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Evidence for Nucleotide Excision Repair as a Modifying Factor of O^6-Methylguanine-DNA Methyltransferase-Mediated Innate Chloroethylnitrosourea Resistance in Human Tumor Cell Lines

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SUMMARY

We examined the O^6-methylguanine-DNA methyltransferase (MGMT) protein as well as MGMT activity levels and the excision repair cross-complementing rodent repair deficiency gene, *ERCC2* (*XPD*), protein levels in 14 human tumor cell lines not selected for chloroethylnitrosourea (CENU) resistance. These results were compared with 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) cytotoxicity and UV light sensitivity. MGMT protein correlated significantly with MGMT activity ($r = 0.9497$, $p = 0.0001$). There was no significant linear correlation between BCNU cytotoxicity and MGMT content as determined by both Western analysis ($r = 0.139$, $p = 0.6348$) and activity assay ($r = 0.131$, $p = 0.6515$). However, MGMT-rich cell lines were found to be more resistant than MGMT-poor cell lines to BCNU ($t = 2.2375$, $p = 0.0225$) but not to UV ($t = 1.1734$, $p = 0.1317$). Furthermore, the most BCNU-sensitive cell lines were all MGMT-poor. UV sensitivity was significantly correlated to BCNU cytotoxicity ($r = 0.858$, $p = 0.0001$). Significant correlations were found between ERCC2 protein levels and BCNU cytotoxicity ($r = 0.786$, $p = 0.0009$) or UV sensitivity ($r = 0.874$, $p = 0.0001$). Our results confirm that MGMT plays an important role in CENU resistance, but not in UV resistance. The correlation of UV sensitivity with BCNU cytotoxicity suggests that nucleotide excision repair is an important modifying factor of MGMT-mediated innate CENU resistance in human tumor cell lines, especially in highly resistant cell lines. *ERCC2* may be implicated in this process.

Anti-cancer drug resistance appears to be multifactorial. Two mechanisms implicated in CENU resistance of human tumors are GST-mediated metabolism of CENUs (1, 2) and MGMT-mediated repair of CENU-induced DNA adducts (3–5). Detoxification of CENUs by GST-μ has been previously associated with CENU resistance (6) and high levels of GST-π may be an important determinant of resistance to many alkylating agents (2).

MGMT can repair O^6-alkylguanine adducts, such as the chloroethyl adduct formed by CENUs, preventing the formation of cytotoxic interstrand cross-links (4). Previous investigations have correlated CENU drug resistance with the presence of MGMT (3, 7, 8). Also inactivation of MGMT sensitizes cells to CENUs emphasizing the importance of MGMT in CENU drug resistance (9).

NER is a multienzyme complex that is responsible for repairing a wide variety of DNA lesions (10). A number of simple lesions, as well as bulky adducts produced by UV light (11) or by some chemotherapeutic agents such as cisplatin (12), are excised by NER. Excision repair cross-complementing rodent repair deficiency gene 2 (*ERCC2*), also known as xeroderma pigmentosum complementation group D (*XPD*), seems to be a DNA helicase (13) and may also be involved in recognition of DNA damage (14). *ERCC2* can correct the NER defect in the UV-sensitive CHO cell line UV5 (15) and cell lines derived from *XPD* patients (16), indicating that *ERCC2* plays an important role in the NER process. Recently, we investigated *ERCC2* gene expression utilizing quantitative competitive polymerase chain reaction, and found a significant correlation between *ERCC2* expression and CENU cy-

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ABBREVIATIONS: CENU, chloroethylnitrosourea; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CHO, Chinese hamster ovary; ERCC, excision repair cross-complementing; GST, glutathione *S*-transferase; MGMT, O^6-methylguanine-DNA methyltransferase; Mer$^+$, O^6-methylguanine-DNA methyltransferase-rich; Mer$^-$, O^6-methylguanine-DNA methyltransferase-poor; NER, nucleotide excision repair; TFIIH, transcription factor IIH; XPD, xeroderma pigmentosum complementation group D; FBS, fetal bovine serum; SRB, sulforhodamine B; CAK, cyclin-dependent kinase-activating kinase.

totoxicity in six established human tumor cell lines (17), suggesting that *ERCC2* may also play an important role in CENU resistance. To further characterize the contribution of DNA repair genes in CENU resistance, we examined ERCC2 protein levels, MGMT protein levels, and MGMT activity in 14 human tumor cell lines. These results were correlated with cytotoxicity to 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and to UV light sensitivity.

Materials and Methods

Cell culture. Fourteen established human tumor cell lines were used in this study. Cell lines A498, HT-29, ACHN, SF-295, 786-0, and CAKI-1 were supplied by National Cancer Institute; T98-G was a kind gift from Dr. D. Yarosh at Applied Genetics, Freeport, NY; SK-MG-1 and SK-MG-4 were obtained from Dr. G. Cairncross at the University of Western Ontario, London, Ontario, Canada. SKI-1 was supplied by Dr. J. Shapiro at Barrow Neurological Institute, Phoenix, AZ; MGR-3 was from Dr. F. Ali-Osman at University of Texas M.D. Anderson Cancer Center, Houston, TX; and SKNSH was from Dr. E. Shoubridge at the Montreal Neurological Institute (Montreal, Quebec, Canada). None of these cell lines was selected for resistance to CENUs. All cell lines were grown and maintained as monolayers of cells using appropriate medium (McCoy's 5A medium supplemented with 10% FBS, RPMI medium 1640 with 5% FBS, or Dulbecco's modified Eagle's medium with 10% FBS) with 10 μg/ml gentamicin, in a humidified 5% CO_2 atmosphere at 37°. All cells were harvested for experiments in the subconfluent to confluent state.

Western blot analysis. Total cellular proteins of each cell line were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules CA) (18). Polyclonal ERCC2 antibody, MER-2, was raised against the whole recombinant ERCC2 protein in mouse (a kind gift from Dr. Larry Thompson, Lawrence Livermore National Laboratory, Livermore, CA) and MGMT antibody, MT 3.1, is a mouse monoclonal antibody. The blot was first hybridized with the primary antibody (ERCC2 at 1:5000 or MGMT at 1:500) overnight at 4°, followed by incubation with secondary antibody (sheep anti-mouse Ig at 1:1000 for ERCC2 or at 1:500 for MGMT) (Amersham Life Science, Buckinghamshire, UK) for 1 hr at 4°. The specific antibody binding was visualized with ECL reagents (Amersham) followed by subsequent exposure of the blot to film (Eastman Kodak Company, Rochester, NY). Intensities of bands on the membranes were quantified by densitometry using Ultroscan XL. LKB 2222-020 Laser Scanner (Pharmacia LKB Biotechnology, Uppsala, Sweden). Protein loading was standardized with α-tubulin. The α-tubulin expression was detected with mouse monoclonal IgG primary antibody at 1:1000 (Amersham) and secondary antibody at 1:1000. Normalized gene expression was obtained by the densitometry reading of each gene divided by the densitometry reading of α-tubulin, for each cell line. Each sample was run at least twice using two different cell extracts. The MGMT protein levels were measured in two independent laboratories with different cell extracts. The Western blot analysis performed in the laboratory of Dr. Brent is as described previously (19) except that MGMT antibody, MT 5.1, and β-tubulin were used (20). The protein levels represent the mean values of at least three individual experiments from two separate cell extracts.

MGMT activity assay. MGMT activity was determined as described previously using a DNA substrate treated with [^{3}H]methylnitrosourea (22 Ci/mmol; Amersham) (21). The activity was measured in five samples of cell extract with increasing protein content, and the mean activity for each cell line was calculated by linear regression analysis of a plot for transferase activity versus protein level.

UV light sensitivity. UV light sensitivity was performed by a modification of the SRB (Sigma, St. Louis, MO) colorimetric anticancer drug-screening assay (17, 22). Briefly, an appropriate amount of cells was placed in a tissue culture dish with surface area of 78.5 cm^2, and exposed immediately to UV light (254 nm) from a 60-Hz, 0.16-A UV lamp at a fixed distance of 19 cm, for different periods of time and subsequently plated on flat bottom 24-well plates with a surface area of 2 cm^2 (day 1). Controls were not exposed to UV light. The cultures were fed with 1.5 ml of appropriate medium on day 5 and incubated for 3 more days. On day 8 cultures were fixed to the plastic substratum by gently adding 1.0 ml of a 10% trichloroacetic acid in 0.9% NaCl solution. The cultures were incubated for 1 hr at 4° and then washed five times with water to remove the trichloroacetic acid. Plates were air-dried and then stained with a 0.4% SRB dissolved in 1.0% acetic acid, for 30 min. SRB was then removed, and the cultures were quickly rinsed five times with 1.0% acetic acid to remove unbound dye. The cultures were air-dried, and bound dye was solubilized with 2 ml of 10 mM unbuffered Tris base (pH 10.5). Absorbance was read using a spectrophotometer at 500–550 nm. The LD_{90} (J/m^2) values were obtained by exponential curve fit of the linear portion of the cytotoxicity curve utilizing CA-Cricket Graph III version 1.01 (Computer Associates International, Islandia, NY). The LD_{90} values represent the mean of at least three separate experiments.

BCNU cytotoxicity. The cytotoxicity of BCNU was performed in a similar fashion (17, 22), with the exception that cells were plated on day 1 and BCNU was added to the culture on day 2 and was not removed. BCNU cytotoxicity is expressed in micromolar as IC_{90} values, which were obtained as described above.

Statistical analysis. The concordances among gene expression, BCNU cytotoxicity, and UV sensitivity were analyzed utilizing linear regression. With concern to MGMT expression, the cell lines were divided into two groups, Mer^+ and Mer^-). The CENU cytotoxicity and UV sensitivity for the Mer^+ and Mer^- groups were analyzed using the Student t test.

Results

UV light sensitivity and BCNU cytotoxicity. UV light-damaged DNA is repaired by NER and thus, UV sensitivity is a major determinant of NER activity. To determine whether NER is involved in CENU resistance, UV light sensitivity and BCNU cytotoxicity were examined in the 14 cell lines. In the 14 cell lines, there is a 6-fold range in UV light sensitivity and 17-fold range in BCNU cytotoxicity (Table 1). Fig. 1 shows representative survival curves of the cell lines to UV or BCNU. There was a significant correlation between UV sensitivity and BCNU cytotoxicity in these cell lines ($r = 0.858$, $p = 0.0001$) (Table 2).

MGMT expression. MGMT is considered to be one of the major mechanisms of CENU resistance. The MGMT protein levels for each cell line (normalized to the highest expression cell line) was determined by two separate laboratories. There was a very good correlation of protein levels between the two separate laboratories ($r = 0.8882$, $p = 0.0001$). The MGMT protein level was also significantly correlated to MGMT activity ($r = 0.9497$, $p = 0.0001$). The mean values of the normalized MGMT protein levels and activity for each of the 14 cell lines are listed in Table 1. Using linear regression analysis, MGMT expression as determined by protein or activity failed to correlate with BCNU cytotoxicity (Table 2, Fig. 2). However, Mer^+ cell lines (MGMT activity >0.1 pmol/mg of protein) were more resistant than Mer^- cell lines (MGMT activity less than 0.1 pmol/mg of protein) to BCNU (Mer^+, 79.71 ± 16.85; Mer^-, 27.64 ± 11.70; $t = 2.2375$, $p = 0.0225$) but not to UV (Mer^+, 16.06 ± 3.53; Mer^-, 10.70 ±

TABLE 1

Gene expression, CENU cytotoxicity, and UV sensitivity in 14 human tumor cell lines

ERCC2 protein level for each cell line was divided by ERCC2 protein level for SKNSH, for each experiment. The result is expressed as the mean ± standard error of at least three separate experiments from two separate total cellular protein extractions for each cell line. MGMT protein levels for each cell line was divided by the MGMT protein level for MCF-7 for each experiment. The result is expressed as the mean ± standard error of at least three separate experiments from two separate total cellular protein extracts for each cell line. Cytotoxicity to BCNU is expressed as the mean ± standard error of at least three separate experiments. UV sensitivity is expressed as the mean ± standard error of at least three separate experiments.

Cell line	ERCC2 protein	MGMT		BCNU IC_{90}	UV LD_{90}
		Protein levels	Activity		
			pmol/mg of protein		*J/m²*
MCF-7 (B)	0.06 ± 0.004	1	3.240	69 ± 8	12.4 ± 1.0
T98-G (G)	0.065 ± 0.012	0.306 ± 0.127	0.645	46 ± 8	9.6 ± 0.86
SKI-1 (G)	0.050 ± 0.026	0.012 ± 0.012	0.024	15 ± 1	8.9 ± 1.4
HT29 (C)	0.002 ± 0.002	0.613 ± 0.089	1.140	55 ± 3	8.9 ± 0.1
SF-295 (G)	0.081 ± 0.021	0.037 ± 0.037	0.065	23 ± 0	8.8 ± 0.7
MGR-3 (G)	0.022 ± 0.022	0.022 ± 0.022	0.038	86 ± 2	16.4 ± 1.2
SK-MG-4 (G)	0.065 ± 0.006	0.052 ± 0.025	0.070	11 ± 1	13.1 ± 1.4
CAKI-1 (R)	0.093 ± 0.026	0.393 ± 0.139	0.883	63 ± 4	7.7 ± 0.7
786-0 (R)	0.062 ± 0.035	0.023 ± 0.019	0.021	17 ± 1	10.3 ± 1.7
SK-MG-1 (G)	0.006 ± 0.004	0	0	14 ± 1	6.6 ± 0.3
SKNSH (N)	1	0.167 ± 0.094	0.435	173 ± 10	38.2 ± 1.9
UW-28 (G)	0.018 ± 0.018	0.134 ± 0.134	0.359	35 ± 6	17.6 ± 1.7
ACHN (R)	0.182 ± 0.042	0.681 ± 0.011	1.200	50 ± 5	9.2 ± 0.7
A498 (R)	0.316 ± 0.016	0.130 ± 0.083	0.155	147 ± 5	25.1 ± 2.3

Types of tumor cell line: B, breast; C, colon; G, glioma; N, neuroblastoma; R, renal.

1.45; $t = 1.1734$, $p = 0.1317$) (Fig. 2). Furthermore, the BCNU-sensitive cell lines were all MGMT-poor.

***ERCC2* expression.** To ensure that *ERCC2* expression as determined by Western blot was independent of protein loading, different amounts of protein, ranging from 10 to 80 μg, were loaded and analyzed. The results showed that increasing protein content from the same extract yielded similar ERCC2 protein levels (normalized to α-tubulin). The ERCC2 protein levels determined from two separate cellular extracts were similar ($r = 0.9899$, $p = 0.0001$). Fig. 3 is a representative Western blot indicating *ERCC2* expression for each of the 14 cell lines. The ERCC2 protein levels for each cell line were normalized by the highest expression cell line for each individual experiment. The mean value of the normalized ERCC2 protein levels for each of the 14 cell lines is listed in Table 1. There is a significant correlation between ERCC2 protein levels and UV sensitivity, and ERCC2 protein levels and BCNU cytotoxicity (Table 2, Fig. 2). The highly BCNU-resistant cell lines were *ERCC2*-rich.

Discussion

A network of DNA repair mechanisms operates in cells to safeguard their genetic material (10). MGMT can repair O^6-alkylguanine adducts, such as the chloroethyl adduct formed by CENUs, preventing the formation of cytotoxic interstrand cross-links (4). This suggests that MGMT-positive tumor cells should be more resistant to CENU damage than MGMT-negative cells. In our investigation, cell lines with high MGMT expression as measured by protein and activity levels were more resistant to BCNU than MGMT-poor cell lines. In addition, BCNU-sensitive cell lines were all MGMT-poor, indicating that MGMT plays a major role in BCNU drug resistance. The role of MGMT *vis-à-vis* clinical BCNU drug resistance is under investigation. O^6-Benzylguanine inhibition of MGMT sensitizes tumor cells, and clinical trials are underway to determine if O^6-benzylguanine can increase the efficacy of BCNU treatment (23).

DNA damage induced by UV light is classically repaired by NER. We examined the role of NER in the 14 cell lines by assessing their UV light sensitivity. In this study, we have demonstrated a significant correlation between BCNU cytotoxicity and UV sensitivity, suggesting that DNA damage induced by both UV and CENU could be at least in part repaired by NER. Thus it is possible that NER is implicated in BCNU resistance. Possible mechanisms by which NER could repair BCNU damage include: (a) repair of the initial adduct (24), (b) repair of the DNA-adduct-MGMT complex (4), and/or (c) most likely, NER involvement in the repair of interstrand cross-links.

NER along with recombination repair is necessary for the removal of DNA cross-links in bacteria and *Saccharomyces cerevisiae* (25). *ERCC1* and *ERCC4* complement CHO cell NER mutants (groups 1 and 4, respectively), which are hypersensitive to bifunctional alkylating agents (26, 27). The ERCC1/ERCC4 complex probably functions as an endonuclease that is implicated in the initial steps of recombination repair (25). Thus, we examined ERCC1 protein levels in the 14 cell lines and did not find any correlation with BCNU resistance (data not shown). Recently, *HsRad51*, a human homolog of *RecA* and *ScRad51*, which are involved in recombination repair in bacteria and *S. cerevisiae*, respectively, has been cloned (25). We did not find a correlation between HsRad51 protein levels and BCNU resistance (data not shown).

NER, specifically *ERCC2*, may be implicated in the mechanism of resistance to CENUs, as functional *ERCC2* is required for the preferential removal of *N*-ethylpurines induced by ethylnitrosourea, in the actively transcribed DNA (28). Transfection of an MGMT expression vector into a CHO UV41 (group 4) mutant cell line having a defective function of an important NER gene (*ERCC4*), increases CENU resis-

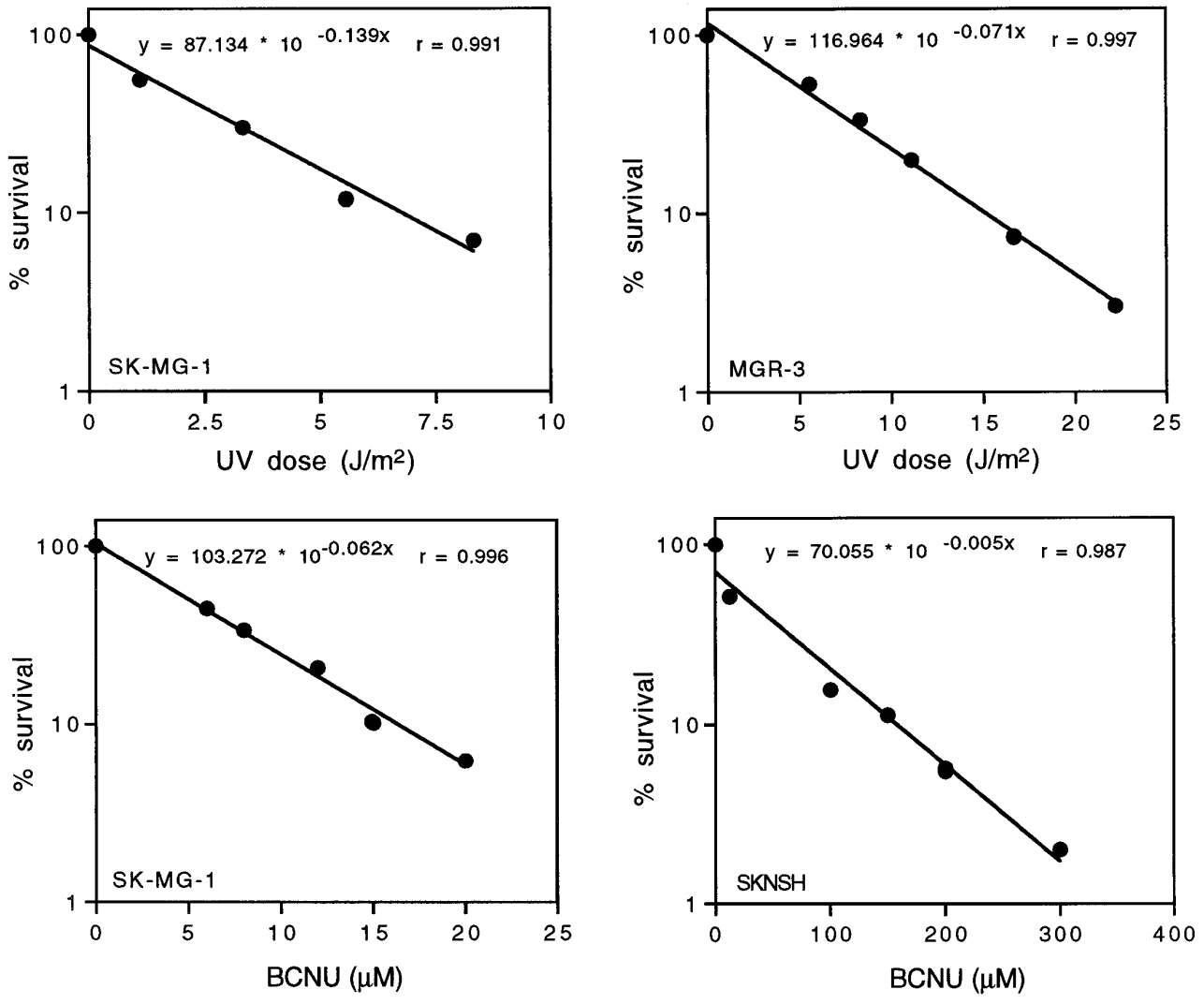

Fig. 1. Representative survival curves of cell lines after exposure to UV or BCNU. The cytotoxicity of BCNU or UV was determined by the SRB assay. The UV sensitivity (LD_{90}) is the UV dose (J/m^2) resulting in 10% cell survival, and the BCNU cytotoxicity (IC_{90}) is the BCNU dose (μM) resulting in 10% cell survival.

TABLE 2

Correlations between UV sensitivity, BCNU cytotoxicity and MGMT or ERCC2 expression in 14 human tumor cell lines

	Regression formula	*r*	*p*
UV-BCNU	$y = 0.15x + 5.2$[a]	0.858	0.0001
UV-ERCC2 (protein level)	$y = 28.7x + 9.7$[a]	0.874	0.0001
UV-MGMT (protein level)	$y = -4.3x + 14.9$[a]	0.153	0.6017
UV-MGMT (activity)	$y = -1.0x + 14.4$[a]	0.103	0.7255
BCNU-ERCC2 (protein level)	$y = 148.7x + 36.5$[b]	0.786	0.0009
BCNU-MGMT (protein level)	$y = 21.8x + 51.9$[b]	0.139	0.6348
BCNU-MGMT (activity)	$y = 7.4x + 53.1$[b]	0.131	0.6515

[a] *y* represents UV sensitivity and *x* represents either BCNU cytotoxicity, ERCC2 protein level, MGMT protein level, or MGMT activity.

[b] *y* represents BCNU cytotoxicity and *x* represents either ERCC2 protein level, MGMT protein level, or MGMT activity.

tance to a similar extent as MGMT-transfected wild-type CHO cells. However, the difference in CENU sensitivity between the two cell lines that is attributable to the NER defect persists in the mutant cell line (29). Furthermore, both MGMT activity and NER are necessary for proficient repair of O^6-ethylguanine lesions resulting from ethylnitrosourea treatment of cell lines (30). Cisplatin is a bifunctional cross-linking anti-cancer agent with activity against a variety of human tumors. The cytotoxicity of cisplatin is believed to be due to the formation of DNA cross-link adducts, and repair of these adducts can occur by NER (12). Expression of genes implicated in NER is increased in clinically resistant ovarian tumors (31). Thus NER may be an important determinant of cisplatin resistance. Our previous investigation indicated a correlation between ERCC2 mRNA levels and CENU resistance in six human tumor cell lines (17). In the present study, two highly BCNU-resistant cell lines, SKNSH and A498, were associated with high ERCC2 protein content and low MGMT expression. This suggests that NER is a modifying factor of MGMT-mediated CENU resistance in these cell lines. However, the importance of *ERCC2* in this process is unclear, because the correlation between UV sensitivity and BCNU cytotoxicity was more significant than that between ERCC2 protein levels and BCNU cytotoxicity.

TFIIH is a multisubunit complex required for transcription

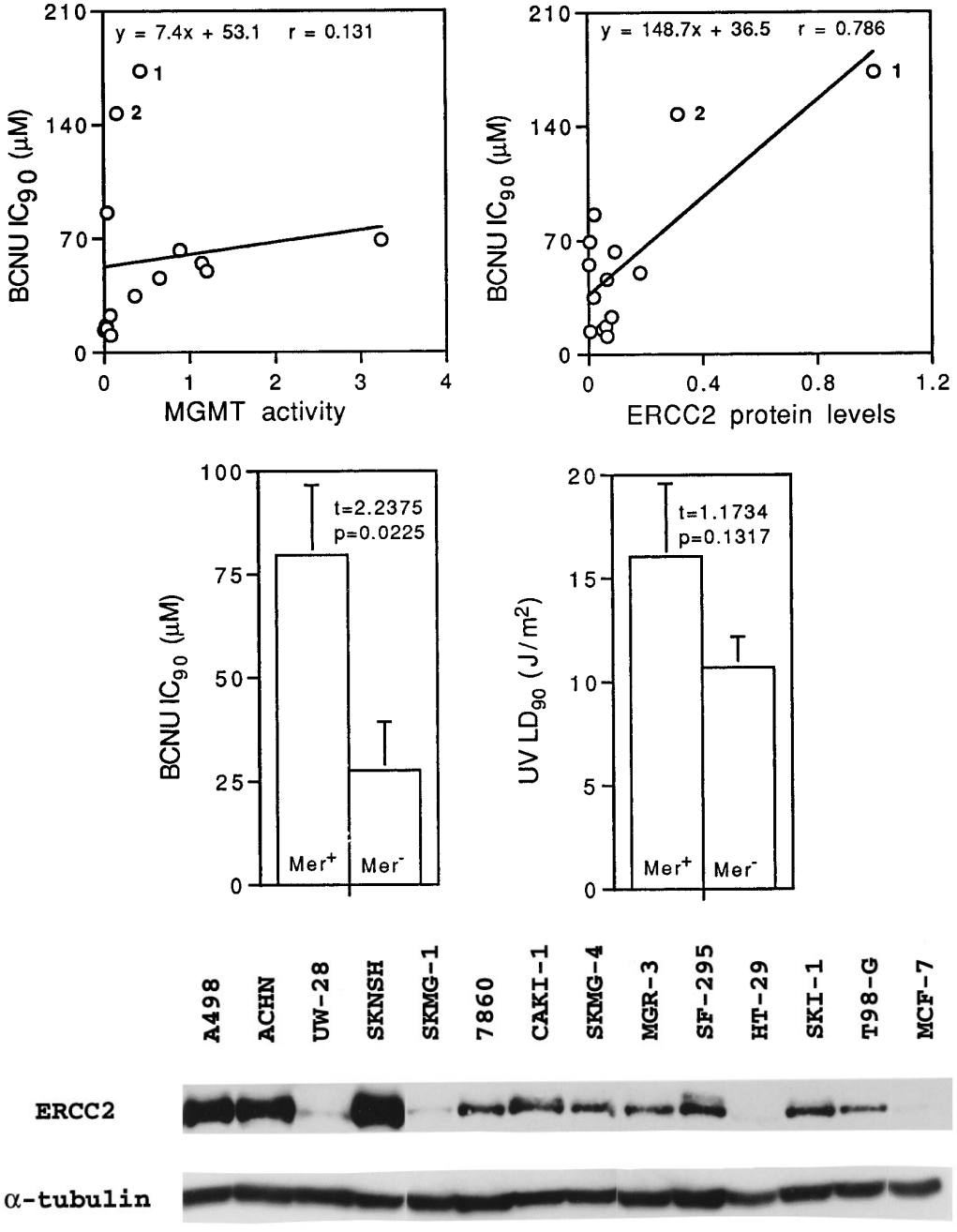

Fig. 2. Linear regression analysis shows no significant correlation between BCNU cytotoxicity and MGMT activity, but a significant correlation between *ERCC2* protein levels and BCNU cytotoxicity. ○ 1, SKNSH cell lines; ○ 2; A498 cell lines. These highly BCNU-resistant cell lines express a low level of MGMT but a high level of *ERCC2*. However, there was a significant difference between Mer^+ and Mer^- cell lines with respect to BCNU resistance, but not to UV light sensitivity.

Fig. 3. A representative Western blot of *ERCC2* and α-tubulin expression for the 14 cell lines.

and for DNA NER (32). TFIIH possesses three enzymatic activities: (a) an ATP-dependent DNA helicase, (b) a DNA-dependent ATPase, and (c) a kinase with specificity for the carboxyl-terminal domain of RNA polymerase II, identified as CAK. Recently, Drapkin *et al.* (33) have isolated three distinct CAK-containing complexes from Hela nuclear extracts, one of which is associated with ERCC2. They found that CAK-ERCC2 can associate loosely with core-TFIIH to reconstitute holo-TFIIH transcription activity, suggesting that ERCC2 may serve as a molecular bridge. These observations suggest that *ERCC2* plays an important but yet undefined role in NER.

Based on our present observation, we conclude that NER may be an important modifying factor of MGMT-mediated CENU resistance in human tumor cells. ERCC2 may be implicated in this mechanism. Future experiments in which ERCC2 protein levels are down-regulated in *ERCC2*-rich cell lines as well as overexpression of *ERCC2* in *ERCC2*-poor cell lines should be useful in further defining the role of *ERCC2* and NER in BCNU resistance.

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