Abstract
Although N-nitroso compounds (NNC) are ubiquitous in the human environment and are known neurocarcinogens in animal models, results of epidemiological studies have not yet convincingly associated NNCs with brain tumor occurrence in humans. Animal studies have suggested that specific codons (12, 13, and 61) in the ras family are mutable by exposure to NNCs. The purpose of this study was to measure the presence of mutations in the ras family of oncogenes in tissue from childhood brain (CB) tumors as a preliminary step toward investigating potential occurrence in humans. Animal studies have suggested that specific codons (12, 13, and 61) in the ras family are mutable by exposure to NNCs. The purpose of this study was to measure the presence of mutations in the ras family of oncogenes in tissue from childhood brain (CB) tumors as a preliminary step toward investigating their potential use as biomarkers of chemical exposure. DNA was extracted from paraffin-embedded formalin-fixed CB tumors from tissues resected during neurosurgical operations. Using the PCR, designed RFLP-screening methods, and sequencing, we attempted to screen brain tumors from 46 children for the presence of H, K, and N-ras mutations at codons 12, 13, and 61. Screening for oncogene mutations using PCR, RFLP methods, and DNA sequencing was successfully completed for a high proportion of the available specimens. Astrocytoma specimens from three children for whom screening with PCR was successfully completed were found to contain CAA → GAA point mutations in K-ras at codon 61. None of the specimens contained mutations at any of the other locations. These results, although preliminary, provide a potential clue for future mechanistic studies of CB tumors. The possible roles of NNCs in inducing this mutation, or of this mutation as an early or late event in tumor progression, however, remain unclear.

Introduction
ras oncogene mutations of varying types have been detected in approximately 20–25% of all human tumors (1–4), with specific mutation patterns more commonly associated with tumors of different types and at different locations (1, 5). NNCs3 have been shown to induce tumors in several animal models (6, 7) and ras activation has been recognized as a potential mutational event in many of these tumors (8–11). This animal evidence, coupled with evidence of specificity of ras mutations by tumor type, suggests that NNCs may be associated with tumorigenesis in humans, and that ras oncogene mutations may have potential as biomarkers of NNC exposure in humans.

Despite the convincing animal evidence of an NNC-brain tumor association via transplacental exposure in young rodents (7, 9, 12), the causes of brain tumors in humans remain largely unknown. If ras oncogene mutations similar to those observed in NNC-induced tumors in rodents are detected in childhood brain tumors, this may provide evidence for an NNC-childhood brain tumor association via a similar pathway.

Materials and Methods
Subjects. Paraffin-embedded formalin-fixed brain tissue specimens were obtained from 46 children who had undergone neurosurgical operations for brain tumors at Children's Hospital Medical Center in Seattle, WA. All pathology specimens were reviewed by a neuropathologist and were classified by histology. A majority of these children (34, or 74%) were also involved in a large, multicenter case-control study of risk factors for childhood brain tumors diagnosed between 1984 and 1990; for these, demographic information was also available. For 12 of the children, however, no demographic details were available because their specimens were accessed from a general pool of tissue blocks comprised of excess specimens obtained routinely during operations and made available for research without access to patient-identifying information. Tumors were classified based on the neuropathology report as astroglial (ICD-9 9380–9384, 9400–9421, and 9424–9442), PNET (ICD 9362, 9470–9473, and 9500), or other. Access to specimens and laboratory procedures for this project was approved by the Institutional Review Boards at the Fred Hutchinson Cancer Research Center and Children's Hospital Medical Center (Seattle, WA).

DNA Extraction. Paraffin-embedded formalin-fixed brain tissue specimens were assayed for mutations in ras genes. In the laboratory, 10–40-μm-thick sections of the formalin-fixed paraffin tissue blocks were cut and stored in sterile Microfuge tubes for DNA extraction. Extraction of DNA involved a modification of a method described previously (13). Briefly, samples were deparaffinized using one extraction with xylene, followed by one rinse with 100% ethanol and then dried down in a 55°C heat block. Tissues were then resuspended in a buffer containing 7.5 mM Tris-HCl (pH 8.5), 37.5 mM KCl, 1 mM MgCl2, 0.5% Tween 20, and 20 μg/ml protease K and incubated in a 55°C heat block for at least 3 h. Samples containing

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1 The abbreviations used are: NNC, N-nitroso compound; PNET, primitive neuroectodermal tumor; NMU, N-nitroso-N-methylurea.

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more tissue were incubated overnight. Samples were then boiled for 10 min, centrifuged, and then used for PCR or stored at 4°C. Some samples were extracted according to a modified protocol (14) including a DNA purification step of an extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and one of chloroform following the digestion step above.

**RFLP and Sequencing to Detect ras Mutations.** Analysis of samples for ras mutations were performed using a modified screening method for mutations in the ras genes (15). This method used PCR and designed primers that created RFLP to distinguish between mutated and nonmutated ras genes. The analysis was designed to look at mutations at the 12th, 13th, and 61st codons of H-, K-, and N-ras. For this study, 1–10 μl of the sample extractions were used to amplify products. Generally, the PCR conditions were initial denaturation at 94°C for 2 min followed by 30–40 cycles of denaturation (94°C for 1 min), annealing (at the specific temperature for the primer set, 1 min), and extension (72°C for 1 min). After PCR, the products were then subjected to the appropriate restriction enzyme digestion and run on 2–4% agarose gels or 8% polyacrylamide gels to observe cutting patterns. This assay was designed so that if there were mutations, the restriction enzyme would not cut the PCR product. Samples that were not cut completely by the restriction enzyme (indicating mutation in one allele) were gel purified and sequenced for confirmation.

The primers originally designed for RFLP to detect mutations at codon 61 of N-ras did not amplify efficiently because there was a mismatch at the 3' end of the sense primer. Because of this problem, we chose to directly sequence the PCR product. A new sense primer was designed which did not contain any mismatches (5'-gat tct tac aga aaa caa gtg-3'). Using this primer and the previous original antisense primer, PCR amplification was performed at an annealing temperature of 54°C. The amplified product was purified by electrophoresis on a 2% agarose gel, excised, and centrifuged in a 0.2 M Spin-X column (Costar). PCR cycle sequencing was accomplished using the dideoxy termination method by end labeling one of the primers (with γ-32P]ATP) using the fmol Sequencing System (Promega). An example of this sequencing is given in Fig. 1.

**Screening for H-ras-61 required a heminest strategy because two mismatches in the internal antisense primer decreased the efficiency of the PCR.** The screening protocol required initial amplification of a larger 285-bp product which was then used as template to amplify the target sequence. It was required initial amplification of a larger 285-bp product which increased the efficiency of the PCR. The screening protocol caused two mismatches in the internal antisense primer and direct sequencing were used for the determination of mutations at this site. A, control DNA without mutation at codon 61 (lymph). B, control DNA with mutation at codon 61 (HL-60). Arrow, A to T mutation.

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**Table 1** Demographic and disease characteristics of 46 children with brain tissue specimens assayed for the presence of ras oncogene mutations

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis* (yr)</td>
<td></td>
<td></td>
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<tr>
<td>&lt;5</td>
<td>16</td>
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</tr>
<tr>
<td>5–9</td>
<td>13</td>
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<td>15+</td>
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<td>5.9</td>
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<td>58.8</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma/glioma</td>
<td>25</td>
<td>54.3</td>
</tr>
<tr>
<td>PNET</td>
<td>12</td>
<td>26.1</td>
</tr>
<tr>
<td>Other</td>
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<td>10.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>8.7</td>
</tr>
</tbody>
</table>

* Information missing for 12 children.
Results

General Characteristics of Subjects. We attempted to assay a total of 56 paraffin-embedded brain tissue specimens from 46 children for the presence of ras family oncogene mutations. Nearly one-half (47.1%) of the children for whom demographic information was available were less than 5 years old; 38.2% were between the ages of 5 and 9 years (Table 1). The majority (58.8%) were male. Over one-half of the specimens were from astrocytomas and glial tumors (54.3%); approximately one-quarter (26.1%) were PNETs.

Results of Assay for ras Mutations. A total of 56 specimens were obtained; 37 of the 46 children had a single tissue block available for assay, 8 children had two specimen blocks, and 1 child had three specimen blocks. Five of the 56 specimens contained nontumor brain tissue resected during the neurosurgical operation.

Although we attempted to screen all specimens from tissue blocks for K-, N-, and H-ras mutations at codons 12, 13, and 61 (a total of 9 oncogene screens which included 11 separate assays for each specimen), not all specimens contained a sufficient quantity or adequate quality of DNA for successful PCR amplification, resulting in a slightly lower number of completed assays for each ras mutation screen (Table 2). Of the 504 total ras mutation screening procedures attempted, 3 (or less than 1%) were aborted because an inadequate quantity of DNA was available for assay. We were successfully able to complete 486 (97%) of the remaining 501 screens; PCR amplification did not occur for 15.

Mutations in the K-ras oncogene at codon 61 were present in three specimens. These were observed in astrocytoma tumor tissues resected in first operations for primary tumors of three children, all female, ages 4, 5, and 12 years. Specimens from the 5- and 12-year olds were both from low-grade fibrillary astrocytomas located in the cerebellum and brainstem, respectively. Specimens from the 4-year-old were from a pilocytic astrocytoma in the ventricular region. The two younger children each had two astrocytoma tumor specimens screened, only one of which contained the K-ras mutation. Testing of these samples was repeated to confirm these results. None of these children had nontumor brain tissue available for assay. The result of the RFLP demonstrating this mutation in one of these specimens is shown in Fig. 2. Subsequent sequencing analysis (Fig. 3) indicated that all three mutations were identical and consisted of CAA → GAA transversions. No N- or H-ras or other K-ras mutations were detected at any of the three codons examined (codon 12, 13, or 61) in any of the other specimens. An example of the RFLP assay is presented in Fig. 4.

Discussion

To complete the screening for mutations in codons 12, 13, and 61 of the H-, K-, and H-ras oncogenes, at least 10 PCR amplifications per specimen were necessary. Some samples would not amplify specific PCR products, possibly due to the presence of badly degraded DNA; however, we were able to complete our battery of assays for 97% of the specimens. Mutations at K-ras-61 were detected in specimens from three children; all contained the same mutation, a C to G transversion in the first nucleotide of codon 61, substituting glutamic acid for glutamine.

The ras family of oncogenes appears to play a role for human tumors at several sites (1), and ras mutations have been shown to result from NNC exposure in laboratory animals (8, 9). Mutated ras proteins have lost the ability to be turned off and thus stimulate growth or differentiation autonomously. Point mutations identified to date that lead to ras gene activation appear to be specific and include those screened at codons 12, 13 and 61 (16). ras oncogene mutations have been detected in varying proportions of human tumors screened, ranging from 10 to 90% depending on tissue type (2). Relatively high prevalences of ras mutation have been observed in adenocarcinomas of the pancreas, colon, and lung (3, 4, 17). Of the three ras family oncogenes, activated K-ras has been detected most often in human tumors, and it has been suggested that this is due to exposure to specific environmental agents (1). The pattern of ras mutations observed in several chemically induced rodent tumors indicates that certain chemicals may exhibit specificity for mutation occurrence at specific sites on the gene (10, 11, 18, 19), raising the potential for use of ras mutations as biomarkers for selected chemical exposures.

Given the variety of oncogene characteristics examined and the wide range of patient ages included in previous studies.

Table 2. Results of ras oncogene mutation assays on 56 brain tissue specimens from children with brain tumors.

<table>
<thead>
<tr>
<th>Codon</th>
<th>N-</th>
<th>K-</th>
<th>H-</th>
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<td>12</td>
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<td>56</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
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</tr>
<tr>
<td>61</td>
<td>56</td>
<td>56</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>56</td>
<td>56</td>
<td>56</td>
</tr>
</tbody>
</table>

Fig. 2. K-ras, codon 61. Four percent agarose gel demonstrating the use of RFLP: Lanes 2, 4, 5, and 8, amplified DNA incubated with the restriction enzyme BclI; Lanes 1, 3, 5, 7, and 9, amplified DNA alone. Lane 1, molecular weight ladder; Lanes 2 and 3, control DNA without mutation at codon 61 (lymph); Lanes 4 and 5, sample 30; Lanes 6 and 7, sample 31; and Lanes 8 and 9, sample 34. Note that Lane 4 has two bands at about half the intensity of Lane 2 or 6, indicating a mutation in one of the alleles of K-ras-61.
of brain tumors, it is difficult to characterize the role of the \textit{ras} oncogenes in childhood brain tumors. Overexpression of \textit{H}- or \textit{N-ras} was reported in 0, 43, and 71\% of low-, intermediate-, and high-grade astrocytomas (20), and increased expression of \textit{N-ras} was measured in all tumors in a series of five human glioblastomas assayed (21). However, amplification or rearrangement of \textit{N-ras} was not observed in a separate series of 65 astrocytic tumors (22). These previous studies were not, however, restricted to childhood tumors. Previous reports suggest that the spectrum of chromosomal abnormalities (23) and oncogene amplification (24) differs between brain tumors of adults and children, although neither of these focused specifically on \textit{ras} oncogenes. To our knowledge, our report represents the first systematic characterization of mutations in the \textit{H}-, \textit{K}-, and \textit{N-ras} oncogenes to date.

Our results suggest that \textit{K-ras-61} mutations may be present in a measurable proportion of childhood brain tumors. Three of the 46 children in our series, or nearly 7\%, had tumor specimens containing \textit{K-ras} mutations; 3 of 25 (12\%) of those with astroglial tumors. These levels appear to be lower than the proportions of \textit{ras} mutations reported for tumors from other sites. In an earlier study, no \textit{ras} mutations were detected in any of 18 neuroblastomas, sarcomas of neural origin affecting mostly infants and children up to age 10 years (25). More recently, a mutation in \textit{K-ras} at codon 12 (G to A transition) was found in a PNET: however, this was thought to be a result of therapeutic radiation to the central nervous system (26). It would be of interest to compare the levels and specific type of mutation we observed with those measured in other series of childhood brain tumors.

It should be noted that two of the three children whose specimens contained mutations each had two tumor specimens screened, of which only one was positive for \textit{K-ras-61} mutations. Although it is possible that the lack of a mutation observed in the second specimen of these two children might be artifactual, perhaps the result of DNA degradation, we excluded from our results specimens for which PCR amplification was unsuccessful, reducing this likelihood. Furthermore, specimens containing mutations were rescreened to verify our results, and thus our positive findings are unlikely to be due to screening error. For these two children with two tumor specimens screened, this suggests that not all of the cells within these tumors contained this mutation, a finding that may be indicative of heterogeneity present within these tumors. Other possible explanations are that this mutation arose as a late event in tumor progression, or that the collection techniques for removing brain tissues also removed some adjacent nontumor tissues. The \textit{K-ras-61} mutation that we did see is, however, compatible with an hypothesis of a C to G change occurring due to NNC-induced mutational changes in C, possibly as a result of 3-alkyl or \textit{O\textsuperscript{2}}-alkyl DNA lesions. It would be interesting to know whether or not similar mutations were also present in the nontumor brain of these three children; unfortunately, such specimens were not available. Mutations were not observed in any of the five specimens containing nontumor brain tissue from other children. Future studies examining the presence of mutations in brain specimens of young neurosurgery patients without tumor, possibly from epilepsy patients, would help to clarify further the role of these mutations in childhood brain tumors. Investigations of the potential relationship between exposure to NNCs and the occurrence of \textit{K-ras-61} mutations would help us understand whether these mutations may have resulted from environmental exposures.

Although the causes of \textit{K-ras-61} and other \textit{ras} mutations in humans have not been identified, it has been suggested that they may be due to exposure to specific agents in the environment such as NNCs (27). For example, the majority of \textit{ras} mutations observed in colon cancer are G to A transitions at the second G of a GG pair, a mutation characteristic of exposure to alkylating agents such as NNC (2). Most \textit{K-ras} mutations in lung cancer are G to T transversions, a finding consistent with mutation patterns associated with mutagenic components in NNC-containing tobacco smoke (2). However, the levels of these mutations occurring in nontumor tissues is less frequently...
considered and of importance as well. One frequently observed H-ras-12 mutation in NMu- induced rat mammary tumors (aGGG to GAA mutation) was also measured in the mammary epithelium of 70% of the untreated rats (28). This level was not significantly altered following NMU exposure, leading the authors to conclude that it was an independent effect of NMU on tumor formation. An epigenetic mechanism for NMU has been proposed whereby NMU-induced alterations in promoter configuration irreversibly deregulated H-ras-1 expression and causing mammary carcinogenesis (29). A similar scenario is certainly possible for the K-ras-61 mutations we observed in brain tumors; unfortunately, we cannot gain no further insight from the five nontumor brain specimens we assayed with negative results. It will be of interest to expand our work to a larger number of children for whom both tumor and normal tissues are available and for whom epidemiological data exist for potential dietary and environmental exposures to NNCs.

Application of this RFLP assay that was developed to screen for ras mutations in tumor cell lines appeared to work for archived formalin-fixed paraffin-embedded tissue sections despite some limitations. Although the majority of the RFLPs were conducted successfully with our specimens, two procedural modifications were necessary. The first modification involved screening mutations at N-ras-61; the primers originally designed did not amplify efficiently due to a mismatch at the 3’ end of the sense primer. Although the original procedure might have amplified DNA from tumor cell lines, it would not do so with formalin-fixed paraffin-embedded tissue sections, likely because of a somewhat degraded quality of DNA. Because of this, we directly sequenced the PCR product. The second modification was necessary to conduct screening for H-ras-61. Originally, a heminested procedure was attempted wherein a larger PCR fragment was first amplified followed by a second amplification using this larger fragment as a template, a procedure that resulted in inefficient amplification. This was dealt with by eliminating the heminested step and directly amplifying the PCR product at a lower annealing temperature, 55°C instead of 60°C. Presumably, this second modification was necessary because of possible degradation of the formalin-fixed paraffin-embedded tissue sections.

Despite the fact that in some instances as many as 10 years had elapsed since the subjects’ neurological operations, the majority (486/504, or 96%) of the screens performed using formalin-esis. Nature (Lond.), 4682-4689, 1989.

References

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