Expression of Ha-ras Oncogene Products in Human Neuroblastomas and the Significant Correlation with a Patient's Prognosis

Takeo Tanaka, Dennis J. Slamon, Hiroko Shimoda, Chikio Waki, Yoshinori Kawaguchi, Yoshito Tanaka, and Noriaki Ida

Division of Clinical Research, Department of Pediatrics, National Kure Hospital, 3-1 Aoyama, Kure City, Hiroshima 737, Japan [T. T., H. S., C. W., Y. K., Y. T.]; Division of Hematology and Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024 [D. J. S.]; and Department of Pediatrics, Mazda Hospital, Mazda Motor Corporation, 2-15 Aosaki-minami, Fuchu-cho, Aki-gun, Hiroshima 735, Japan [N. I.]

ABSTRACT

Neuroblastomas represent a spectrum of diseases categorized by histological subtypes, age of the patient, and extent of tumor (stage) at diagnosis. In this study we analyzed Ha-ras p21 (protein with molecular weight of 21,000) expression immunohistochemically on 47 primary human neuroblastomas resected at diagnosis. The data demonstrate that the amount of the Ha-ras product correlates with a favorable prognosis (P < 0.001) and early stages of disease at diagnosis (P < 0.05). These findings from unmanipulated human neuroblastomas indicate that the Ha-ras gene product (p21) might play a role in the mechanism(s) controlling aggressiveness in this type of tumor in vivo and that the Ha-ras p21 detected by a simple and reproducible immunohistochemical procedure may be of clinical importance in predicting prognosis in patients with this malignancy.

INTRODUCTION

The ras protooncogenes are conserved across a broad span of evolution. Some data indicate that the ras gene products may be important in transduction of membrane signals necessary for certain cell functions (1-4). In yeast, ras proteins appear to activate adenylate cyclase (5). In mammalian cells, however, their function remains uncertain. It is well known that point mutation of ras genes produces a malignant phenotype of cell (6, 7). Recently, several reports demonstrated that ras p21 genes promote the morphological differentiation of rat pheochromocytoma cell line (PC 12) into neuron-like cells and that this differentiation is accompanied by cessation of cell division (8-10). In addition, we have shown that Ha-ras p21 is expressed at high levels in normal rat brain (11). These findings suggest that the Ha-ras gene products might have a functional association with the differentiation of neural tissues.

Neuroblastomas are malignant tumors believed to be derived from the sympathetic nervous system of children. These tumors have been variously classified as neuroblastomas, ganglioneuroblastosmas, and ganglioneuromas. The prognosis for patients varies, and it is not always obvious at diagnosis what affects survival in patients with the disease. Previous studies have shown that histopathological findings, clinical stage of the disease, and age of the patient at diagnosis are factors relating to the prognosis (12-14). More recently, amplification of the N-myc protooncogene was shown to correlate directly with aggressiveness of the disease and to be an intrastage prognostic factor in human neuroblastoma (15).

Given the association of the Ha-ras gene with differentiation in neural tissue (8-11), we decided to evaluate the expression of the Ha-ras gene in neuroblastomas.

MATERIALS AND METHODS

Patients with Neuroblastomas

Forty-seven patients with adequate clinical and histological materials for analysis enrolled in this study. They were treated during 1972 to 1985. The neuroblastomas were staged according to standard clinical criteria at the time of diagnosis as follows (14): Stage I, tumors confined to the organ or structure of origin; Stage II, tumors extending in continuity beyond organ or structure of origin but not across the midline, regional nodes on homolateral side may be involved; Stage III, tumors extending in continuity beyond the midline, regional nodes may be involved bilaterally; Stage IV, large primary tumors with remote disease involving bone, bone marrow, organs, soft tissues, distant lymph nodes; Stage IVs, patients who would otherwise be in Stages I or II but who have remote disease confined only to one or more of the following sites: liver, skin, or bone marrow (not bone). No attempt was made to grade the neuroblastomas according to the histopathological criteria (13) relating to the prognosis. No case with Stage IVs was included in this analysis because the favorable prognosis could be predicted. This study was retrospective and the therapy was not controlled. The methods of treatment have varied over more than 10 years and depended on the clinical stage estimated at diagnosis. The principal treatment has been surgical excision and post-surgical therapies were followed using any of several regimens. When patients with Stage I disease are younger than 1 year of age, no treatment is followed after surgical resection. Patients with Stage I disease who were older than 1 year of age and patients with Stage II disease were treated with radiation to the tumor bed without/with chemotherapy using vincristine and cyclophosphamide. The majority of patients with Stage III and IV diseases was treated with multiagent chemotherapy and radiotherapy after tumor resection. In the current study, there was only one case receiving neither chemo- nor radiotherapy after surgical resection because the patient was in Stage I disease and younger than 1 year of age. Patients without evidence of the disease for more than 2 years after diagnosis were considered long-term survivors. Twenty-three (49%) of 47 cases examined were long-term survivors. The number of disease-free survivors is compatible with the rates reported by others (12, 15-17).

Summary of cases studied is shown in Table 1. The median age of the cases was 2.9 years of age (range, 1 month to 8 years of age).

Antibodies against ras Gene Products

Antibodies used in this study were generated to synthetic peptides corresponding to different domains of the predicted amino acid sequence of ras p21, as reported previously (18, 19). An antibody specific to Ha-ras p21 was generated to a 20-amino acid sequence corresponding to the variable region (C terminal; residues 160 to 179) of v-Ha-ras p21. The amino acid sequence is homologous to c-Ha-ros p21 but different from Ki-ras and N-ras p21s in this domain. The second antibody was generated against a 16-amino acid sequence (residues 29 to 44) of v-Ha-ras p21. A strong sequence homology of this domain in the ras gene family provided a broad reactivity of the antibody with all ras p21s. The specificity of two antibodies used in immunoblot and immunohistochemical analyses were reported previously (11, 20).
Ha-ras GENE PRODUCT IN NEUROBLASTOMA

Detection of Ha-ras Gene Product (p21) in Neuroblastomas

All tissues examined in the current study came from untreated primary neuroblastomas removed surgically for diagnostic and/or therapeutic reasons.

Western Blotting Analysis. Details of electrophoresis and immunoblot used have been described elsewhere (11, 19). Extracts from tumors were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel containing 4% stacking gel. After electrophoresis, the nitrocellulose paper was incubated with antibody against ras p21. [3H]Staphylococcal protein A followed by autoradiography was used to visualize p21 bands.

Immunohistochemistry. The primary antibody for immunohistochemical analysis was purified by antigenic peptide-binding affinity chromatography. Nonimmunized IgG at the same concentration of the antibody was used simultaneously as a negative control. ABC immunoperoxidase staining (Vectastain ABC Kit, Vector Lab.) was performed on 5-μm sections from formalin-fixed, paraffin-embedded tumor tissues (20). Deparaffinized and hydrated tissue sections were pretreated with 0.3% H2O2 in methanol to inactivate endogenous peroxidase activity. An affinity purified primary antibody (5 μg of IgG/ml of Dulbecco’s phosphate buffered saline, pH 7.6) to Ha-ras p21 was incubated with the tissue section. After incubating with a biotinylated second antibody, the tissue sections were then incubated with avidin-biotin-linked peroxidase. Diaminobenzidine-4-HCl (0.5 mg/ml) with 0.01% H2O2 in 50 mM Tris buffer, pH 7.2, was used as the substrate for detecting localization of antibody binding. Sections were then lightly counterstained with hematoxylin.

Statistical Analysis

Analysis (χ2) was performed to evaluate the significant effect of the Ha-ras gene product detected in neuroblastoma on patient’s prognosis. Age and stage of disease at the time of diagnosis were also examined individually for the Ha-ras gene products in tumors.

RESULTS

Forty-seven paraffin-embedded tumor tissues were examined immunohistochemically. The immunohistochemical reaction products were detected in tumor cells with varieties among the cases (Fig. 1). Eleven tumors were frozen at −70°C separately and could be available to identify the product as Ha-ras p21 by using Western blot analysis (Fig. 2). The results obtained by both immunohistochemical and Western blot analyses correlated well. However, there were a few exceptional cases, such as a case with Stage I disease (lane 3 in Fig. 2). The amount detected by Western blotting was as low as the level of tumor from Stage IV disease (lane 6 in Fig. 2) but immunohistochemical analysis on the case showed a considerable amount of Ha-ras p21 in the tumor cells. On the other hand, there was no case with the high amount of Ha-ras p21 detected by Western blotting and low expression detected by immunohistochemistry. Therefore, Western blot analysis provides only limited information about the amount of p21 found in individual tumors for at least two reasons. First, tumors in vivo are composed of a heterogeneous cell population and can contain varying (sometimes large) numbers of nonmalignant cells such as stromal elements and inflammatory cells. Specific protein analysis based on lysis of such a heterogeneous cell population can result in a dilution or augmentation of various macromolecules, including p21. Secondly, biochemical analysis alone, does not usually take into account the viability of the cells examined and if nonviable or necrotic tissue is used, protein degradation could account for spurious decreases in the amount detected. After testing the specificity of the antibody, immunohistochemical analysis circumvents both of these problems in that the specific cells of interest can be identified and the overall quality of the specimen can be assessed simultaneously.

For these reasons, all 47 neuroblastomas in this study were assayed immunohistochemically for Ha-ras p21 using antibody purified by affinity chromatography. There was no definite diversity of the immunohistochemical reaction in the tumor cells of a case, even though the variety was observed among cases. Immunohistochemical reactivity in malignant cells was scored using the following scale; negative (−), weak staining (1+), definite staining (2+), and intense staining (3+) as shown in Fig. 1. Using this system 80% (38/47) of tumors contained detectable amounts of Ha-ras protein. There was no significant difference in the incidence of cases in which Ha-ras protein was detected when comparing the two major histological subtypes in this study, neuroblastomas and ganglioneuroblastomas (83% and 76%, respectively). In the two cases of benign ganglioneuroma examined, there was definite (2+) expression of Ha-ras protein in the more differentiated ganglion-like cells (data not shown). Similarly, stratification of the tumors by patient age at diagnosis (younger than 1 year of age, 1–3 years of age, and older than 4 years of age) failed to show significant difference in the amount of the Ha-ras protein (3+ to −) detected in tumor cells ($\chi^2 = 8.402$, degree of freedom (6), not significant). There were 57% (4/7), 43% (10/23), and 47% (8/17) of disease-free survivors who had been diagnosed before 1 year of age, between 1 and 3 years of age, and after 4 years of age, respectively. These data indicate that Ha-ras gene expression is independent of patient’s age at diagnosis. However, there was a statistically significant correlation of Ha-ras protein detected in tumor cells with clinical outcome of the patient and stage of disease at diagnosis (Fig. 3). In patients with Stage I and II disease, nine of 10 (90%) had high levels of Ha-ras protein expression in their tumor cells (2+ to 3+) and all patients are alive and free of disease. Fifteen (60%) of 25 tumors from patients with Stage IV disease, however, had negative or weakly detectable (− or 1+) amount of Ha-ras protein and these patients had a much poorer prognosis with only one out of 15 (6%) surviving without disease. Of 10 patients with Stage IV and (2+) expression of Ha-ras protein in tumor cells, four cases (40%) survive without disease for more than 2 years. Patients with Stage III disease were of interest in that five of five (100%) cases with high levels (more than 2+) of Ha-ras protein were all alive and disease free. Conversely, five of seven (71%) Stage III patients who had succumbed to their disease or were alive with active disease,
Fig. 1. Immunoperoxidase staining of neuroblastomas with Ha-ras p21 specific antibody. A, neuroblastoma (Stage I) demonstrates "intense" staining (3+) for Ha-ras p21 compared to those stained with control IgG; B, Neuroblastoma (Stage III) demonstrated "definite" staining (2+) for Ha-ras p21; C, neuroblastoma (Stage IV) shows detectable but "weak" staining (1+) for Ha-ras p21; D, neuroblastoma (Stage IV) shows no reaction product to the antibody "negative" staining (−).
had negative or weakly detectable levels (− or 1+) of the Ha-ras gene products. Taken together, these data indicate that relatively high levels (2+ or greater) correlate significantly with a better clinical prognosis \( \chi^2 = 13.427 \), degree of freedom (1), \( P < 0.001 \) and a lesser extent of disease among Stages I to IV at diagnosis \( \chi^2 = 7.982 \), degree of freedom (3), \( P < 0.05 \).

**DISCUSSION**

It is known that aberrant expression of ras oncogenes, either at the quantitative or qualitative levels, can result in malignant transformation and/or block differentiation (21-25). Thus, the correlation of Ha-ras protein expression in human neuroblastosomas with a favorable prognosis and earlier stage of disease is somewhat enigmatic. It is still uncertain whether the Ha-ras gene products in tumor cells determine primarily a biological behavior of the tumor cells or whether the expression of the gene is a secondary phenomenon resulting from the cellular differentiation. The current study suggests that the Ha-ras gene product may play a role in decreasing the aggressiveness of neuroblastomas cells in vivo. This hypothesis is supported, in part, by observations that the ras gene products (p21) directly induce morphological differentiation of rat pheochromocytoma cells (PC 12) to neuron-like cells in the absence of certain growth factors (8, 9). This differentiation is accompanied by cessation of PC 12 cell division. Furthermore, microinjection of antibody against ras p21 into PC 12 cells inhibits differentiation induced by nerve growth factor (10), again implying a role for p21 in the differentiation process. Consistent with a possible role of Ha-ras p21, we recently reported that a high amount of the protein is expressed specifically in nervous tissues of normal rats (11, 20). These data suggest a new function of the Ha-ras gene product relating to cellular differentiation. However, it remains to be elucidated whether correlation of the Ha-ras gene product with the differentiation is restricted in tissues and/or tumors relating to the nervous system.

Recently, amplification of N-myc DNA has been reported as a prognostic factor of neuroblastomas (15). We have been examining whether the augmentation or negative expression of the Ha-ras gene product in neuroblastomas comes from alterations of the Ha-ras gene at DNA levels. It is still preliminary, however. Of nine cases examined, five cases with weak or negative expression of Ha-ras p21 died of progressive disease and four with the expression more than (2+) are alive without disease for more than 2 years. There was no significant alteration of their Ha-ras gene levels detected by Southern blot analysis. On the other hand, the amplification of the N-myc gene was identified in 40% (two of five) of cases with a poor prognosis (unpublished data). It seems likely that a certain unknown factor affects the transcriptional and/or translational mechanism(s) for the expression of Ha-ras gene products.

Further attention should be focused on a large number of tumors to determine the relative significance of Ha-ras gene product with the association of factor(s), such as other oncogenes. We conclude from the current study that the expression of Ha-ras p21 in neuroblastoma cells correlates significantly with a better overall prognosis in patients with neuroblastomas indicating that this gene product may play a role in the biological behavior of the tumor in vivo. Finally, the simple and reproducible immunohistochemical analysis presented here could provide important information pertinent to patient care.

**ACKNOWLEDGMENTS**

We thank H. Muneoka, R. Ooka, and R. Nakatani (Department of Clinical Pathology, Mazda Hospital) for the technical assistance. We also thank Drs. A. Komiyama (Department of Pediatrics, Shinshu University, Japan), M. Yanai (Department of Pediatrics, Nagasaki University, Japan), H. Mugishima, F. Shimizu, K. Suwa (Department of Pediatrics, Hiroshima University, Japan), T. Ichikawa, M. T. Yokoyama (First Department of Surgery, Hiroshima University), and M. Kobayashi (Department of Pediatrics, Hiroshima University) for providing tumor samples and cooperation with this study.

**REFERENCES**


---

**TUMORS**

<table>
<thead>
<tr>
<th>HaNRK(1X2)(3)(4)(5)(6)</th>
<th>p21</th>
</tr>
</thead>
<tbody>
<tr>
<td>lane 1: neuroblastoma (Stage IV); lane 2: ganglioneuroma (Stage I); lane 3: neuroblastoma (Stage I); lane 4: neuroblastoma (Stage II); lane 5: neuroblastoma (Stage III); lane 6: ganglioneuroblastoma (Stage IV).</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Expression of ras p21 in neuroblastomas. "NRK, extract of normal rat kidney cells transformed by Harvey MSV. Lane 1, neuroblastoma (Stage IV); lane 2, ganglioneuroma (Stage I); lane 3, neuroblastoma (Stage I); lane 4, neuroblastoma (Stage II); lane 5, neuroblastoma (Stage III); lane 6, ganglioneuroblastoma (Stage IV). |

Fig. 3. Correlation of Ha-ras p21 expression to stage of disease at diagnosis and the patient's prognosis. The immunohistochemical reactivity (staining) of tumor cells to Ha-ras p21 specific antibody was estimated and scored "negative (−)" to "intense (3+)" as was shown in Fig. 1. Symbols, patient outcome: O, a disease-free survivor for more than 2 years; •, a case dead from progressive disease; O, a patient with progressive disease resistant to chemotherapy, who is alive at the time of this report. |


Expression of Ha-ras Oncogene Products in Human Neuroblastomas and the Significant Correlation with a Patient's Prognosis

Takeo Tanaka, Dennis J. Slamon, Hiroko Shimoda, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/4/1030

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.