).

Discussion

The present study is the first to demonstrate the presence of *ras* mutations in human adrenal cortical tumors. We found N-*ras* mutations at codon 61 in 7 of 56 (12.5%) primary adrenal cortical tumors (16% from American patients) but in none of six cases of hyperplasia. In the previous literature, 47 adrenal cortical lesions had been analyzed for all possible *ras* mutations, and none was detected [5, 16]. Ohgaki et al. [5] analyzed 15 cancers and 18 adenomas by direct sequencing of PCR products and found no *ras* mutations. Yoshimoto et al. [16] found no *ras* mutations in 12 adenomas and 2 hyperplasias by PCR single strand conformation polymorphism (PCR-SSCP) analysis. One possible explanation for this discrepancy might be that different methods were used by each group. In this study we employed sequence-specific oligonucleotide hybridization

Table 2. N-*ras*/codon 61 mutations in primary adrenocortical tumors.

Patient	Age (years)	Sex	Diagnosis	Outcome
Cancer				
M.K.	46	M	Nonfunctioning	Died of cancer, 8 months
M.C.K.	41	F	Cushing & virilizing syndrome	Died of cancer, 2 months
B.S.	29	F	Virilizing syndrome	Died of operative complications, 1 day
Adenomas				
H.K.	55	F	Aldosterone-producing	Lost to follow-up
V.G.	51	F	Aldosterone-producing	Alive, no recurrence at 4 years
B.H.	43	F	Nonfunctioning	Alive, no recurrence at 3 years
H.C.	10	M	Virilizing syndrome	Alive, no recurrence at 3 years

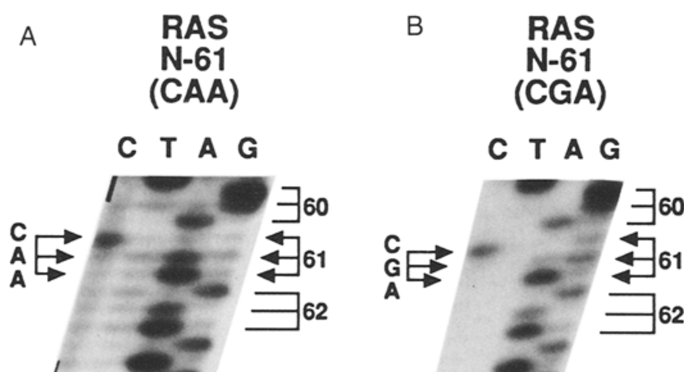

Fig. 2. Autoradiography of dsDNA sequencing. The sequences of a normal and mutated allele of the N-*ras* gene, exhibited by the DNA from the spleen (normal) and adrenal cortical carcinoma (mutated) of patient M.K. **A.** Reading from top to bottom, the spleen DNA sequence for N-*ras* gene is the normal (5′. . .GGACAAGAA. . .3′) sequence. **B.** Reading from top to bottom, the adrenocortical carcinoma sequence for N-*ras* gene has an *A* to *G* transition at codon 61 (5′. . .GGAC*G*AGAA. . .3′).

(SSOH), which has the sensitivity to detect a point mutation if more than 10% of the total DNA analyzed possesses a mutant allele [17]. This sensitivity is the same as that measured by PCR-SSCP [18] or direct sequencing of PCR products [19]. For every point mutation we found at initial hybridization, we repeated both the amplification of tumor DNA and the hybridization process at least once more to confirm our results. Furthermore, we confirmed an A to G transition at the second position of N-*ras* codon 61 by directly sequencing the DNA-PCR product of one adrenal cortical carcinoma (patient M.K.: Table 2, Fig. 2).

A second possibility for the discrepancy is a regional or geographic variation. The prevalence and pattern of *ras* mutations in other endocrine tumors appears to be influenced by environmental or genetic factors. For instance, iodine deficiency has been shown to be associated with a high prevalence of H-*ras* mutations at codon 61 in thyroid tumors [20]. Of interest in this regard is the fact that N-*ras* mutations were identified in tissue from benign tumors in 20% of American patients (4 of 20) but in none of 12 Japanese patients. The reasons for these differences remain to be clarified.

In this study N-*ras* mutations were found in both benign and malignant adrenal cortical neoplasms. This finding is not peculiar to adrenal cortical tumors, as *ras* mutations have been detected in humans in both adenomas and carcinomas such as those of the thyroid [20, 21] and colon [2, 3]. The incidence of *ras* mutations in the adenomas (20%) of American patients was slightly higher than that in carcinomas (12.5%), which suggests that *ras* mutations occur at an early stage in adrenal cortical tumorigenesis.

Table 3. Relation of *ras* mutations to outcome.

Outcome	*ras*-positive	*ras*-negative
Dead of tumor	2/2[a]	11/21
Survival time	2, 8 months	8.2 ± 10.7 years (0.16–45.0 years)[b] 4.5 ± 4.59 years (0.2–15.0 years)[c]

[a]One additional patient died 1 day postoperatively of operative complications.
[b]All 21 patients: mean ± SD and range.
[c]Patients dead of tumor ($n = 11$).

The clinical significance of *ras* mutations in adrenal cortical tumors remains unclear. The *ras* mutations do not differentiate benign from malignant tumors. Furthermore, because of the small number of patients, little can be deduced concerning the relation of a *ras* mutation to tumor virulence or aggressiveness (Table 3).

This series of adrenal cortical tumors is the largest one to be analyzed for mutations of the $G_i2\alpha$ gene. Although the amplification of tumor DNA at codons 179 and 205 was successful, no mutations were found. Our data concur with those of Reincke et al. [10] and differ with those of Lyons et al. [9], who reported mutations at codon 179 in 3 of 11 adrenal cortical tumors. The SSOH method, which we used, does not allow examination of other codons, so we cannot rule out the possibility that another codon(s) could be mutated. We conclude from our data that point mutations of the $G_i2\alpha$ gene at codons 179 or 205 are not involved in the development of adrenal cortical tumors.

Finally, the importance of other oncogenes or tumor suppressor genes in the development of adrenal cortical cancers remains to be clarified. Yano et al. [22], in an attempt to identify tumor suppressor genes, analyzed nine adrenal cortical cancers. Using polymorphic DNA probes, evidence of deletions were noted in the chromosomal arms 17p, 11p, and 13q in six of six, four of six, and three of six cancers, respectively. None of these changes was found in adenomas or hyperplastic lesions. Ohgaki et al. [5] reported that point mutations of the p53 tumor suppressor gene occurred in 3 of 15 (20%) sporadic adrenal cortical cancers. Perhaps other tumor suppressor genes or oncogenes are involved in the pathogenesis of benign and malignant adrenal cortical neoplasms.

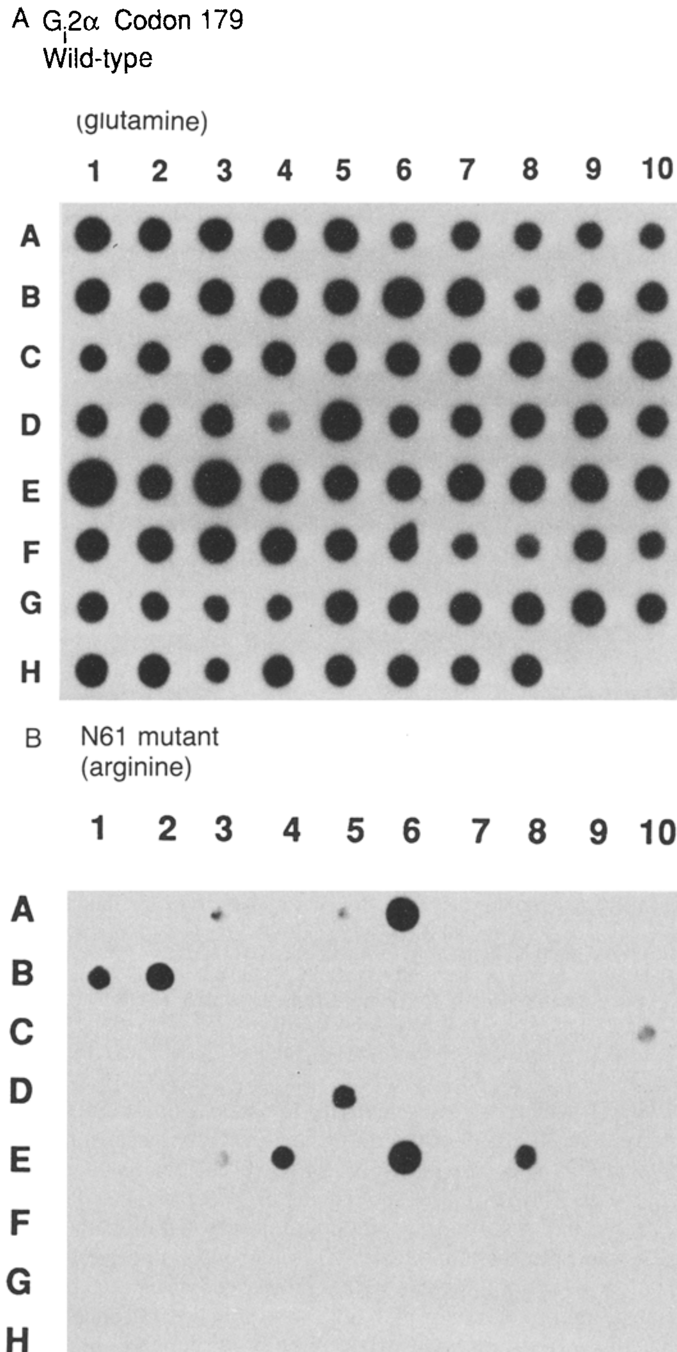

Fig. 3. Autoradiography of $G_i2\alpha$ hybridization. Tumor tissue DNAs were amplified, applied to nylon membrane, and hybridized with sequence-specific oligonucleotide probes. The signal is strong and fairly uniform for all samples, indicating that amplification of the $G_i2\alpha$ gene at codons 179 and 205 was successful. **A.** Wild-type $G_i2\alpha$ codon 179 (CGC: arginine). **B.** Wild-type $G_i2\alpha$ codon 205 (CAG: glutamine).

Résumé

On a étudié les mutations ponctuelles des gênes ras (K-, H-, et N-ras) au niveau des codons 12, 13 et 61 ainsi que des gênes Gi2α au niveau des codons 179 et 205 dans 56 tumeurs primitives et 6 cas d'hyperplasie de la corticosurrénale. Des 56 tumeurs, il y avait 24 cancers et 32 tumeurs bénignes. Les 24 cancers et 20 des tumeurs bénignes provenaient de patients d'origine américaine, alors que les autres tumeurs bénignes provenaient de patients japonais. Des tumeurs bénignes, 12 étaient des adénomes produisant le cortisol, 15 étaient des adénomes produisant l'aldostérone, 3 n'étaient pas sécretants et deux étaient des adénomes associés à un syndrome de virilisation. L'ADN tumoral obtenu sur des pièces par fixation à la paraffine et au formol a été amplifié par une réaction en chaîne polymérase et les mutations ponctuelles ont été détectées par la séquence spécifique d'hybridation d'oligonucléotides. Des mutations actives ont été retrouvées chez 7 parmi 56 (12.5%) tumeurs: 3 des 24 (12.5%) cancers et 4 des 32 (12.5%) adénomes. Parmi les adénomes de la population américaine, 4 sur 20 (20%) avaient des mutation ras+, alors qu'aucun des Japonais en avait. Toutes les mutations détectées étaient des substitutions d'adénine en guanine à la position du codon 61 N-ras, entrainant une conversion de glutamine en arginine. Il n'y avait aucune mutation des gênes Gi2α. Ces résultats démontrent que les mutations N-ras au niveau du codon 61 peuvent être impliquées dans la genèse des tumeurs bénignes et malignes de la corticosurrénale. En revanche, aucune mutation des gênes Gi2α n'a été identifiée dans l'hyperplasie corticosurrénale.

Resumen

Se estudiaron las mutaciones puntuales de los genes ras (K-, H- y N-ras) en los codones 12, 13 y 61 y del gen Gi2α en los codones 179 y 205 en 56 tumores adrenocorticales y en 6 hiperplasias suprarrenales. De los tumores, 24 eran carcinomas y 32 neoplasmas benignos. Los 24 carcinomas y 20 de los neoplasmas benignos eran de pacientes norteamericanos; los 12 adenomas restantes provenían de pacientes japoneses. Entre los tumores benignos, 12 eran adenomas productores de cortisol, 15 eran adenomas productores de aldosterona, 3 eran adenomas no funcionantes y 2 eran adenomas que habían producido un síndrome de virilización.

El ADN obtenido tejido fijado en formol, contenido en bloques de parafina o fresco congelado, fue amplificado por medio de la reacción en cadena de la polimerasa y se procedió a detectar las mutaciones puntuales mediante hibridación de secuencia específica de oligonucleótidos.

Se encontraron mutaciones ras activantes en 7 de 56 (12.5%) de la totalidad de los tumores: 3 de 24 (12.5%) carcinomas y 4 de 32 (12.5%) adenomas. Entre los adenomas de la población norteamericana, 4 de 20 (2%) exhibieron mutaciones ras positivas, en tanto que no se hallaron en los tumores de los pacientes japoneses. La totalidad de las mutaciones detectadas fueron transiciones adenina a guanina en la segunda posición del codón N-ras 61, resultante en la conversión de glutamina a arginina. No se hallaron mutaciones en los genes K-ras o H-ras; tampoco se identificaron mutaciones del gen Gi2α.

Tales hallazgos demuestran que las mutaciones N-ras en el codón 61 pueden contribuir a la génesis de los tumores suprarrenales, tanto de los benignos como de los malignos. Finalmente, no se detectaron mutaciones de los genes Gi2α en los tejidos suprarrenales hiperplásicos.

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Invited Commentary

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The *ras* and the inhibiting guanyl nucleotide regulatory proteins ($G_i2\alpha$ protein) are both involved in signal transduction and the regulation of cell growth, division, and differentiated function in a wide variety of tissues. $G_i2\alpha$ is a member of the large family of G proteins that function as "on–off" switches in coupling membrane-associated receptors to intracellular regulatory enzymes. The normal role of $G_i2\alpha$ is to inhibit the accumulation of the intracellular second messenger cAMP. A mutation of the arginine 179 codon constitutively activates this function of $G_i2\alpha$, enhancing the inhibition of cAMP [1] and resulting in neoplastic transformation in some experimental cell systems [2]. Another, more extensively studied G protein mutation, termed *gsp* for its occurrence in $G\alpha$ stimulatory proteins, results in the constitutive production of cAMP and is associated with adenoma growth in many tissues in vivo. The *ras* mutation, commonly found in carcinomas of the colon, pancreas, and lung, is among the most extensively studied of all tumor-associated mutations. The *ras* gene product is located on the inner side of the plasma membrane and is structurally homologous to the α subunit of the G protein. Mutant *ras*, like mutant G proteins, fails to "turn off" and is tumorigenic in many cells. *ras* Activation in some malignant cells may help them to invade and metastasize.

Yashiro et al. have sought evidence of the activation of *ras* and $G_i2\alpha$ oncogenes in tumors of the adrenal cortex. There are few reports of the examination of adrenal cortical tumors for these or other oncogenes. In the initial report of the $G_i2\alpha$ oncogene, it was found in one adrenal cortical carcinoma and two adrenal adenomas of 11 adrenal tumors studied [3], yet was found in none of 62 specimens studied by Yashiro et al. or in any of 12 specimens in the only other report in the literature. Conversely, *ras* mutations had not previously been found in any