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Differential Oncogene Amplification in Tumor Cells from a Patient Treated with Cisplatin and 5-Fluorouracil

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Abstract—*Peritoneal cells were derived from a patient (PK) with adenocarcinoma of the colon during the course of cisplatin/5-fluorouracil (5-FUra) treatment. Resistance to cisplatin and 5-FUra, characterized by a lack of response to chemotherapy and continued growth of the tumor, was concomitantly associated with a 2–4-fold increase in DNA copy number for dTMP synthase and dihydrofolate reductase. There was a corresponding amplification in DNA copy number of the c-*myc (2×), H-ras (4×), *and c-*fos (15×) *oncogenes. Cytogenetic studies revealed an iso (13q) chromosome, but failed to show any double minutes or homogeneously staining regions. In addition, drug-resistant tumor cells from PK and another patient (HG) displayed enhanced expression of dTMP synthase, c-*fos *and DNA polymerase* β *when compared to normal colon tissue and the HCT8 human colon carcinoma cell line. These results suggest that elevated oncogene DNA and gene expression may be involved in the development of cisplatin resistance.*

INTRODUCTION

EVEN THOUGH cisplatin is widely used in the treatment of carcinomas, its mechanism of action remains unclear [1, 2]. Resistance to this and other chemotherapeutic agents represents one of the main obstacles in cancer chemotherapy [3, 4]. Recent evidence from cell culture experiments [5] has pointed to the action of cisplatin on the dTMP synthase cycle, part of the pathway for synthesis of deoxythymidine triphosphate, in addition to its DNA binding capacity. 5-Fluorouracil (5-FUra) is metabolized to 5-fluorodeoxyuridine monophosphate, which can bind and inactivate dTMP synthase [6]. Cisplatin and 5-FUra are used in combination *in vivo* and have been shown to achieve complete responses against human neoplasms [1]. The basis for this synergy lies at the level of dTMP synthase [5]. Moreover, cisplatin-resistant human carcinoma cells grown in culture exhibit increased messenger RNA and enzyme activity of dTMP synthase and DNA repair enzymes [7–11]. The oncogenes c-*fos* and c-*Hras* have also been implicated in cisplatin resistance *in vitro* [2]. Therefore, it would be of interest to examine the expression of these genes in patient cells resistant to cisplatin and 5-FUra.

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PATIENT'S CASE REPORT

The cells of a 51-year-old white female (PK) with colon cancer metastatic to the peritoneum were studied for their DNA at diagnosis, then during and after treatment with cisplatin and 5-FUra (Table 1).

PK had initially received intravenous 5-FUra alone in January and February of 1986 and a single dose of Lomustine (CCNU) late in February, and demonstrated progression of peritoneal metastases. The effects of treating the patient with 5-FUra prior to the combination chemotherapy are unknown. However, we examined changes that occurred in both the DNA and RNA of tumor cells specific to the dTMP synthase cycles and oncogenes during the course of cisplatin/5-FUra treatment.

PK had multiple large intra-abdominal tumor masses as well as large volumes of malignant ascites.

Table 1. *Treatment protocol and tumor cell sampling for patient PK*

Date	Treatment	Samples	Cytology
4 April	—	PK_1	—
8 May	Cisplatin (125 mg) 5-FUra (1.04 g)	—	+, Adenocarcinoma
30 May	Cisplatin (125 mg) 5-FUra (1.56 g)	—	+, Adenocarcinoma
4 June	—	PK_3	—
27 June	Cisplatin (124 mg) 5-FUra (1.56 g)	—	— Not diagnostic
29 July*	—	PK_5	+, Adenocarcinoma
27 September	—	PK_7	—
5 October	Expired	—	—

*Tumor showed a doubling in size on CT scan.

The latter required repeated paracenteses for comfort. An intraperitoneal Port-a-Cath was placed for chemotherapy in April 1986. On 4 April 1986, prior to cisplatin-containing chemotherapy, washings from the peritoneum were obtained (PK_1). The patient then received intraperitoneal cisplatin and 5-FUra on 8 May, 30 May and 27 June (Table 1). During this time her disease remained stable with no evidence of tumor response. The definite evidence of tumor progression (from CT scan) occurred on 29 July after three courses of intraperitoneal chemotherapy. Her subsequent course was complicated by dehydration and bowel obstruction. The patient expired on 5 October 1986. Patient HG had colon carcinoma, with large volumes of malignant ascites, that had failed cisplatin/ara-C (1-β-D-arabinofuranosylcytosine) treatment. The HG cells could not be maintained in culture, so the experiments were performed with cells taken directly from the patient's ascites. Normal colon tissue (NCT) was obtained from Professor R. Nalick, University of Southern California Cancer Center, Los Angeles, CA. The HCT8 human colon carcinoma cell line was obtained from Dr J. Bertino at Memorial Sloan-Kettering, NY [12].

MATERIALS AND METHODS

Cell culture

Cells obtained from the patient were either prepared for DNA or RNA extraction or cultured on Falcon Primaria flasks (to inhibit fibroblast growth) and maintained in (folic acid-free) RPMI-1640 medium with 10% fetal calf serum and folinic acid (10^{-8} M) [8]. No fibroblasts were detected under these cell culture conditions. Only the PK_7 sample grew stably in culture. The failure to establish PK_1, PK_3, and PK_5 made it impossible to amass sufficient cells in order to examine gene expression or the cytogenetics in these samples.

DNA isolation and slot blotting

DNA was extracted from cells using the SDS/proteinase K digestion method [7] and characterized on Genatran filters as previously described [7].

RNA isolation and northern blotting

RNA was extracted from the cells by the guanidinium/cesium chloride method [13] and blotted onto Genatran filters as described [7].

Preparation of cloned inserts

The dTMP synthase (TS) and dihydrofolate reductase (DHFR) cDNAs were obtained from T. Seno and G. Attardi as previously described [7]. The thymidine kinase (TK) and DNA polymerase β cDNAs were obtained from P. Deininger and S. Wilson as previously described [11]. The cDNA for pXBR-1, a tandemly repeated sequence on the X-chromosome [14] used as the control was a gift of Dr Thomas Yang at the Beckman Research Institute, City of Hope. Plasmids containing cloned human oncogenes were purchased from American Type Culture Collection (Rockville, MD). The following fragments from oncogene cDNAs were used as probes in these studies: *H-ras*, 0.6 kb (BamHI, Xma III); c-*myc*, 1.5 kb (EcoRI, ClaI); and c-*fos*, 1.4 kb (EcoRI, XhoI) [11].

Cytogenetic studies

The tumor cells were grown in culture for 1 month with three passages before harvesting for chromosome analysis. Twenty-four hours after trypsinization for the fourth passage, colcemid (GIBCO, Grand Island, NY) was added for 35 min in a final concentration of 0.1 μg/ml. The cells were detached with trypsin-EDTA (NaCl, 137 mM; KCl 5 mM; dextrose 5.5 mM; $NaHCO_3$ 7 mM; EDTA 0.5 mM; trypsin 0.5%) and treated with hypotonic KCl for 20 min. Fixative (3 parts methanol to 1

part glacial acetic acid) was added directly to the hypotonic solution. Air-dried slides were banded using trypsin–Giemsa [15]. One hundred banded metaphase spreads were examined at the 400–550 band stage, and five full karyotypes were prepared.

RESULTS

Patient DNA was placed on slot blots and probed using dTMP synthase and DHFR cDNA. The results in Fig. 1 indicate increases in gene copy number as the patient developed resistance to the anti-cancer agents. Densitometric analysis of dTMP synthase cycle genes revealed that resistance to cisplatin and 5-FUra resulted in a 4-fold increase in dTMP synthase and 2-fold in DHFR DNA from the time the patient was initially treated with chemotherapy to the stage of disease associated with lack of response to drugs and doubling of tumor size. The PK_7 sample also exhibited amplification of three oncogenes tested (Fig. 1). Specifically, there was a 15-fold enhancement of c-*fos* DNA, 4-fold of *H-ras*, and 2-fold of c-*myc* upon resistance to the treatment regimen. However, there were no differences in the levels of c-*erb-B*, the epidermal growth factor receptor gene, and c-*fms*, whose product represents the putative macrophage CSF-1 receptor (data not shown). Finally, there was no change in the amount of pXBR-1 DNA, a tandemly repeated sequence on the X-chromosome designated as the control in the serial samples. A quantitative summary of the results appears in Table 2.

Even though the copy number of control (pXBR-1) and two oncogenes remained unaltered, cytogenetic studies were undertaken to ensure that the observed amplification occurred in association with resistance to chemotherapy and not a general increase in cellular DNA content. Karyotype analysis of trypsin–Giemsa banded PK_7 chromosomes (Fig. 2) characterized the cells as 46,X,X,+i(13q). The presence of iso (13q) in all the tumor cells resulted in tetrasomy 13q. The second X-chromosome, presumably the inactive one, was missing. A fraction (10%) of the tumor cells were monosomic for chromosomes 6 and 12. However, no double minutes or homogeneously staining regions were identified in the PK_7 chromosomes. Thus, the cytogenetic analysis was not specific for a region of gene amplification. The tetrasomy 13 revealed by the karyotype analysis is probably not directly related to the phenomenon described here since none of the genes we analyzed map to 13q.

However, this chromosome does carry a gene for the repair of u.v. damage [16] and abnormalities have also been demonstrated for this gene in retinoblastoma patients, suggesting a possible role for suppressor genes [17]. The significance of this with respect to our studies is unknown.

Finally, we investigated expression of the aforementioned genes in drug-resistant colon cells versus normal colon tissue and the human colon carcinoma cell line HCT8. Northern blot analysis (Fig. 3) reveals that mRNA for dTMP synthase, c-*fos*, *H-ras* and c-*myc* was 2–10-fold higher in two colon carcinoma patients that failed cisplatin treatment than in normal colon tissue. In contrast, DHFR mRNA doess not appear amplified in the patient cells. Since the dTMP synthase cycle enzymes help comprise a multi-enzyme complex involved in DNA synthesis, it was of interest to examine whether the expression of dTMP synthase cycle genes alone was altered in drug-resistant cells or whether the entire complex was amplified. To this end, the expression of two additional genes, thymidine kinase (TK) and DNA polymerase β, part of the DNA synthesis and repair complex but not the dTMP synthase cycle, was also studied. Both genes were more highly

Table 2. Gene copy number in PK cells

Genes dTMP synthase cycle	Relative number of gene copies*			
	PK_1	PK_3	PK_5	PK_7
TS	<1	1	2	4
DHFR	—	1	1	2
Control				
pXBR-1	—	1	1	1
Oncogenes				
c-*fos*	—	1	< 3	15
c-*H-ras*	—	1	2	4
c-*myc*	—	1	1	2

*Slot blots were exposed to Kodak X-omat AR film at −70°C and intensifying screens for varying periods of time so that the signal intensities obtained between different probes were comparable and fell within the linear response range of the film. Intensities were quantitated with a scanning densitometer and normalized as the ratio of PK_7 or PK_5 DNA to that of PK_3 cells. In most cases, the samples were analyzed on at least two filters so that the average reported is from six to 12 different determinations.

expressed in the patient cell lines as compared to both normal colon and HCT8 cells. These patient cells also exhibit corresponding amplification of the DNA copy number for these genes when compared to normal colon cells (data not shown).

DISCUSSION

There are inherent difficulties in conducting an investigation of *in vivo* drug resistance. Multiple samples are required for proper chronological analysis. The samples (taken from the patient's ascites) usually yield very few cells, making it difficult to perform all the necessary hybridization experiments. Recently, this problem has been circumvented somewhat by the development of the polymerase chain reaction (PCR), modified and tailored for the dTMP synthase gene [9] and other DNA repair genes [10]. In addition, investigation of the cells' molecular characteristics is subject to the vagaries of tumor cell heterogeneity, in which a clear pattern may emerge with difficulty. Moreover, difficulties exist in showing a low level of amplification in patient cell lines. The PK_7 cells did not possess double minutes or homogeneously staining regions upon cytogenetic analysis. This may be explained by the discovery of submicroscopic elements or episomes [18], which go undetected by cytogenetic methods, as additional manifestations of gene amplification. The use of normal colon tissue (Fig. 3) as a background measure of gene expression is also problematic due to the essentially non-proliferating nature of the normal tissue and inclusion of nonepithelial cell types in the sample. Yet no other suitable control exists for the studies described here. Finally, it is difficult to uncouple the effects of tumor progression from those of drug resistance on gene expression in tumor cells *in vivo*.

Nevertheless, the results presented here corroborate evidence pointing to the same phenomenon in three ovarian carcinoma patients who, having failed cisplatin/5-FUra treatment, exhibit gene amplification of c-*fos* as well as the dTMP synthase cycle genes [19]. Furthermore, elevated gene expression of dTMP synthase, TK, oncogenes and DNA repair enzymes upon cisplatin resistance has been demonstrated in A2780 ovarian carcinoma [2, 7–9, 20] and HCT8 colon carcinoma cells (9–11, 20], where the appropriate control experiments have been conducted. Finally, the PCR assay has extended this hypothesis to the messenger level by showing elevated dTMP synthase, DNA polymerase β, and *fos* mRNA as well as gene copy number in ovarian and colon (including PK_7) patient cells resistant to cisplatin and 5-FUra [9, 10]. The data in this manuscript are significant since they use colon tumor cells *in vivo* to show a progressive amplification of the genes in question using the three different samples. The data on patient HG was included because it allows us to uncouple the effects of resistance to cisplatin from those to 5-FUra in PK. Secondly, achieving the same results in terms of mRNA amplification in HG cells discounts any influence growth of PK cells in culture may have had on the results since HG cells were not propagated in culture.

The notion linking gene amplification and drug resistance is not new. In fact, resistance to methotrexate, a folate antagonist, is accompanied by elevated copies of DHFR [21], and colon tumor cells resistant to 5-FUra/leucovorin also exhibit dTMP synthase gene amplification [22]. The proposed link between oncogenes and drug resistance is supported by studies in which transfection of *H-ras* into NIH-3T3 cells renders them more resistant to cisplatin [23]. Finally, c-*fos* is induced in the cellular response to DNA damaging agents, including cisplatin in Chinese hamster ovary cells [24].

The importance of the dTMP synthase cycle in cisplatin resistance lies in two observations, namely that it represents the sole *de novo* source of thymidine, and that the availability of thymidine for incorporation into DNA is rate-limiting in DNA synthesis [25]. The link between dTMP synthase and oncogenes may be supported by Pardee's concept of a multi-enzyme complex or replitase required for DNA synthesis [26, 27]. This complex includes enzymes of DNA precursor synthesis (e.g. dTMP synthase and TK), as well as those with a direct role in DNA synthesis and repair (DNA polymerase α and β, topoisomerase I, etc.). We have recently shown that c-*fos* and the DNA synthesis genes are induced following cisplatin administration in a sequential fashion in A2780 cells resistant to cisplatin [2]. The link between c-*fos* and DNA synthesis is supported by the studies which show that its gene product may act as a transcriptional activator through its DNA binding capacity [28]. Finally, the increased DNA polymerase β mRNA suggests enhanced capability for repair of cisplatin-induced DNA damage [2, 10, 11], itself a proposed mechanism of resistance to cisplatin [29]. We have tested this hypothesis in culture with human colon cells and have inhibited cisplatin-induced DNA repair with AZT (azidothymidine) [11].

In conclusion, data presented here with two adenocarcinoma patients are consistent with previous work focusing on the dTMP synthase cycle as both a site of action for the synergy of the cisplatin/5-FUra combination as well as a site used by resistant tumor cells to circumvent this synergy. Moreover, this study implicates both oncogene amplification and its enhanced expression in the development of high levels of drug resistance and poses an indirect connection between oncogenes and dTMP synthase.

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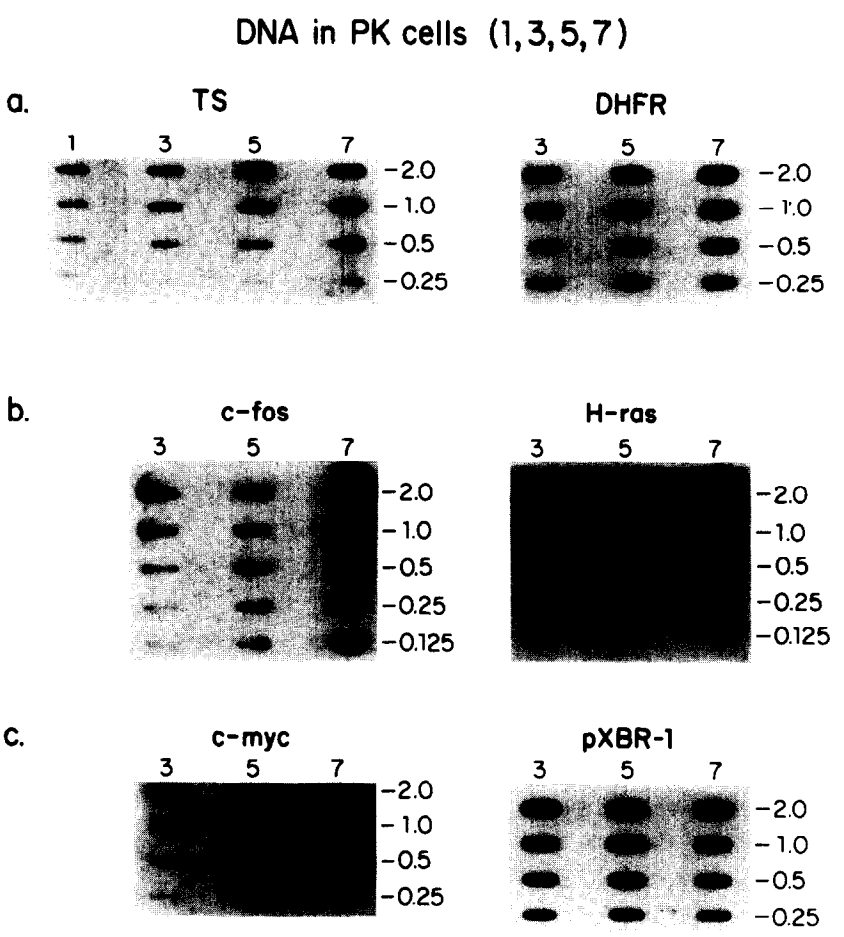

*Fig. 1. DNA analysis of PK cells. Panel A depicts slot blots of TS and DHFR DNA in the serial samples Panel B shows DNA copy number of c-*fos, H-ras, *c-*myc *and pxBR-*1.

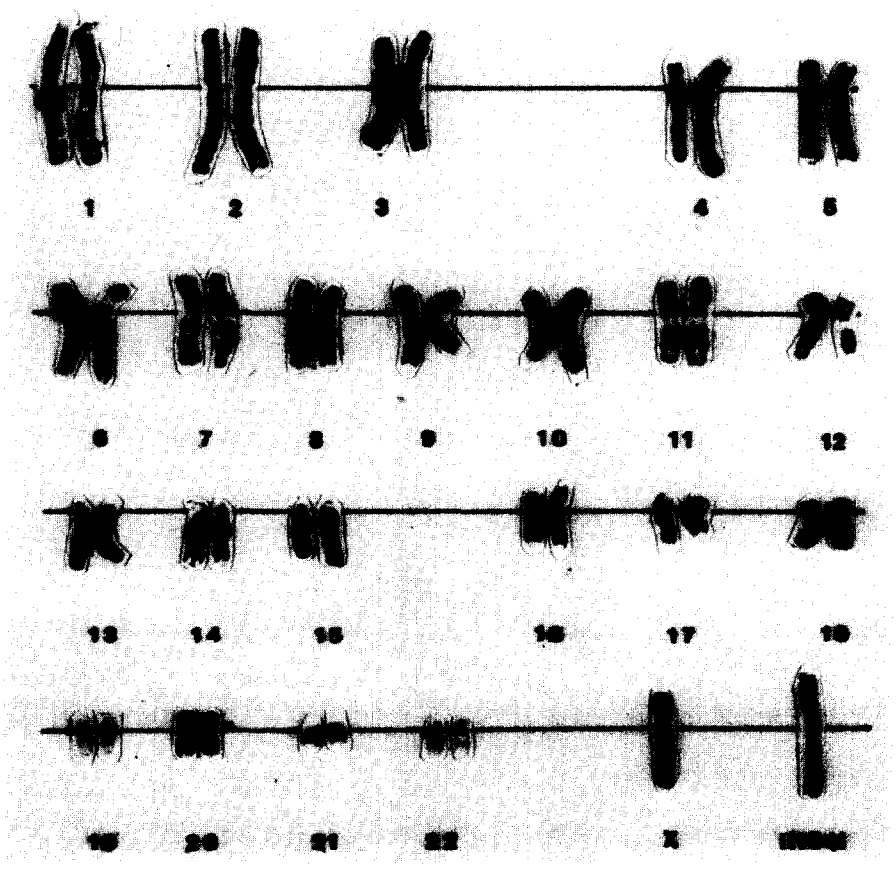

Fig. 2. Karyotype of PK cells: 46,*X*,−*X*,+*i*(13*q*).

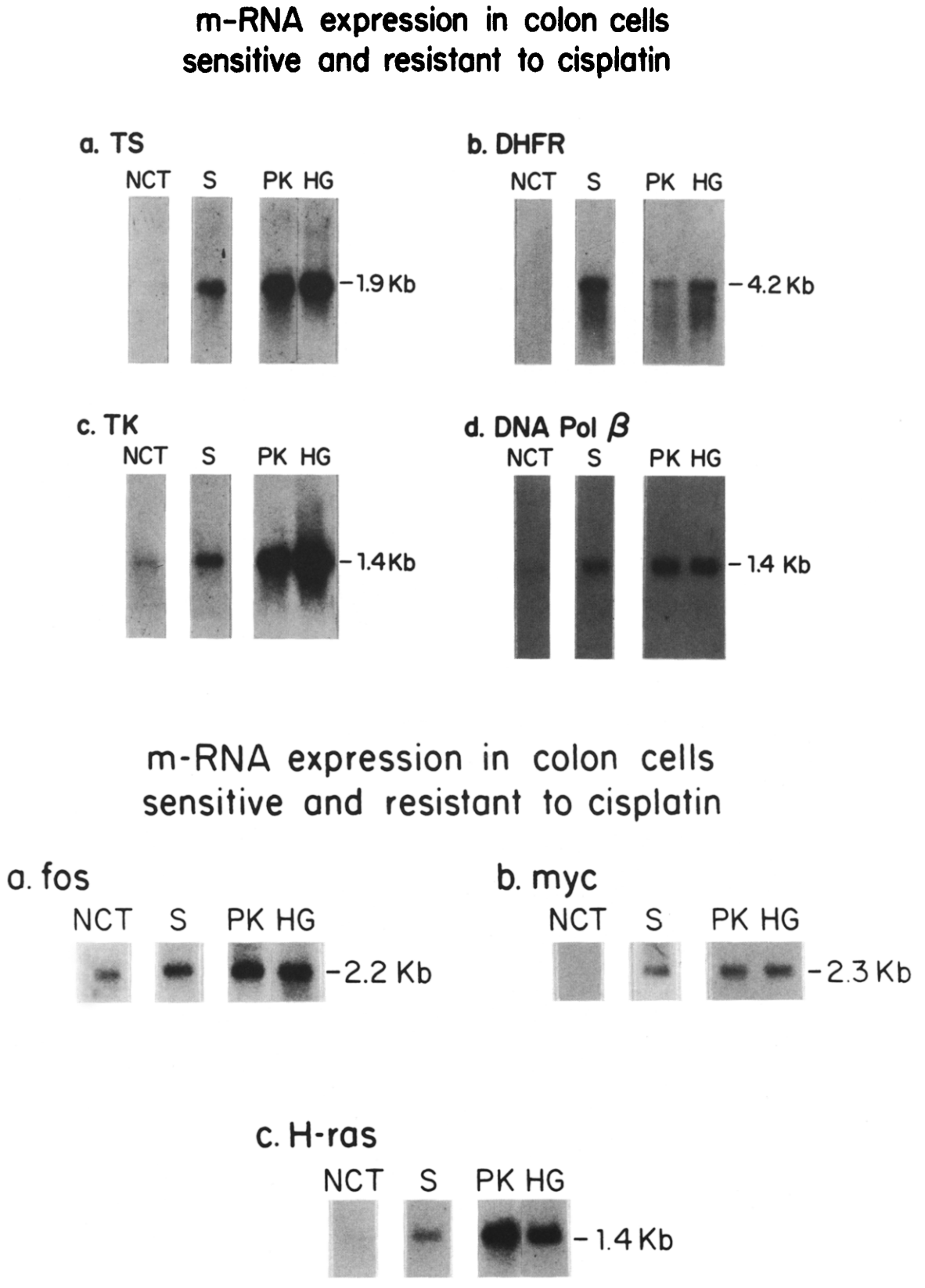

Fig. 3. Northern analysis of mRNA (10μg) from two patients with colon carcinoma (PK_7 and HG), HCT8 cells sensitive to chemotherapy agents (S) and normal colon tissue (NCT).

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