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Establishment of a Human Cell Line Secreting Neuron-specific Enolase from a Primitive Neuroectodermal Tumor of the Retroperitoneal Cavity

Jun Nakashima,[1,3] Yutaka Horiguchi,[1] Munehisa Ueno,[1] Kaoru Nakamura,[1] Masaaki Tachibana,[1] Jun-ichi Hata[2] and Hiroshi Tazaki[1]

[1]*Department of Urology and* [2]*Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160*

Primitive neuroectodermal tumor (PNET) is one of the small round cell malignancies of presumed neural crest origin for which an effective treatment has not yet been established. In the present study, a human cell line, designated KU-9, was established from a 27-year-old male patient with PNET of the retroperitoneal cavity and has been successfully maintained in nude mice and in culture. On histological examination, the primary tumor was composed of poorly differentiated small round cells arranged in clusters showing a variety of mitotic changes, and contained Homer-Wright rosettes. The histopathological appearance of the KU-9 xenografts was similar to that of the primary tumor. Electron microscopy revealed neurosecretory granules and cytoplasmic processes in the xenograft. No significant amplification of N-*myc* gene was observed in the KU-9 cells. The KU-9 cells showed chromosome numbers ranging from 56 to 61 with consistent structural abnormalities being add(2)(q31), +add(11)(p11.2), +add(13)(p11.1), and +del(22)(q12). Cultured KU-9 cells grew exponentially with a doubling time of about 50 h and a time-dependent increase in medium levels of neuron-specific enolase (NSE) was noted. Serum levels of NSE in KU-9 tumor-bearing nude mice were significantly elevated and a linear relationship between the serum NSE levels and the tumor NSE content or tumor volume was observed, suggesting that serum levels of NSE may reflect the PNET tumor burden and tumor extent. These results indicate that the KU-9 cell line provides a reproducible model system which could be useful in gaining some insight into the histogenesis and oncogenesis of PNET and in establishing an effective treatment for PNET.

Key words: Primitive neuroectodermal tumor — Neuron-specific enolase — Cell culture — Nude mice

Primitive neuroectodermal tumor (PNET) is a rare, highly malignant soft tissue tumor of presumed neural crest origin that arises outside the central and sympathetic nervous systems. The first report in the literature concerning PNET was made by Stout in 1918, describing the microscopic appearance of a tumor that had arisen in the ulnar nerve.[1)] Since then many reports have appeared describing this tumor's unique variety of histopathological traits. However, very few reports on PNET of the retroperitoneal cavity have been published. The treatment strategy for PNET has thus far been based on its similarity to other small round cell tumors and, to date, there has been no uniform and effective treatment approach in ameliorating the conditions associated with it.[2–4)] Since further laboratory studies are still required to characterize PNET, and in order to develop a treatment strategy, reproducible *in vivo* and *in vitro* model systems are needed. The present study was undertaken to establish and characterize a human cell line, KU-9, derived from a PNET of the retroperitoneal cavity which secretes neuron-specific enolase.

MATERIALS AND METHODS

Case report We have previously reported on a rare case of PNET arising from the retroperitoneal cavity.[5)] Briefly, a 27-year-old man presented with left flank pain of sudden onset. Computerized tomography and ultrasonography revealed a round heterogeneous mass sized at 10×11 cm, protruding anteriorly from the left kidney and extending over the Gerota's fascia. There was also paraaortic lymph node swelling. Angiography showed only a slight deviation of the left renal artery presumably due to tumor compression. No feeding vessels of the tumor were observed from the left renal artery. The tumor was extirpated en block with the left kidney and paraaortic lymph nodes. Pathological diagnosis was PNET. Considering the very poor prognosis of PNET, postoperative adjuvant chemotherapy was undertaken. The tumor recurred in the left renal fossa, at which point the patient underwent a course of radiation. Unfortunately, death ensued three months postoperatively.

[3] Present address : Department of Urology, Urawa Municipal Hospital, 2460 Mimuro, Urawa, Saitama 336. To whom requests for reprints should be addressed.

Cell culture technique A portion of the surgical specimen was minced and rinsed with cold Eagle's minimal essential medium. The resulting cell suspension was passed through a 25-gauge needle to dissociate the cell aggregates. The cells were maintained in 25-cm^2 culture flasks (Corning) containing Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G, and 200 μg/ml of streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C as previously reported.[6] The culture medium was renewed every 2–3 days. This cell line was designated as KU-9.

Transplantation of PNETs into nude mice A single-cell suspension of KU-9 cells (approximately 1×10^7 cells) was inoculated subcutaneously on the back of BALB/c athymic nude mice (5 to 6 weeks of age). When tumor growths of more than 1 cm in diameter developed, they were aseptically removed, minced into 2 mm fragments, and transplanted subcutaneously on the back of other nude mice using a trocar needle as previously reported.[7] Tumor widths (a) and lengths (b) were measured using micrometer calipers. Tumor volume (V) was estimated according to the formula[8] $V = (a^2 \times b)/2$ and expressed in mm^3.

Preparation of samples from KU-9 tumor-bearing nude mice Blood and tissue samples from KU-9 tumor-bearing nude mice were prepared for NSE determinations according to a method reported previously.[6, 9] Blood samples were drawn via cardiac puncture when the animals were killed. Serum samples were taken after centrifugation and were frozen at −80°C until used for the neuron-specific enolase (NSE) assay. The tumors from nude mice were weighed, minced, and placed in cold phosphate-buffered saline immediately after removal. These preparations were then washed with cold phosphate-buffered saline and homogenized for 90 s at 4°C using a Polytron homogenizer (Ibaraki) in 0.02 M cold phosphate-buffered saline at a ratio of 5 ml to 1.0 g of tissue. This mixture was centrifuged for 30 min at 12,000 g at 4°C and the supernatant was collected and frozen until used for the NSE assay.

Enzyme immunoassay for NSE The levels of NSE in the mouse serum, tumor extract, and culture medium were determined by using an enzyme immunoassay kit (EIA) for NSE (Amano Pharmaceutical Co., Ltd., Nagoya).

Histological examination The primary tumor and the xenograft were examined microscopically, immunochemically, and by electron microscopy. The tumors were fixed with 20% formalin and stained with hematoxylin-eosin (H & E) and periodic acid-Schiff (PAS). Immunohistochemical staining was carried out on the primary tumor and KU-9 xenograft using antibodies specific to NSE (DAKO Corporation, Carpinteria, CA), neurofilament (2F11), epithelial membrane antigen (EMA), leucocyte common antigen (LCA), CD45RO (UCHL1), CD20 (L26) (Dakopatts, Glostrup, Denmark), neurofilament (N21-8-4) (Kyowa Medex, Tokyo), keratin (Dakopatts, Santa Barbara, CA), pankeratin (Immunon, Pittsburgh, PA), muscle actin (HHF-35) (Enzo Diagnostics, New York, NY), and myoglobin (Cappel Laboratories, Cochranville, PA). Antibody specifically reactive with Ewing's sarcoma (5C11)[10] was also employed. For electron microscopy, the xenograft was fixed with 2.5% glutaraldehyde, dehydrated in graded alcohol and embedded in Epon 812.[11]

Cytogenetic analysis Cytogenetic analysis was carried out on the established cell line. The medium was renewed the day before processing. Colcemid (GIBCO, Grand Island, NY) at a final concentration of 0.1 μg/ml was applied to the cells for a period of 2 h before harvesting. The cells were detached with trypsin, centrifuged for 5 min at 1000 rpm, then incubated in potassium chloride at a concentration of 0.075 M for 10 min at 37°C as hypotonic treatment. After centrifugation for 5 min at 1000 rpm, the cells were fixed in methanol: acetic acid (3:1) solution and trypsin-Giemsa staining was performed.

Oncogene analysis For the determination of N-*myc* amplification, 1.5 and 0.75 μg portions of DNA isolated from KU-9 cells were spotted on a nitrocellulose membrane using a slot blot apparatus. The membrane was hybridized with ^{32}P-labeled human N-*myc* probes (Oncor, Inc., Gaithersburg, MD). A β-globin probe was used for controls. The DNA levels were determined by use of a scintillation counter. Amplification was estimated by liquid scintillation counting as follows; sample

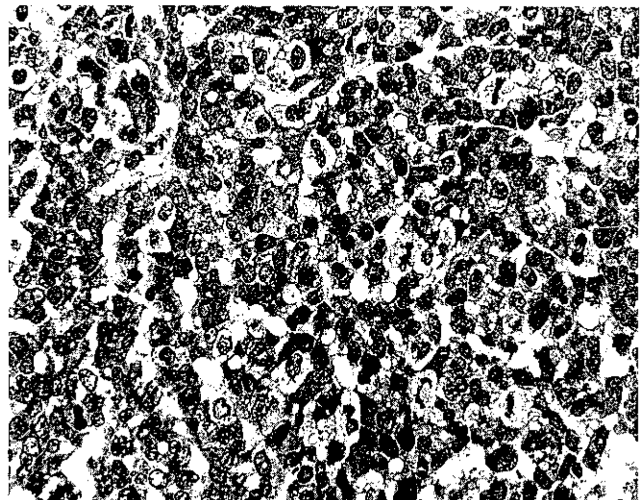

Fig. 1. Light-microscopic appearance of the KU-9 xenografted tumor (H & E, ×540). Small round, closely packed cells were noted.

cpm (N-*myc*)/negative control cpm (N-*myc*) × negative control cpm (β-globin)/sample cpm (β-globin)

The positive control of N-*myc* amplification consisted of DNAs isolated from a human neuroblastoma cell line, IMR-32. Blood from healthy volunteers served as the negative control.

RESULTS

Pathological examination revealed that the primary tumor was composed of poorly differentiated small round cells arranged in clusters showing a variety of mitotic changes, and contained Homer-Wright rosettes as previously reported.[5] The KU-9 xenograft also contained closely packed small round cells with an abundance of mitotic figures and showed similar figures to the primary tumor (Fig. 1), although Homer-Wright rosettes were not apparent in the xenograft. Both the primary tumor and xenograft contained glycogen. This was proved by a positive reaction with PAS stain which was eliminated with diastase. Immunohistochemical staining was strongly positive for NSE in the primary tumor and faintly positive in the xenograft. In contrast, both the primary tumor and xenograft revealed negative staining for neurofilament, epithelial membrane antigen, leucocyte common antigen, CD45R0, CD20, keratin, pankeratin, muscle actin, and myoglobin. In addition, the primary tumor and xenograft did not react with 5C11, which is the antibody specifically reactive with Ewing's sarcoma.[10] Electron microscopy demonstrated neurosecretory granules, cytoplasmic processes and sparse glycogen granules in the xenograft (Fig. 2, A and B).

KU-9 xenografts were successfully maintained in nude mice. The *in vivo* xenograft doubling time was approximately 7 days (Fig. 3). KU-9 cells at approximately 1×10^7 cells inoculated under the skin produced tumors within 6 weeks and an approximately 80% take rate was obtained. Serum levels of NSE were significantly elevated in KU-9 tumor-bearing nude mice, while the levels in control nude mice were less than 3 ng/ml. Fig. 4A illustrates the relation between total tumor NSE levels and serum NSE levels in the KU-9 tumor-bearing nude mice. A linear correlation (R=0.829) between the two parameters was found. In addition, a linear correlation

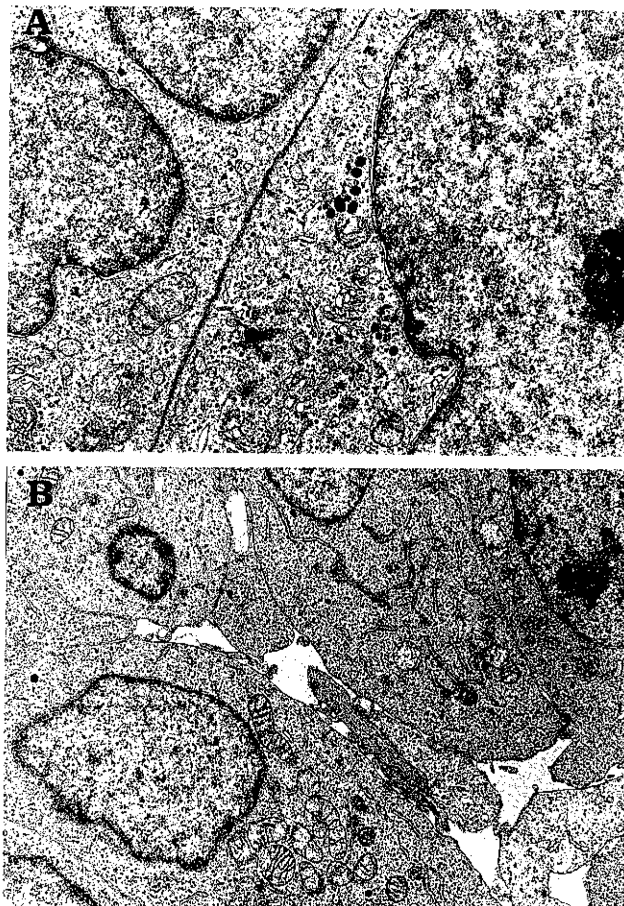

Fig. 2. Ultrastructure of the KU-9 xenografted tumor. (A) Neurosecretory granules are present (×13804). (B) demonstrates the cytoplasmic process (×9388).

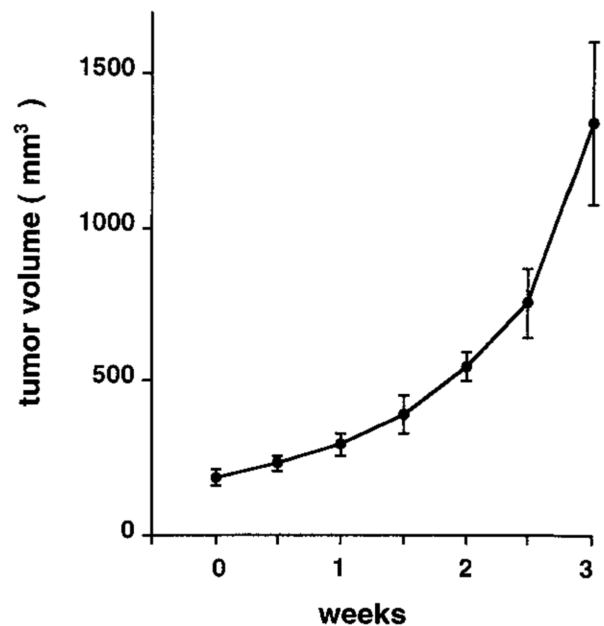

Fig. 3. Growth curve of the KU-9 xenografts in nude mice. The xenografts were transplanted subcutaneously on the back of nude mice. The tumor volume was determined using calipers. Each value represents the mean±SE from eight nude mice.

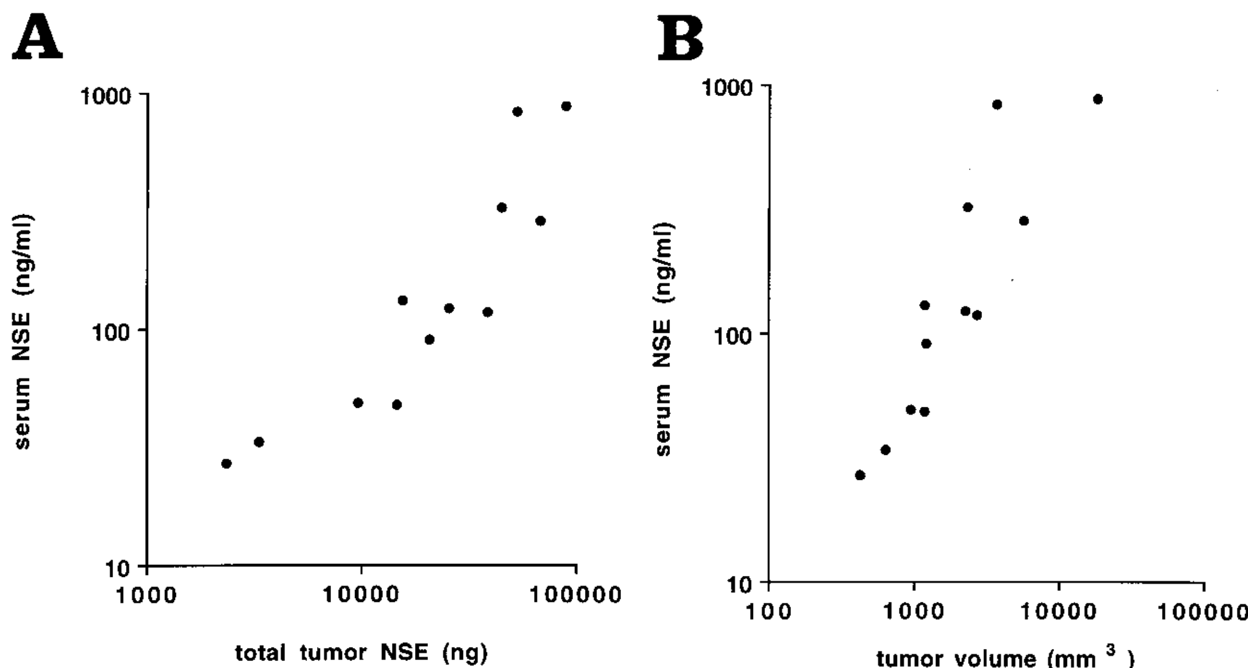

Fig. 4. (A) Total tumor NSE and serum NSE levels in the KU-9 tumor-bearing nude mice. The total tumor NSE levels were measured as described in "Materials and Methods." Each dot represents the value of NSE from one animal (n=12). The linear regression coefficient was 0.829. (B) Tumor volume and serum NSE levels in the KU-9 tumor-bearing nude mice. Each dot represents the value of NSE from one animal (n=12). The linear regression coefficient was 0.775.

(R=0.775) was demonstrated between tumor volume and serum NSE levels (Fig. 4B). It seems likely that serum levels of NSE depend on the tumor volume and tumor NSE content in KU-9 tumor-bearing nude mice.

The correlation between growth of KU-9 cells and NSE levels in 2-day spent culture medium was studied. Fig. 5 demonstrates that KU-9 cells grew exponentially *in vitro*, with a doubling time of about 50 h, reaching confluency 6 days after 1×10^5 cells were seeded into 24 multiwell plates. NSE in the culture medium became detectable on the second day in culture, and increased in association with the increase in cell number.

In the cytogenetic analysis of KU-9 cells, 20 metaphases were counted. The number of chromosomes varied from 56 to 61 and the modal number was 58. The representative G-banded karyotype was 58,X,−Y,+X, +1, +1, add(2)(q31), +7, +8, +add(11)(p11.2), +12, +add(13)(p11.1), +18, +18, +20, +20, +del(22)(q12) (Fig. 6). The consistent structural abnormalities were add(2)(q31), +add(11)(p11.2), +add(13)(p11.1) and +del(22)(q12).

Slot blot analysis was carried out to determine N-*myc* amplification in the KU-9 cells. No significant amplification of N-*myc* gene was observed in the KU-9 cells (Fig. 7).

DISCUSSION

Stefanko *et al.* have summarized data from nine reports[12] on 49 patients with PNETs. In their summary, 33 of 48 patients had died of recurrent disease at the time of reporting and the median survival of these 33 was 9 months. This suggests that PNET is a highly malignant tumor with a very poor prognosis. PNET arises in the soft tissues of the chest wall or peripheral lung and is also known as Askin's tumor.[2] PNET which arises adjacent to the spine is rare. To date only a few permanent PNET cell lines are available. The new human cell line, KU-9, is an extremely rare example of a cell line representing a PNET originating from the retroperitoneal space.

Histopathological examination of the primary tumor removed at surgery revealed small round closely packed cells with abundant mitotic figures and Homer-Wright rosettes. The histopathological appearance of the KU-9 xenografts was similar to that of the primary tumor. The abundance of mitotic figures suggested a rapid rate of cell division. Immunohistochemical staining was strongly positive for NSE in the primary tumor and faintly positive in the xenograft. Serum levels of NSE in KU-9 tumor-bearing nude mice and medium levels of NSE in

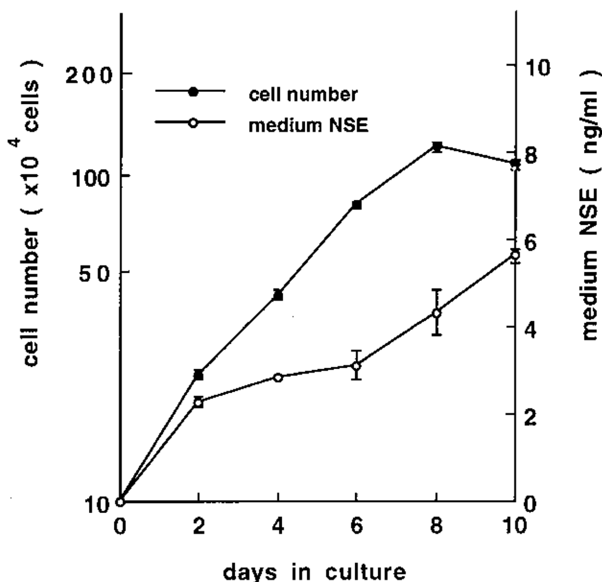

Fig. 5. Correlation between the growth of KU-9 cells and the NSE levels of 2-day spent culture medium as measured by EIA. Each value represents the mean±SE from three samples.

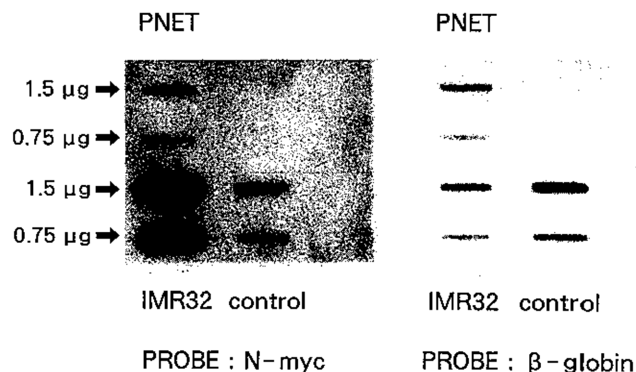

Fig. 7. Slot blot hybridization experiments. N-*myc* gene amplification in the KU-9 cells was determined as described in "Materials and Methods." The positive and negative controls were IMR32 (neuroblastoma cells) and white blood cells from healthy volunteers. DNA samples from KU-9 cells (PNET), neuroblastoma cells (IMR32) and white blood cells from healthy volunteers (control) were spotted onto a nitrocellulose filter and sequentially hybridized to ^{32}P-labeled human N-*myc* and β-globin probes; 1.5 and 0.75 μg of DNA were spotted on the upper and lower slots of each group, respectively.

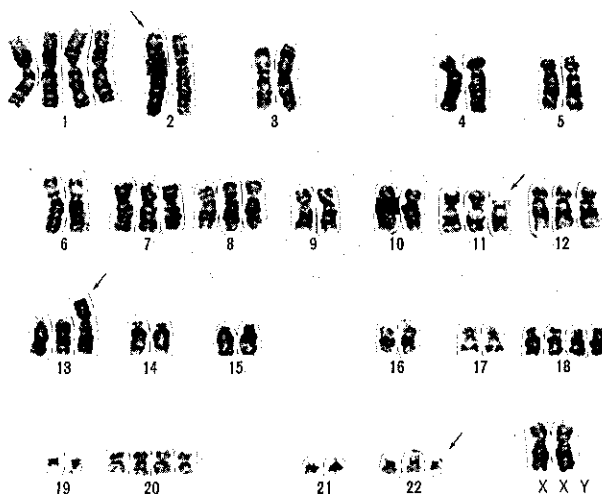

Fig. 6. Karyotype of a KU-9 cell. The structural abnormalities in a representative G-banded karyotype from KU-9 cells are indicated by arrows.

the culture of KU-9 cells were elevated. The primary tumor and xenograft did not react with an antibody specifically reactive with Ewing's sarcoma. Electron microscopic examination demonstrated cytoplasmic processes, neurosecretory granules, and sparse glycogen granules. The microscopic and immunohistochemical findings of the primary tumors and KU-9 xenografts as described earlier suggest that Ewing's sarcoma, neuroblastoma, rhabdomyosarcoma, and lymphoma were less likely diagnoses. Based on the criteria recently proposed by Marina *et al.*,[3] the final pathologic diagnosis was PNET. Genetic abnormalities associated with neuroectodermal tumors include amplification of the cellular oncogene N-*myc* in neuroblastoma tumors.[13] In the present study, no significant amplification of the N-*myc* gene was found in DNA from KU-9 cells. No immunoreactivity was detected in KU-9 tumors with antibody specific for neurofilament, which suggests that KU-9 cells do not express an antigen typically found in differentiated neurons. These results suggest that malignant transformation may occur during the process of neural development prior to commitment to a neuronal lineage, with persistence of undifferentiated neuroectodermal stem cells.

Both Aurias *et al.*[14] and Turc-Carel *et al.*[15] have reported that the cytogenetic finding of a reciprocal translocation, (11;22)(q22;q12) may be a reliable and unique marker for Ewing's sarcoma. The same abnormality has also been found in extraskeletal Ewing's sarcoma[16] and peripheral neuroepithelioma.[17] These findings may suggest that these entities have a common histogenesis or a similar oncogenesis. Potluri *et al.* have reported that no consistent deletions or translocations could be identified in their study of five PNET cases and that additional 1q material [either as extra chromosome

#1 or i(1q)] was found in three of five cases and monosomy 13 in two.[18] Recently, Gorman *et al.* summarized cytogenetic reports from 21 different PNET samples and cell lines.[19] There were few consistent chromosome rearrangements or numerical aberrations. Chromosomes 5, 8, and 12 were most frequently involved in numerical changes and of the 21 cases reported, 11 had the t(11; 22). In contrast, eight samples did not contain chromosome rearrangements involving either 11q24 or 22q12. Two samples showed some rearrangement or deletion of the 11q24 and/or 22q12 chromosomal regions. Numerical aberrations of chromosomes 1, 8, and 12 were found in the KU-9 cells. Structural abnormalities linked to chromosomes No. 11 and No. 22 (+add(11)(p11.2) and +del(22)(q12)) were also found. While no translocation t(11;22)(q24;q12) was identified in KU-9 cells, the numerical aberrations of chromosomes and the structural abnormalities found in the KU-9 cells were compatible with those mentioned in the cytogenetic report on PNET by Gorman *et al.*[19] A chromosome abnormality of deletion of 1p,[20] which was identified in more than 66% of neuroblastomas, was not identified in KU-9 cells.

In 1981, Tapia *et al.* reported that NSE is produced by neuroendocrine tumors.[21] Subsequently the clinical usefulness of serum NSE as a marker for small cell lung cancer, neuroblastoma, and other neuroendocrine tumors has been discussed.[21–24] The PNET patient in this study who exhibited elevated preoperative serum levels of NSE showed a rapid decrease of the levels after the operation. The serum levels of NSE then increased in parallel with the tumor recurrence.[5] In this study, it was demonstrated that the KU-9 cells grow exponentially *in vitro* and that the medium levels of NSE increase in association with the increase in cell number. Serum levels of NSE in KU-9 tumor-bearing nude mice were significantly elevated when compared with control nude mice. In addition, serum NSE levels correlated well with tumor NSE levels and tumor volume in KU-9 tumor-bearing nude mice. Taken together, these facts suggest that serum levels of NSE may reflect the PNET burden and tumor extent. This supports the view that monitoring NSE serum level trends in patients with retroperitoneal PNET may be useful in evaluating the effectiveness of treatment and in assessing tumor reccurrence.

These results indicate that the NSE-secreting KU-9 cell line provides a novel and reproducible *in vitro* and *in vivo* model system which should be useful in gaining insight into the histogenesis and oncogenesis of PNET and in establishing an effective treatment for PNET.

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