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Low Frequency of *ras* Gene Mutations in Neuroblastomas, Pheochromocytomas, and Medullary Thyroid Cancers[1]

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ABSTRACT

Little is known about the prevalence and significance of *ras* gene activation in neural crest tumors such as neuroblastomas, pheochromocytomas, and medullary thyroid cancers (MTCs). Therefore, we analyzed DNA from 10 human neuroblastoma cell lines and 10 primary human pheochromocytomas for activating mutations in N-*ras*, H-*ras*, and K-*ras*. We also studied DNA from 24 primary neuroblastomas and 10 MTCs for N-*ras* mutations. *ras* genes were analyzed by direct sequencing of specific DNA fragments amplified by the polymerase chain reaction. With the exception of the SK-N-SH cell line, the examined *ras* gene sequences were normal in all the neuroblastomas, pheochromocytomas, and MTCs tested. A single point mutation was identified at codon 59 ($GCT^{ala} \rightarrow ACT^{thr}$) in one N-*ras* allele in an SK-N-SH subline. Interestingly, this mutation is different from the activating codon 61 mutation which resulted in the initial identification of N-*ras* from SK-N-SH DNA. Therefore, we analyzed the sequences of earlier passages and sublines of the SK-N-SH cell line, but mutations at codon 59 or 61 were not detected, suggesting that neither mutation was present in the primary tumor. Our results indicate that N-*ras* mutations may occur spontaneously during *in vitro* passage of cell lines but rarely, if ever, occur in primary neuroblastomas, pheochromocytomas, and MTCs. In addition, we have not found H-*ras* or K-*ras* mutations in any neuroblastoma cell line or primary pheochromocytoma.

INTRODUCTION

Activating mutations in the protooncogenes H-*ras*, K-*ras*, and N-*ras* have been identified in a wide variety of human cancers. Their prevalence has been estimated to be between 15 and 30% in general (1–3), although in specific tumors the prevalence varies from <5% in Hodgkin's disease (4) to almost 90% in pancreatic cancers (5). Activating mutations of N-*ras*, H-*ras*, and K-*ras* in human tumors have generally been described as occurring at codons 12, 13, or 61 (1–3). Amplification of normal or mutated *ras* genes appears to be a rare mechanism of *ras* activation (6–8).

The N-*ras* protooncogene was first identified as the transforming gene of cells transfected with DNA from the SK-N-SH human neuroblastoma cell line. The N-*ras* gene was initially described as having a mutation at codon 61 in SK-N-SH cells (9). Mutations in N-*ras* have also been found in up to 20% of malignant melanomas (10–12), which, like neuroblastomas, are tumors of neural crest origin. This suggests that *ras* genes may play a more important role in cutaneous melanomas. Preliminary studies indicated that N-*ras* mutations may occur in a very small number of neuroblastomas (13, 14), but the prevalence and significance of *ras* gene mutations in this and other tumors of neural crest origin, such as pheochromocytomas and medullary thyroid cancers, have not been exhaustively studied.

In this study, we analyzed 10 neuroblastoma cell lines and 10 primary pheochromocytomas for mutations in the first and second exons of N-*ras*, K-*ras* and H-*ras*. We also examined 24 primary neuroblastomas and 10 MTCs[3] for mutations in the first and second exons of N-*ras*. We used the PCR with direct sequencing of amplified DNA fragments instead of dot blot screening using specific oligonucleotides, since the latter approach may not screen for mutations in all relevant codons. We found that *ras* mutations did not occur *de novo* in these neural crest tumors but that a mutation did develop with time in a cultured cell line.

MATERIALS AND METHODS

Tumors and Cell Lines. Neuroblastoma cell lines were obtained from the originators of the lines as well as from the ATCC. The following neuroblastoma cell lines were used: NGP, NMB, NLF (15–17), NAB (a gift from Dr. James Casper), and SMS-KAN, SMS-KCN, SMS-SAN, SMS-LHN, and LA-N-6, which were gifts from Dr. C. Patrick Reynolds (18). Five sublines or clones of SK-N-SH were analyzed for *ras* mutations. These included DNA samples derived from (*a*) several passages of an SK-N-SH subline maintained in the laboratory of G. M .B., (*b*) early and late passages (transfers 3, 6, 11, and 49) of the original SK-N-SH cell line maintained in the laboratory of J. L. B. (19–21), (*c*) SH-SY5Y, a subclone with a neuroblastic phenotype derived from SK-N-SH in the laboratory of J. L. B. (22, 23), (*d*) SH-EP-1, a subclone with an epithelial phenotype derived from SK-N-SH in the laboratory of J. L. B. (22, 23), and (*e*) an SK-N-SH subline from the ATCC. Primary neuroblastoma tissue was obtained as part of a Pediatric Oncology Group study (G. M. B.). Pheochromocytoma and medullary thyroid tumor tissues were obtained from the Department of Surgery, Washington University School of Medicine, St. Louis, MO (J. F. M., S. A. W.). All primary tumor tissue examined was taken from residual tissue obtained at the time of surgery. Tumor tissue was, in all cases, from the primary and not from a metastatic site. Tumors were frozen in liquid nitrogen within 5 min of removal from the patient and stored at −70°C.

Genomic DNA Analysis. DNA was prepared by standard detergent-proteinase K lysis, followed by organic extraction and dialysis, as previously described (24, 25). DNA was quantitated by a 4,6-diamidino-2-phenylindole fluorometric assay (26, 27). Hybridization of DNA from the SK-N-SH sublines to the hypervariable D1Z2 probe (28) after a *Taq*I digest was done as a "DNA fingerprint" to confirm the common origin of all these lines. The D1Z2 probe was generously provided by Dr. Michael Litt.

PCR. DNA segments of interest were amplified by the PCR technique (29). Oligonucleotides flanking the regions of interest in the first and second exons of N-*ras*, H-*ras*, and K-*ras* were prepared by standard oligonucleotide synthesis. The following oligonucleotides were used: N-*ras* first exon, CTGGTGTGAAATGACTGAGT (sense) and

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[3] The abbreviations used are : MTC, medullary thyroid cancer; PCR, polymerase chain reaction; ATCC, American Type Culture Collection.

GGTGGGATCATATTCATCTA (antisense); N-*ras* second exon, GTTATAGATGGTGAAACCTG (sense) and ATACACAGAGGAAGCCTTCG (antisense); H-*ras* first exon, GCAGGAGACCCTGTAGGAGG (sense) and TATAGTGGGGTCGTATTCGT (antisense); H-*ras* second exon, GATTCCTACCGGAAGCAGGT (sense) and AAAGACTTGGTGTTGTTGAT (antisense); K-*ras* first exon, ACCTTATGTGTGACATGTTC (sense) and GTTGGATCATATTCGTCCAC (antisense); K-*ras* second exon, TCTCAGGATTCCTACAGGAA (sense) and CCAGTCCTCATGTACTGGTC (antisense).

Cloning. SK-N-SH second exon N-*ras* PCR products were subcloned into the phagemid vector Bluescript (Stratagene) to confirm the presence of a heterozygous mutation. PCR fragments were blunt-end ligated into Bluescript at the *Sma*I restriction site. Colonies containing recombinant plasmids were selected and the inserts were sequenced by the dideoxy technique (30) using plasmid T3 and T7 primers.

Sequencing. PCR products were separated from primers by either Centricon-100 (Amicon) or by acrylamide gel extraction (31). The fragments were then run on a minigel for quantitation, and 200 ng was sequenced by the dideoxynucleotide chain termination technique of Sanger (30). The oligonucleotides used for PCR were also used as sequencing primers.

RESULTS

Neuroblastomas. With the exception of SK-N-SH, none of the neuroblastoma cell lines had detectable *ras* mutations, as determined by PCR and sequencing. Sequence analysis of first and second exons of N-*ras* also did not reveal any mutations in 24 primary neuroblastomas.

However, a heterozygous point mutation in codon 59 ($GCT^{ala} \rightarrow ACT^{thr}$) was found in N-*ras* sequences derived from the SK-N-SH subline maintained in the laboratory of one of us (G. M. B.). This point mutation occurs in a *Pvu*II restriction site which is present in the normal, germline DNA. Thus, *Pvu*II digestion of plasmids containing SK-N-SH-derived, second exon N-*ras* PCR fragments produced either two smaller fragments for the normal, unmutated sequence, whereas only one fragment was seen in subclones containing the mutant N-*ras* allele (data not shown). Sequencing of the PCR-amplified fragments from genomic DNA reveals both an A and a G band in the first position of codon 59, indicating that the cell line is heterozygous for the mutation (Fig. 1, *top*). This mutation was confirmed by cloning and sequencing the PCR fragments (Fig. 1, *bottom*). Sequencing of these cloned fragments demonstrated either normal (GCT) or mutant (ACT) codon 59 sequences, confirming the presence of two alleles. Interestingly, we did not find a codon 61 mutation, which had previously been reported in another subline of SK-N-SH (9) (see below).

Therefore, we analyzed several samples of the original SK-N-SH cell line, maintained in the laboratory of the originator (J. L. B.), which had been frozen at early and late passages (T-3, T-6, T-11, and T-49) (19–21). We also analyzed two clonal sublines of SK-N-SH, SH-SY5Y, and SH-EP-1 (22, 23), as well as SK-N-SH acquired from the ATCC. No *ras* mutations were found in any of these cells. We did DNA fingerprinting with a hypervariable probe D1Z2 (28) after *Taq*I digestion of representative samples from the five SK-N-SH-derived cell lines, which indicated that all had an identical pattern for all lines (Fig. 2 and data not shown), which confirmed that the difference was not based on cell line contamination. Thus, the difference we identified in the DNA sequence of the N-*ras* gene apparently was an independently acquired mutation and was not present in the original tumor.

Pheochromocytomas and MTCs. In 10 primary pheochromocytomas, the first and second exons of N-*ras*, H-*ras*, and K-*ras* were sequenced, but no mutations were noted. Finally, sequence analysis of first and second exons of N-*ras* did not reveal any mutations in 10 medullary thyroid cancers.

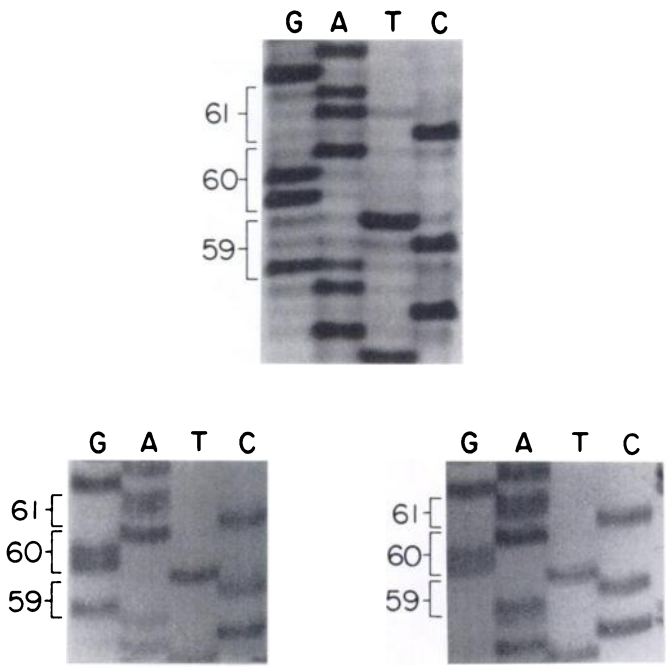

Fig. 1. *Top,* results of direct dideoxynucleotide sequencing of the PCR-amplified DNA fragment of the second exon N-*ras* DNA from the SK-N-SH cell line maintained in the laboratory of G. M. B. The sequence of codons 59–61 in SK-N-SH DNA are shown. PCR fragments were separated from primers and sequenced by the Sanger method. The first position of codon 59 contains two bands, G and A, reflecting heterozygosity for a G → A mutation. Note that there are no abnormalities in codon 61. *Bottom,* results of subcloning and sequencing of PCR fragments from the second exon of N-*ras* from the SK-N-SH cell line described above. The sequences of a normal and mutated allele of N-*ras* in SK-N-SH are shown. The sequence on the *left* has the expected normal sequence (GCTGGACAA, reading bottom to top) for N-*ras*, whereas the sequence on the *right* (ACTGGACAA, reading bottom to top) has a point mutation at the first position of codon 59 ($GCT^{ala} \rightarrow ACT^{thr}$). *Ordinates,* codons.

DISCUSSION

We have demonstrated that N-*ras*, H-*ras*, and K-*ras* mutations rarely, if ever, occur in neuroblastoma cell lines and primary pheochromocytomas and that N-*ras* mutations are rare in primary neuroblastomas and MTCs. Previous analyses of N-*ras* genes in neuroblastomas have been reported. No mutations were found in 18 tumors from one study (13), whereas 2 of 15 tumors had mutations in another study (14). The identification of mutations in only 2 tumors of 57 studied, combining our results with the two other previous reports (13, 14), virtually rules out an important role for *ras* activation in the genesis of neuroblastomas.

H-*ras* expression in neuroblastomas was analyzed in another report (32). It was found that H-*ras* expression was higher in more differentiated tumors and in earlier stages of disease, so increased *ras* expression at the mRNA level appeared to be a favorable sign. Indeed, expression of normal or transforming *ras* genes has been shown to induce neuronal differentiation in the PC12 rat pheochromocytoma cell line (33, 34) and in cultured human MTC cells (35), and microinjected antibodies to *ras* proteins inhibit differentiation in induced PC12 cells

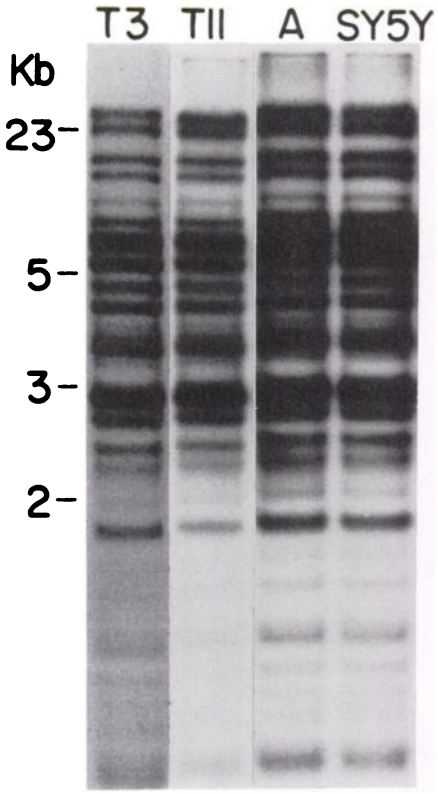

Fig. 2. Results of hybridization of various SK-N-SH subline DNAs with the hypervariable probe D1Z2 (28) after digestion with *TaqI*. Four cell line DNAs are shown, which include passages 3 and 11 from the original SK-N-SH cell line (19, 21), an SK-N-SH-subline (*lane A*) maintained in the laboratory of G. M. B. (which is heterozygous for the codon 59 mutation), and the SH-SY5Y neuroblastic subclone of the SK-N-SH cell line. The resulting DNA fingerprints show an identical pattern, indicating that all the SK-N-SH-derived cell lines used in this study were derived from the same patient. Kb, kilobase.

(36). Thus, expression of normal or mutated forms of *ras* genes in some neuroectodermal cells may be associated more with differentiation than with transformation.

Several other types of genetic lesions have been identified in neuroblastomas, such as deletions of the short arm of chromosome 1 and cytogenetic manifestations of gene amplification (15, 16, 21, 37–39). Molecular analysis indicates that deletions of the short arm of chromosome 1 most consistently involve the region from 1p36 (40, 41). Amplification of the N-*myc* protooncogene is characteristic of some primary neuroblastomas and most cell lines, and it is strongly correlated with advanced stages of disease and rapid disease progression (17, 24, 25, 39). Less is known about pheochromocytomas and MTCs, but both tumors appear to be characterized by deletion or loss of heterozygosity of the short arm of chromosome 1 (42–44), similar to that described for neuroblastomas. In addition, MTCs frequently overexpress the N-*myc* oncogene (45), although amplification has not been described. Despite the characteristic activation of a particular *myc* gene, our data indicate that activation of a complementing *ras* gene rarely if ever occurs in these tumors.

The fact that an N-*ras* codon 59 mutation was found in a late passage SK-N-SH subline is interesting, since neither this mutation nor a previously described codon 61 mutation was found in early passage SK-N-SH cells. This suggests that either the mutant cells were present in small numbers in the original tumor, and became predominant with further passage of the cells in our laboratory (G. M. B.), or that the mutations occurred during *in vitro* passage. The phenomenon of late passage cells acquiring an N-*ras* mutation at codon 12 has been described previously (46). Our results suggest either that two independent mutations occurred in sublines of SK-N-SH during growth *in vitro* or that the mutations were present in a small population of cells in the original tumor, and under *in vitro* conditions these mutations conferred a selective advantage which resulted in their predominance.

It has been demonstrated that codon 59 mutations are activating and contribute to cellular transformation (47). Indeed, codon 59 mutations have been described in the viral H-*ras* analogue, Harvey murine sarcoma virus (48), and in the viral K-*ras* analogue, Kirsten murine sarcoma virus (5). In addition, they have been described in the N-*ras* gene in a small number of human leukemias (49) but have not been described as occurring spontaneously in H-*ras* or K-*ras* genes in any human tumor. It is assumed that a codon 59 mutation resulting in an amino acid change (ala → thr) would be activating in K-*ras* and N-*ras*, because this has been shown to be activating in H-*ras* (48). Indeed, it is of interest that a base pair change in H-*ras* intron sequences has been reported which apparently causes stabilization or increased half-life of the mRNA (50). This is another potential mechanism of *ras* gene activation whose prevalence and significance is not known.

Most studies done to detect *ras* mutations in human tumors have used the slot blot technique, with differential hybridization of oligonucleotides of known sequence to genomic DNA or PCR fragments of unknown sequence (51). Published reports of *ras* gene analysis using this technique have not screened for codon 59 mutations and have been limited to the use of oligonucleotide probes to determine mutations in codon 12, 13, or 61. Thus, the true incidence of codon 59 mutations in human tumors is not known. The technique of PCR amplification and direct sequencing of PCR products has the advantage of giving definitive information about the codons of interest and their surrounding sequences.

In summary, we have demonstrated that N-*ras* mutations may occur spontaneously during *in vitro* passage of cell lines but rarely, if ever, occur in primary neuroblastomas, pheochromocytomas, and MTCs. In addition, we have not found H-*ras* or K-*ras* mutations in any neuroblastoma cell line or primary pheochromocytoma. In the one case in which an N-*ras* mutation was found, it involved codon 59. Since such mutations would not be identified by most studies which rely on oligonucleotide hybridization, either additional oligonucleotides or a sequencing approach should to be used to detect these mutations. Finally, caution should be used in interpreting the significance of *ras* mutations identified in cell lines that have been in continuous culture.

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Note Added in Proof

A codon 61 mutation in H-*ras* was noted in 1/18MTCs by Takai *et al.*, Henry Ford Hosp. Med. J., *37:* 112–115, 1989.

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