An Increase in the Expression of Ribonucleotide Reductase Large Subunit 1 Is Associated with Gemcitabine Resistance in Non-Small Cell Lung Cancer Cell Lines

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Abstract

The mechanisms of resistance to the antimetabolite gemcitabine in non-small cell lung cancer have not been extensively evaluated. In this study, we report the generation of two gemcitabine-selected non-small cell lung cancer cell lines, H358-G200 and H460-G400. Expression profiling results indicated that there was evidence for changes in the expression of 134 genes in H358-G200 cells compared with its parental line, whereas 134 genes in H358-G200 cells exhibited 233 genes that appeared to be under- or overexpressed compared with H460 cells. However, only the increased expression of ribonucleotide reductase subunit 1 (RRM1), which appeared in both resistant cell lines, met predefined analysis criteria for genes to investigate further. Quantitative PCR analysis demonstrated H358-G200 cells had a greater than 125-fold increase in RRM1 RNA expression. Western blot analysis confirmed high levels of RRM1 protein in this line compared with the gemcitabine-sensitive parent. No significant change in the expression of RRM2 was observed in either cell line, although both gemcitabine-resistant cell lines had an approximate 3-fold increase in p53R2 protein. A partial revertant of H358-G200 cells had reduced levels of RRM1 protein (compared with G200 cells), without observed changes in RRM2 or p53R2. In vitro analyses of ribonucleotide reductase activity demonstrated that despite high levels of RRM1 protein, ribonucleotide reductase activity was not increased in H358-G200 cells when compared with parental cells. The cDNA encoding RRM1 from H358-G200 cells was cloned and sequenced but did not reveal the presence of any mutations. The results from this study indicate that the level of RRM1 may affect gemcitabine response. Furthermore, RRM1 may serve as a biomarker for gemcitabine response.

Introduction

Gemcitabine (2'-2'difluorodeoxycytidine) is an efficacious cytotoxic agent that currently has marketing approval in the United States for the treatment of non-small cell lung and pancreatic cancers (1–5). This nucleoside analogue has a variety of activities thought to contribute to its antitumor effect. The antiproliferative activity is believed to be dependent on the incorporation into DNA of its triphosphate metabolite (gemcitabine triphosphate) and subsequent function as a DNA synthesis chain terminator (6). Other reported activities include the inhibition of CTP synthetase and dCMP deaminase (DCTD; Refs. 8 and 9). In contrast to the related deoxyctydine (dCyd) analogue cytarabine, gemcitabine diphosphate also inhibits ribonucleotide reductase, resulting in a decrease of competing nucleotide pools (10, 11).

Ribonucleotide reductase is the rate-limiting step in DNA synthesis, because it is the only known enzyme that converts ribonucleotides to deoxyribonucleotides, which are required for DNA polymerization and repair. Ribonucleotide reductase holoenzyme consists of dimerized large and small subunits ribonucleotide reductase subunits 1 and 2 (RRM1 and RRM2), respectively (12). The pairing of RRM1 and RRM2 is essential for scheduled DNA synthesis to occur. RRM1 has also been shown to function with an RRM2 homologue regulated by p53, known as p53R2, that is important in DNA repair secondary to genotoxic stress (13, 14).

Ribonucleotide reductase is the target for the clinically useful antitumor agent, hydroxyurea. Hydroxyurea interferes with the required tyrosyl free radical of RRM2, resulting in a loss of ribonucleotide reductase activity (15). In this regard, cell lines selected for resistance to hydroxyurea have been shown to possess increased RRM2 protein levels and ribonucleotide reductase activity (16–18). Other more recently characterized inhibitors also predominantly inhibit RRM2 (19, 20). By contrast, previous studies with bacterial ribonucleotide reductase have shown that 2'-substituted diphosphate nucleosides may directly interfere with the RRM1 subunit. In fact, a 2'-fluoro-methylenyl derivative appears to form a covalent interaction with RRM1 from Escherichia coli that permanently inactivates that subunit (21). The physical relationship between gemcitabine and mammalian ribonucleotide reductase has not been well characterized, however, data support the hypothesis that the RRM1 subunit is the most likely intracellular target of gemcitabine diphosphate (22).

Studies examining the effects of the levels of expression of RRM1 using both transfection and antisense approaches have shown that higher levels of RRM1 may be associated with growth suppression. These same studies have not directly evaluated the relationship of RRM1 expression and cytotoxic drug sensitivity (23, 24). Loss of heterozygosity of the chromosomal region containing RRM1 (11p15.5) was found in 48% of lung tumors examined, suggesting that RRM1 may act like a tumor suppressor, particularly at the early stages of carcinogenesis (25).

In this study, we have evaluated gene expression changes in two different gemcitabine-selected non-small cell lung cancer (NSCLC) cell lines compared with their cell lines of origin. The most dramatic change associated with the resistant phenotype is an increase in the expression of RRM1, without an accompanying change in ribonucleotide reductase activity. These data suggest that RRM1 may represent a critical target for the antitumor activity of gemcitabine.

Materials and Methods

Cell Culture. H358 and H460 were obtained from the American Type Culture Collection. The cells are maintained in RPMI with t-glutamine and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum, 0.15% sodium bicarbonate, 1 mM sodium pyruvate, 0.45% glucose, and 50 ng/ml gentamicin. Gemcitabine-resistant H358 and H460 cells were generated by exposure to...
gradually increasing concentrations of drug. The first selection concentration used was 3 nM. When cells adapted to the drug, gemcitabine concentration was doubled. Final selection was in 200 nM and 400 nM gemcitabine for H358 and H460 and is indicated in each cell line reference, i.e., H358-G200 and H460-G400. Resistant cell lines are maintained in their final selection concentration.

Before additional experiments, cells were passaged at least once in the absence of drug. H358-G200REV cells are H358-G200 cells that were passaged in the absence of drug for approximately 2 months (9–15 passages).

**Cytotoxicity Assays.** Cells, 5000/well, were seeded in a 96-well plate. H358 and related resistant cells were continuously exposed to drug for 96 h. H460 and H460-G400 cells were exposed to drug for 72 h. Cytotoxicity assays were performed with Cell Titer 96 (Promega) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent. After at least 2 h of incubation, plates were scanned at a wavelength of 490 nm. Resulting absorbances were converted to percent survival by comparing treatment with untreated (100% survival) cells. IC50s are defined as the concentrations of drug that result in 50% cell survival compared with untreated cells. The Student’s t test function in SigmaPlot was used to calculate P values.

**RNA Preparation for Expression Profiling.** Cells were seeded at a density of 1 × 10^4 cells/flask and allowed to adhere for 24 h. They were then exposed to vehicle or gemcitabine (24 nM) for an additional 6 or 24 h. All samples collected were in log-phase growth. RNA was isolated using the TRizol (Invitrogen) method.

**Oligonucleotide Array Hybridization and Data Analysis.** RNA labeling was performed according to the manufacturer’s recommendations (Affymetrix, Santa Clara, CA). In brief, double-stranded cDNA was synthesized using 10 μg of total RNA primed with a T7-oligo (dT)x primer (Genset, La Jolla, CA), and transcribed into biotinylated cRNA using the BioArray High Yield transcripti

**Cloning and Sequencing of RRM1 from H358-G200 Cells.** Using the same reverse transcription-PCR conditions described above, RRM1 was cloned from H358-G200 total RNA. The following primers were used for cloning: forward, 5'-aaagatagctggagtaaag-3' and reverse, 5'-aaagatatcgatgcatgtgatcaagcaag-3'. The data have been normalized by the expression of actin using TaqMan PDAR Actin Control Reagent (Applied Biosystems) to control the quality and quantity of the extracted RNA.

**Western Blotting.** Cells were lysed in 0.02 m Tris-Cl (pH 7.2) + protease inhibitor mixture (Roche Molecular Biochemicals) by freeze-thawing three times. Cell nuclei and debris were pelleted by spinning at 14,000 rpm in a refrigerated tabletop microcentrifuge for 10 min. Protein concentrations were determined using the Bio-Rad DC Protein Assay kit. Protein extracts were run on Novex precast 10% Tris-glycine gels (Invitrogen) in 1× Tris-glycine running buffer (Invitrogen). Samples were then transferred to polyvinylidene difluoride membrane (Invitrogen) in 20% methanol/1× Tris-glycine transfer buffer (Invitrogen). Membranes were blocked overnight in 3% nonfat milk in 1× Dulbecco’s PBS (Invitrogen) and 0.05% Tween 20 (Sigma). Primary antibody incubations were done in blocking buffer for 2–3 h at room temperature. Blots were washed several times with 1× Dulbecco’s PBS and 0.05% Tween 20 before secondary antibody exposure (1 h at room temperature). Antibodies used were: anti-RRM1 (Chemicon International); anti-p53R2/NT (ProSci, Inc.); anti-actin (Boehringer Mannheim); antimouse HRP conjugate (KPL); and antirabbit HRP conjugate (KPL). Detection was done using the Enhanced Western blotting reagents (Amersham Pharmacia Biotech).

**Ribonucleotide Reductase Activity Determination.** Each cell line was seeded onto 100-mm tissue culture plates at a density of 3.5 × 10^5 cells/plate and settled overnight. Six to nine plates were seeded per cell line per experiment. The cells were then scraped from the plates, pelleted, and washed twice in 1× Dulbecco’s PBS (Life Technologies). The cells were resuspended in 1 ml of lysis buffer [0.02 m Tris-HCl (pH 7.2), 0.01 m β-mercaptoethanol, and 1× protease inhibitor mixture; Roche] and passed through two freeze-thaw cycles. Lysates were then incubated with G-25 Sephadex (equilibrated in lysis buffer) at 4°C for 15 min on a tube rocker. The concentrations of protein in each sample were then determined using the Coomassie Plus Assay Reagent (Pierce). Only freshly isolated lysates were used for ribonucleotide reductase activity assessment. The total ribonucleotide reductase reaction volume was 150 μl. One hundred μg of protein from each cell lysate were used in each reaction, using lysis buffer for volume adjustment to 100 μl. Final concentration of ribonucleotide reductase substrate mononucleotides was 12 μM 2′-deoxyuridine-5′-triphosphate (dUTP), 10 μM 2′-deoxycytidine-5′-triphosphate (dCTP), 10 μM thymidine-5′-triphosphate (dTTP), 5 μM 5′-deoxyadenosine-5′-triphosphate (dATP), and 1 μM [3H]dCTP (22 Ci/mmol; Amersham Pharmacia Biotech). The reactions were incubated at 37°C for 30 min. They were then boiled for 10 min to stop the reaction. Next, the samples were spun for 10 min to pellet the precipitated protein. The supernatants were transferred to a fresh tube and treated with calf intestinal alkaline phosphatase (Amersham Pharmacia Biotech) for 3 h. The samples were boiled for 5–10 min to end the reaction. One μmol of dCDy and 1 μmol of cytidine (C) were added to each reaction as carriers. High pressure liquid chromatography was done using 10 mm NH4H2PO4 (pH 7.4) as the running buffer and a reversed-phase C18, 300A,
Table 1  

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Cell line</th>
<th>RR(^a) (± SE)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Gemcitabine</td>
<td>H358-G200</td>
<td>61.6 (± 28.2)(^b)</td>
<td>4</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>H358-G200REV</td>
<td>2.88 (± 0.72)</td>
<td>3</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>H460-G400</td>
<td>1100 (± 250)</td>
<td>3</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>H358-G200</td>
<td>0.40 (± 0.3)</td>
<td>2</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>H358-G200</td>
<td>0.57 (± 0.21)</td>
<td>2</td>
</tr>
</tbody>
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\(^a\) RR, relative resistance.  
\(^b\) See Ref. 26.

Fig. 1. Semiquantitative reverse transcription-PCR expression analysis of \(dCK\), CTP synthetase, CDA, and DCTD. A, relative to \(\beta\)-actin expression, there is no measurable change in \(dCK\) expression in H358-G200 cells compared with H358. There is a decrease in \(dCK\) in H460-G400 cells compared with H460. However, this difference was determined by densitometry to be less than 2-fold. B, CTP synthetase (CTP) levels remained unaltered in both gemcitabine-resistant cell lines. C, CDA expression did not change in H358-G200 cells but did decrease in H460-G400 cells, compared with their parental lines H358 and H460, respectively. D, the expression level of DCTD did not change in either H358-G200 or H460-G400 cell lines.

Fig. 2. Reverse transcription-PCR analyses validating the increased expression of RRM1. The H358-G200 and H460-G400 samples were found to express RRM1 higher than their sensitive parental cell lines.

Gemcitabine is an effective therapeutic agent for non-small cell lung and pancreatic cancers. To date, there have been no detailed studies reported identifying gemcitabine resistance mechanisms in NSCLC. The objective of this study was to generate gemcitabine-resistant NSCLC tissue culture models and to evaluate the potential molecular changes associated with this phenotype.

Gemcitabine-Selected NSCLC Cell Lines. Two tissue culture models were generated to study gemcitabine resistance. H358-G200 cells, which were selected in a final gemcitabine concentration of 200 \(\mu\)M, are 62-fold (± 28) less sensitive to gemcitabine compared with the parental cell line H358 (Table 1; Ref. 26). H358-G200REV are cells that were cultured for 10–15 passages in the absence of gemcitabine and have retained only about a 3-fold level of resistance to gemcitabine compared with H358 cells (Table 1). H460-G400 cells were determined to be more than 1000-fold resistant to gemcitabine. Despite prolonged passage in the absence of drug, we were unable to revert the gemcitabine-resistant phenotype of H460-G400 cells.

Both H358-G200 and H460-G400 cell lines were assessed for their response to cytarabine, a related dCyd analogue. H358-G200 cells remained sensitive to cytarabine (Table 1). Both H460 and H460-G400 cells are insensitive to the cytotoxic effects of cytarabine, with IC\(_{50}\) greater than 1 mM. The gemcitabine-selected variants were also assessed for their response to hydroxyurea. H358-G200 cells remained sensitive to hydroxyurea exposure (Table 1). When taken together, these data suggest that a resistance mechanism distinct from those associated with either hydroxyurea or cytarabine must be present in the H358 drug-selected cell lines.

Analysis of \(dCK\), CDA, CTP Synthetase, and DCTD RNA Expression. Several potential mechanisms of gemcitabine resistance have been previously reported from cell culture models. Expression and activity level of \(dCK\) have been shown to influence cellular response to gemcitabine (27). The phosphorylation of gemcitabine by \(dCK\) is rate-limiting in the intracellular activation of the drug (28). Thus, we compared the expression level of \(dCK\) in both resistant cell lines but did not observe a difference in its expression in H358-G200 cells compared with the H358 level. There was a minimal decrease (less than 2-fold) in \(dCK\) RNA in H460-G400 compared with H460 (Fig. 1A). Although this difference may partially contribute to the gemcitabine-resistant phenotype of H460-G400 cells, it is unlikely to confer the very high levels of resistance observed in this cell line.

The increased expression of CTP synthetase is another potential mechanism of gemcitabine resistance, because this effect would result in a larger dCTP pool that would compete with gemcitabine triphosphate for DNA integration. In addition, gemcitabine triphosphate is known to inhibit CTP synthetase (8). Using semiquantitative reverse transcription-PCR, we did not observe a change in the expression of this gene in either gemcitabine-resistant cell line, demonstrating that an expression change of this enzyme is not likely to be involved in the phenotype (Fig. 1B).

Both CDA and DCTD have the ability to inactivate gemcitabine or its metabolites by irreversible deamination (9, 29). CDA is known to

4.6 × 250-mm column (GraceVydac, Hesperia, CA). The data are presented as the ratio of dCyd/C for each data set. SE was determined by dividing the SE for each data set by the number of lysates tested. The Student’s unpaired t test was used to calculate P values using SigmaPlot software.

Results and Discussion

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Both CDA and DCTD have the ability to inactivate gemcitabine or its metabolites by irreversible deamination (9, 29). CDA is known to
be the primary enzyme responsible for the in vivo inactivation of gemcitabine. In this regard, transfection studies, where CDA was expressed at high levels, resulted in cells with reduced gemcitabine sensitivity (30, 31). Thus, we assessed whether these genes were expressed at high levels, resulted in cells with reduced gemcitabine resistance in these cell lines. Criteria were established to identify gene changes worthy of additional study. First, an identified change must have been consistently altered at both 6- and 24-h time points, and the change(s) must be replicated on both hybridized microarrays for each sample. In addition, the predicted expression changes must be at least 5-fold. Changes in gene expression that were less than 5-fold but otherwise fulfilled the above criteria were also included in the analysis comparing H358-G200 and H460-G400 profiles.

Using this strategy, 134 total expression changes were identified in H358-G200 cells compared with the original H358 cells. Fourteen decreases and 17 increases were identified that exhibited greater than 5-fold change in gene expression in H358-G200 cells compared with H358. H460-G400 cells were found to exhibit 233 alterations compared with H460 cells. Twenty-two genes were down-regulated, and the expression of 34 genes increased to a level at least 5-fold.

To determine those changes most likely to be important in the gemcitabine-resistant phenotype, the results of both analyses were compared. Cross-comparison of expression profiles revealed seven genes with changes in expression common to both H358-G200 and H460-G400 cell lines. Two of the genes were down-regulated in both cell lines, the ES/130 ribosome receptor and F-actin, whereas forkhead (FKHR), pyruvate dehydrogenase complex protein X (ProX), Tat-interacting protein 30 (TIP30), and myo-inositol monophosphate 2 (IMPA2) all exhibited increases in expression. However, all of these changes were less than 5-fold. The up-regulation of RRM1 was the only identified change that represented at least a 5-fold change in expression in both resistant variants.

**RRM1 Analysis.** Reverse transcription-PCR that was performed in a semiquantitative manner was initially used to evaluate RRM1 RNA expression relative to β-actin. Results of these experiments supported the cDNA profiling results that showed RRM1 expression to be increased in both H358-G200 and H460-G400 cells, regardless of time in culture or acute exposure to gemcitabine compared with parental controls (Fig. 2). Additional analysis with real-time, quantitative PCR (Taqman) demonstrated that H358-G200 cells had an approximately 125-fold increased level of RRM1 message compared with parental cells (Fig. 3A). Examination of H358 sublines selected in lower concentrations of gemcitabine revealed that beginning with cells selected in 12 nM drug, there was a proportional increase in RRM1 message with increasing gemcitabine exposure (Fig. 3A). Profiling results were further verified by examining the levels of RRM1 protein in the resistant variants as compared with their cell lines of origin. Western blot analysis confirmed the RNA expression results. Significant increases in RRM1 protein were found in both H358-G200 and H460-G400 compared with H358 and H460, respectively (Fig. 3B).

The results appeared to be independent of growth phase of the cell, because similar results were observed when cell lysates were obtained from either confluent or logarithmically growing cultures (data not shown). A gradual increase in RRM1 protein levels was
observed as H358 cells were selected in increasing concentrations of gemcitabine (Fig. 3B). In addition, the level of RRM1 protein decreased in H358-G200 cells that had partially restored sensitivity to gemcitabine (H358-G200REV; Fig. 3B). In that regard, prolonged passage in the absence of drug resulted in additional lowering of the levels of RRM1 protein in cell extracts (data not shown). These results demonstrate that the level of RRM1 protein correlates with gemcitabine sensitivity and exposure.

To assess whether RRM1 from H358-G200 cells possessed a mutation, the corresponding cDNA was isolated, cloned, and sequenced. The cDNA sequence was identical to GenBank accession no. X59543, demonstrating that no mutations had occurred to the RRM1 gene during the prolonged exposure to gemcitabine (data not shown).

Ribonucleotide Reductase Small Subunit Analysis. Because the large and small subunits are required for ribonucleotide reductase activity, we evaluated the expression level of RRM2 mRNA using semiquantitative reverse transcription-PCR (Fig. 4). RRM2 was not detected on Western blots, despite loading up to 150 μg of protein. Thus, the expression level was determined by semiquantitative reverse transcription-PCR (Fig. 4). Results showed no difference in RRM2 expression in H358-G200 or H460-G400 cell lines compared with H358 or H460 cells, respectively. However, a modest increase (less than 3-fold) in p53R2 protein as H358 cells were selected in 6–200 nM gemcitabine was observed (Fig. 4B). A higher level of p53R2 was also observed in H460-G400 compared with H460 cells. Densitometry of the bands indicated that H358-G200 and H460-G400 cells have less than a 3-fold up-regulation of p53R2 protein levels compared with their parental cell lines. However, H358-G200REV cells maintained the higher level of p53R2 expression found in H358-G200 cells. Thus, the level of p53R2 expression does not directly correlate with gemcitabine sensitivity. These results demonstrate that RRM1 is the only known up-regulated ribonucleotide reductase subunit in two genetically distinct NSCLC cell lines that correlates with gemcitabine response.

Ribonucleotide Reductase Activity. We measured the ability of ribonucleotide reductase to convert C to dCyd in H358 and H358-G200 cellular lysates to determine whether a change in activity correlated with gemcitabine sensitivity. Reactions were performed in triplicate, with three to four independently isolated lysates tested for each cell line. The mean dCyd/C for H358 lysates was determined to be 0.225 (±0.109). The dCyd/C mean for H358-G200 was 0.085 (±0.009). Although the mean dCyd/C value for H358 lysates was 2.6-fold higher than for H358-G200 extracts, this result was not statistically significant (P > 0.05). These results, however, are consistent with the maintenance of H358-G200 sensitivity to hydroxyurea.

When examined in their entirety, these data demonstrate that the increased expression of RRM1 is associated with the gemcitabine-resistant phenotype in two independently generated NSCLC models. There is a direct correlation between the level of RRM1 protein and gemcitabine exposure in H358 cells. Surprisingly, the data demonstrate that the increased RRM1 expression does not alter ribonucleotide reductase holoenzyme activity. In fact, there is a trend toward diminished holoenzyme activity in the highest RRM1-expressing cell line (H358-G200). One possibility that these results suggest is that RRM1 may be acting as a “molecular sink” for gemcitabine, whereby the drug binds to RRM1 and irreversibly inactivates that subunit. At least one study, in bacterial systems, has suggested that gemcitabine can directly interfere with RRM1 within the context of the ribonucleotide reductase holoenzyme (22). There appeared to be direct binding of gemcitabine diphosphate to RRM1, and this interaction may be irreversible. As such, both drug and protein may be effectively inactivated by such an interaction.

Our data suggest that a similar mechanism may be operative in these NSCLC cells selected for gemcitabine resistance; as cells are exposed to progressively higher concentrations of gemcitabine, the drug irreversibly interacts with and inactivates its target, RRM1. The cell subsequently increases the expression of RRM1 in a coordinate fashion to maintain viability and the required level of ribonucleotide reductase holoenzyme activity. Removal of gemcitabine from the culture results in a relatively rapid return of RRM1 protein levels to near baseline, demonstrating that the response is a direct effect of gemcitabine exposure.

Although the importance of RRM1 to the activity of gemcitabine in patients with NSCLC has not been fully evaluated, the results from this study demonstrate that RRM1 protein expression levels correlate with gemcitabine sensitivity. Results from one retrospective clinical study indicate RRM1 levels may influence both response and survival of NSCLC patients to gemcitabine/cisplatin combination therapy (32). These data suggest RRM1 is a novel, informative biomarker for predicting and monitoring the responses of NSCLC patients to gemcitabine.

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References


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