Activated N-ras Oncogenes in Human Neuroblastoma

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ABSTRACT

Fifteen primary neuroblastomas and four bone marrow samples from neuroblastoma patients, representing clinical Stages I to IV, have been screened for mutations in codons 12, 13, and 61 of N-ras. Neuroblastoma DNAs were subjected to the polymerase chain reaction to amplify the relevant sequences and were then hybridized with specific oligonucleotides to locate and identify point mutations.

The results show that one Stage I tumor contained an N-ras gene that was activated by a GC-CG transversion of the first base of codon 61, while in one Stage II tumor, activation of N-ras involved a GC-CG transversion of the first position of codon 13. N-ras activations were not detected in the six Stage III tumors and eight Stage IV tumors that were examined.

INTRODUCTION

Activating mutations in cellular ras genes have been implicated in the development of a wide range of human solid tumors and haematological malignancies (1). Three closely related protooncogenes, Ha-ras, Ki-ras, and N-ras, are all expressed in normal cells and encode homologous M, 21,000 GTP-binding proteins (p21), which are localized at the inner surface of the cell membrane and are thought to be involved in growth signal transduction (2). The ras proteins acquire transforming potential upon replacement of a single amino acid, resulting from a point mutation in codons 12, 13, or 61 of the gene (3–5). There is evidence to suggest specificity of activation of the ras oncogenes in particular tumor types. Ha-ras is activated in bladder and urinary carcinomas (6), Ki-ras in lung and colon carcinoma (7–11), and N-ras, which was originally isolated from the neuroblastoma cell line SK-N-SH (12), in a significant percentage of haemopoietic malignancies (4, 13, 14).

Neuroblastoma represents, after brain tumors, the most common solid tumor of childhood, with an annual incidence of 8 per million children under 15 yr of age, and with most occurring in the first 3 yr of life. The prognosis varies from spontaneous regression of congenital tumors and almost 100% survival of children with localized tumors to less than 10% survival with nonlocalized, disseminated tumors in children older than 1 yr of age (15). In addition to the known prognostic factors such as age and stage of tumor (16), it has been recently demonstrated that increased copy number of the protooncogene N-myc (17, 18) or its increased expression (19) is associated with progressive growth of the tumor.

In contrast to the role of N-myc, several reports associate expression of ras oncogene M, 21,000 proteins with the morphological differentiation of neural tissue (20–22). More recently, Tanaka et al. (23) showed that expression of the Ha-ras protein product correlated with a favorable prognosis in neuroblastoma. Given the possible involvement of ras in differentiation of tissues and/or tumors relating to the nervous system and the initial isolation of the N-ras gene from a neuroblastoma cell line, it was decided to screen primary neuroblastomas for mutations in the N-ras gene.

The occurrence of mutationally activated N-ras in tumor sample DNAs was investigated using a highly specific assay based on oligonucleotide hybridization (24), following in vitro amplification of the sequences of interest using the polymerase chain reaction technique (25, 26). A series of 15 primary neuroblastomas and 4 bone marrow aspirates from neuroblastoma patients have been examined in this study.

MATERIALS AND METHODS

Tumor Samples and DNA Isolation. Human tumor tissue was obtained from surgical specimens removed either at diagnosis, or following chemotherapy, from various Children's Hospitals in New Zealand and Australia as part of a tumor referral program in conjunction with the Australian and New Zealand Childhood Cancer Study group (see Table 1). Specimens were either snap frozen in liquid nitrogen immediately following removal or transported at room temperature in RPMI medium. Specimens were stored at -70°C until DNA isolation. Four samples received were bone marrow aspirates from Stage IV patients in whom metastatic infiltration of the marrow had occurred.

DNA was isolated using a modified phenol extraction technique (27). Briefly, a portion of tissue, with a volume of approximately 0.5 cm³, was placed in 1 ml of ice-cold 1.5 M KH₂PO₄-8.0 mM Na₂HPO₄-135 mM NaCl-2.7 mM KCl, pH 7.4, and subjected to disruption using a 20- to 30-s pulse from a laboratory disperser (Ystral X 10/20). Following the addition of 15 ml of lysis solution [20 mM Tris-HCl (pH 8.0)-0.5 mM EDTA-1 M NaCl-0.5% sodium dodecyl sulfate], 1 ml of proteinase K solution (3 mg/ml) was added, and the lysate was incubated, with shaking, at 37°C for 16 h. The mixture was then extracted with 15 ml of a mixture containing phenol (500 g), m-cresol (70 ml), water (55 ml), and 8-OH-quinoline (0.5 g), by stirring at room temperature for 10 min. After centrifugation at 10,000 rpm for 10 min, the aqueous layer was removed, and the DNA was ethanol precipitated. DNA was stored as ethanol precipitates at -20°C until required.

Polymerase Chain Reaction. Samples of genomic DNA (1 μg) from tumor specimens were subjected to the polymerase chain reaction as described by Saiki et al. (25). Pairs of 20-mer oligonucleotide primers corresponding to regions on either side of the 12th and 61st codons of the human N-ras gene were synthesized using the solid phase phosphoramidite method (Applied Biosystems; DNA Synthesizer 381A).

The sequences and regions spanned by the primers are as described by Verlaan-de Vries et al. (26). Following 25 cycles of denaturation, annealing, and extension using the Klenow fragment of DNA polymerase I (Amersham International, Amersham, Bucks, United Kingdom), the amplified DNA samples were denatured (98°C, 5 min), and 10-μl aliquots were applied to Gene Screen Plus (New England Nuclear) nylon membranes using a dot-blotting apparatus (Bio-Dot; Bio-Rad, Watford Herts, United Kingdom). The membranes were sequentially treated with denaturing (0.2 M NaOH-0.6 M NaCl) and neutralizing (0.8 M Tris-HCl-0.6 M NaCl (pH 7.5)) solutions, air dried, and exposed to short-wave UV radiation for 5 min prior to prehybridization.

Oligonucleotide Hybridizations. 20-mer oligonucleotides spanning the regions of codons 12 and 61 were synthesized corresponding to the wild-type sequences and to the sequences of all mutations possible at codons 12, 13, and 61 of the human N-ras gene. Sequences of the probes are listed in Table 2. The probes were labeled, and prehybridization, hybridization, and autoradiography of the filters were performed exactly as described by Ireland et al. (28). Briefly, each of the filters was hybridized to 32P end-labeled oligonucleotide probes corresponding to the wild-type sequence of the amplified codon and to a full panel of...
of the samples screened, two were found to contain activated synthetic oligonucleotide probes, following in vitro amplification of the regions surrounding codons 12 and 61 of the gene. The first, Sample NB01, contained a GC-CG transversion mutation in the first base of codon 61, causing incorporation of arginine instead of glycine in the ras protein product. Samples NB10 and NB15 also bound the wild-type probes for codons 61 and 13, respectively, indicating the presence of a normal allele in the cells containing an N-ras mutation or the inclusion of some normal tissue, with two copies of the normal gene, in the initial tumor sample. The relative intensities of the signals detected after hybridization of replicate filters with wild-type and mutant-specific probes suggest that, in the case of the codon 13 mutation (NB15), there are fewer copies of the mutant ras oncogene than of the normal ras allele in the DNA of the primary tumor. In contrast, Sample NB10 hybridized more strongly with the mutant probe than the wild type, suggesting that the activated oncogene is present in a higher copy number than the normal allele in this DNA. None of the other 17 DNA samples exhibited hybridization to probes corresponding to mutations at codons 12, 13, or 61 of N-ras, showing binding only to the wild-type probes for these codons.

Table 1 contains the patient sample details together with several recognized prognostic indicators for neuroblastoma, namely, age, tumor stage, N-myc copy number, and vanillmandelic acid:homovanillic acid ratios. Six of the tumor samples studied contained more than one copy of the N-myc gene. Three Stage III tumors displayed low to moderate amplification levels (3- to 20-fold), while three Stage IV tumors were found to contain >32-fold amplification of the gene. These results are in keeping with the predicted association of high N-myc copy number with late disease stage and poor prognosis (18).

Of the two samples shown to contain a mutation in N-ras, one represents the only Stage I tumor studied (NB10), and the other, one of four Stage II tumors (NB15). None of the six Stage III or the eight Stage IV tumors in the study were found to contain an N-ras mutation. Whereas the H-ras and K-ras genes are the cellular homologues of the Harvey and Kirsten sarcoma viruses (29), N-ras
has no such viral counterpart. N-ras was first discovered as the transforming gene present in the neuroblastoma cell line SK-N-SH (12). Analysis of the gene in these cells localized the transforming activity to a mutation in codon 61, which causes replacement of glutamine by lysine in the N-ras protein product (30). Subsequently, activated N-ras genes have been reported in both cell lines and primary tumor samples. Codon 61 mutations have been shown to occur in lung carcinoma (31), fibrosarcoma (32), rhabdomyosarcoma (33), and rectal carcinoma (34) cell lines and in primary thyroid tumors (35) and acute myeloid leukemias (36, 37). Codons 12 and 13 have been found to be activated in primary retinal tumors (38), thyroid cancers (35), and in acute myeloid leukemias (36, 37). The data presented in this paper represent the first example, to date, of N-ras activation in primary neuroblastoma tissue.

Both mutations in the current study are GC-GC transversions, one in codon 61 and one in codon 13. Of the mutations detected previously in N-ras at these codons, this type of mutation has been reported only once, for codon 13, in a primary retinal tumor (38).

N-ras mutations appear to occur relatively infrequently in primary neuroblastoma. Ballas et al. (39) reported no ras mutations in 18 neuroblastomas, and mutations were observed in only 2 of 19 samples examined here. It is of interest, however, to note that, of the 19 cases studied in this report, the 2 mutations occurred within the group of 5 Stage I and Stage II tumors. Despite the limited numbers available in this study, such a result is perhaps suggestive of a possible relationship between N-ras gene mutation and the early stages of neuroblastoma. Several lines of evidence link expression of activated ras proteins with differentiation in neural tissues. Bar-Sagi and Feramisco (21) demonstrated the effect of activated versus normal human Ha-ras protein in inducing morphological differentiation of rat phaeochromocytoma (PC12) cells, and Noda et al. (20) attributed the nerve growth factor-like activities of Kirsten and Harvey murine sarcoma viruses in the same cell line to the viral oncogenes v-Ki-ras and v-Ha-ras. Furthermore, Hagg et al. (22) reported the inhibition of nerve growth factor-induced differentiation in PC12 cells by microinjection of a monoclonal antibody which recognized all rat M, 21,000 protein species.

While none of these studies specifies the involvement of the N-ras protein, the results presented here suggest that N-ras, as well as the Harvey and Kirsten ras genes, may play a role in neural tissue differentiation.

It is obvious from the well-documented involvement of the oncogene N-myc in the progression of neuroblastoma (17–19) and the more recent demonstration of the linkage of a chromosome 1 deletion with poor prognosis in the disease (15) that at least two genes regulate the growth and characteristics of this tumor type. Interpretation of whether the results presented here represent two exceptional cases of N-ras activation in primary neuroblastoma tissue or a more general phenomenon of ras gene involvement in decreasing the aggressiveness of neuroblastoma or other neuroectodermal tumors in vivo awaits the investigation of a larger number of tumors, which is currently under way in this laboratory.

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