Stability of c-K-ras Amplification during Progression in a Patient with Adenocarcinoma of the Ovary

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ABSTRACT

We have identified a case of serous cystadenocarcinoma of the ovary in which the tumor cells display an amplification (from 10- to 20-fold) of the cellular oncogene K-ras. Normal cells purified from the malignant ascites did not show such amplification.

Five consecutive samples were obtained by paracentesis over a 9-month period during which the patient received chemotherapy and underwent clinical progression. The level of c-K-ras amplification in the tumor cells did not change during this period. In studies of the tumors of 6 additional patients with adenocarcinoma of the ovary and 5 cell lines of the same histology, we have detected no other example of significant c-K-ras amplification.

INTRODUCTION

Gene amplification has been proposed as one of the mechanisms by which c-onc genes can play a role in the development of cancers (1). Several reports have been made regarding c-onc amplification in human tumor cells. These include cell lines derived from leukemias, lung cancers, breast carcinomas, neuroblastomas, retinoblastomas, and colon carcinomas (1). Although such studies provide circumstantial evidence of a relationship between the oncogenic process and c-onc amplification, it cannot be ruled out that amplification occurs in the course of the establishment of continuous cell lines (2).

Recently the first examples of c-onc amplification in primary human tumor samples have been reported. These include amplifications of c-H-ras in a bladder carcinoma (3), c-myb in a case of acute myeloblastic leukemia (4), c-myc in a case of chronic granulocytic leukemia (5), and several cases of N-myc amplification in neuroblastoma (6, 7) and retinoblastoma (8). In the case of neuroblastoma it has been proposed that the amplification may be related to the progression of these tumors by conferring selective advantage to cancer cells through overproduction of the N-myc gene product (6, 7).

We report here an additional example of c-onc amplification in primary tumor cells. We have identified a patient with serous cystadenocarcinoma of the ovary in which the tumor cells display an amplification for c-K-ras. Normal cells isolated from the same primary tumor cells. We have identified a patient with serous cystadenocarcinoma of the ovary and had received chemotherapy. Selection of patients for study was based on the availability of multiple biopsies and on an elevated tumor cell percentage (>90%). Cells were harvested by centrifugation (600 x g for 10 min), resuspended in McCoy's medium containing fetal calf serum (10%), and passed through needles of decreasing size to 25 gauge. Cells were frozen in McCoy's medium with dimethyl sulfoxide (10% v/v) and fetal calf serum (20% v/v) for storage at -80°C.

Clinical Case Report. The patient from whom the c-K-ras-amplified tumor was obtained was a 51-year-old woman who presented with Stage III serous cystadenocarcinoma of the ovary. Following bilateral salpingo-oophorectomy and partial omentectomy bulky residual disease remained and chemotherapy was initiated with melphalan 8 mg/m2 p.o. for 4 days every 4 weeks. After 8 courses a partial remission had been achieved but ascites was still present. Paracentesis was performed (Sample a) and the ascites was shown to contain adenocarcinoma cells.

We then received i.v. cyclophosphamide (400 mg/m2), doxorubicin (40 mg/m2), and cis-platinum (40 mg/m2) every 21 days. During this period Samples b and c were obtained. After 4 courses of this combination chemotherapy she had evidence of disease progression with rapid accumulation of ascites and required further paracentesis (Sample d). She then received vincristine (4 mg/m2) i.v. but failed to respond (Sample e) and died 1 month later.

Cell Lines. Cell lines of ovarian carcinoma origin [HOC-7, HEY, Skov-3, CaOV3 (9), and OVCAR (10)] were maintained in a-minimal essential medium containing 10% fetal calf serum.

Isolation of DNA and Southern Blotting. High molecular weight genomic DNA was isolated by using sodium dodecyl sulfate-protease K lysis, organic extraction, and NaCl-ethanol precipitation (11). DNA was digested with EcoRI, electrophoresed on 0.8% agarose, and transferred to a Zetabind membrane (AMF CLUNO, CT) essentially as described by Southern (12). Hybridizations were performed using standard procedures and high stringent conditions. The c-K-ras probe was a 2.5-kilobase EcoRI-XbaI fragment from the first exon of the human K-ras gene (13); it was nick translated with dCTMP as described (14). λDNA digested with HindIII was used as a size marker.

Cytotufluorimetric Analysis of DNA Ploidy and Cell Sorting. Hoechst dye No. 33342 (Calbiochem, Los Angeles, CA) was used as a DNA stain; cells (2 x 106) were exposed to Hoechst dye (10 µM) in phosphate-buffered saline containing Triton X-100 (0.1% v/v) for 30 min at 4°C. The cells were then analyzed by flow cytometry on a Coulter Epics V flow cytometer. Normal human leukocytes were used as diploid controls in mixing experiments for each sample.

RESULTS

We screened DNA from 5 ovarian tumor cell lines and from 7 noncultured ovarian tumors from different patients for amplification of seven different c-onc genes: myc; myb; H-ras; N-ras; K-ras; fos; and erb-B. We found only one case with a significant
level of amplification. A Southern blot of EcoRI-restricted DNA from four tumors and normal human fibroblasts hybridized with the c-K-ras probe is shown in Fig. 1; the lane corresponding to DNA from patient 3 shows an amplified 6.6-kilobase band.

Four additional biopsies of this patient's tumor were available; these were obtained over a period of 9 months during which the patient received chemotherapy and underwent a disease progression (see description of patient in "Materials and Methods").

We therefore studied by Southern blot analysis the stability of the c-K-ras amplification over this time period. Fig. 2 shows that the DNA from all 5 samples presents a significant degree of amplification and that the intensity of the c-K-ras band in Samples c–e is slightly decreased from that in Samples a and b. Rehybridization of the same filter with a human c-erb-B probe demonstrated that the lesser intensities of the c-K-ras bands in Samples c–e were not due to differences in the amount of DNA loaded (data not shown).

When serially diluted DNAs from Samples a and e were hybridized with the c-K-ras probe and the intensities of the bands were compared with normal human fibroblasts, the c-K-ras band of Samples a and e was found to be from 10- to 20-fold and from 5- to 10-fold, respectively, more intense than the band corresponding to the fibroblasts (Fig. 3).

A possible explanation of this result is the presence of different percentages of tumor cells in the different ascitic samples. Our selection of this patient for initial study was based on the fact that Sample 1 ascites was considered to contain >90% tumor cells by cytological assessment. In order to characterize the cellular components of all 5 samples the ploidy of the cells was examined by flow cytometry after Hoechst No. 33342 vital dye staining. The fluorescence spectra are shown in Chart 1 for patient Samples a to e. Two peaks were evident in all samples, one coinciding with the position of control normal leukocytes in mixing experiments and one showing a hyperdiploid DNA content. The hyperdiploid cells had a low incidence of G2 and S-phase cells. The proportion of cells in the aneuploid peak in the five consecutive ascitic fluids was 95.0, 89.9, 65.3, 29.5, and 29.5%, respectively.

It is clear therefore that the decreased intensity of hybridization seen in DNA from Samples c–e (Fig. 2) is related to the proportion of tumor cells in the population. When corrected for proportion of aneuploid cells the level of c-K-ras amplification was essentially constant in all samples of ascites.

In order to confirm that the diploid cells in the ascitic samples were normal and also to allow assessment of the c-K-ras gene in normal tissue, diploid and aneuploid cells were sorted from one of the ascitic tumors (Sample d) on the basis of ploidy after Hoechst dye staining. The sorted diploid population was reas-
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...essed for ploidy (Chart 1F) to confirm the proportion of diploid cells (93%). Representative fields of hematoxylin and eosin-stained cytcentrifuge slides prepared from the sorted diploid and aneuploid populations are shown in Fig. 4. The diploid cells consisted entirely of lymphoid and histiocytic cells while the aneuploid cells were large, anaplastic adenocarcinoma cells, confirming that the diploid cells were indeed of normal origin. The low viability of the cryopreserved cells precluded a more detailed analysis of the phenotype of the lymphoid cells.

DNA was extracted from the normal cells and Southern blot analysis was performed using the c-K-ras probe. It can be observed that the c-K-ras gene is not amplified in the normal cells (Fig. 2, Lane f).

DISCUSSION
c-K-ras amplification has been described in a mouse adrenocortical tumor cell line (15), and at low levels in a human colon cell line (16), a human small cell carcinoma cell line (16), and a human lung giant cell carcinoma line (17). We report here an example of in vivo amplification of this gene in a case of human adenocarcinoma of the ovary.

Our study suggests that amplifications of proto-oncogene sequences in human ovarian cancer are rare. This is in distinction to the situation in neuroblastoma and retinoblastoma; in these cancers a high proportion of tumors display N-myc amplification (6, 8).

Since we were unable to derive normal cells by live harvest we purified diploid cells from the malignant ascites using a fluorescence-activated cell sorter, taking advantage of the hypodiploid DNA content of the tumor. Cytological assessment (Fig. 4) confirmed that the diploid cells were hemopoietic in origin. Since we found that the c-K-ras gene was not amplified in these normal cells (Fig. 2, Lane f) our data are consistent with the amplification of c-K-ras in this patient occurring as a somatic rather than germinal mutation.

The significance of c-K-ras amplification for the pathogenesis of this disease cannot be adequately assessed. However, it is tempting to speculate on the basis of this information and that of the finding of an activated K-ras gene in one case of ovarian carcinoma (18) that the c-K-ras product may be involved in some aspect of cellular behavior in a small proportion of cases of ovarian adenocarcinoma.

In this particular patient the degree of c-K-ras amplification in the tumor cells did not change over a period of 9 months despite clinical progression of the tumor and chemotherapy suggesting that the genetic abnormality that generated the amplification was stable or that a constant selective pressure over a homogeneously amplified tumor cell population was present during the 9-month period. A relationship has been proposed between tumor progression and N-myc amplification in neuroblastoma (6, 7) based on degrees of amplification in tumors of different stages from different individuals. Since the development of ascitic tumor is a relatively late event in the clinical history, we are unable to speculate as to the relationship of the c-K-ras amplification to tumor genesis and early growth. It may be significant that by the time of initial paracentesis (Sample a), the patient had received chemotherapeutic treatment with an alkylating agent; it has been suggested that agents that effect DNA synthesis or cause DNA damage may facilitate gene amplification (2).
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The low viability of the cryopreserved cells has unfortunately made it impossible for us to look for karyotypic evidence of gene amplification. It is possible that cytogenetic studies of a cell line that we have recently derived from the cryopreserved patient material will allow us to obtain such evidence.

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REFERENCES

Fig. 4. Staining of sorted cell populations. Cells from ascites Sample d were sorted on the basis of diploid DNA content (A) or aneuploid DNA content (B). H & E, x 3000.
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