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ORIGINAL ARTICLE

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Characterization of MLH1 and MSH2 DNA mismatch repair proteins in cell lines of the NCI anticancer drug screen

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Abstract *Purpose and methods*: The lack of a functional DNA mismatch repair (MMR) pathway has been recognized as a common characteristic of several different types of human cancers due to mutation affecting one of the MMR genes or due to promoter methylation gene silencing. These MMR-deficient cancers are frequently resistant to alkylating agent chemotherapy such as DNA-methylating or platinum-containing compounds. To correlate drug resistance with MMR status in a large panel of human tumor cell lines, we evaluated by Western blot the cellular levels of the two MMR proteins most commonly mutated in human cancers, MLH1 and MSH2, in the NCI human tumor cell line panel. This panel consists of 60 cell lines distributed among nine different neoplastic diseases. *Results*: We found that in most of these cell lines both MLH1 and MSH2 were expressed, although at variable levels. Five cell lines (leukemia CCRF-CEM, colon HCT 116 and KM12 and ovarian cancers SK-OV-3 and IGROV-1) showed complete deficiency in MLH1 protein. MSH2 protein was detected in all 57 cell lines studied. Absence of MLH1 protein was always linked to resistance to the methylating chemotherapeutic agent temozolomide. This resistance was independent of cellular levels of O^6-alkylguanine DNA alkyltransferase. Based on data available for review in the NCI COMPARE database, cellular levels of MLH1 and MSH2 did not correlate significantly with sensitivity to any standard anticancer drug or with any characterized molecular target already tested against the same panel of cell lines. *Conclusion*: Based on evaluation of 60 tumor cell lines in the NCI anticancer drug screen, MLH1 deficiency was more common than MSH2 deficiency and was always associated with a high degree of temozolomide resistance. These data will enable correlations with other drug sensitivities and molecular targets in the COMPARE database to evaluate linked processes in tumor drug resistance.

Key words DNA mismatch repair · NCI anticancer drug screen · DNA repair · Drug resistance · Temozolomide

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Introduction

DNA replication results in occasional base pair errors mainly due to the insertion of a mismatched base or the slippage of the replication complex in regions of simple nucleotide repeats. All organisms require a functional DNA mismatch repair (MMR) system able to correct the replicative mismatches. The central role played by this system in the maintenance of genomic stability is evident in the appearance of mutator phenotypes and microsatellite instability (MSI) in prokaryotic or eukaryotic cells carrying a defective MMR gene [1]. Interest in the role of MMR in the etiology and progression of cancer has increased because of the finding that defects in one of the five human MMR proteins are present in hereditary nonpolyposis colon cancer (HNPCC) [2–4], as well as many sporadic cancers [5–8].

Three polypeptides (MSH2, MSH3 and MSH6/GTBP) are homologues of the bacterial protein MutS and are all involved in the initial phase of recognizing and binding the targeted mismatch. MSH2 can heterodimerize either with MSH6 to give hMutSα or with MSH3 to give hMutSβ, each of which is characterized by different substrate specificity [9, 10]. These heterodimers recruit to the repair reaction another heterodimer,

hMutLα, formed by two homologues of the bacterial protein MutL, MLH1 and PMS2. This heterodimer completes the assembly of the repair complex and proceeds with the excision and the resynthesis of the mismatched DNA region [11].

Beyond its role as a postreplicative repair system, MMR proteins appear to be able to recognize and bind a wide variety of DNA adducts including those induced by clinically used anticancer drugs such as simple methylating agents, platinum-containing compounds and several different alkylating agents. Cells lacking an efficient MMR pathway tolerate the presence in their DNA of adducts induced by cancer chemotherapy agents such as temozolomide (TMZ), cisplatin, 6-thioguanine, procarbazine, busulfan, doxorubicin and etoposide [12].

The correlation between MMR defects and drug resistance phenotypes has so far been investigated only in a limited number of human cancer cell lines or clinical specimens. Data have not been available concerning correlations between MMR defects and the outcome of the novel anticancer agent screen performed by NCI in its Anticancer Drug Screen Program. The Developmental Therapeutics Program (DTP) of the NCI has used 60 human cell lines derived from nine major histological types (bone marrow, lung, skin, ovary, colon, central nervous system, kidney, prostate and breast) to evaluate the eventual anticancer activity of new compounds from synthetic and natural origins [13]. To date, the approximate number of compounds tested by NCI is 60,000, and 10,000 new compounds are added to the program annually. For each compound tested in the assay, there is a pattern of anticancer activity represented by the GI_{50} values, which are the concentrations able to inhibit cell growth by 50% of the vehicle control sample after a 48-h continuous exposure, obtained from different cell lines. The so-called mean graph represents the display technique used to characterize each compound's growth inhibitory potential [14]. The COMPARE algorithm, developed by NCI, can be used to search the available database for compounds with similar activity pattern or compounds whose activity correlates to a certain extent with a molecular target measured in the same array of cell lines.

The present study is the first characterization of two components of the MMR pathway most frequently defective in human malignancies, MLH1 and MSH2, in the NCI anticancer drug screen cell lines. Not only did we want to correlate MMR deficiency with drug resistance to methylating agents, as we have previously reported with two MMR-deficient cell lines [15], but we also wanted to determine whether resistance or sensitivity to other drugs would be correlated with deficiency or relative expression of MLH1 and MSH2. In addition, since this panel has been characterized for several other molecular pathways important in drug resistance [16–21], our study was also designed to correlate MLH1 and MSH2 levels with other molecular targets.

Materials and methods

Cell lines and cell survival assay

Cell pellets (1×10^7 cells each) from the 58 cell lines included in the study were obtained from Dr. D. Scudiero and Mr. R. Camalier of the NCI DTP. For cytotoxicity experiments, cells were grown in RPMI-1640 supplemented with 5% fetal bovine serum and 5 m*M* L-glutamine. TMZ cytotoxicity was evaluated by plating, according to the inoculation density suggested by NCI-DTP, the different cell lines in six-well clusters 16 h before exposure to the drug. Cells were exposed to TMZ for 2 h, and incubated for 5 days at 37 °C in drug-free medium. Some samples were pretreated for 2 h with O^6-benzylguanine (BG) (kindly provided by Dr. R. Moschel, FCRDC-NCI) and further incubated for 5 days in the presence of BG after the removal of the drug. Cells were trypsinized and counted with a hemocytometer.

Western blotting

Cell pellets were resuspended in ice-cold lysis buffer containing 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1% NP40 and 1 m*M* EDTA, 1 m*M* PMSF, 0.01 mg/ml aprotinin and 0.01 mg/ml leupeptin. Samples were sonicated twice, 10 s each time, and the total protein concentration evaluated by the Bradford assay (Bio-Rad, Hercules, Calif.). Total protein (20 μg for each cell line) was separated on an 8% SDS-polyacrylamide gel and transferred to a PVDF Immobilon membrane (Millipore, Bedford, MA). Detection of MLH1 and MSH2 proteins was performed by using specific monoclonal antibodies recognizing the two human mismatch proteins: anti-MLH1 no. 13271A (Pharmingen, San Diego, Calif.) and anti-MSH2 no. 26-100UG (Oncogene Research/Calbiochem, Cambridge, Mass). Equal loading of the different samples was monitored by probing the filters with monoclonal anti-actin antibody no. N350 (Amersham Piscataway, NJ). Relevant bands were visualized by hybridization of the membranes to an anti-mouse IgG antibody (Amersham) which was peroxidase-linked and detected with the ECL system (Amersham). Comparison between individual blots was accomplished including in each gel 20 μg of total cell protein extracted from the MMR wild-type colon cancer cell line, SW480. MLH1 and MSH2 levels in SW480 cell extracts were found to vary by no more than 10% between the different blots analyzed (Fig. 1). MLH1 and MSH2 levels are expressed throughout as SW480-relative densitometry units. Similar standard errors were found when the MLH1 and MSH2 ratios between individual cell line extracts and SW480 were determined in at least two independent experiments.

COMPARE methodology

The available data concerning the chemosensitivity profiles of the cell lines from the NCI anticancer drug screen can be accessed through the world-wide web at http://dtp.nci.nih.gov/. The NCI screening procedures have been already described [22]. Briefly, each compound included in the screening was evaluated for its cell growth inhibition activity using a 48-h assay with sulforhodamine B [23]. The growth-inhibitory power of each agent is expressed as the GI_{50} value. All the GI_{50} values obtained for a given compound can be graphically represented through the mean graph created by plotting positive and negative values from a set of GI_{50} values along a vertical line representing the mean response of all the cell lines in the panel to the test agent. Positive values represent cellular sensitivities to the test agent that exceed the mean, whereas negative values represent sensitivities to the test agent that are less than the mean.

The expression of molecular targets in the cell lines can be correlated with the effects of the test compounds through the aid of the COMPARE algorithm [14], which analyzes the different databases (standard anticancer agents, synthetic compounds, natural compounds and molecular targets) to search for the highest ranked

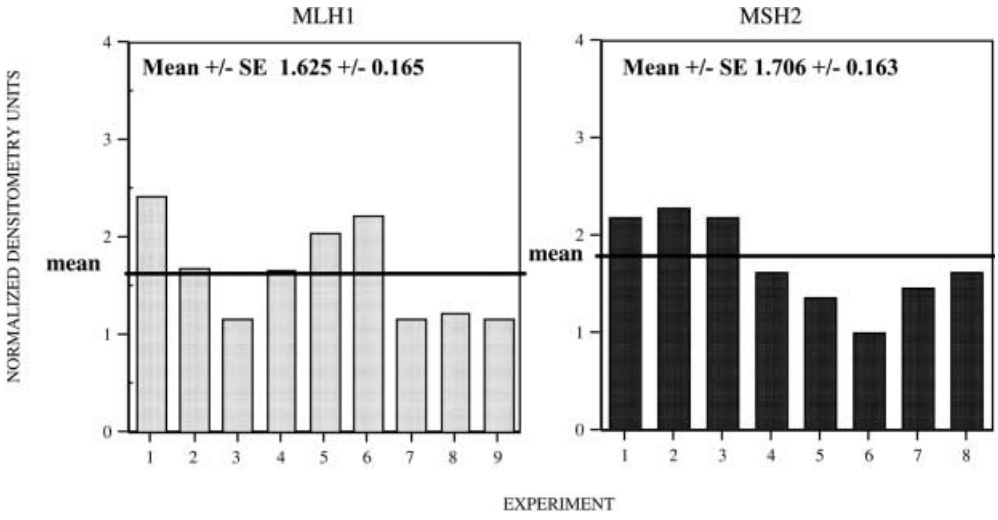

Fig. 1 Levels of expression of MLH1 and MSH2 in SW480 cell extract as evaluated in nine different Western blotting experiments. Raw values obtained by densitometry of the bands were normalized to the distribution of MLH1 or MSH2 expression among the samples loaded in each single gel

compounds or molecular targets matching the distribution of the input data across the panel of cell lines. The COMPARE analyses are reported as rank-ordered lists of compounds and each of the selected agents is characterized by the Pearson correlation coefficient which measures the yield of similarity between the input and that specific compound.

Results

Five cell lines of the NCI anticancer drug screen do not express MLH1 protein

To identify defects in the DNA MMR pathway in the human cancer cell lines included in the NCI anticancer drug screen, we determined the levels of expression of MLH1 and MSH2. Levels of MLH1 (Fig. 2) and MSH2 (Fig. 3) in the 58 cell lines were assayed by Western blotting and then quantified by densitometry. We compared MLH1 and MSH2 expression with the colon cancer cell line SW480, which is known to be MMR^+, to establish a standardized assay of relative activity. Figure 1 shows the expression of MLH1 and MSH2 in SW480 as evaluated in nine different Western blots. The standard error for both was about 10%. Figure 4 shows the standard curves for the Western blots obtained by loading different amounts of SW480 cell extract and probing for MLH1 or MSH2 expression. The relationship between the total amount of proteins loaded and densitometry scanning of the resulting bands was linear for MLH1 and MSH2. The majority of the cell lines expressed MMR proteins, although at levels lower than the reference cell line SW480 (Figs. 5 and 6). The mean value of the distribution of MLH1 levels in the cells was 0.42 SW480-relative densitometry units (range 0.01–0.92). Five cell lines (leukemia CCRF-CEM, colon cancers HCT116 and KM12 and ovarian cancers IGROV-1 and SK-OV-3) did not express any detectable MLH1. The expression of MSH2 varied among the 57 cell lines analyzed showing a mean of 0.59 SW480-relative densitometry units (range 0.03–3.763).

MMR-deficient cell lines are resistant to the methylating agent TMZ independent of O^6-alkylguanine-alkyltransferase

We searched the NCI database for available data regarding the methylating agent TMZ (NSC 362856) because we have previously shown [15] that colon cancer cell lines carrying mutations in MMR proteins are resistant to this drug. The NCI chemosensitivity profile for TMZ showed a lack of activity at the highest concentration used (100 μM) in a majority of cell lines tested. To reevaluate TMZ sensitivity, we performed cytotoxicity assays in the cell lines expressing and lacking MLH1 expression. Figure 7 shows the TMZ-induced cytotoxicity profiles of three cell lines (H-460, HT29 and HL-60) which are MMR wild-type (Fig. 7A) and four cell lines (CCRF-CEM, SK-OV-3, KM12 and IGROV-1) which are MMR-deficient (Fig. 7B). Cells were exposed to TMZ alone or following pretreatment with 10 μM BG, which depletes cellular O^6-alkylguanine-alkyltransferase (AGT). Figure 7 and Table 1 show that BG potentiates TMZ cytotoxicity in MMR wild-type cells positive for AGT protein, whereas all MMR-deficient cells studied were much more resistant to TMZ alone and to BG-mediated sensitization. This resistance to TMZ in MMR-deficient cell lines was independent of AGT levels. When eight MHL1-deficient cell lines were compared with seven MMR-proficient cell lines (Table 1), the MMR-deficient lines were 8- to100-fold more resistant to BG plus TMZ than the MMR-proficient cells.

Expression of MLH1 and MSH2 does not correlate with activity patterns of standard anticancer agents

According to the COMPARE program [14], the activity profile of a given compound tested against the 60 cell lines included in the anticancer drug screen can be graphically represented by the "mean graph". This is

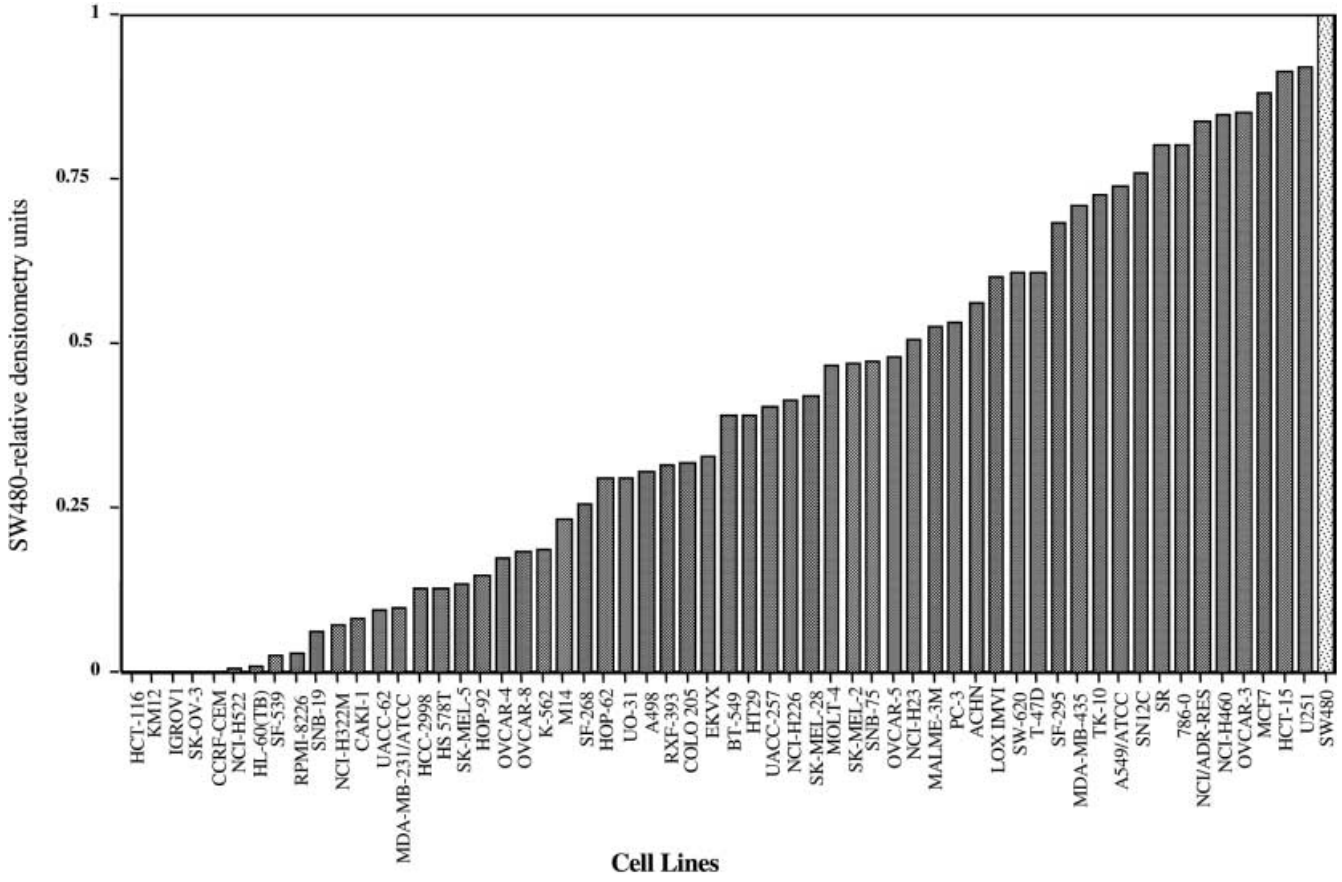

Fig. 2 Summary of MLH1 levels in 58 cell lines of the NCI Anticancer Drug Screen panel. A value of 1 has been assigned to the relative protein level evaluated in extracts of the colon cancer cell line SW480 that was run on each blot

generated by plotting positive and negative values along a vertical line representing the mean values either of the cytotoxicity or the molecular target in the cell lines. Positive values indicate cell lines with expression of the target exceeding the mean, whereas negative values indicate cell lines in which the expression is less than the mean. The ''fingerprint'' pattern resulting from MLH1 and MSH2 analysis was used by the COMPARE algorithm to select compounds whose activity pattern was the closest possible to the one obtained for either MMR protein.

We first evaluated a ''fingerprint'' of MLH1 or MSH2 values against the GI_{50} values of several standard anticancer agents across the entire panel of cell lines. This analysis did not lead to any significant correlation (data not shown). Next, we correlated MLH1 levels with drugs to which MMR-deficient cell lines have been reported to be resistant. Figure 8 shows the results obtained by plotting the growth-inhibitory potencies (expressed as $-\log GI_{50}$) of these drugs in each single cell line versus MLH1 levels. Three of the five MLH1-negative cell lines were moderately resistant to cisplatin while two MLH1-negative cell lines were moderately resistant to dacarbazine. However, the distribution of the GI_{50} values was not significantly different between MLH1-positive and -negative cell lines.

Discussion

Evaluation of MLH1 protein levels is a reliable parameter for screening MMR status in cancer cell lines

The presence of pathogenic germ-line mutations in *hMLH1* and *hMSH2* genes has been identified in 50–60% of families affected by HNPCC, the most common hereditary colon cancer syndrome [24–26]. More recently, a number (10–20%) of sporadic colon cancers have been identified as carrying germ-line or somatic mutations for either the *MLH1* or *MSH2* gene [27, 28]. Cancers carrying mutations in MMR genes are characterized by an increase in a specific type of genomic instability, that is MSI, which has been correlated with the lack of expression of MLH1 or MSH2 proteins [29]. A number of MSI cancers which lack MLH1 expression do not have any mutation in the genes, but instead have hypermethylation silencing of the MLH1 promoter [30–32]. We chose to screen the large number of cell lines in the NCI bank for expression of MMR proteins rather than to measure gene expression by mRNA or genomic mutations because levels of stable protein expression have been shown to

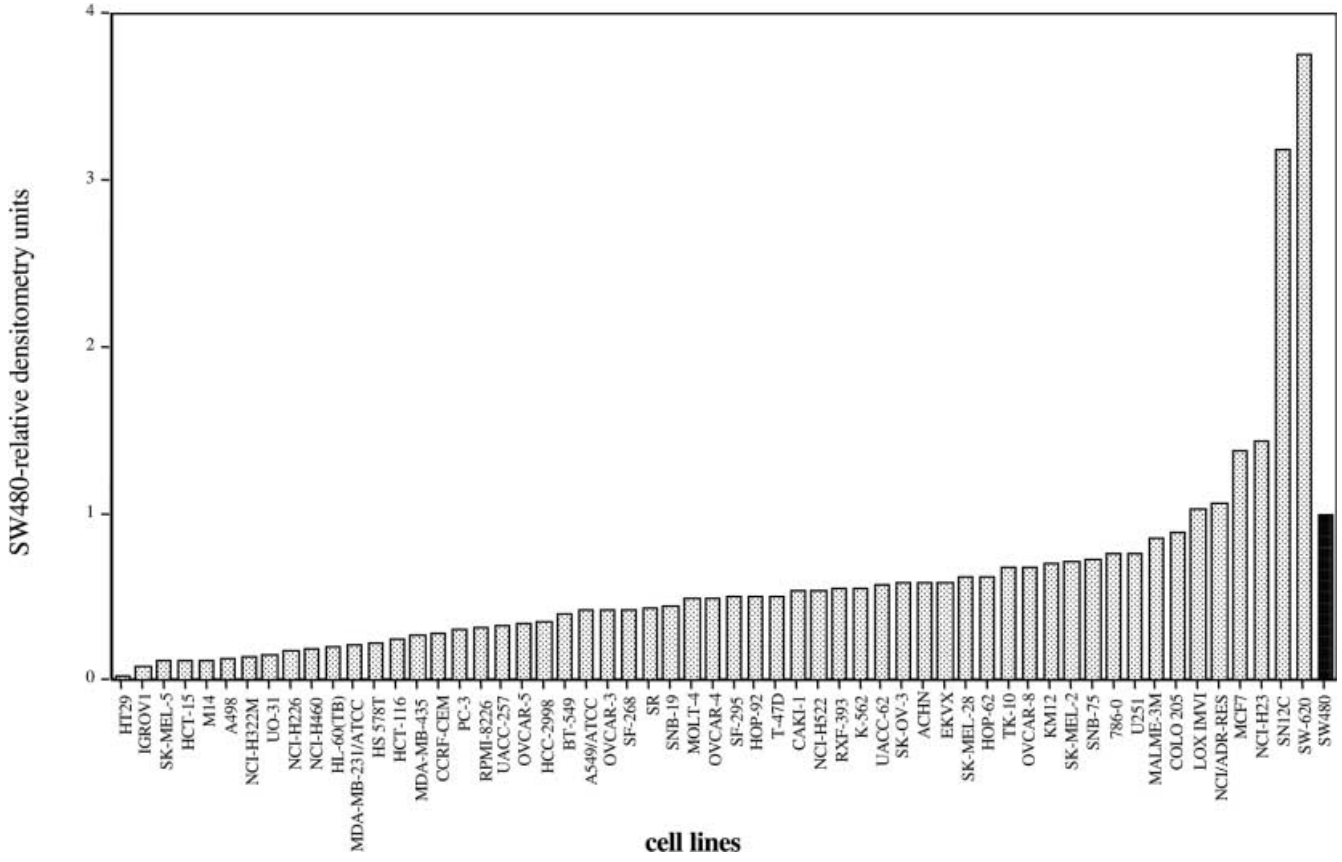

Fig. 3 Summary of MSH2 levels in 57 cell lines of the NCI Anticancer Drug Screen panel. For explanation see legend to Fig. 2

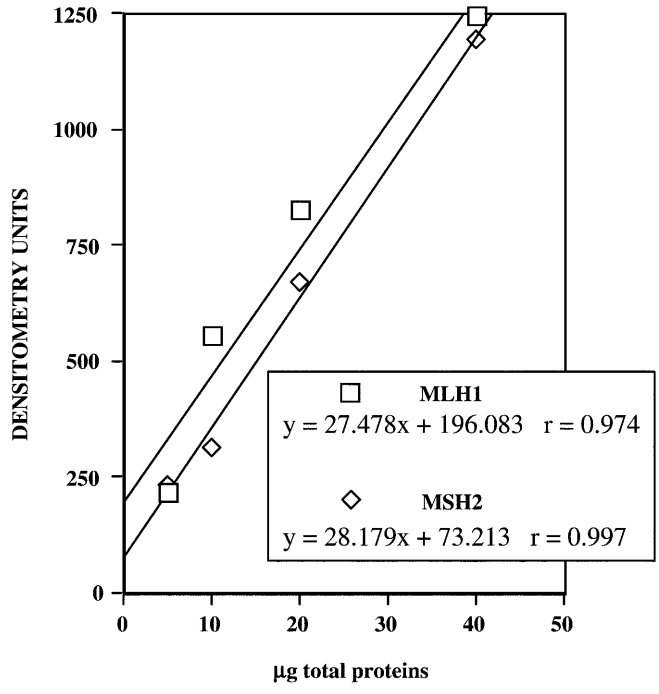

Fig. 4 Standard curves of MLH1 and MSH2 proteins in SW480 extracts. Increasing amounts of total cell proteins were loaded and probed for expression of MMR proteins. Linear regression equations and regression coefficients are indicated for each protein

accurately predict MMR functionality [33, 34]. As previously shown for the T-lymphocytic leukemia CCRF-CEM [35], cell lines heterozygous for mutations affecting MMR genes can produce full-length message without expression of functional protein.

Of the 58 cell lines analyzed, 5 (9%) were characterized as MMR-deficient based on MLH1 levels: the leukemia CCRF-CEM, the two colon cancers HCT116 and KM12 and the ovarian cancers SK-OV-3 and IGROV-1. Our results are consistent with previously published data for CCRF-CEM, HCT116 and SK-OV-3 [35–37]. We also found for the first time that KM12 and IGROV1 are lacking in MLH1. CCRF-CEM and HCT116 lack MLH1 protein due to mutations affecting exon 9 of the relevant gene, whereas SK-OV-3 carries a larger deletion involving exons 4–19 of the same gene. These defects of the *hMLH1* gene result in complete absence of the transcript and genomic instability. This phenotype is linked to tolerance to methylating agent-induced DNA damage and it has been shown [38] that transformation of HCT116 cells with a normal chromosome 3 carrying the *hMLH1* normal gene restores a wild-type MMR function and reduces the tolerance to DNA damage after treatment with methylating agents.

The MSH2 protein was present in all the extracts prepared from the cell lines of the NCI panel and this was confirmed in repeated preparations. Of note, the MMR-proficient cell line HT29 expresses low MSH2 levels. This suggests that only the complete absence of MSH2 can be considered as a valid index of MMR

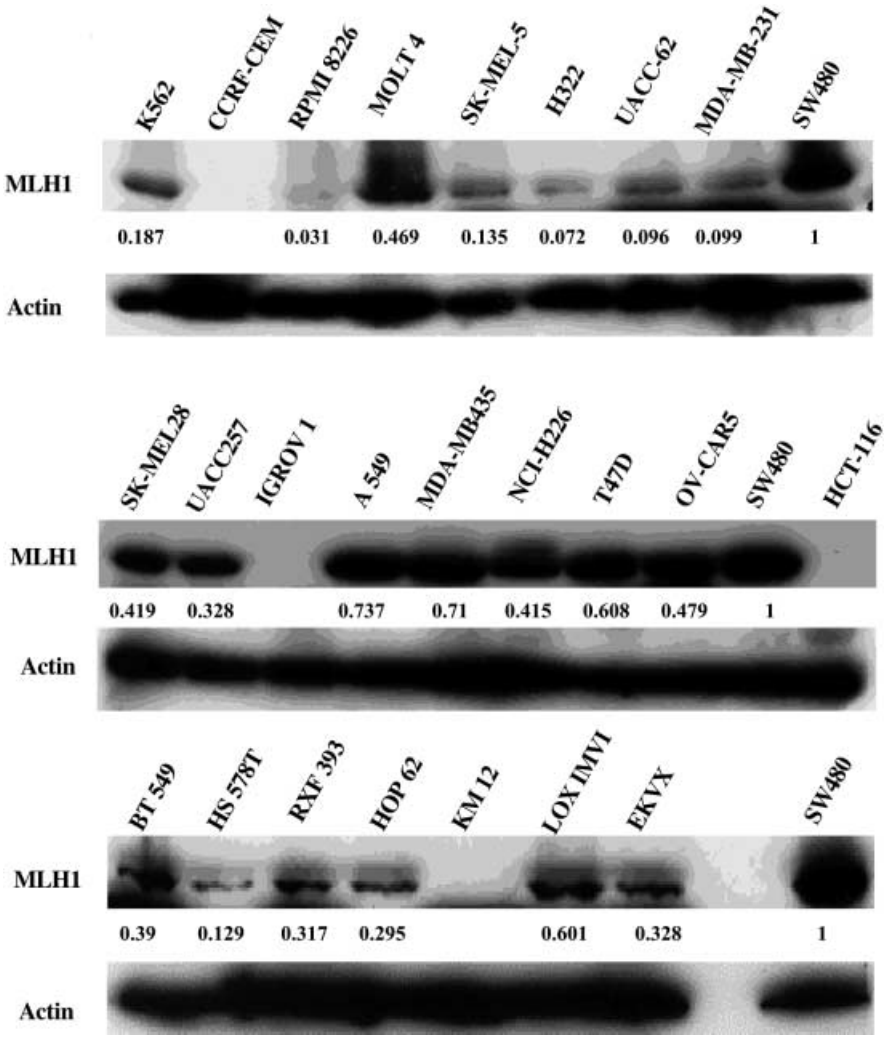

Fig. 5 Expression of MLH1 in some cell lines of the NCI anticancer drug screen. Numbers represent the SW480-relative densitometry units for the indicated cell lines

deficiency whereas very low levels of the same protein can support a functional MMR pathway [39].

MMR characteristics of the cell lines of the NCI anticancer drug screen are not correlated with patterns of chemosensitivity

In the course of this study, we took advantage of the COMPARE database to determine whether relative expression of MLH1 and MSH2 could affect resistance or sensitivity to more than 60,000 other anticancer drugs and to correlate the expression with other molecular targets.

Our study did not show any correlation in excess of 0.5 between overall MLH1 or MSH2 expression in the cell lines included in the NCI panel and the growth-inhibitory activity of any standard anticancer agent. We confirmed that individual cell lines lacking MLH1 protein are characterized by a relative resistance to platinum-containing compounds and by tolerance to DNA damage induced by methylating agents. From this we conclude either that the drug sensitivity testing is not stringent enough to identify such correlations, or that the complexity of drug resistance mechanisms overrides the contribution of variable levels of MLH1 or MSH2. On the other hand, we can draw a relationship between lack of MMR and AGT-independent resistance to the methylating agent TMZ. MLH1-positive and -negative cell lines showed similar sensitivities when exposed to TMZ alone, whereas the distributions of IC_{50} values for BG plus TMZ clearly separated the two groups (Table 1).

MMR defects have been linked to resistance to a number of anticancer drugs either due to cellular tolerance to relevant DNA damage or to selection of drug-resistant variants as a result of genomic instability [12, 40]. It is, therefore, important to identify new drugs characterized by a selective activity against tumors with MMR defects. The use of information technology paired with the evaluation of MMR deficiency can be a novel approach for screening potential anticancer agents whose activity may relate to the molecular characteristics of the cells. As an example, Tables 2 and 3 show the ten compounds whose activity profiles best correlated with the lowest MLH1- or MSH2-expressing cell

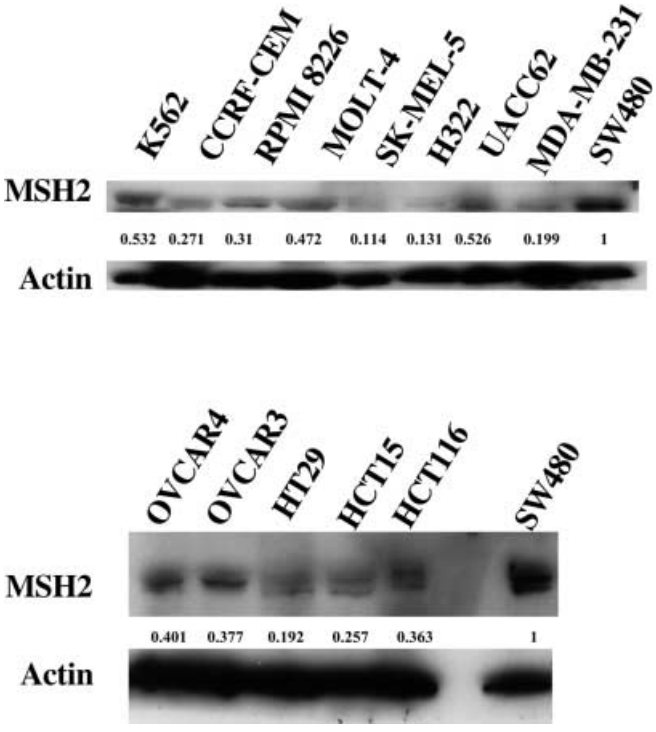

Fig. 6 Expression of MSH2 in some cell lines of the NCI anticancer drug screen. Numbers represent the SW480-relative densitometry units for the indicated cell lines

Table 1 Cytotoxicity of TMZ compared to MMR proficiency in human cancer cell lines. The relationship between TMZ-induced cytotoxicity and MMR status in human cancer cell lines. IC_{50} values for TMZ alone or TMZ + BG are shown for cell lines of the NCI anticancer drug screen (bold) and colon cancer cell lines of our laboratory collection (*italic*). AGT activity was evaluated as previously described [41]

Cell line	MMR defect	TMZ IC_{50} (μM)	10 μM BG + TMZ IC_{50} (μM)	AGT (fmol/μg DNA)
CCRF-CEM	MLH1	600	300	4.6
KM12	MLH1	600	600	0.3
SK-OV-3	MLH1	1200	500	2.8
IGROV-1	MLH1	1600	1200	4.7
HCT 116	MLH1	875	875	6.9
VACO 6	*MLH1*	*300*	*300*	*18.4*
RKO	*MLH1*	*1400*	*1300*	*39.5*
VACO 481	*MLH1*	*105*	*105*	*7.2*
RPMI 8226	wt	90	30	0.4
H460	wt	160	10	2.4
HT29	wt	700	13	18.4
HL-60	wt	160	40	1.7
VACO 411	*wt*	*12.5*	*12.5*	*0.1*
VACO 8	*wt*	*41*	*41*	*0.1*
SW 480	*wt*	*350*	*25*	*6.4*

lines, respectively. However, the Pearson correlation coefficients associated with these compounds were not particularly high, reaching a maximum value of 0.587 and 0.530 for the two compounds best correlated with MLH1 and MSH2 distribution, respectively. Links to pertinent chemical and activity characteristics of these compounds can be freely obtained at http://dtp.nci.nih.gov.

In conclusion, our study is the first comprehensive assessment of the MMR status of 58 cell lines included in the NCI anticancer drug screen. Those cell lines recognized as MMR-deficient due to the absence of the MLH1 protein were all further characterized by an

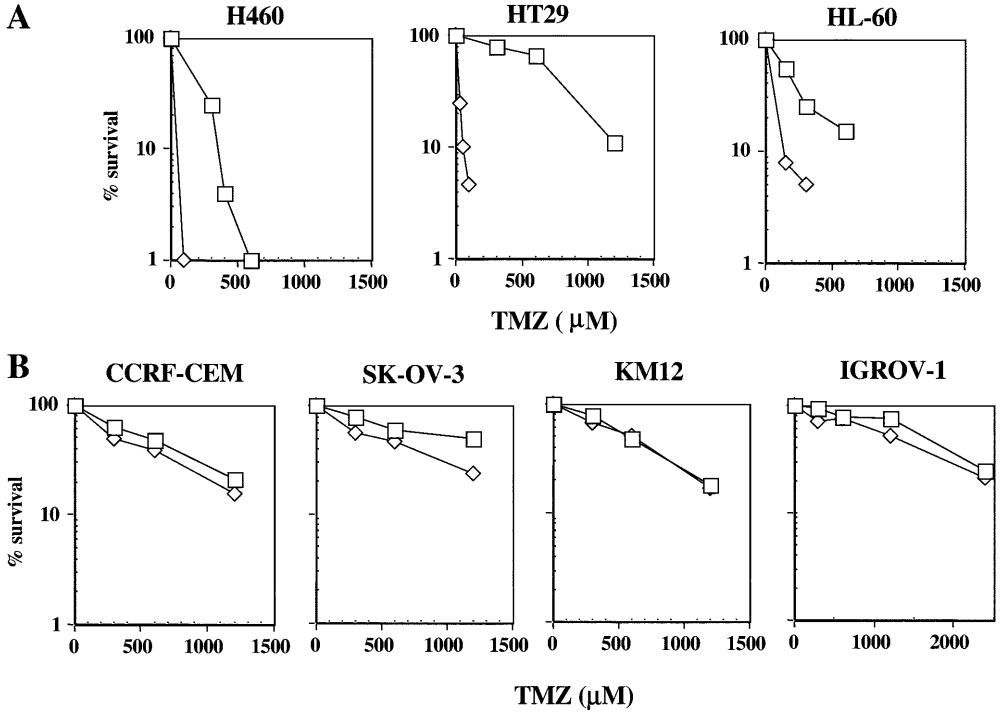

Fig. 7A,B TMZ-induced cytotoxicity in MMR wild-type (**A**) and MMR-deficient (**B**) cell lines of the NCI anticancer drug screen. Cells were treated for 2 h with the indicated concentrations of TMZ (□) or were pretreated for 2 h with BG (open diamonds); and then exposed to TMZ in the presence of BG. Cells were incubated in fresh medium or in medium containing BG for 5 days and counted using a hemocytometer

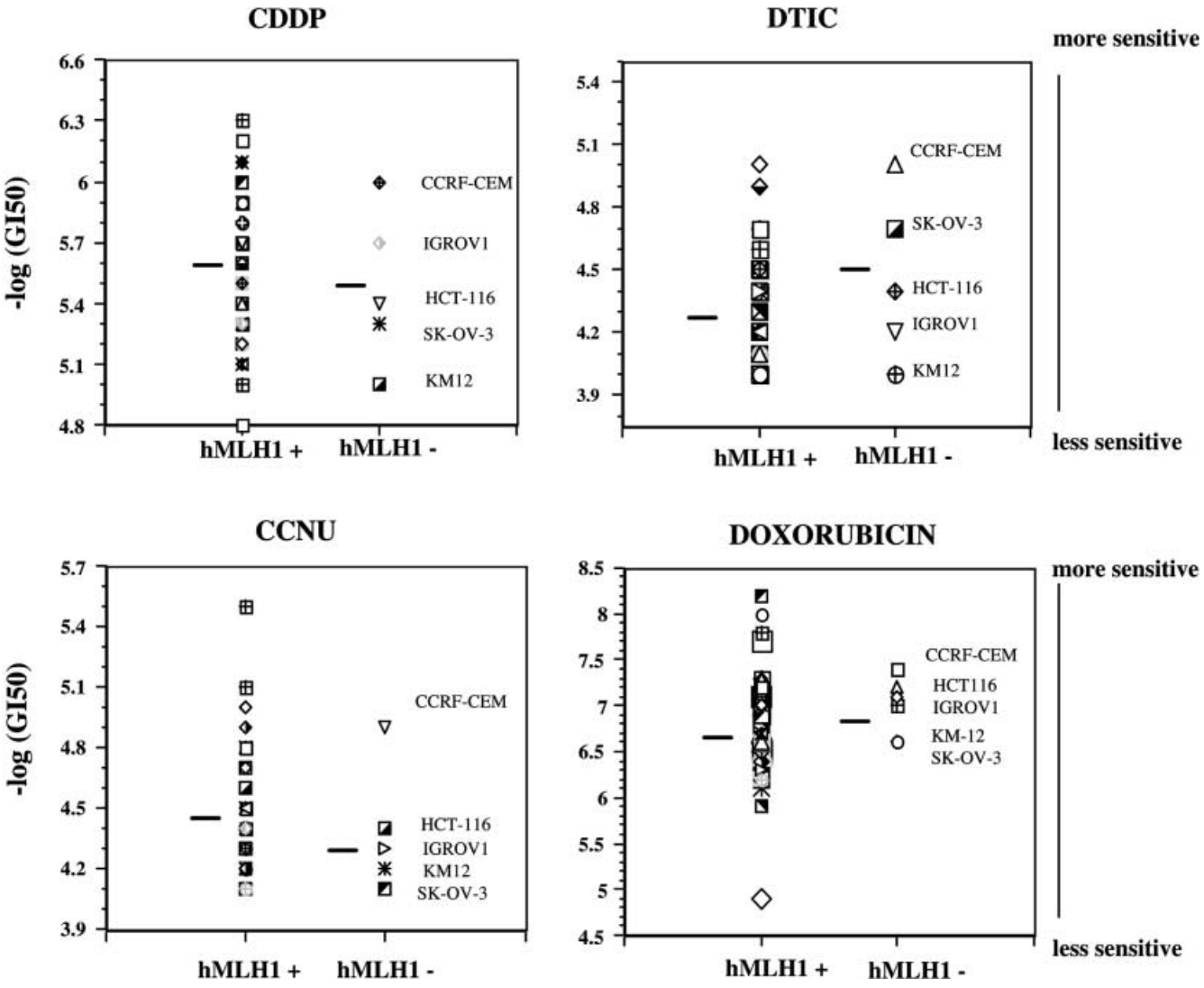

Fig. 8 Relationship between MLH1 expression and cytotoxicity induced by the indicated anticancer drugs in 58 cell lines of the NCI anticancer drug screen. Cell lines were plotted according to proficiency in MLH1 expression as evaluated by Western blotting. Sensitivities to the different drugs are plotted as $-\log GI_{50}$

Table 2 List of compounds identified by COMPARE analysis when MLH1 values of all the cell lines were used as seed

Pearson correlation coefficient	NSC number (name)
0.587	622357
0.503	88381
0.458	668430
0.447	655101
0.446	643170 (ethyl 3,4-dimethoxy-α-benzoyl cinnamate)
0.445	665006
0.440	622356
0.438	642649
0.433	611439 [(±)-3-benzyloxymethyl-4,5-isopropylidene-2-cyclo]
0.433	635299

Table 3 List of compounds identified by COMPARE analysis when MSH2 values of all the cell lines were used as seed

Pearson correlation coefficient	NSC number (name)
0.530	632916
0.491	354646 (morpholino-ADR)
0.483	633658 (D-Phe-Ala-Tyr-D-Trp-Lys-Val-Ala-D-Nal-NH_2)
0.474	622707
0.469	633662 (D-Phe-Cpa-Tyr-D-Trp-Lys-Thr-Phe-Thr-NH_2)
0.443	622918
0.438	140395
0.417	657586
0.406	683905
0.395	642406

evident tolerance to the DNA damage induced by the methylating agent TMZ. On the other hand, we did not observe in these cell lines any significant correlation between MMR defects and resistance to other currently used anticancer drugs or coordination with other molecular targets characterized in the same panel of cell

lines. This suggests a clear lack of coordination between the MMR pathway and other known mechanisms of drug resistance. This study was intended to provide an initial tool for exploring possible MMR-drug interactions. We confirm a high correlation between lack of MLH1 and resistance to BG plus TMZ. This is a clear example of protein expression predicting drug resistance for a useful pharmacological profiling of human cancers. Other relationships may be further explored and elucidated with the aid of the comprehensive approaches described in this study.

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