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Increase in tumor *GADD153* mRNA level following treatment correlates with response to paclitaxel

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Abstract *Purpose*: We investigated the relationship between the basal and treatment-induced change in the tumor expression of the drug resistance gene *MDR1* and the cellular injury response gene *GADD153,* and clinical response to paclitaxel treatment. *Methods*: *MDR1* and *GADD153* mRNA levels were measured by reverse transcriptase polymerase chain reaction (RT-PCR) in tumor samples obtained by fine needle aspiration biopsy from 14 patients before and 24 h after paclitaxel infusion. *Results*: There was no difference between responders and non-responders with respect to either the basal *MDR1* mRNA level or the change in *MDR1* mRNA level at 24 h after treatment ($P = 0.464$). Likewise, there was no difference in basal *GADD153* mRNA level between responders and non-responders. However, there was a significantly greater increase in *GADD153* mRNA at 24 h in responders compared with non-responders ($P = 0.005$). An increase in *GADD153* mRNA level of 1.5-fold or higher predicted response with a sensitivity of 86% and a specificity of 100%. *Conclusions*: An increase in *GADD153* mRNA level reflects chemotherapy-induced damage sufficient to be manifest as a clinically detectable reduction in tumor volume. Measurement of the change in *GADD153* mRNA level successfully identified patients destined to respond as early as 24 h post-treatment.

Key words *GADD153* · *MDR1* · Paclitaxel

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Introduction

There is a clinical need to detect response quickly after administration of chemotherapeutic drugs and an even a greater need to predict likelihood of response. *GADD153*, a cellular injury response gene, is a member of a family of genes that undergo a coordinate increase in mRNA level in response to growth arrest and to DNA-damaging agents [6, 7, 16]. This gene encodes the nuclear protein CHOP 10 (C/EBP homologous protein) that acts as a negative modulator of C/EBP transcriptional factors, which participate in the process of terminal differentiation and growth arrest in adipose tissue, to inhibit cell cycle progression [29]. CHOP 10 itself cannot bind to DNA, although it is able to form stable heterodimers with other C/EBP proteins to prevent their binding to DNA or promote their binding to nonclassical CCAAT sites [32]. It was originally isolated by subtractive hybridization on the basis of rapid induction by UV radiation in Chinese hamster ovary (CHO) cells [23]. However, it was subsequently shown to be induced by a wide variety of DNA-damaging agents such as cisplatin, ormaplatin, mitomycin C, actinomycin D, doxorubicin, etoposide, and paclitaxel, which injure cells by different mechanisms [3, 5–9]. Moreover, it has been demonstrated in cell lines and in xenograft systems that

there is a good correlation between the extent of cellular injury and treatment-induced increase in *GADD153* message level after exposure to certain of these DNA-damaging agents [2, 3, 9, 10, 17, 18, 20].

The *MDR1* gene product P-glycoprotein functions as an energy-dependent efflux pump, which decreases the intracellular accumulation of many types of chemotherapeutic agents [11, 12, 15, 24, 26, 28]. Over-expression of *MDR1* is one cause of resistance to paclitaxel and other drugs that are substrates for the P-glycoprotein pump. Resistance to paclitaxel often emerges during treatment and is a major obstacle to successful cancer therapy (reviewed in reference [26]) and there is evidence that resistance to paclitaxel can be mediated by P-glycoprotein [30].

We conducted a study in patients receiving paclitaxel in combination with high dose progesterone (used as a P-glycoprotein modulator) to determine whether change in the level of either *GADD153* or *MDR1* mRNA could be used as a molecular marker for the detection or prediction of clinical response. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on mRNA isolated from needle biopsies of tumor obtained immediately prior to and at 24 h after the start of paclitaxel administration. Recent developments in PCR technology allow us to determine, semi-quantitatively, relative levels of gene expression by comparing the ratio of RT-PCR products derived from the gene of interest, in this case *GADD153* or *MDR1*, with an endogenous internal standard gene, in this case *β-ACTIN* [14, 25].

Materials and methods

Cell culture conditions and northern blotting

The human ovarian serous adenocarcinoma cell line 2008 [4] were grown as monolayer cultures in a humidified incubator at 37 °C and 5% CO_2. 2008 cells were maintained in complete RPMI 1640 supplemented with 5% fetal calf serum (Irvine Scientific, Santa Ana, Calif.) and 2 m*M* L-glutamine.

Total RNA was extracted from the cells by the isothiocyanate/cesium chloride method [1] and electrophoresed on a 1% formaldehyde agarose denaturing gel. The RNA was then transferred to Magnagraph nylon membranes (Micron Separations, Westboro, Mass., USA) by capillary transfer. The RNA was immobilized by baking at 80 °C for 30 min. Probes were labeled with (^{32}P)-dCTP using the Amersham Multiprime kit (Amersham, Arlington Heights, Ill., USA). The blots were prehybridized, hybridized, and washed according to Sambrook et al. [31]. The blots were then analyzed and quantitated by the Molecular Imager System (Bio-Rad, Hercules, Calif., USA). The human *GADD153* probe was a gift of Dr. N.J. Holbrook (NIA, NIH, Baltimore, Md.). Lane loading differences were corrected for by comparison with the same blot hybridized with a *β-ACTIN* probe.

Patient characteristics, treatment, and tumor tissue procurement

Nineteen patients were entered on this study, all of whom gave written informed consent before study entry in accordance with Federal, State, and local guidelines. Both at time zero and 24-h samples containing sufficient mRNA were obtained from eight and 14 patients, respectively, for analysis of the change in *MDR1* and *GADD153* mRNA levels. mRNA was evaluable for basal *MDR1* expression prior to therapy from a total of 14 patients. The biopsy samples from the other patients yielded mRNA that was insufficient in either amount or quality for these PCR reactions. Table 1 provides information on the characteristics of the patients entered on this study. All patients had measurable disease, and all had been heavily pretreated with other chemotherapy regimens. Responses were defined as either partial (> 50% reduction in tumor volume) or minimal (> 25% but < 50% reduction in tumor volume). No patient attained a complete response. Progesterone was first dissolved in ethanol at a concentration of 66.67 mg/ml and stored in 50-ml glass vials. On the day of treatment, the progesterone-ethanol stock solution was diluted 1:11 in Intralipid 20% and administered as a 24-h continuous infusion concurrently with 125 mg/m^2 paclitaxel (Taxol, Bristol Myers, Syracuse, N.Y.). Tumor tissue specimens were obtained by fine needle aspiration immediately before treatment and at 24 h after the start of drug infusion. The biopsy was added to Buffer D (4 *M* guanidine isothiocyanate, 25 m*M* sodium citrate, pH 7, 0.5% Sarcosyl, and 0.1 *M* 2-mercaptoethanol), homogenized, and prepared for RNA extraction.

RNA extraction, cDNA synthesis, and quantitation

The procedure followed for RNA extraction was similar to that described by Sambrook et al. [31]. Following extraction, the RNA pellet was dissolved in DEPC-treated water and treated with DNAse I (Epicenter Technologies, Madison, Wis., USA) at 37 °C for 30 min. The DNAse was later denatured by heating the tubes to

Table 1 Characteristics of the patients. *PD* progressive disease, *MR* minimal response (25–50% reduction in tumor size), *PR* partial response (> 50% reduction in tumor size)

Patient number	Cycle	Patient response	*GADD153* induction after 24 h	Primary tumor
1	1	PD	–	Ovarian adenocarcinoma
2	3	PD	–	Endometrial adenocarcinoma
3	1	PD	–	Squamous cell cancer of the throat
4	1	PD	–	Squamous cell cancer of the lung
5	9	MR	+	Ovarian adenocarcinoma
5	1	PR	+	Ovarian adenocarcinoma
6	1	PD	–	Squamous cell cancer of the mandible
7	1	MR	+	Squamous cell cancer of the tongue
8	1	PD	–	Ovarian adenocarcinoma
9	1	PD	–	Breast cancer
10	1	MR	+	Ovarian adenocarcinoma
11	1	PR	–	Large cell lymphoma
12	1	PR	+	Colon cancer
13	1	PD	–	Breast cancer
14	5	MR	+	Colorectal cancer
15	1	PD	–	Nasopharyngeal cancer

70 °C for 10 min. MMLV-RTase mix (RNAguard, 0.1 *M* DTT, 5X MMLV buffer, 10 m*M* dNTP's, BSA, random primers – Gibco/BRL) was added to the samples, followed by MMLV-RTase enzyme (Gibco/BRL-2000 U/ml). The reaction was incubated for 10 min at room temperature, then for 45 min at 42 °C, and finally for 3 min at 90 °C. Another aliquot of MMLV-RTase mix was added and the reaction was incubated for 45 min at 42 °C and 10 min at 90 °C (Perkin-Elmer Thermocycler, Norwalk, Conn., USA). RNAse A (2.5 mg/ml) was added and incubated at 37 °C for 30 min, to remove remaining RNA from the hybrid. The samples were then stored in 0.5-ml microfuge tubes at −20 °C.

The amount of cDNA made from tumor mRNA was estimated by ethidium bromide analysis as previously described [31]. This semi-quantitative data was used to select a range of four dilutions for the PCR reaction (vide infra). If cDNA concentrations were barely detectable by this method, an additional PCR amplification of *β-ACTIN* was performed to determine whether enough cDNA was present to quantitate gene expression in these samples. Sufficient cDNA was present to quantitate change in *MDR1* and *GADD153* message levels in eight and 14 patients, respectively, and for measurement of basal *MDR1* message level in 14 patients.

PCR conditions and quantitation of PCR product

A modification of the method of Horikoshi et al. [14] was used for PCR. A stock solution was prepared by adding 20 µl of the cDNA sample isolated from the previous procedure to 80 µl sterile water in 0.5-ml tubes, and dilutions ranging from 1:2 to 1:2500 were made from this stock solution. To new sterile 0.5-ml centrifuge tubes were added 1.52 m*M* Taq mix for *β-ACTIN* (10X Taq Buffer, without $MgCl_2$; 25 m*M* $MgCl_2$; sterile water; 10 m*M* dNTP's; 0.5 *M* Spermidine) or 2 m*M* Taq mix for *GADD153* and *MDR1* (same as Taq buffer for *β*-ACTIN but using different amounts of 25 m*M* $MgCl_2$ and sterile water), and their respective 3′ and 5′ primers. The primers used are listed below and were synthesized by the Molecular Core Facility of the UCSD Cancer Center.

Four cDNA dilutions were chosen based on the ethidium bromide quantitation and triplicate aliquots of each were added to the tubes. The dilutions for *GADD153* and *MDR1* were always lower than those for *β*-ACTIN because the mRNA level of *GADD153* and *MDR1* averaged approximately 1000-fold and 20-fold less than that of *β*-ACTIN, respectively [18–20]. A "hot start" was done to denature the cDNAs; sterile water, 1× Taq Buffer, Ampli-Taq polymerase, and 1.26 µCi ^{32}P-dCTP were then added to each tube. The PCR cycles were 2 min at 94 °C before the first cycle, then 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C for 45 cycles. The PCR products were then stored at 4 °C.

Five microliters of polyacrylamide gel loading dye was added to each tube of PCR product from the previous procedure and these mixtures were electrophoresed on an 8% polyacrylamide gel. The bands of interest were quantified on a Bio-Rad Molecular Imager using the Biorad Molecular Analyst software. The amount of PCR product is a function of input cDNA. The amounts of product from the four serial dilutions were used to fit regression lines for *GADD153*, *MDR1*, and *β*-ACTIN and the results were expressed as the ratio of the slope of the regression line for either *GADD153* or *MDR1* to that for *β*-ACTIN.

Statistics

Fisher's exact test was used to evaluate contingency tables of *GADD153* message levels versus patient response (data not shown). The sensitivity of this test is the number of partial/minimal responders with an increase in *GADD153* message levels after 24 h, over the total number of partial/minimal responders. The specificity is defined as the number of non-responders without an increase in *GADD153* mRNA levels after 24 h divided by the total number of non-responders. The Student's *t*-test was used to evaluate differences in basal *MDR1* levels.

Results

Correlation between *GADD153* message level and magnitude of injury caused by paclitaxel

Human ovarian carcinoma 2008 cells were exposed to paclitaxel for 24 h, over a concentration range corresponding to 1–10 times the concentration required for 50% growth inhibition (IC_{50}), after which the cells were harvested for RNA extraction and determination of survival in a clonogenic assay. Figure 1A shows that there was a progressive increase in the *GADD153*

GADD153:	CATACATCACCACACC (sense);	TGACCACTCTGTTTCC (antisense)
MDR1:	CCCATCATTGCAATAGCAGG (sense);	TGTTCAAACTTCTGCTCCTGA (antisense)
β-ACTIN	GAGCGGGAAATCGTGCGTGACATT (sense);	GATGGAGTTGAAGGTAGTTTCGTG (antisense)

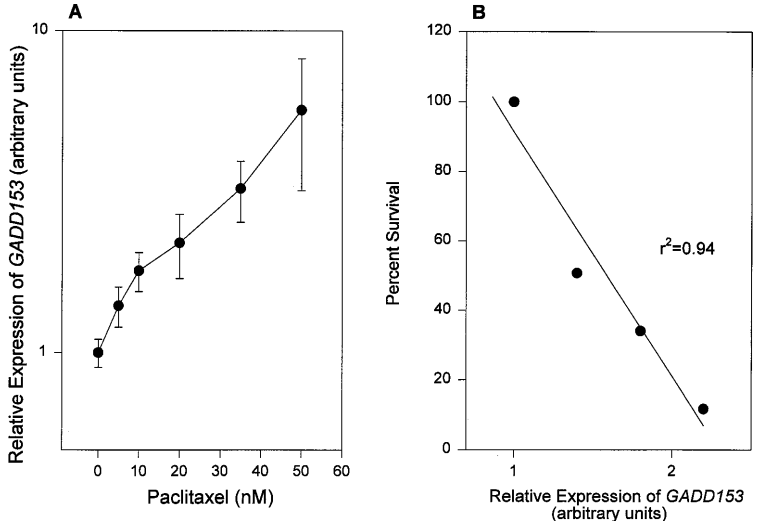

Fig. 1A,B Effect of exposure to paclitaxel on the level of *GADD153* mRNA and tumor cell kill. **A** Fold change in *GADD153* mRNA level as a function of paclitaxel concentration measured by northern blot analysis in cells harvested at 24 h after the start of drug exposure. **B** Correlation between the change in expression of *GADD153* mRNA and tumor cell survival after a 24-h exposure to paclitaxel. Each data point represents the mean of three experiments performed with triplicate cultures. *Vertical bars* standard error of the mean (SEM)

mRNA level as a function of paclitaxel concentration. At a concentration 10 times the IC_{50} there was a 5.7 ± 2.5 (SD)-fold increase in *GADD153* mRNA level at 24 h. Fig. 1B shows the correlation between fold increase in *GADD153* mRNA and cell survival over 2 logs of tumor cell kill. There was a very good correlation between these two parameters ($r^2 = 0.94$). These results provide validation of the change in *GADD153* mRNA level as a measure of cellular injury for paclitaxel.

Change in *GADD153* mRNA level and tumor response

Among the 19 patients sampled, RNA adequate for analysis from the paired biopsies was obtained from 14. The average amount of cDNA present in the sample was 0.4 ± 0.5 (SD) ng. Figure 2 shows a representative analysis of *β-ACTIN*, *GADD153*, and *MDR1* levels along with digitized images of the gels.

Among the 14 patients in this study from whom adequate mRNA was available, seven had progressive disease on the cycle of therapy analyzed, and seven had evidence of a biological response. In three of these seven patients it was manifest as a partial response (> 50% reduction in the volume of measurable disease) and in four of seven a minimal response (> 25% but < 50% reduction in volume). Figure 3 shows that there was no difference between the responders and non-responders with respect to the basal level of *GADD153* message (data not shown). However, as shown in Fig. 3, there

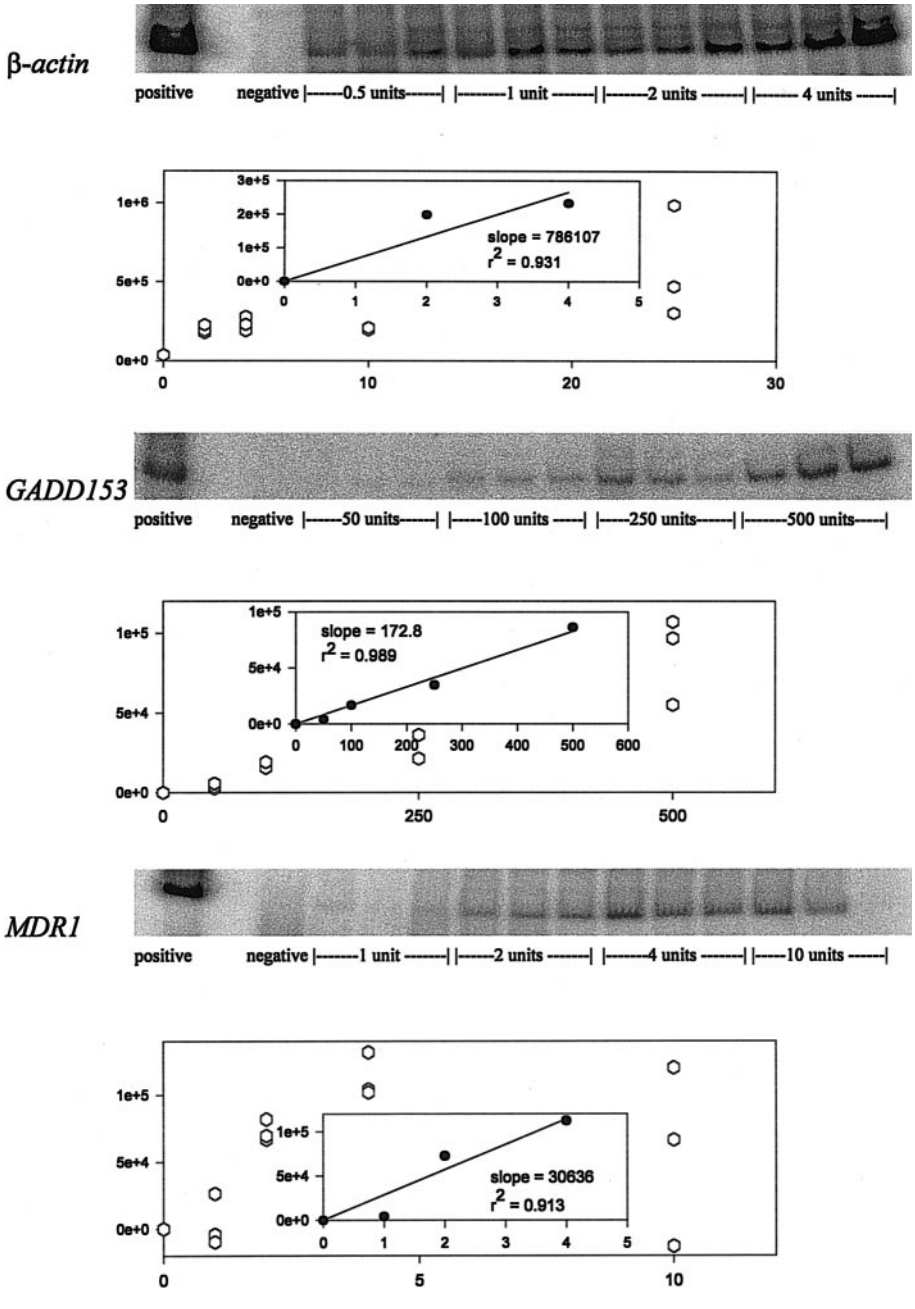

Fig. 2 Representative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *GADD153* and *MDR1* mRNA levels relative to that of *β-ACTIN*. cDNA samples were serially diluted, and triplicate PCR reactions were run for each amount of input cDNA. Each graph shows the amount of PCR product, determined using a phosphoimager, as a function of the amount of input cDNA. The inset in each graph shows the linear regression performed on the mean value for each cDNA dilution on the linear portion of the curve for determination of slope

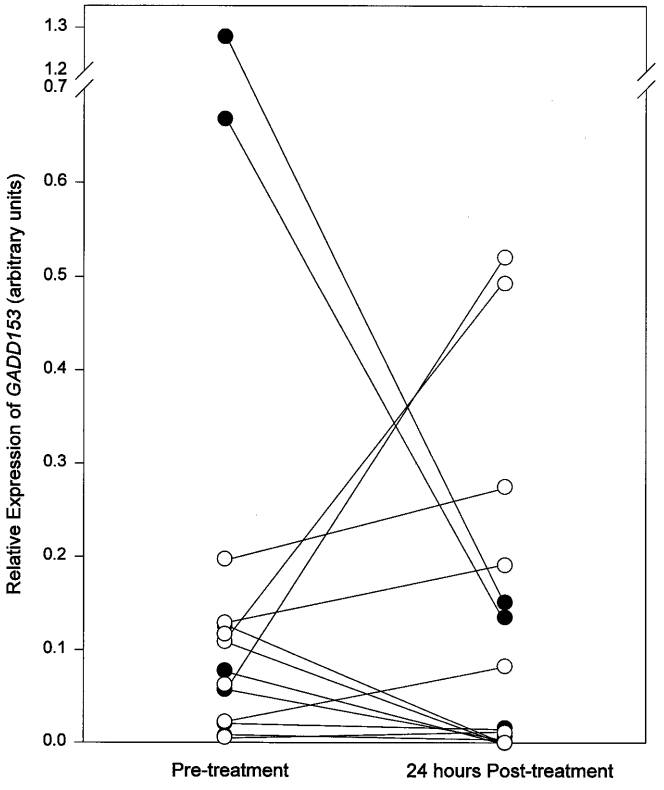

Fig. 3 Expression of *GADD153* mRNA in the tumor relative to that of *β-ACTIN* before and at 24 h after paclitaxel infusion in patients attaining either a minimal or partial response (*open circles*) or no response (*filled circles*)

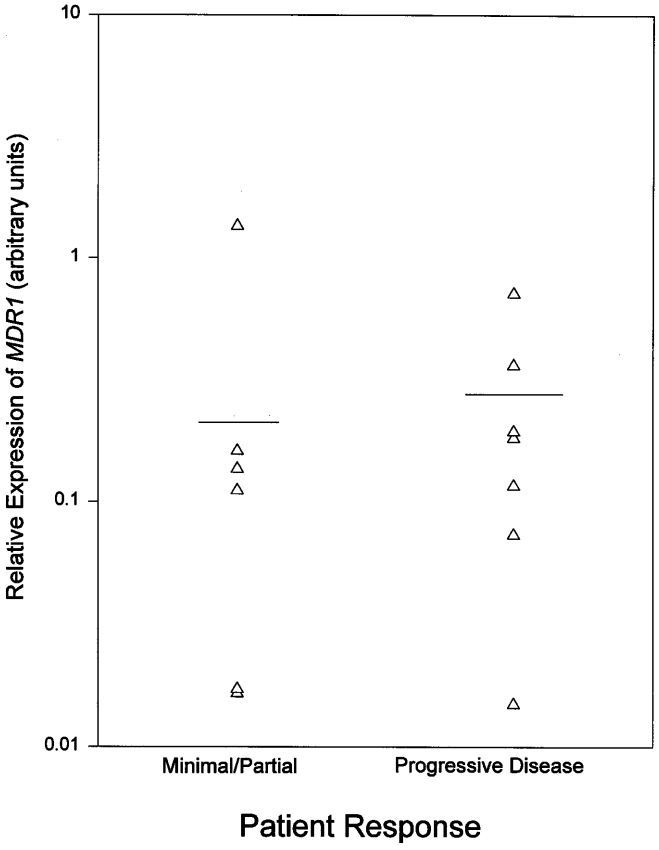

Fig. 4 Comparison of pre-treatment *MDR1* mRNA level, relative to that of *β-ACTIN*, in tumors attaining a minimal or partial response versus no response to paclitaxel infusion. Mean value for responders 0.218; mean value for patients with progressive disease 0.285 ($P = 0.66$, Student's *t*-test)

was a difference with respect to the treatment-induced change in *GADD153* mRNA level. Seven of seven patients with progressive disease had no increase in *GADD153* mRNA levels 24 h after treatment compared with the sample obtained immediately before paclitaxel treatment. In contrast, six of seven patients with either a minimal response or a partial response had an increase in *GADD153* mRNA level 24 h after treatment, which ranged from 1.4 to 8.2-fold ($P = 0.005$, Fisher's exact test).

MDR1 message level and tumor response

A relation between the basal level of the *MDR1* gene mRNA level and tumor response was sought among the 14 patients with evaluable mRNA samples obtained prior to the start of paclitaxel infusion. Figure 4 shows the ratios of *MDR1* to *β-ACTIN* message in patients with progressive disease and patients with either a minimal or partial response. The mean value of the basal *MDR1*/β-ACTIN ratio in patients exhibiting progressive disease was 0.2 ± 0.2 (SD), while the mean for patients sustaining either a minimal or partial response was 0.3 ± 0.5 (SD) ($P = 0.66$). Thus, the basal level of *MDR1* message was not predictive of tumor response to paclitaxel. It is of interest that the values measured in the tumors reflect an increase in *MDR1* levels between two- to three-fold higher than those found with drug-sensitive cell lines in vitro using the same quantitation technique [25]. This may be related to the fact that this group of patients had been heavily pretreated with other chemotherapy regimens and suggest that, in this group of patients, the basal level of *MDR1* message was not predictive of tumor response to paclitaxel.

To determine whether an increase in *MDR1* message level over the first 24 h after the start of paclitaxel infusion was related to response, the *MDR1*/*β-ACTIN* mRNA expression ratio was determined in biopsies taken immediately before and at 24 h after the start of the paclitaxel infusion. Figure 5 shows that there was no consistent change in *MDR1* message and thus no relation between change in *MDR1* mRNA level and response.

Discussion

Under circumstances where several different modalities of therapy can be given, identification of techniques that permit rapid assessment of whether the treatment administered has in fact produced sufficient damage to the tumor to result in a clinically useful response would be

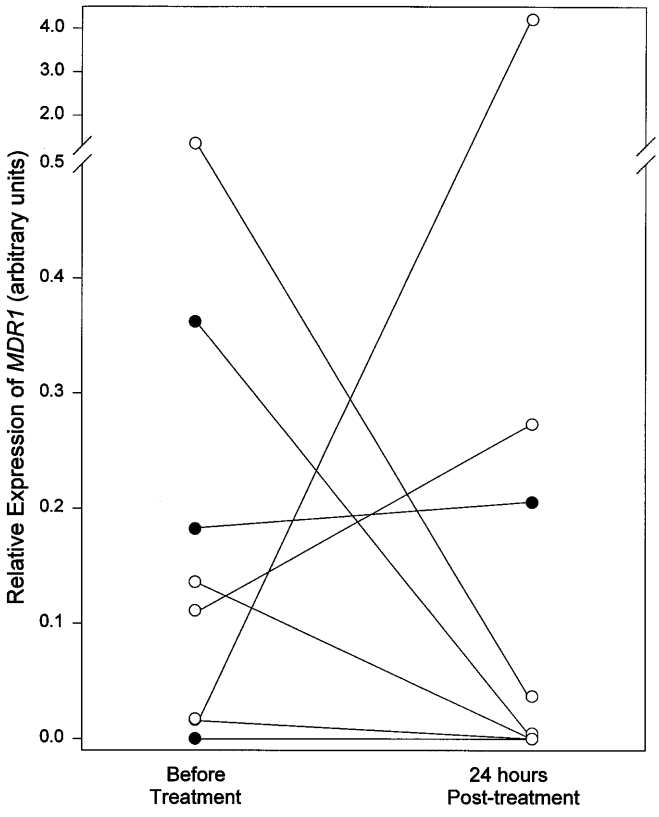

Fig. 5 Expression of *MDR1* relative to that of *β-ACTIN* before and 24 h after infusion of paclitaxel in patients attaining either a minimal or partial response (*open circles*) or no response (*filled circles*)

of great help in clinical decision-making. Identification of molecular markers that accurately predict the likelihood of response to specific agents would be of even greater clinical utility. Our approach to this goal is based on the observation that the damage induced by many chemotherapeutic agents and types of radiation causes an increase in the mRNA levels of genes activated during the cellular injury response [14a].

GADD153 was selected as a candidate molecular marker of tumor injury based on observations that its message level increases markedly following injury produced by many different kinds of agents [2, 3, 5–10, 13, 17, 21]. Using cell culture systems and human tumor xenografts we have previously demonstrated that, in the case of *GADD153*, there is a good correlation between the magnitude of the increase in message level and the extent of tumor cell kill for cisplatin [9, 17, 21]. In addition, we also demonstrated that *GADD153* message levels were increased in biopsies of responding head and neck patients treated with cisplatin, indicating that *GADD153* message levels might be used as a molecular marker for tumor response in a clinical setting [21]. The results presented in this report now provide validation of this relationship for another cytotoxic drug, paclitaxel, a drug with a different mechanism of action compared with cisplatin, and suggest that that it may also be possible to extend this approach for this drug to the clinic.

In this small study, none of the seven patients with progressive disease had an increase in *GADD153* message level 24 h after treatment, whereas six of the seven patients having some evidence of a biological response to the paclitaxel therapy had an increase in *GADD153* mRNA level of 1.4- to 8.2-fold. Two of the non-responding patients actually had a decrease in *GADD153* mRNA level following treatment, the etiology and significance of which remain unknown. Despite the fact that the number of patients entered on this pilot study was limited, these results are supportive of the use of *GADD153* as a molecular marker for detection of response. First, the study of the combination of paclitaxel and high-dose progesterone, which was under study as a potential modulator of P-glycoprotein function, produced only minimal and partial responses. Nevertheless, the change in *GADD153* mRNA levels at 24 h was sensitive enough to distinguish between those tumors that had no clinically detectable response at all and those having any evidence that the treatment was effective. The magnitude of the observed change in *GADD153* mRNA level in the samples from tumors sustaining these minimal but detectable responses agrees well with the data available on the relationship between paclitaxel-induced tumor cell kill and *GADD153* induction from in vitro studies [10]. Exposure of 2008 ovarian carcinoma cells in vitro to a concentration of paclitaxel twice the IC_{50} produced a two-fold increase in *GADD153* mRNA (Fig. 1A). It is likely that when used on tumors that demonstrate greater clinical responsiveness, or with drug programs that produce a higher complete response rate, this marker will have greater discriminatory power, such as in the case of cisplatin in head and neck cancer [21].

We have demonstrated a similar relationship between the magnitude of increase in *GADD153* message and clinical response rate in patients with head and neck carcinoma after cisplatin administration [21]. In this study of 32 patients, seven patients who had no response also had no increase in *GADD153* mRNA level. The eight patients attaining a partial response had an increase in *GADD153* mRNA that averaged 1.6-fold, whereas in the 17 patients who attained a complete remission the mean increase was 2.6-fold. Larger studies and trials with other tumor types and drugs are needed to confirm this promising lead.

The relationship between the message level of *MDR1* and the clinical responsiveness of solid tumors treated with paclitaxel remains uncertain, despite the fact that in vitro P-glycoprotein has been reported to mediate paclitaxel resistance [24]. In this small study, we found no relationship between response and either the basal level of mRNA expression of *MDR1* or the change in *MDR1* mRNA level at the end of the 24-h paclitaxel treatment. Because of the limited sample size a robust estimate of intra-patient variance could not be obtained. Thus, these data do not permit any firm conclusion regarding the clinical significance of basal *MDR1* mRNA

levels. However, it appears substantially less likely that *MDR1* mRNA level will turn out to be as good a molecular marker of the extent of cellular injury as the change in *GADD153* level.

The ability to use *GADD153* and potentially other damage responsive genes as molecular predictors or markers of response is based on refinement of PCR-based techniques that now permit semi-quantitative measurement of mRNA levels with as few as the 100–1000 cells obtainable from needle-aspiration biopsies [14]. The amount of heterogeneity within a solid tumor, and hence in separate needle biopsies from the same tumor mass, is an obvious concern when using this approach. As part of our previous study of patients with head and neck cancer, in a total of four patients we demonstrated that when five separate biopsies were obtained from the same tumor mass the coefficient of variation ranged between 26% and 29% [19]. In the current study, four needle biopsies from patient number 3 with a head and neck (throat) carcinoma yielded a coefficient of variation of 24.5%. This indicates that, relative to the magnitude of the increase in message level produced by effective treatment, the message level of *GADD153* in a single tumor biopsy is sufficiently representative of the whole tumor mass to permit detection of cellular injury of a degree that has clinical relevance. In addition, using an immunohistochemical staining approach to quantitate *GADD153* protein levels in frozen xenografted tumors, we have also found that intratumoral variation in *GADD153* levels was approximately 30 to 40% [17].

Another issue of concern with this approach is the fact that an aspiration biopsy inevitably contains some normal tissue elements as well as tumor cells. The actual fraction of tumor cells in the sample cannot practically be determined without sacrificing most of the sample, and it is not known how *GADD153* induction differs in the normal and malignant elements of the biopsy. However, in another study using cisplatin as a cytostatic drug, the cDDP-DNA adduct formation and increase in *GADD153* protein level in both tumor and stromal cells in a xenograft model was analyzed. The result was that approximately 25-fold more *GADD153* protein per cDDP-DNA adduct was formed in tumor cells than in stromal cells [17]. The latter indicates that the contribution of non-tumor cells to the level of *GADD153* expression within biopsies is minimal, at least in cases where more than 30% of the cells biopsied are tumor cells. While more information is needed to resolve this issue, the correlation between the measured increase in *GADD153* mRNA and the clinical response in this study, argues that operationally useful information on the extent of tumor injury can be obtained.

There are a number of other challenges that must be faced in assessing the utility of *GADD153* as a molecular marker of response. One is whether the relationship between the magnitude of the increase in *GADD153* message and the extent of tumor cell kill is the same for all drugs and tumor types. In all probability it is not, but experience is limited. In the case of cisplatin and paclitaxel, despite the fact that their mechanism of action is very different and that they activate the *GADD153* promoter through different signal transduction pathways [9, 10, 22, 27], the clinical results thus far are favorable and suggest that it may be possible to use the increase in mRNA level of *GADD153* as a molecular diagnostic test with which to assess the extent of tumor injury produced by agents with different mechanisms of action.

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