Detection of Drug Resistance in Human Tumors by in Vitro Enzymatic Amplification


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ABSTRACT

Both acquired and natural resistance to chemotherapy agents have proved problematic in the treatment of neoplasia. Thymidylate synthase, which catalyzes the synthesis of thymidine precursors, has been shown to be amplified in response to a variety of chemotherapeutic agents. The detection of such amplification could prove beneficial in the development of alternative clinical protocols. In this study we report the use of existing enzymatic amplification methods in order to detect incipient amplification of the thymidylate synthase gene upon resistance to cisplatin. The assay utilizes a modification of the polymerase chain reaction in which a sequence of the thymidylate synthase gene is amplified including two flanking oligonucleotides acting as primers for DNA synthesis. This method exhibits greater sensitivity than conventional nucleic acid detection methods and requires less than 100 ng of total RNA from patient tumors and no in vitro culturing of patient cells.

INTRODUCTION

The TS cycle plays a central role in DNA biosynthesis since it represents the sole source of de novo thymidine and the availability of thymidine is rate-limiting in DNA synthesis (1). As a result, the TS cycle has been the target of many chemotherapeutic agents, such as methotrexate, 5-FUra, and 5-fluorodeoxyuridine, among others (2). We have recently shown that cisplatin, initially thought to kill tumor cells exclusively by binding to DNA (3), also exerts an influence on the TS cycle by causing elevations in the intracellular pools of folate cofactors, thereby rendering ovarian carcinoma cells more sensitive to 5-FUra (4). Moreover, we have shown that tumor cells resistant to cisplatin display increased levels of TS, whether through elevated gene expression (in vitro) (5, 6) or gene amplification (in vivo) of TS cycle genes (7). Hence if TS gene amplification represents one mechanism of resistance to cisplatin, 5-FUra, and 5-fluorodeoxyuridine (8, 9), then the development of an assay to detect this amplification in patient cells could aid in the clinical monitoring of drug resistance. In this study we describe such an assay using existing methods of in vitro enzymatic amplification (10, 11) to detect levels of TS gene expression.

MATERIALS AND METHODS

Tumor Cells. The human ovarian carcinoma cell line, A2780, was obtained from Dr. R. Ozols at the National Cancer Institute (5), and a human colon carcinoma cell line, HCT8, was obtained from Dr. J. R. Bertino, at Memorial Sloan-Kettering, New York, NY (12). These cells were maintained in RPMI 1640 media (folate acid free) with 10% dialyzed fetal calf serum and 10^-4 M folic acid (6). Patient DNA or RNA from two colon carcinomas (P. K., H. G.) that failed cisplatin chemotherapy and three ovarian carcinomas (M. D., D. M., and T. M. S.) patients who had failed cisplatin and 5-FUra treatment (7) were analyzed in the PCR assay. Normal ovarian tissue, normal colon mucosa tissue, and moderately differentiated adenocarcinoma of the sigmoid colon untreated with chemotherapeutic agents or radiation were obtained from the Tissue Procurement Service at the University of Alabama at Birmingham. DNA was purified by the proteinase K/SDS method (5). RNA was extracted by the guanidine/cesium chloride method (5).

PCR Assay. Total RNA from both A2780 cells and patient cells (P. K., H. G., T. M. S., D. M., and M. D.) was incubated in 1× amplification buffer [10 mM Tris-HCl (pH 8), 6.6 mM MgCl2, 100 pmol of Primer 2 and Primer 3, 6.0 mM β-mercaptoethanol, 60 mM NaCl], 1 mM DTT and 1.5 mM dNTPs (Pharmacia) in a final volume of 0.1 ml. The Mg2+ should be titrated for the optimum enzyme activity (1.5 mM). Samples were heated to 95°C for 5 min and cooled to 37°C for 2 min, at which time 2 units of avian myeloblastosis virus reverse transcriptase (Life Sciences) were added and incubated for an additional 2 min. Samples (1 μg of DNA or 100 ng of total RNA) were denatured and cooled again, and in Round 2 both reverse transcriptase and 0.5 units Klenow DNA polymerase (New England Biolabs) were added, followed by a 2-min incubation (37°C). In Round 3, samples were heated to 95°C for 2 min. Reverse transcriptase treatment was discontinued after two rounds, and an additional 0.5 units DNA polymerase were included in Rounds 10 and 20. In Round 3, 0.09 μg RNase A (Sigma) degraded the RNA following the denaturation step. In Round 3, samples were heated to 95°C (2 min) and cooled to 72°C for 3 min. This cycle of heating, annealing, and polymerization was continued for 25 min with 0.5 units of Thermus aquaticus DNA polymerase (New England Biolabs). The calculated error rate for DNA polymerase is 1 in 5000 bases and higher temperatures minimize mispriming.2 All enzymes were diluted in 1× amplification buffer + 1 mM DTT. The polymerization time was increased with increasing number of cycles (i.e., from 1.5 to 30 min, and from 10 to 25 cycles). After completion of the last round, samples were stored at 4°C and electrophoresed in 1.8% agarose gel, 1 μg/ml ethidium bromide, 0.5X TBE buffer, and 0.5X SYBR Green I (Molecular Probes) stained. The 200 bp band was cut out and the DNA was extracted with phenol/chloroform/isoamyl alcohol (1:1:1) and precipitated with 2 volumes of 95% ethanol. The DNA was resuspended in 1× amplification buffer and stored at -20°C. When desired, the amplified DNA was cleaved with 100 units PstI (BRL) in the presence of 1× reaction buffer [50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 50 mM NaCl] and 0.1 mg/ml bovine serum albumin for 1 h at 37°C.

Received 2/9/88; revised 5/18/88; accepted 7/20/88.

1This work was supported by the American Cancer Society (CH265) and Bristol-Myers Co.
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3The abbreviations and trivial name used are: TS, thymidylate synthase; 5-FUra, 5-fluorouracil; cisplatin, cis-diamminedichloroplatinum(II); PCR, polymerase chain reaction; DTT, dithiothreitol.
5Unpublished data.
RESULTS AND DISCUSSION

In preparation for the modified PCR assay, tumor cells are obtained from patients’ tissue or peritoneal fluid, and total RNA is isolated as described (5, 6). With the use of oppositely oriented primers (designated as TS nos. 2 and 3) a 171-basepair region of the TS gene is replicated repeatedly in vitro, flanked by the two oligomers. Fig. 1 schematically portrays the position of the oligomers vis-à-vis the rest of the TS gene (13).

Experimentally, the oligomers are annealed to the RNA template, and prime the first strand polymerization using reverse transcriptase. The samples then undergo cycles of denaturation, annealing, and polymerization by addition of heat-stable DNA polymerase. This treatment is continued for 25 rounds, and inclusion of ribonuclease A in Round 3 helps eliminate RNA which may compete for oligonucleotide binding. The advantages of using RNA instead of DNA as the template for amplification have been previously discussed (11). Briefly, the lower sequence complexity of RNA should enhance priming efficiency, and totally intact RNA is not required for successful enzymatic amplification since the target sequence of amplification is relatively short (171 basepairs). In some tumors, only enhanced gene expression has been demonstrated for drug resistance in the absence of gene amplification (5-7). Most importantly, the use of RNA allows us to extend the concept of gene amplification upon drug resistance to the messenger level, a phenomenon we were previously unable to investigate due to the inordinate number of patient cells required for RNA analysis (7). The PCR assay allows us to circumvent that requirement, since the use of as little as 100 ng total patient RNA is sufficient to generate a strong signal after completion of 25 rounds of amplification.

Upon termination of the amplification reaction, the samples are electrophoresed in 0.5× TAE and alkaline blotted onto Zeta Probe filter membranes. The amplified DNA segment is detected by probing with a third oligomer (designated as TS no. 4) complementary to a region within the amplified sequence (Fig. 1). Amplification of RNA or DNA is shown by comparison of levels of TS in patients with known clinical resistance to cisplatin/5-FUra and reported gene amplification (7) to two stable DNA polymerase. This treatment is continued for 25 rounds, and inclusion of ribonuclease A in Round 3 helps

The PCR product derived from 25 rounds of amplification was then subjected to Pstl digestion and probed with TS no. 4. The PCR product derived from 25 rounds of amplification was then subjected to Pstl digestion and probed with TS no. 4. Theoretically, such a treatment should cleave the amplified sequence to be amplified was selected so that it contained a cleavage site for a restriction endonuclease, in this case Pstl. The PCR product derived from 25 rounds of amplification was then subjected to Pstl digestion and probed with TS no. 4. Differences between the concentration of TS mRNA from A2780S and A2780DDP cells can be detected as early as the tenth round in this assay. This product from the PCR assay was further identified as the digested probe. As shown in Fig. 1, the sequence to be amplified was selected so that it contained a cleavage site for a restriction endonuclease, in this case Pstl. The PCR product derived from 25 rounds of amplification was then subjected to Pstl digestion and probed with TS no. 4. Theoretically, such as treatment should cleave the amplified sequence into two fragments, only one of which (the 72-basepair moiety) should be detected by autoradiography (see Fig. 1). Fig. 2A detects the progressive exponential amplification of the desired 171-basepair segment in A2780 cells between 10 and 20 rounds. The amplified fragment of A2780S mRNA is not yet detected at the end of 10 rounds of the PCR assay (lane A), but the 15-round sample does yield a noticeable band (lane B), and marked amplification is observed upon completion of twenty rounds (lane C). Differences between the concentration of TS mRNA from A2780S and A2780DDP cells can be detected as early as the tenth round in this assay. This product from the PCR assay was further identified as the defined sequence.

Before examining levels of TS mRNA in drug-resistant cells, potential practical problems of the PCR assay must be considered. First, mRNA degradation may affect the true amplification seen in the samples. However, an amplified sequence of sublines of A2780 ovarian carcinoma cells sensitive and three-fold resistant to cisplatin (5, 6). Previous studies have shown a direct relationship between the amount of mRNA and TS enzyme activity (5, 6).

Fig. 2A essentially portrays control experiments demonstrating the successful adaptation of the assay using the modifications alluded to above. Fig. 2A detects the progressive exponential amplification of the desired 171-basepair segment in A2780 cells between 10 and 20 rounds. The amplified fragment of A2780S mRNA is not yet detected at the end of 10 rounds of the PCR assay (lane A), but the 15-round sample does yield a noticeable band (lane B), and marked amplification is observed upon completion of twenty rounds (lane C). Differences between the concentration of TS mRNA from A2780S and A2780DDP cells can be detected as early as the tenth round in this assay. This product from the PCR assay was further identified as the defined sequence. As shown in Fig. 1, the sequence to be amplified was selected so that it contained a cleavage site for a restriction endonuclease, in this case Pstl. The PCR product derived from 25 rounds of amplification was then subjected to Pstl digestion and probed with TS no. 4. Theoretically, such as treatment should cleave the amplified sequence into two fragments, only one of which (the 72-basepair moiety) should be detected by autoradiography (see Fig. 1). Fig. 2A depicts the results of this digestion as a segment shorter in length hybridized to the radiolabeled probes.

Before examining levels of TS mRNA in drug-resistant cells, potential practical problems of the PCR assay must be considered. First, mRNA degradation may affect the true amplification seen in the samples. However, an amplified sequence of
only 171 basepairs will minimize the effect of mRNA degradation. Defined concentrations of standard TS mRNA are also run concurrently in the PCR assay for detection of any degradation. Secondly, DNA contamination of the samples may lead to amplification of TS DNA sequences as well as RNA (Fig. 3A). Amplification of the mRNA sample in the absence of reverse transcriptase demonstrates a faint band suggesting possible DNA contamination. Treatment with DNase (5 μg for 5 min) and removal of reverse transcriptase from the assay eliminates most of the DNA contamination. DNA contamination of the mRNA samples was determined to be less than 0.1% of the sample. Finally, we examined the PCR assay for linearity of amplification using TS mRNA generated from a pTZU18 (T7 RNA polymerase) vector (BRL) containing the TS cDNA. There was a linear increase in the product of transcription when different concentrations of TS mRNA ranging from 1 to 1000 pg were subjected to the PCR assay (Fig. 3B).

The next step concerns the examination of elevated TS gene expression upon resistance to cisplatin/5-FUra in six different ovarian tissue samples: the aforementioned A2780S ovarian cell line sensitive to cisplatin; a subline (termed A2780DDP) threefold resistant to cisplatin (Fig. 4, A and B); and tumor cells from three ovarian carcinoma (M. D., D. M., and T. M. S.) patients (7) failing treatment with (and therefore resistant to) the cisplatin/5-FUra combination (Fig. 4, D-F). Previous work with the A2780DDP cells established a 3.6-fold enhancement of TS mRNA and enzyme activity (without gene amplification) as compared to the parent line (5, 6). With respect to the ovarian tissue samples, resistance to the chemotherapeutic regimen correlated with a 4-fold amplification of mRNA and DNA in the M. D., D. M., and T. M. S. samples (7). This information allows us to test the utility of the PCR assay as a method of detecting failure to chemotherapy, since a small degree of elevation in TS expression could be magnified and detected definitely after 25 rounds of the polymerization reaction. To this end, total RNA from all six samples was incubated with the oligonucleotides in the PCR assay. The results, shown in Fig. 4A, point to a 3.8-fold difference in the PCR product of the A2780DDP cells as opposed to the A2780 cells. The ratio of the in vitro enzymatic amplification was similar to that measured by northern blot analysis (5, 6). The three patients (Fig. 4, D-F) M. D., D. M., and T. M. S. showed 9.6-, 9.0-, and 8.2-fold increases, respectively, in TS mRNA when compared to normal ovarian tissue (NOT) (Fig. 4C). The in vitro enzymatic amplification of RNA in the patient cells confirmed the previously described increase in TS gene expression (7). These results show that in the three patients, amplification of TS was not limited to the level of the gene, but that cells failing treatment with cisplatin/5-FUra exhibit higher expression of TS mRNA.

In human colon samples, the PCR product of HCT8DDP cells was 4.1-fold greater than the drug-sensitive HCT8S cells (Fig. 4, A', B'), which is similar to the difference found using Northern analysis (14). Normal colon tissue (Fig. 4C) and human colon carcinoma tissue untreated with cancer chemotherapeutic agents (Fig. 4, D', E') contained similar amounts of TS mRNA. In contrast, two colon carcinoma patients (P. K. and H. G.) who failed cisplatin treatment also had elevated TS mRNA (3.7- and 9.6-fold increases, respectively; Fig. 4; F', G'). Furthermore, ovarian DNA from normal tissue and a patient (T. M. S.) sample was subjected to the original PCR assay. The results, which appear in Fig. 5, demonstrate gene amplification in the TS patient sample, corroborating previously reported data (7). Thus, we have shown that the PCR technique allows for the detection of drug-resistant tumor cell lines and ovarian tissue samples.
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assay can duplicate results obtained from conventional nucleic acid detection methods, but it far exceeds the latter due to its greater sensitivity and the insignificant amount of patient RNA required for successful enzymatic amplification.

We have thus developed a technique in order to monitor gene or mRNA amplification in patient cells. This method could employ normal tissue and untreated carcinoma tissue as a standard, representing drug-sensitive cells, as we have done here. Alternatively, cells obtained from the patient prior to treatment with the anticancer agents could be stored and used as an internal standard. The level of TS gene expression in the patient's tumor cells following multiple courses of chemotherapy could then be compared to the pretreatment standard and other patient samples (such as P. K., H. G., T. M. S., D. M., and M. D.) which could represent the drug-resistant "standards." One patient that has responded to the cisplatin/5-FUra combination has not shown an increase in TS gene amplification.

"In tumors, it is likely that a common mechanism underlies the amplification of genes which confer drug resistance and genes which give a growth advantage to the tumor" (15). We chose to develop the PCR assay for TS since, because of its significance in DNA biosynthesis, enhanced TS gene expression (a) may contribute such a selective growth advantage when cells become drug resistant and (b) is one of the initial steps in cisplatin resistance (5–7). As it stands, this assay could aid in the detection of early failure to cisplatin, 5-FUra, leucovorin, and 5-fluorodeoxyuridine, which alone or in combination with each other or other drugs are used in colon, ovarian, testicular, and head-and-neck carcinomas, among others. Thus a "positive" result in the PCR assay could prompt a much earlier investigation of drug resistance clinically in order to switch to alternative treatment protocols. It is important to note, however, that this assay can be tailored to monitor the cellular response to other agents in which gene amplification or increased gene expression is a mechanism of drug resistance. For instance, we are currently in the process of synthesizing oligo-primers specific to dihydrofolate reductase, which has been shown to be amplified in response to methotrexate (16). Finally, oncogene amplification may play a role in achieving high levels of resistance clinically (7) and correlate with overall survival in breast cancer (17). The use of the PCR assay (modified for specific oncogenes such as fos) could be beneficial in the clinical monitoring of tumor progression as well as drug resistance (7).

In conclusion, data presented here shows that the modified PCR assay can be an effective device in the detection of early failure to chemotherapy agents since it has confirmed data achieved by conventional molecular biology techniques while circumventing practical problems of time and sensitivity of detection as well as number of patient cells required for this detection. Moreover, the data generated by the PCR assay has enabled us to analyze TS mRNA expression in tumor samples where previously we were limited to detecting only gene amplification in response to cisplatin and 5-FUra.

ACKNOWLEDGMENTS

We wish to thank Dr. L. Leong and Dr. D. Blayney for providing the patient cells, Dr. B. Kaplan for synthesizing the oligoprimer, Dr. L. Carlini for quantitation of the PCR products, and P. Ure for preparing the manuscript.

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