Gene expression analysis of gallium-resistant and gallium-sensitive lymphoma cells reveals a role for metal-responsive transcription factor-1, metallothionein-2A, and zinc transporter-1 in modulating the antineoplastic activity of gallium nitrate

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Abstract

Several clinical trials have shown gallium nitrate to be an active agent in the treatment of lymphoma. Whereas gallium is known to target cellular iron homeostasis, the basis for lymphoma cell resistance to gallium is not known. Understanding mechanisms of resistance may suggest strategies to enhance the clinical efficacy of gallium. In the present study, we used a focused DNA microarray to compare the expression of genes related to metal metabolism in gallium-resistant and gallium-sensitive lymphoma cell lines developed by us. Gallium-resistant cells were found to display a marked increase in gene expression for metallothionein-2A and the zinc transporter ZnT-1. Cells exposed to gallium nitrate displayed an increase in the binding of metal-responsive transcription factor-1 to metal response element sequences involved in the transcriptional regulation of *metallothionein* and *ZnT-1* genes. Gallium nitrate induced metallothionein-2A and ZnT-1 expression in cells. A role for metallothionein in modulating the antineoplastic activity of gallium was confirmed by showing that the induction of metallothionein expression by zinc provided partial protection against the cytotoxicity of gallium and by showing that the level of endogenous metallothionein in lymphoma cell lines correlated with their sensitivity to gallium nitrate. Immunohistochemical staining of lymphomatous tissues revealed metallothionein protein to be variably expressed in different lymphomas. Our studies show for the first time that gallium acts on pathways related to zinc metabolism and that metal-responsive transcription factor-1 activity and metallothionein expression contribute to the development of gallium drug resistance. Furthermore, the endogenous level of metallothionein in lymphoma may be an important determinant of clinical response to gallium nitrate. [Mol Cancer Ther 2007;6(2):633–43]

Introduction

Over the past two decades, several clinical studies have shown the group IIIa metal salt gallium nitrate to have efficacy in the treatment of non-Hodgkin's lymphoma (1–5). More recently, its activity in lymphoma was confirmed in a multicenter phase 2 clinical trial where significant therapeutic responses were noted in patients with non-Hodgkin's lymphoma whose disease had relapsed following treatment with various chemotherapeutic regimens, including stem cell transplantation (6).

The mechanisms of cytotoxic action of gallium nitrate are only partly understood. Prior studies have shown that, in the circulation, gallium binds to transferrin resulting in its preferential targeting to transferrin receptors that are frequently expressed on lymphoma cells *in vivo* (7–12). Inhibition of cellular proliferation by gallium is due in part to disruption of intracellular iron homeostasis, including an inhibition of the iron-dependent activity of ribonucleotide reductase (13–15). Other mechanisms of cytotoxicity may also be involved but are less well defined (16, 17).

A major obstacle to the successful treatment of malignancy is the development of tumor cell resistance to chemotherapy. An understanding of the mechanisms of drug resistance is important because it may suggest strategies to circumvent such resistance and may reveal novel processes that could serve as therapeutic targets. To investigate the mechanisms of tumor resistance to gallium in non-Hodgkin's lymphoma, a gallium-resistant cell line was developed in our laboratory from a gallium-sensitive CCRF-CEM lymphoma cell line. Although the pathways involved in the development of gallium resistance in these cells are not well understood, we have shown previously that they display a decrease in iron and gallium uptake relative to the parental gallium-sensitive cells (18, 19).

Received 9/11/06; revised 11/1/06; accepted 12/15/06.

Grant support: United States Public Health Service RO1 CA109518 (C.R. Chitambar).

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Conflict of interest statement: C.R. Chitambar has served previously as a consultant for Genta, Inc., the manufacturer of gallium nitrate.

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doi:10.1158/1535-7163.MCT-06-0557

To gain further insight into the basis for lymphoma cell sensitivity and resistance to gallium nitrate, we have now used a DNA microarray analysis to identify genes related to metal homeostasis that may be differentially expressed in gallium-resistant and gallium-sensitive cells. We show that gallium-resistant cells display a significant increase in ZnT-1, a membrane-based zinc efflux transporter, and in metallothionein-2A (MT2A), a low-molecular weight cysteine-rich intracellular protein involved in the cellular handling of zinc, cadmium, and certain other metals, but not in the cellular handling of iron (20–22). In gallium-sensitive cells, gallium induces MT2A and ZnT-1 expression via activation of the zinc finger transcription factor metal-responsive transcription factor-1 (MTF-1). The level of endogenous and exogenously induced expression of metallothionein in cells affects their sensitivity to gallium nitrate. We also show that metallothionein protein is expressed in some lymphomas *in vivo*, thus suggesting that our observations in cell lines may have a bearing on the clinical efficacy of gallium nitrate.

Materials and Methods

Materials

GEArray Q Series Human Metal Transport and Homeostasis Gene Array and the reagents required for its use were purchased from SuperArray Bioscience Corp. (Frederick, MD). Zinc sulfate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Corp. (St. Louis, MO). Gallium nitrate was obtained from Genta, Inc. (Berkely Heights, NJ). Murine monoclonal antibody E9 to metallothionein was purchased from DAKO Corp. (Carpinteria, CA). [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from Perkin-Elmer Life Sciences (Boston, MA).

Cell Lines

The development of a gallium-resistant cell line (GnR cells) from a parental gallium-sensitive CCRF-CEM cell line (GnS cells) has been reported previously by us (18). Human T-lymphoblastic leukemia/lymphoma CCRF-CEM cells were obtained from American Type Culture Collection (Manassas,VA). The GnR cell line was developed by continuous exposure of CCRF-CEM cells to incremental concentrations of gallium nitrate over the course of several months. GnR cells were routinely grown in medium containing 150 μmol/L gallium nitrate (18). Two additional daughter cell lines were derived from the GnR cells after gallium nitrate was removed from the culture medium. The first cell line (termed GnR-R cells) retained resistance to gallium nitrate even in the absence of gallium nitrate, whereas the second cell line (termed Gnr-S cells) reverted to a gallium-sensitive phenotype. DoHH2, HBL-2, Granta, JVM-2, NCEB-1, and Z138C lymphoma cell lines were obtained from the British Columbia Cancer Agency (Vancouver, British Columbia, Canada; ref. 23). All cell lines were grown in RPMI 1640 supplemented with 10% FCS in an atmosphere of 5% to 6% CO_2 at 37°C.

Cell Proliferation Assay

The effects of gallium nitrate on cell proliferation were measured by MTT assay as previously described by us (18, 24). In some experiments, cells were preincubated with zinc sulfate and then washed and examined for growth inhibition by gallium nitrate. Cells (2×10^5/mL) were plated in 96-well plates (100 μL/well) and growth assessed after 48 or 72 h of incubation at 37°C by the addition of MTT to the wells. The absorbance of the wells was measured by spectrophotometer at dual wavelengths of 570 and 630 nm using an EL310 microplate autoreader (Biotech Instruments, Winooski, VT).

RNA Isolation

Total RNA was extracted from cells using Trizol, as recommended by the manufacturer (Invitrogen, Carlsbad, CA). RNA was treated with RNase-free DNase I to remove any residual genomic DNA. The quality and integrity of the RNA was assessed by agarose gel electrophoresis before use in DNA microarray analysis and reverse transcription-PCR.

DNA Microarray Analysis

Differentially expressed genes related to metal transport and homeostasis in gallium-sensitive and gallium-resistant cells were identified by DNA microarray using a GEArray focused DNA microarray according to the manufacturer's protocol. Five micrograms of total RNA isolated from gallium-sensitive and gallium-resistant cells were used as a template for the synthesis of corresponding biotin-16-dUTP-labeled cDNA probes. The annealing mixture was prepared with GEA primer mix and total RNA. The annealing mixture was placed in a thermal cycler at 70°C for 3 min and at 42°C for 2 min before the addition of the master mixture (GEA labeling buffer, biotin-16-dUTP, RNase inhibitor, reverse transcriptase, and RNase-free water) and incubating the mixture at 42°C for 90 min. The labeling efficiency of the biotin-labeled cDNA probes was determined by spotting 1 μL from serial dilutions of the reaction mixture onto HyBond nylon membrane and developing the membrane using a chemiluminescent detection reagent (SuperArray Bioscience). cDNA probes detectable at a 1,000-fold dilution or greater were used for hybridization.

The respective cDNA probes were denatured and hybridized to the microarray membranes overnight at 60°C in GEAhyb hybridization solution. Membranes were then washed and the hybridization signals on the membrane were detected using chemiluminescent detection reagents and methodology provided by the manufacturer. This involved sequential treatment of the membranes with GEAblocking Solution Q, alkaline phosphatase-conjugated streptavidin in buffer F, and washing buffer F. Membranes were finally incubated in a solution containing CDP-Star chemiluminescent substrate. The chemiluminescent array image on the membrane was captured using an AlphaInnotech FluorChem Model 8900 Imaging System and array analysis software. The signal intensity of each spot on the membrane was measured and the signal intensity from each gene was normalized to the signal intensity of the *β-actin* gene.

Semiquantitative Reverse Transcription-PCR

First-strand cDNA synthesis was carried out using the manufacturer's protocol for SuperScript II Reverse Transcriptase (Invitrogen). The reaction mixture containing 2 μg DNA-free total RNA, 0.5 μg oligo(dT) 12 to 18 primer, 0.5 mmol/L deoxynucleotide triphosphates, and RNase inhibitor was incubated at 42°C for 50 min. The reaction was inactivated by an additional incubation at 70°C for 15 min. The cDNA product was diluted serially to yield 1:4, 1:16, and 1:64 dilutions and 2 μL from each dilution were used for PCR amplification. The primer pair sequences for MT2A, metallothionein-3 (MT3), and β-actin used for PCR have been reported previously by others (25, 26). For MT2A, the forward primer sequence was 5′-CCGACTCTAGCCGCCTCTT-3′ and the reverse primer sequence was 5′-GTGGAAGTCGCGTTCTTTACA-3′. For MT3, the forward primer sequence was 5′-CCGTTCACCGCCTCCAG-3′ and the reverse primer sequence was 5′-CACCAGCCACACTTCACCACA-3′. The forward primer sequence for β-actin was 5-CGAGGACTTTGATTGCAC-3 and the reverse primer sequence was 5-TATCACCTCCCCTGTGTG-3. PCR products were resolved on a 1.2% agarose gel, which was stained with ethidium bromide to visualize the bands.

cDNA Probes for Northern Blotting

The pDNR-LIB plasmid containing MT2A cDNA and the pT7T3D-Pac1 plasmid containing sequence-verified ZnT-1 cDNA were obtained from American Type Culture Collection. Plasmids were purified using a Qiagen Plasmid kit (Valencia, CA). The 451-bp MT2A cDNA fragment was excised from the respective plasmid with *Sfi*, whereas a 600-bp ZnT-1 cDNA-containing fragment was excised from the respective plasmid with *Xho*1 and *NSi*1. cDNA probes were labeled with ^{32}P (specific activity, 1×10^9 to 2×10^9 cpm/μg) using a RadPrime DNA Labeling System (Life Technologies, Gaithersburg, MD).

Northern Blotting

Fifteen micrograms of total RNA isolated from cells under different experimental conditions were analyzed by Northern blotting as previously described (18). MT2A and ZnT-1 mRNAs were detected by hybridization of the membranes at 68°C overnight with 1.2×10^6 cpm/mL of the specific ^{32}P-labeled cDNA probe in QuikHyb Hybridization solution (Stratagene, La Jolla, CA). Autoradiography of the membranes was done using BioMax X-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen at −80°C for 4 to 24 h. Membranes were stripped and reprobed with a ^{32}P-labeled β-actin probe to confirm equal RNA loading on the gel.

Western Blotting

Gallium-sensitive and gallium-resistant cells were analyzed for metallothionein protein using an enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL). The protein content of supernatants from cell lysates was measured by bicinchoninic protein assay (Pierce, Rockford, IL). Samples were diluted in SDS sample buffer without 2-mercaptoethanol as described by Aoki et.al. (27) and resolved by SDS-PAGE as described by Laemmli (28). Proteins on the gel were transferred onto a polyvinylidene difluoride membrane using a Transblot system (Bio-Rad, Richmond, CA). The metallothionein band on the membrane was detected with E9 antibody (1:200 dilution) and horseradish peroxidase–labeled secondary antibody. Membranes were developed in enhanced chemiluminescence detection solution and exposed to BioMax film for autoradiography.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from 5×10^6 cells incubated with or without gallium nitrate for 4 to 24 h as described previously (29). The binding of MTF-1 in the nuclear extracts to radiolabeled MRE-s was examined by electrophoretic mobility shift assay as reported previously (30), using a Gel Shift Assay System as recommended by the manufacturer (Promega, Madison WI). MRE-s is a designed metal response element (MRE) oligonucleotide that has a high-binding affinity for MTF-1 (30, 31). ^{32}P-labeled double-stranded oligonucleotide for MRE-s (sequence reported in ref. 30) was prepared in a reaction mixture containing the oligonucleotide, [γ-^{32}P]ATP, T4 kinase, and kinase buffer [70 mmol/L Tris-HCl (pH 7.6)/10 mmol/L $MgCl_2$/5 mmol/L DTT] and purified using a Sephadex G25 spin column. The MTF-1-MRE-s binding reaction mixture consisted of nuclear proteins (10 μg), 5× binding buffer (Promega), and ^{32}P-labeled MRE-s (8×10^6 cpm/moL; 125 fmol/μL). Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel at 190 V for 2.5 h at 4°C. The gel was dried and bands were identified by autoradiography.

Immunohistochemical Analysis

To examine the expression of metallothionein in lymphoma cells *in vivo*, immunohistochemical staining with E9 antibody (1:50 dilution) was done on formalin-fixed, paraffin-embedded archived tissue samples of non-Hodgkin's lymphoma using a previously described method (32). All slides were stained using an automated immunostainer (DAKO). Following incubation with E9 antibody, slides were incubated for 15 min in labeled streptavidin-biotin for linking and then labeled with diaminobenzidine chromogen. Microscopy was done using an Olympus BX41 microscope (Center Valley, PA) and images were captured as described (32).

Results

Gallium-Resistant and Gallium-Sensitive Cell Lines and Response to the Growth-Inhibitory Effects of Gallium Nitrate

Gallium-resistant GnR cells were developed from the parental gallium-sensitive CCRF-CEM cell line (GnS), whereas GnR-R and Gnr-S cells arose from GnR cells over time following removal of gallium nitrate from the culture medium. The effects of gallium nitrate on the proliferation of these cells after 48 and 72 h of incubation are shown in Fig. 1A and B, respectively. As shown in these figures, gallium nitrate inhibited the growth of both GnS and Gnr-S cells in a similar manner, indicating that the latter cells had reverted to a gallium-sensitive phenotype from a

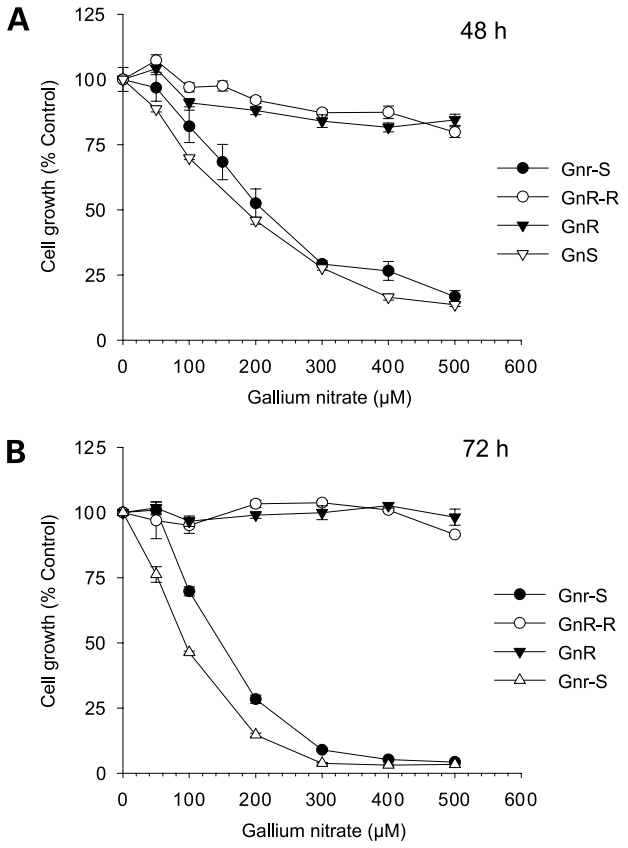

Figure 1. Effect of gallium nitrate on the proliferation of gallium-sensitive and gallium-resistant CCRF-CEM cell lines. Gallium-sensitive (GnS and Gnr-S) and gallium-resistant (GnR and GnR-R) cells were plated in 96-well plates (0.2×10^6/mL) in the presence of increasing concentrations of gallium nitrate. Cell growth was measured by MTT assay after 48 h (**A**) and 72 h (**B**) of incubation. *Points,* mean ($n = 3$); *bars,* SE.

previously gallium-resistant phenotype. In contrast, GnR-R cells have maintained a gallium-resistant phenotype for years in the absence of gallium nitrate and, as shown in Fig. 1A and B, their growth, like that of GnR cells, was not inhibited by gallium nitrate. The proliferation of either gallium-resistant cell line was not inhibited with gallium nitrate concentrations up to 4,000 μmol/L (data not shown).

Identification of Differentially Expressed Genes in Gallium-Resistant and Gallium-Sensitive Cells

Our prior studies have shown that the development of drug resistance to gallium nitrate is associated with alterations in cellular iron homeostasis; however, the molecular basis for this resistance is not known (18, 33). Therefore, to gain further insight into whether gallium resistance is related to alterations in genes involved in metal metabolism, an analysis was done using the GEArray Q series human metal transport and homeostasis gene array. The 96 genes represented on this array encode for proteins involved in the transport, metabolism, and storage or sequestration of iron, copper, zinc, and selenium. This array includes antioxidant enzymes whose prosthetic groups contain these metals and other metal-responsive stress proteins and transcription factors. The gene list is available online.[3] Total RNA from GnS, GnR, Gnr-S, and GnR-R was used to generate cDNA probes, which were each hybridized to DNA array membranes, and the hybridization signals were detected by chemiluminescence autoradiography. Figure 2A (*1–4*) shows the membrane autoradiographs obtained with cDNA probes from the individual gallium-sensitive and-resistant cell lines. The intensity of the hybridization signals on each membrane reflects the expression level of different genes in the respective cell line. Hybridization signals were quantitated and normalized to the intensity of the β-actin gene signal on the same membrane. The gene expression ratios between GnS and GnR cells and between Gnr-S and GnR-R cells were calculated. Gnr-S cells were compared with GnR-R cells because both cell lines were derived from GnR cells and had been maintained in culture without gallium nitrate. Table 1 lists the genes that were found on DNA array to be differentially expressed between GnR and GnS cells and GnR-R and Gnr-S cells. Comparison of gene expression between GnR with GnS cells revealed 20 genes with a GnR/GnS gene expression ratio of >2 or ≤0.5, indicating a 2-fold difference in expression of the respective gene. Of these 20 genes, 14 were differentially expressed between GnR and GnS cells but not between GnR-R and Gnr-S cells (Table 1). In contrast, comparison of GnR-R and Gnr-S cells revealed 18 genes to be differentially expressed between the two cell lines. Of these 18 genes, 12 were differentially expressed between GnR-R and Gnr-S cells but not between GnR and GnS cells (Table 1). As shown in the lower section of Table 1, genes that displayed a consistently increased expression in both GnR and GnR-R cells relative to gallium-sensitive cells were the genes for iron-responsive element binding protein 2 (iron regulatory protein-2), MT2A, MT3, MTF-1 (a zinc finger transcription factor), and solute carrier family 30 member 1 (ZnT-1, a zinc transporter). Of these, the genes for MT2A, MT3, and ZnT-1 displayed the highest expression in gallium-resistant cells (Table 1).

Confirmation that Metallothionein and ZnT-1 Gene Expression Is Increased in Gallium-Resistant Cells

The results of the DNA array suggested that the expression of both MT2A and MT3 isoforms was increased in gallium-resistant cells. It is known, however, that these two metallothionein isoforms share 65% nucleotide sequence homology (34). Therefore, to determine whether the findings on DNA array represented an increase in one or both of these metallothionein isoforms, a semiquantitative PCR was carried out with stringent primer pairs chosen from the divergent 5′ and 3′ untranslated regions of *MT2A* and *MT3* genes as described by Mididoddi et. al. (25).

As shown in Fig. 2B, MT2A gene expression was detected by PCR in both GnR-R and Gnr-S cells, and

[3] http://www.superarray.com

higher levels of MT2A expression were observed in GnR-R cells, consistent with the results of the DNA array analysis. In contrast, MT3 gene expression could not be detected by PCR in GnS, GnR, Gnr-S, or GnR-R cells (data not shown). These results indicate that the increased level of gene expression in GnR and GnR-R cells corresponding to *MT2A* and *MT3* genes on the DNA array actually represents an increase in the *MT2A* gene alone. Further analysis of the gallium-sensitive and gallium-resistant cell lines by Northern and Western blotting confirmed a higher level expression of MT2A mRNA and protein in GnR and GnR-R cells (Fig. 2C and D). Northern blot analysis was done to confirm the increase in ZnT-1 detected in gallium-resistant cells seen in the microarray studies. As shown in Fig. 2E, there was a marked increase in ZnT-1 transcripts in GnR-R cells relative to gallium-sensitive Gnr-S cells. As reported previously (35), Northern blotting revealed the existence of more than one transcript for ZnT-1. Gallium-resistant GnR cells also displayed increased levels of ZnT-1 mRNA expression when compared with GnS cells (data not shown).

The results of the above studies collectively confirm that gallium resistance is associated with a high level of MT2A and ZnT-1 gene expression.

Induction of MT2A and ZnT-1 Expression by Gallium Nitrate

The induction of metallothionein gene expression by certain divalent metals, notably zinc and cadmium, and the protection conferred by metallothionein against the cytotoxicity of these metals have long been recognized (20, 21). However, an effect of gallium on the expression of the metallothionein or ZnT-1 genes has not been reported previously. In the studies shown in Fig. 3A and B, MT2A gene expression was examined in Gnr-S cells following exposure to gallium nitrate. As shown in Fig. 3A, incubation of cells with increasing concentrations of gallium nitrate for 24 h produced a dose-dependent increase in MT2A mRNA. This increase was detected within 4 h of continuous exposure of cells to 100 μmol/L gallium nitrate (Fig. 3B). In addition to the increase in MT2A expression, cells incubated with gallium nitrate displayed a dose-dependent increase in ZnT-1 mRNA expression (Fig. 3C).

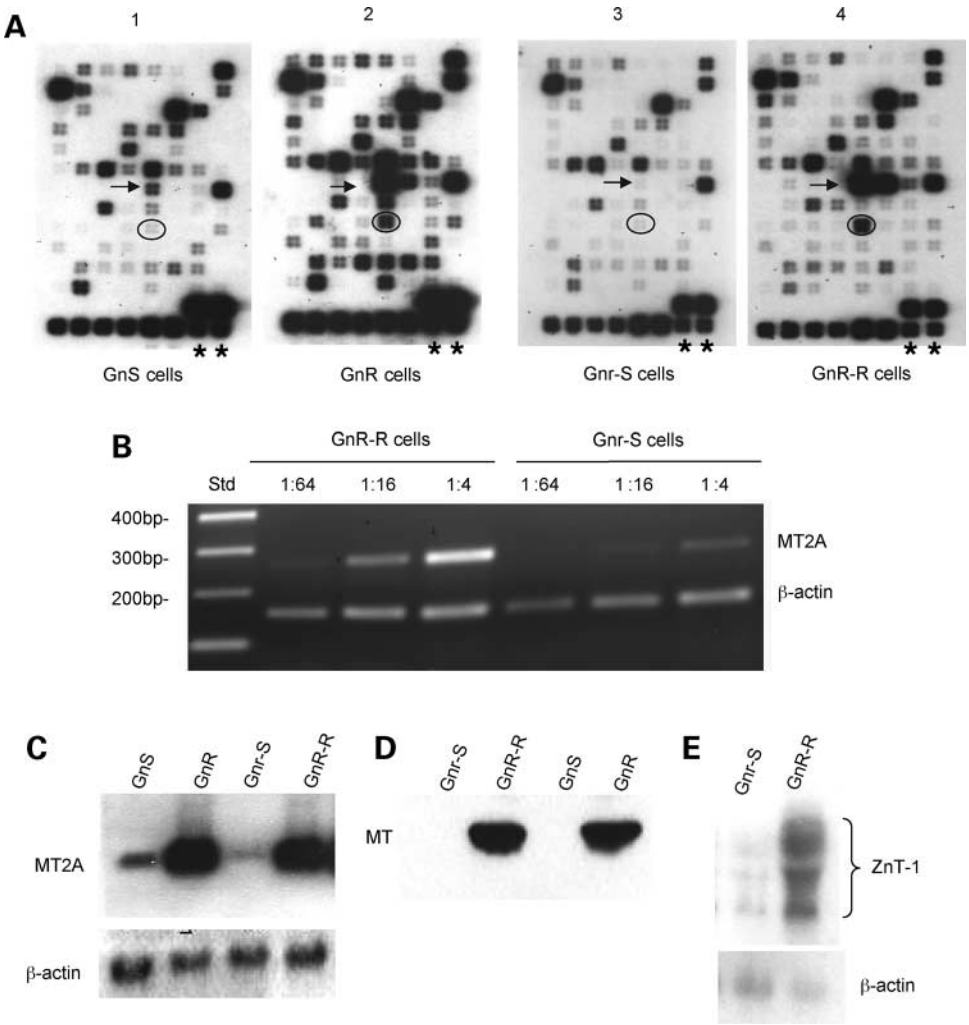

Figure 2. MT2A and ZnT-1 gene expression is increased in gallium-resistant cells. **A,** DNA arrays showing differential gene expression in gallium-sensitive and gallium-resistant CCRF-CEM cells. Biotinylated cDNA probes prepared from GnS, GnR, Gnr-S, GnR-R cells were hybridized to human metal transport and homeostasis gene array membranes and hybridization signals were detected as detailed in Materials and Methods. *Arrows,* location of the *MT2A* and *MT3* genes; *circles,* location of the *ZnT-1* gene; *asterisks,* location of the *β-actin* gene. **B** to **D,** verification that MT2A expression is increased in gallium-resistant cells. **B,** semiquantitative PCR for MT2A. PCR was done as described in Materials and Methods. **C,** Northern blot analysis. Gallium-sensitive GnS and Gnr-S cells and gallium-resistant GnR and GnR-R cells were analyzed for steady-state MT2A mRNA by Northern blotting. **D,** Western blot analysis. Metallothionein levels in gallium-sensitive GnS and Gnr-S cells and gallium-resistant GnR and GnR-R cells were measured by immunoblotting using E9 murine monoclonal antibody against metallothionein. *MT,* metallothionein. **E,** Northern blot analysis. Gallium-sensitive Gnr-S cells and gallium-resistant GnR-R cells were analyzed for steady-state ZnT-1 mRNA by Northern blotting.

Table 1. Differentially expressed genes in gallium-resistant and gallium-sensitive cells

Genbank accession no.	Gene	Gene symbol	GnR/GnS	GnR-R/Gnr-S
NM_004299	*ATP-binding cassette, subfamily B, member 7*	ABCB7	0.43	—
NM_000052	*ATPase, Cu transporting, α polypeptide*	ATP7B	0.44	—
NM_005125	*Copper chaperone for superoxide dismutase*	CCS	0.42	—
NM_001539	*DnaJ(Hsp40) homologue, subfamily A, member 1*	DNAJA1	5.47	—
NM_006894	*Flavin containing monooxygenase 3*	FMO3	0.40	—
NM_005345	*Heat shock 70 kDa protein 1A*	HSPA1A	2.16	—
NM_080430	*Selenoprotein SelM*	SELM	2.12	—
NM_178858	*Sideroflexin 2*	SFXN2	3.23	—
NM_014579	*Solute carrier family 39 (zinc transporter), member 2*	SLC39A2	0.49	—
NM_173596	*Solute carrier family 39 (metal ion transporter), member 5*	SLC39A5	2.54	—
NM_014585	*Solute carrier family 40 (iron-regulated transporter), member 1*	SLC40A1	3.18	—
NM_000454	*Superoxide dismutase 1*	SOD1	2.18	—
NM_003330	*Thioredoxin reductase 1*	TXNRD1	2.77	—
NM_006440	*Thioredoxin reductase 2*	TXNRD2	0.54	—
NM_004048	*β-2-microglobulin*	B2M	—	2.013
NM_001752	*Catalase*	CAT	—	3.34
NM_001964	*Early growth response 1*	EGR1	—	3.52
NM_000146	*Ferritin, light polypeptide*	FTL	—	2.23
NM_000058	*Glutathione peroxidase 1*	GPX1	—	2.47
NM_000178	*Glutathione synthetase*	GSS	—	3.31
NM_001530	*Hypoxia-inducible factor 1, α subunit*	HIF1A	—	2.07
NM_002133	*Heme oxygenase*	HMOX1	—	2.57
NM_006644	*Heat shock 105 kDa protein 1*	HSPH1	—	2.08
NM_000941	*P450 (cytochrome) oxidoreductase*	POR	—	2.74
NM_017767	*Solute carrier family 39 (zinc transporter), member 4*	SLC39A4	—	2.33
NM_006979	*Solute carrier family 39 (zinc transporter), member 7*	SLC39A7	—	2.11
NM_004136	*Iron-responsive element binding protein 2*	IREBP2	2.14	2.33
NM_005953	*Metallothionein-2A*	MT2A	10.17	32.56
NM_005954	*Metallothionein-3*	MT3	6.37	28.46
NM_005955	*Metal-regulatory transcription factor 1*	MTF1	2.06	2.29
NM_021194	*Solute carrier family 30 (zinc transporter), member 1*	SLC30A1	3.78	7.65
NM_001063	*Transferrin*	TF	0.52	0.42

NOTE: Genes showing at least a 2-fold difference in expression ratio between GnR and GnS or GnR-R and Gnr-S cells (ratio, >2 or ≤0.5) were considered significant. Differences in gene expression ratios not considered significant are represented by —.

MTF-1 Binding to MRE Is Increased in Cells Exposed to Gallium Nitrate

The expression of both *MT2A* and *ZnT-1* genes can be activated by the binding of MTF-1 to MRE consensus sequences present on promoter regions of both genes. Electrophoretic mobility shift assay was used to determine whether gallium nitrate induced the expression of *MT2A* and *ZnT-1* genes by increasing the interaction of MRE with MTF-1. Nuclear extracts from cells incubated with gallium nitrate for various times were examined for MTF-1 binding to radiolabeled MRE. As shown by the band intensity in Fig. 3D, only a low level of binding of MTF-1 to MRE was seen when cells were analyzed immediately after the addition of 500 μmol/L gallium nitrate (0 h time point). In contrast, a marked increase in MTF-1 binding activity was seen after 4 h of incubation of cells with gallium nitrate (Fig. 3D). This suggests that gallium nitrate produced an increase in nuclear MTF-1 levels, possibly by inducing the translocation of MTF-1 from the cytoplasm to the nucleus. Interestingly, MRE-MTF-1 binding returned to baseline levels after 24 h of incubation of cells with gallium nitrate. As a control, MRE-MTF-1 binding was also examined in cells treated with zinc sulfate for 4 h, and as expected, these cells displayed an increase in MRE-MTF-1 interaction (Fig. 3D).

Induction of Metallothionein Expression by Zinc Results in a Decrease in Cell Growth Inhibition by Gallium Nitrate

Because MT2A expression was found to be increased in gallium-resistant cells, we tested the hypothesis that the induction of metallothionein expression in gallium-sensitive cells would protect them against the cytotoxicity of gallium nitrate. Gnr-S cells were first exposed to zinc sulfate for 72 h to induce high levels of metallothionein expression and then examined for growth inhibition by gallium nitrate. As shown in Fig. 4A (*lane 2*), MT2A mRNA expression was markedly increased in cells that had been incubated with zinc sulfate (Fig. 4A, compare *lane 2* with *lane 1*). However, following a 24-h incubation of these zinc-treated cells in medium without zinc sulfate, MT2A expression decreased significantly but still remained elevated above basal levels

(Fig. 4A, *lane 3*). With further incubation for 48 and 72 h without zinc sulfate, MT2A levels in these cells returned to low baseline levels (Fig. 4A, *lanes 4* and *5*).

The zinc-induced increase in metallothionein significantly diminished the cytotoxicity of gallium nitrate, with the greatest protective effect seen when cells were incubated with gallium nitrate immediately after exposure to zinc sulfate. As shown in Fig. 4B, in cells not exposed to zinc sulfate, 200 μmol/L gallium nitrate inhibited cell proliferation by 52% at 48 h of incubation. In contrast, in cells that displayed a high level of metallothionein expression following exposure to zinc sulfate for 72 h (corresponding to Fig. 4A, *lane 2*), 200 μmol/L gallium nitrate inhibited cell proliferation by 22% (Fig. 4B).

The protective effect of zinc against gallium cytotoxicity was less striking when zinc-treated cells were first incubated in fresh medium for 24 h to allow for a decrease in their metallothionein content and then examined for response to gallium nitrate. Under these experimental conditions, zinc-treated cells were ~10% less sensitive to growth inhibition by 200 μmol/L gallium nitrate than cells that had not been exposed to zinc sulfate (data not shown).

Endogenous MT2A Levels in Lymphoma Cells Correlate with Sensitivity to Gallium Nitrate

Because the above studies showed that the cytotoxicity of gallium nitrate could be altered by exogenously induced changes in metallothionein expression, we considered whether the level of endogenous metallothionein in cell lines would correlate with their individual sensitivity to gallium nitrate. To examine this, a panel of B-cell lymphoma cell lines was screened for MT2A mRNA expression and for growth inhibition by gallium nitrate. As shown in Fig. 4C, apart from the GnR-R cells, the highest levels of MT2A mRNA were expressed in Granta and NCEB-1 cells, whereas the lowest levels were expressed in JVM-2, DoHH2, and Z138C cells; intermediate levels of MT2A mRNA were expressed in HBL-2 cells. As shown in Fig. 4D, the growth-inhibitory effects of gallium nitrate in these individual cell lines closely paralleled their expression of MT2A. Granta and NCEB-1 cells were markedly less sensitive to gallium nitrate (IC_{50} of 485 and 305 μmol/L, respectively) than JVM-2, DoHH2, and Z138C (IC_{50} of 12.5, 12.5, and 35 μmol/L, respectively), whereas HBL-2 cells displayed intermediate sensitivity to gallium nitrate (IC_{50} of 142 μmol/L).

Expression of Metallothionein in Non-Hodgkin's Lymphoma

To examine whether metallothionein is expressed in lymphomas, a random sample of biopsies from different lymphomas were examined by immunohistochemical staining for this protein. Examples of staining patterns are shown in Fig. 5. Strongly positive staining is shown for an

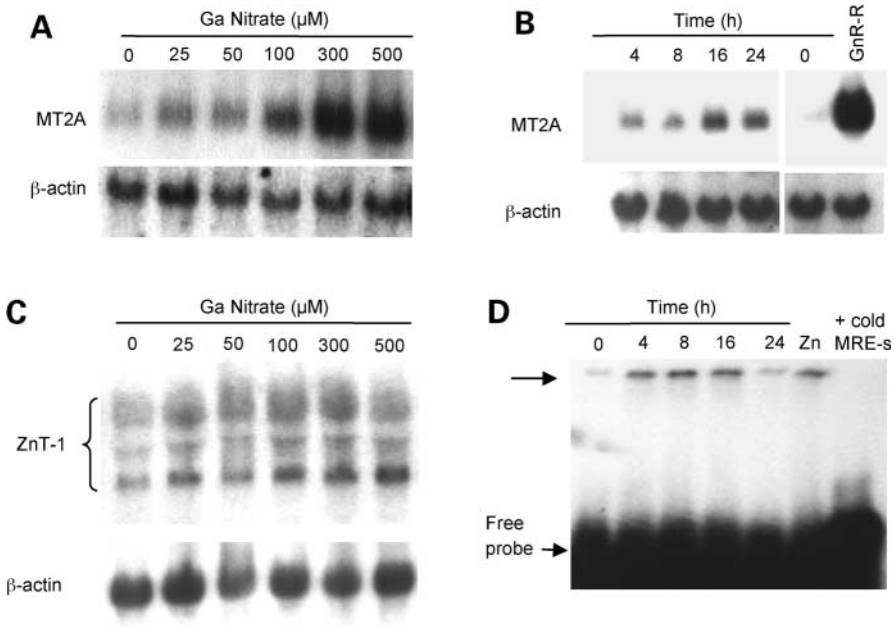

Figure 3. Gallium nitrate induces metallothionein and ZnT-1 expression and MRE-MTF-1 interaction. **A,** dose-dependent increase in MT2A expression by gallium nitrate. Gallium-sensitive Gnr-S cells were incubated with increasing concentrations of gallium nitrate for 24 h and analyzed for MT2A mRNA levels by Northern blotting. **B,** time-dependent induction of MT2A by gallium nitrate. Gnr-S cells were incubated with 100 μmol/L gallium nitrate for 0 to 24 h and then analyzed for MT2A mRNA levels by Northern blotting. MT2A mRNA expression in GnR-R cells incubated without gallium nitrate is shown for comparison. The bands shown for 0 h and GnR-R are from the same gel as the bands shown for the 4- to 24-h time points. **C,** dose-dependent increase in ZnT-1 gene expression by gallium nitrate. Northern blot gallium-sensitive GnS cells were incubated with increasing concentrations of gallium nitrate for 24 h and analyzed for ZnT-1 mRNA levels by Northern blotting. **D,** electrophoretic mobility shift assay detection of MTF-1 binding to MRE sequence. GnS cells were incubated with 500 μmol/L gallium nitrate for the times shown and analyzed for MTF-1 binding to ^{32}P-labeled MRE-s by electrophoretic mobility shift assay as described in Materials and Methods. *Arrow,* MRE-MTF-1 complex. *Lane 6,* MRE-MTF-1 in cells incubated with 500 μmol/L Zn sulfate for 4 h. ^{32}P-MRE binding to MTF-1 was competitively inhibited by a 100-fold excess of nonradioactive (cold) MRE-s (*lane 7*). Data are from representative experiments. Similar results were obtained in three separate experiments.

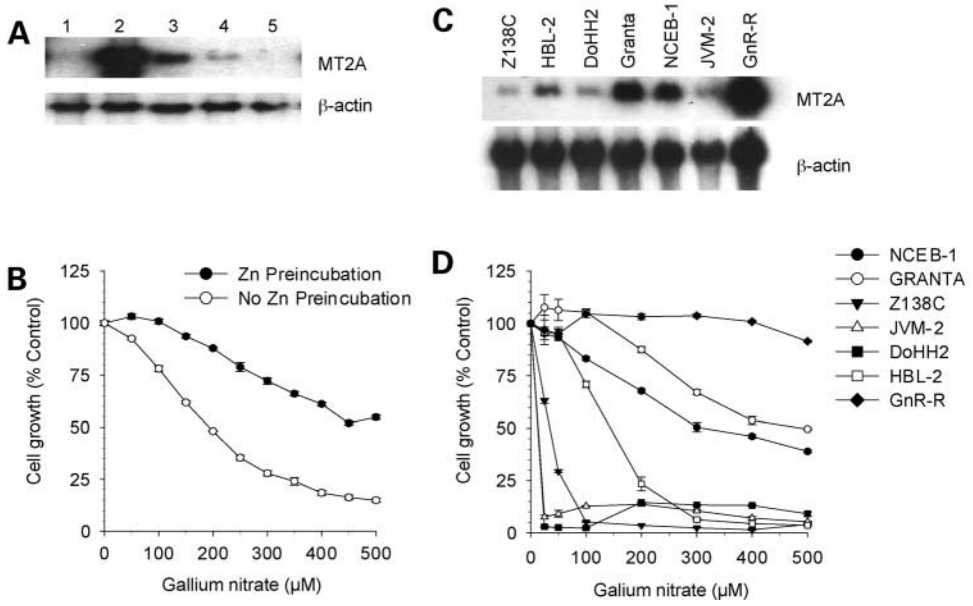

Figure 4. Cellular metallothionein modulates the cytotoxicity of gallium nitrate. **A,** Northern blot showing induction of MT2A expression by zinc. Gallium-sensitive Gnr-S cells were incubated with 100 μmol/L zinc sulfate for 72 h to induce high levels of MT2A expression. Cells were then washed and analyzed by Northern blotting either immediately or following an additional 24 to 72 h of incubation in fresh medium without zinc sulfate. *Lane 1,* control cells, no zinc exposure; *lane 2,* MT2A expression immediately following a 72-h incubation of cells with zinc sulfate; *lanes 3* to *5,* MT expression in cells after 24, 48, and 72 h of reincubation in fresh medium without zinc sulfate. **B,** preexposure of cells to zinc sulfate diminishes their sensitivity to growth inhibition by gallium nitrate. Cells incubated with 100 μmol/L zinc sulfate for 72 h were washed and incubated with increasing concentrations of gallium nitrate. Cell growth was measured by MTT assay after a 48-h incubation. *Points,* mean ($n = 3$); *bars,* SE. **C,** steady-state endogenous MT2A mRNA levels in a panel of lymphoma cell lines. Mantle cell lymphoma (Z138C, HBL-2, Granta, NCEB-1, and JVM-2), follicular lymphoma (DoHH2), and gallium-resistant GnR-R cell lines were analyzed by Northern blotting for MT2A mRNA expression. **D,** differences in the growth-inhibitory effects of gallium nitrate among the different lymphoma cell lines. Cells were plated in 96-well plates (0.2×10^6/mL) in the presence of increasing concentrations of gallium nitrate. Cell growth was measured by MTT assay after a 72-h incubation. *Points,* mean ($n = 3$); *bars,* SE. Note the correlation between MT2A mRNA levels and the inhibition of cell growth by gallium nitrate.

anaplastic large cell lymphoma and two cases of diffuse large B-cell lymphoma. Interestingly, in one of the cases of diffuse large B-cell lymphoma (DLBCL-1) shown in Fig. 5, metallothionein expression seemed to be heterogeneous, with some cells showing positive staining whereas others showing negative staining for this protein (Fig. 5). Heterogeneous staining for metallothionein was also seen in additional cases of DLBCL (data not shown). In contrast, metallothionein was not detected in the samples of Burkitt's, mantle cell, or follicular lymphoma (Fig. 5). These results show that metallothionein is expressed in lymphomas and that its expression is likely to vary among the different lymphomas and may even be heterogeneous within a given lymphomatous tumor.

Discussion

Knowledge of factors that may influence the efficacy of a chemotherapeutic agent is important because such information may be used to identify patients more likely to benefit from treatment with that drug. In the present study, we have attempted to identify genes whose expression might be of value in determining the sensitivity of lymphoma cells to gallium nitrate. Using a metal homeostasis focused DNA gene array to analyze unique gallium-resistant and gallium-sensitive lymphoma cell lines, we show for the first time that the level of MT2A expression in cells is an important modulator of their sensitivity to the cytotoxic effects of gallium nitrate. Whereas our studies also revealed that metallothionein gene expression was induced by gallium, gallium-resistant cells, whether grown in the presence (GnR cells) or absence (GnR-R cells) of gallium nitrate, displayed high levels of MT2A relative to gallium-sensitive cells. This finding suggests that metallothionein levels per se are of importance in modulating cell sensitivity to gallium nitrate. Support for this notion was provided by experiments, in which (*a*) the induction of metallothionein expression by zinc produced a decrease in the cytotoxicity of gallium, a protective effect that diminished as the cellular levels of metallothionein decreased and (*b*) differences in the growth-inhibitory effects of gallium nitrate among different lymphoma cell lines correlated with differences in their endogenous levels of metallothionein.

Next to metallothionein, the highest level of gene expression in gallium-resistant cells was that of solute carrier family 30 member 1 or ZnT-1, a zinc efflux transporter located primarily on the plasma membrane (36, 37). The transcription of metallothionein and ZnT-1 mRNAs is regulated by similar mechanisms that involve

the binding of MTF-1 to MREs present on the promoter regions of their respective genes (38, 39). Hence, we examined whether MTF-1 played a role in the up-regulation of both genes in response to gallium. These studies revealed that cells exposed to gallium nitrate displayed an increase in MRE-MTF-1 binding and thus provided a unifying explanation for the parallel increase in both metallothionein and ZnT-1 expression following exposure of cells to gallium nitrate.

The presence of high levels of MT2A and ZnT-1 in gallium-resistant cells and the ability of gallium nitrate to induce MT2A and ZnT-1 gene expression through MTF-1 activity was an unexpected and surprising finding. Increases in metallothionein expression are associated with exposure of cells to divalent metals, commonly (but not exclusively) zinc and cadmium. The binding of metallothionein to zinc enables it to serve as a storehouse that both sequesters and releases this metal as needed for cellular functions; its binding to cadmium on the other hand protects cells against cadmium toxicity (20, 38, 40). But, gallium, unlike zinc and cadmium, is a trivalent metal that does not exist in a divalent state and its ability to induce metallothionein expression or to potentially interact with metallothionein in cells has not been reported previously. Moreover, gallium (III) is known to share chemical properties with iron (III), a metal whose metabolism is not associated with metallothionein. Given these known properties of gallium, other mechanisms of action for gallium nitrate unrelated to iron metabolism need to be considered to explain the activation of MTF-1 and subsequent expression of metallothionein and ZnT-1. Recent studies have shown that MTF-1 can be activated by zinc, heat shock, and reactive oxygen species (30, 41–43). Whereas certain metals may induce metallothionein gene transcription, this seems to be mediated through their action on zinc proteins resulting in MTF-1 activation by released zinc (41). It is possible therefore that gallium nitrate may activate MTF-1 through action on zinc pathways. Alternatively, a role for oxidative stress in gallium-induced MTF-1 activation may also exist because our preliminary studies have revealed that gallium-induced apoptosis is accompanied by ROS production (44). Further studies are planned to examine the mechanisms of MTF-1 activation gallium and the potential interaction of gallium with cellular zinc metabolism.

Because GnR cells were developed by continuous exposure of GnS cells to gallium nitrate, it would seem that the early events in the development of gallium resistance included a gallium-induced increase in metallothionein and ZnT-1 gene expression in GnS cells. How metallothionein and ZnT-1 proteins function to confer gallium resistance to cells and whether other processes are also involved remains to be determined. Metallothionein may protect cells by sequestering gallium or by acting as an antioxidant in response to gallium-induced ROS production. The contribution of increased ZnT-1 to gallium resistance is less clear. Our earlier studies showed that gallium-resistant cells had a decreased accumulation of gallium (18). Whether this is could be due to increased gallium efflux from cells and whether ZnT-1 might play a role in this process poses an intriguing question that will also be addressed in further studies.

Several clinical trials have shown the efficacy of gallium nitrate in the treatment of non-Hodgkin's lymphoma (1–5). One of the interesting aspects of the antineoplastic activity of gallium has been the observation that patients who have previously failed to respond to other chemotherapeutic drugs may respond dramatically to gallium nitrate (45). Our immunohistochemical analysis of a limited number of randomly selected lymphomas shows that metallothionein expression varies among different lymphomas. Further immunohistochemical studies are in progress to examine

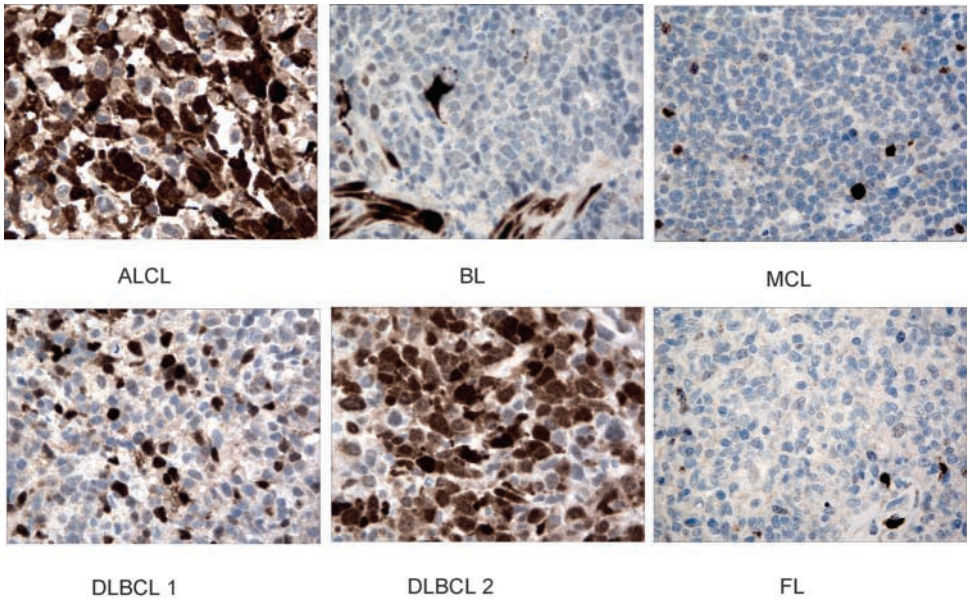

Figure 5. Metallothionein is variably expressed in non-Hodgkin's lymphoma. Immunohistochemical staining with E9 monoclonal antibody to metallothionein was done on tissue samples of different non-Hodgkin's lymphomas. *ALCL,* anaplastic large cell lymphoma; *BL,* Burkitt's lymphoma; *MCL,* mantle cell lymphoma; *DLBCL,* diffuse large B-cell lymphoma; *FL,* follicular lymphoma.

metallothionein expression in a larger cohort of lymphomas and the results will be presented in a subsequent report. We postulate that the level of endogenous metallothionein expression in lymphoma will be an important determinant of clinical response to treatment with gallium nitrate. Moreover, because metallothionein expression within a given lymphoma may be heterogeneous, only those cells with low or absent levels of metallothionein would be expected to be preferentially killed by gallium nitrate.

The results of our investigation provide new insights into pathways involved in the mechanisms of action of gallium and have obvious implications for the use of gallium nitrate in the treatment of lymphoma. Consideration should be given not only to the level of endogenous metallothionein expression in lymphomas being treated with gallium nitrate but also to the possibility that increased dietary intake of zinc supplements by patients may raise tumor metallothionein levels and diminish the response to treatment. Studies are in progress to define the mechanisms of action of gallium compounds on zinc-related pathways and to identify additional processes involved in the antineoplastic activity of this interesting metallodrug.

Acknowledgments

We thank Janine P. Wereley for technical assistance with cell lines and Vincent Graffeo, M.D. and Glen Dawson for assistance with the immunohistochemistry studies.

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Gene expression analysis of gallium-resistant and gallium-sensitive lymphoma cells reveals a role for metal-responsive transcription factor-1, metallothionein-2A, and zinc transporter-1 in modulating the antineoplastic activity of gallium nitrate

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Mol Cancer Ther 2007;6:633-643.