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Expression of P-Glycoprotein mRNA in Human Gastric Tumors

Yoshikazu Sugimoto,[1] Noriko Asami[1] and Takashi Tsuruo[1,2]

[1]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170 and* [2]*Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113*

We have isolated a cDNA clone, pCA12-2, from a λ gt11 cDNA library of an adriamycin-resistant subline of human myelogenous leukemia K562 (K562/ADM) by plaque hybridization with the 2.6 kb genomic probe of P-glycoprotein reported previously. The cDNA pCA12-2 was identified as the 3′-part of P-glycoprotein cDNA by dideoxy sequencing. By using the cDNA probe, expression of P-glycoprotein mRNA was examined in human gastric xenograft lines transplanted in nude mice and clinical samples of human gastric normal tissues and tumors. Five gastric tumor xenograft lines expressed low but significant levels of P-glycoprotein mRNA. The extent of expression was higher in some cases than that observed for R1-3, a weakly drug-resistant subline of K562. Normal gastric tissues from three patients expressed similar levels of P-glycoprotein mRNA and the extent of expression was slightly higher than that of R1-3. Two of three gastric tumor samples expressed higher levels of mRNA than normal gastric tissues. These results suggest that the intrinsic insensitivity of human gastric cancers to chemotherapy could be partly explained by the expression of P-glycoprotein.

Key words: Multidrug resistance — P-Glycoprotein — Human gastric tumor — Cancer chemotherapy

Chemotherapy has been most effective in the treatment of hematologic malignancies, ovarian cancers and small cell lung cancers. Anticancer agents have been less effective in the treatment of colon, kidney, liver and stomach cancer because of the intrinsic insensitivity of individual tumor cells to the anticancer agents.

In the hematologic malignancies, treatment failure often occurs because of the emergence of drug-resistant tumor cells, and thus the development of drug resistance. Some tumor cells show resistance to multiple drugs. This type of resistance is called pleiotropic drug resistance or multidrug resistance (MDR[3]).[1-4] MDR cell lines show cross-resistance to structurally unrelated drugs which were not used for the induction of resistance.[1-4] The MDR cells show lowered accumulation and enhanced efflux of the drugs.[5,6] This change in drug transport in the MDR cells correlates with increased expression of a membrane glycoprotein (P-glycoprotein) with a molecular mass of 170 to 180 kilodaltons.[7-9] Recently, genes corresponding to P-glycoprotein have been isolated.[10-17] Sequence analysis of the cDNA clones[18-20] and functional analysis of P-glycoprotein[21-25] strongly suggest that P-glycoprotein acts as an energy-dependent efflux pump of various anticancer agents in multidrug-resistant cells.

We have established multidrug-resistant sublines of human myelogenous leukemia selected for vincristine (K562/VCR)[26] and for adriamycin (K562/ADM).[27] In the previous report we cloned a fragment of a human multidrug-resistance gene of K562/ADM by using DNA-mediated gene transfer.[13] In this study, we have isolated cDNA clones of the gene from a cDNA library of K562/ADM and confirmed by dideoxy sequencing that a cDNA clone (pCA12-2) codes a 3′-part of the 4.5 kb mRNA of P-glycoprotein.

By using the cDNA probe, we have examined the expression of P-glycoprotein mRNA in human gastric xenograft lines transplanted in nude mice and clinical samples of human gastric normal tissues and tumors. Gastric tumors, which are the most prevalent type of tumors in Japan, are intrinsically rather insensitive to various chemotherapeutic agents. To explore molecular mechanisms of the insensitivity of gastric cancer, we carried out intensive examinations of P-glycoprotein expression in human gastric tumors. In the present study, we found that P-glycoprotein is expressed significantly in gastric tumors. The levels of expression were similar to those observed for the weakly resistant subline of K562.

MATERIALS AND METHODS

Animals and tumor cells Human myelogenous leukemia K562 cells resistant to adriamycin (K562/ADM) and a revertant clone Rl-3 were established in our laboratory as described previously[27] and were maintained in RPMI

[2] To whom correspondence and requests for reprints should be addressed.

[3] The abbreviations used are: MDR, multidrug resistance; cDNA, complementary DNA; K562/ADM, human myelogenous leukemia K562 cells resistant to adriamycin; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

Table I. Profiles of Human Gastric Adenocarcinoma Xenograft Lines

Tumor lines	Age	Sex	Histology	Prior radio- or chemotherapy	Tumor mass doubling time (day)	Expression of P-glycoprotein mRNA[a]
SC-2	24	F	poorly differentiated	—	6.1	3.5
St-4	45	F	poorly differentiated	—	6.6	1.5
SC-6	22	M	poorly differentiated	—	4.1	8
SC-9	83	F	poorly differentiated	—	6.8	1
St-40	—	—	well differentiated	—	4.9	6
R1-3						1

a) Levels in P-glycoprotein mRNA expression were determined by densitometry of the autoradiograms with an RFT scanning densitometer. These values were compared with that of R1-3, which was assigned a value of 1.

1640 medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin. Five human gastric tumor lines established as xenografts in nude mice were used. Their profiles and characteristics are presented in Table I. These tumors have been maintained by serial subcutaneous transplantation of 2×2×2 mm cube fragments in the right subaxillary region of athymic BALB/c-*nu/nu* mice (Clea Japan, Inc., Tokyo) approximately every month. Mice were housed in autoclaved filter-capped cages with autoclaved food and bedding. All cages were kept in laminar-air-flow units in our laboratory. Six- to eight-week-old female mice weighing about 25 g were used for tumor transplantation. When the tumor volume reached about 300 mm^3, the solid tumor was removed and used as a source of DNA and RNA.

P-glycoprotein cDNA clone Total cellular RNA was extracted following disruption of tumor cells in guanidium isothiocyanate followed by CsCl density gradient fractionation.[28] Polyadenylated mRNA was obtained by fractionation on oligodeoxythymidylate cellulose.[28] A cDNA library was constructed in bacteriophage vector λ gt11 starting with mRNA from K562/ADM.[29,30] Phages were plated and screened with 2.6 kb *Eco*RI insert of λ KA2.6 isolated from a genomic library of K562/ADM as described previously.[13]

Among positive phages, a cDNA insert, 1.2 kb in length, was subcloned into the *Eco*RI site of pUC13 vector. The recombinant plasmid, designated pCA12-2, was used for further analysis. This gene was highly amplified in K562/ADM, and hybridized with a 4.5 kb mRNA overexpressed in K562/ADM cells.[13]

The nucleotide sequence of the 1.2 kb *Eco*RI fragment of pCA12-2 was determined by the dideoxy method[31] using M13 vectors.[32] The sequence data were identical to the sequence data of the 3′-part of *mdr*1 cDNA.[18] The 1.2 kb *Eco*RI insert coded the residues 3051-last of the *mdr*1 cDNA. Therefore, the gene isolated by DNA transfection[13] was identified as the P-glycoprotein gene.

Simultaneous isolation of DNA and RNA from solid tumors and tissues Freshly isolated samples of solid tumors or tissues from nude mice or tumor patients were minced, solubilized immediately in guanidium isothiocyanate and centrifuged over a cesium chloride cushion. After centrifugation, RNA was recovered from the pellet in the bottom of the tube[28] and DNA formed a high-viscosity layer in the middle of the tube. The DNA layer was recovered and dialyzed in 10 m*M* Tris-HCl, 1 m*M* EDTA pH 8.0. The DNA solution was extracted with phenol and precipitated with ethanol.

DNA and RNA blot analysis High-molecular-weight DNA from cultured cell lines was extracted as previously described[13] and digested with 10 units of *Eco*RI per μg of DNA for 16 h under the conditions recommended by the supplier. After ethanol precipitation, DNA samples (10 μg/well) were electrophoresed in a 1% agarose gel and transferred to a nitrocellulose filter by the method of Southern.[33] The P-glycoprotein cDNA probe was labeled to give a specific activity of 10^9 cpm/μg by oligolabeling[34] by using [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham Japan Ltd., Tokyo). The filter was hybridized for 16 h at 65°C in a hybridization mixture containing 6×SSC (1×SSC=0.15 *M* NaCl, 0.015 *M* sodium citrate pH 7.2), 5×Denhardt's solution (1×Denhardt's solution = 0.2 g Ficoll 400, 0.2 g polyvinylpyrrolidone, 0.2 g bovine serum albumin per liter), 1% SDS, 100 μg/ml sonicated and denatured *E. coli* DNA, 40 μg/ml poly-

adenylate, and 10^7 cpm of ^{32}P-labeled probe. Filters were washed twice (30 min each) at 65°C in 2×SSC and twice in 0.5×SSC, 0.1% SDS, and then autoradiographed at −70°C with intensifying screens.

For RNA blot analysis, RNA samples (20 μg/well) were run on a 1% agarose gel in 40 m*M* 3-(N-morpholino)propanesulfonic acid-10 m*M* sodium acetate-1 m*M* EDTA, pH 7.0, containing 2.2 *M* formaldehyde and transferred to a nitrocellulose membrane. Hybridization was carried out as described above. Filters were washed in 0.1×SSC, 0.1% SDS.

RESULTS

Expression of P-glycoprotein in human gastric tumors transplanted in nude mice As the genomic DNA and polyadenylated RNA were simultaneously extracted from each solid tumor implanted in nude mice, the contamination of normal mouse cells in the human solid tumors was checked by Southern blot analysis using pCA-12-2. As shown in Fig. 1, hybridization signals of *Eco*RI fragments of five DNA preparations isolated from five human gastric tumors were different in size from those of colon 26, a murine tumor line used as a control. This result indicates that none of the solid tumor used for polyadenylated RNA preparation contained a detectable amount of normal murine cells. In this experiment, the washing condition was moderately stringent (0.5×SSC) in order to detect murine P-glycoprotein gene. For the Northern blot analysis of human P-glycoprotein mRNA, the washing condition was highly stringent (0.1×SSC, 0.1% SDS). This condition is suitable to eliminate the contaminating signal of murine P-glycoprotein mRNA.

Polyadenylated RNA isolated from human gastric tumor cell lines transplanted in nude mice was electrophoresed on an agarose gel, transferred to a nitrocellulose and hybridized to ^{32}P-labeled P-glycoprotein cDNA. Figure 2 shows that these gastric tumors expressed significant amounts of P-glycoprotein mRNA. The amount of RNA loading was confirmed by the control hybridization with β-actin.[35] K562/ADM was used as a positive control expressing very high amounts of P-glycoprotein mRNA. R1-3 is a spontaneously obtained revertant derived from K562/ADM, and shows 3- to 4-fold resistance to adriamycin and vincristine as compared to the parental K562.[35] R1-3 was reported to express a low but significant amount of P-glycoprotein mRNA, which was 5 times higher than that of the parental K562.[35] Therefore, R1-3 was used as another control cell line showing a low level of multidrug resistance and expressing a low level of P-glycoprotein. As shown in Fig. 2, St-4 and SC-9, poorly differentiated gastric adenocarcinoma xenograft lines, expressed an amount of P-glycoprotein mRNA almost equal to that of Rl-3, a weakly resistant cell line. Three other tumor xenograft lines expressed higher levels of P-glycoprotein mRNA. SC-2 and SC-6 are poorly differentiated adenocarcinoma lines and St-40 is a well differentiated line. Among these five adenocarcinoma lines, there is no direct correlation between the stage of differentiation and the expression of P-glycoprotein mRNA. According to Inaba *et al.*,[36] most human gastric tumor xenografts exhibited low response rates to adriamycin or vincristine in nude mice experiments. Among them, SC-9 showed significant sensitivity to ADM (tumor growth inhibition was 63% under the regimen reported previously[36]) and this tumor expressed a relatively low amount of P-glycoprotein.

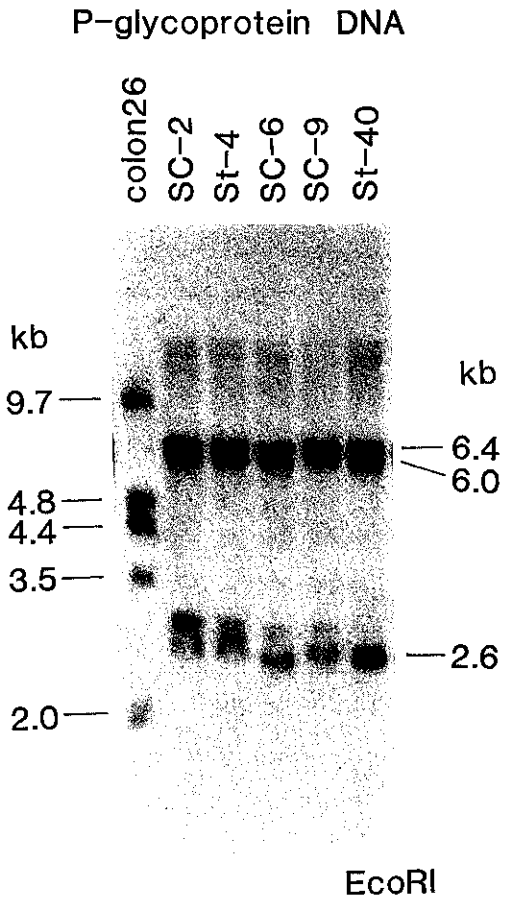

Fig. 1. Southern blot analysis of genomic DNA from human gastric tumor xenograft lines with pCA12-2. Genomic DNA (10 μg) was digested with *Eco*RI, electrophoresed in a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled pCA12-2 probe. Colon 26 is a murine colon adenocarcinoma line. Others are human gastric tumor lines transplanted in nude mice.

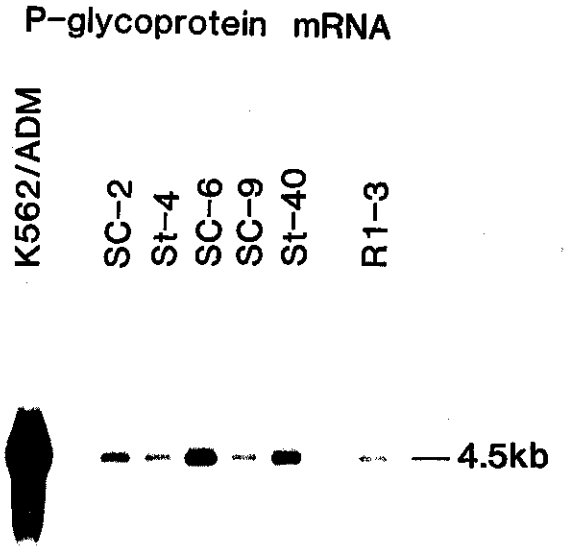

Fig. 2. Northern blot analysis of whole cell mRNA of human gastric tumor xenograft lines with pCA12-2. Polyadenylated mRNA (20 μg) was electrophoresed in a formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled pCA12-2 probe. K562/ADM is a highly multidrug-resistant line. R1-3 is a spontaneously obtained revertant of K562/ADM showing a low level of resistance as compared to the parental K562. Others are human gastric tumor lines transplanted in nude mice.

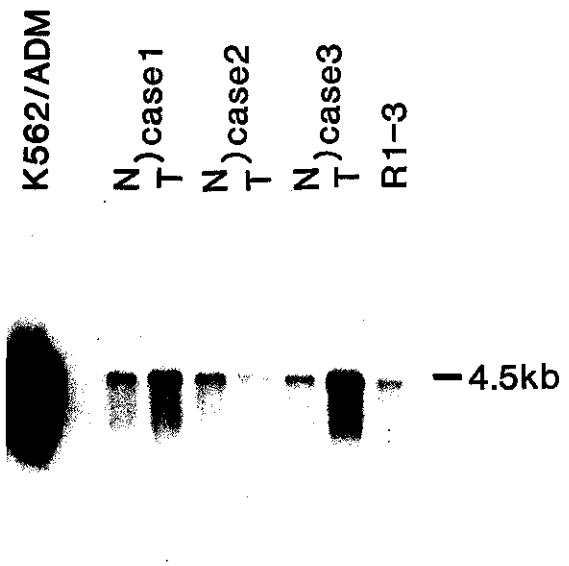

Fig. 3. Northern blot analysis of whole cell mRNA of human gastric normal tissues and tumors with pCA12-2. Polyadenylated mRNA (20 μg) was electrophoresed in a formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled pCA12-2 probe. K562/ADM is a highly multidrug-resistant line. R1-3 is a spontaneously obtained revertant of K562/ADM showing a low level of resistance as compared to the parental K562. Others are human gastric normal tissues and tumors from 3 patients. N, normal tissue; T, tumor.

Expression of P-glycoprotein mRNA in human gastric tumors and normal gastric tissues from untreated patients

The levels of P-glycoprotein mRNA were measured in three gastric cancer patients who had not received any chemotherapy. Northern blot analysis was carried out using pCA12-2 as a probe (Fig. 3). Normal gastric tissues (N) expressed a low level of P-glycoprotein mRNA. The levels among the three patients were very similar and slightly higher than that of R1-3. In 3 gastric tumor samples (T), expression of P-glycoprotein mRNA was different; one sample (case 2) was lower and the other two samples (cases 1 and 3) were higher than that of normal gastric tissues.

DISCUSSION

It has been found that P-glycoprotein is expressed in normal colon, kidney, liver and adrenal tissues of humans, at readily detectable levels by using cDNA clones or antibodies of P-glycoprotein.[37] Expression of P-glycoprotein was also found in secretory epithelium of the uterus during pregnancy[38] or brain endothelial cells at blood-brain barrier sites,[39] suggesting the physiological function of P-glycoprotein as a transport carrier. The P-glycoprotein mRNA was also expressed in several human tumors derived from adrenal gland and colon. In addition, an increased expression of P-glycoprotein was detected in a few tumors at the time of relapse following initial chemotherapy.[37] Several human tumor cell lines originated from kidney were shown to be intrinsically resistant to anticancer drugs at least partly because of the overexpression of P-glycoprotein.[40] In the clinical situation, some human chronic myelogenous leukemia samples at blast crisis expressed P-glycoprotein mRNA.[41] These results suggest that P-glycoprotein is responsible for drug insensitivity of intrinsically drug-resistant tumor cells such as colon, liver and kidney, and of clinically developed drug-resistant leukemia cells.

In western countries, notably in the United States, stomach cancer is ranked as one of the rare cancers. The Japanese, at present, have the highest incidence of stomach cancer, although the incidence has apparently been declining since around 1960, in part due to a recent change in dietary habits away from traditional Japanese

foods towards western-style foods.[42–44] However, the Japanese still show the highest mortality and incidence of stomach cancer in the world at the present time.[42–45] Mass screening and surgical treatment are effective in decreasing stomach cancer mortality, but chemotherapy is usually ineffective.[36]

In our present study, human stomach tumors expressed low but significant levels of P-glycoprotein, which may be sufficient to explain the low level of resistance, similar to that of R1-3. In Fig. 3, P-glycoprotein mRNA levels in the tumors of three patients or in nearby normal stomach tissues are shown. The three normal tissues expressed similar levels of P-glycoprotein mRNA. On the contrary, the expression levels in the three stomach tumors were different. The expression levels of two samples were higher and that of one sample was lower than the expression level of normal tissue. These results suggest that the heterogeneity of P-glycoprotein mRNA expression is caused by the heterogenous nature of the original cells, or alterations of mRNA expression during tumor progression.

Recently we have developed monoclonal antibodies against P-glycoprotein.[46] These antibodies, MRK16 and MRK17, can recognize the cell surface domain of P-glycoprotein, and therefore are suitable for the clinical diagnosis of P-glycoprotein expression especially in the hematological malignancies. By using the monoclonal antibodies, we have detected P-glycoprotein in a cultured line of human gastric tumor (unpublished results). In the present study, we have isolated cDNA probes and applied them for the determination of the level of P-glycoprotein mRNA expression. Now we are trying to detect P-glycoprotein of human stomach by using the monoclonal antibodies. These two tools are effective in the measurement of P-glycoprotein expression, which could be a valuable guide for the prediction of insensitivity of tumors to chemotherapeutic agents.

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