Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines

Rank	Gene	Genbank ID	*r*	*P*
A. MMC				
Sensitive				
1	*SF1*	D26121	0.566	0.001
2	*CBR3*	Ab004854	0.486	0.006
3	*EMS1*	M98343	0.480	0.010
4	*JUN*	J04111	0.473	0.015
5	*SFRS9*	U30825	0.448	0.010
6	*NMBR*	M73482	0.428	0.012
7	*RBMX*	Z23064	0.419	0.012
8	*SOD1*	M13267	0.418	0.024
9	*NOL1*	X55504	0.415	0.025
10	*PELP1*	U88153	0.405	0.019
11	*ARHA*	L25080	0.404	0.030
12	*AARS*	D32050	0.398	0.018
13	*NME1*	X17620	0.398	0.032
14	*HNRPA2B1*	M29065	0.390	0.044
15	*NME2*	L16785	0.378	0.025
16	*VAT1*	U18009	0.376	0.031
17	*SERPINB10*	U35459	0.372	0.028
18	*KIAA0436*	AB007896	0.353	0.041
19	*DRPLA*	D31840	0.350	0.049
20	*MC3R*	L06155	0.346	0.049
Resistant				
1	*SPTBN1*	M96803	−0.450	0.013
2	*PET112L*	AF026851	−0.425	0.027
3	*CAPN1*	X04366	−0.421	0.032
4	*MEL*	X56741	−0.414	0.028
5	*PACE*	X17094	−0.380	0.035
6	*DVL2*	AF006012	−0.370	0.034
7	*LOC54543*	AJ011007	−0.366	0.022
8	*PAPOLA*	X76770	−0.351	0.033
9	*RPLP2*	M17887	−0.345	0.049
10	*ARF4L*	L38490	−0.340	0.042
B. Vinorelbine				
Sensitive				
1	*ARHA*	L25080	0.534	0.003
2	*NME2*	L16785	0.521	0.001
3	*VIL2*	X51521	0.463	0.015
4	*YWHAQ*	X56468	0.450	0.011
5	*HK1*	M75126	0.449	0.016
6	*SATB1*	M97287	0.439	0.006
7	*CAMLG*	U18242	0.439	0.007
8	*CARS*	L06845	0.433	0.007
9	*CCNB1*	M25753	0.427	0.013
10	*U2AF1*	M96982	0.424	0.022
11	*PTMA*	M26708	0.423	0.018
12	*MLC1SA*	M31211	0.397	0.022
13	*NME1*	X17620	0.393	0.035
14	*SARS*	X91257	0.386	0.032
15	*CDC20*	U05340	0.385	0.029
16	*PPP4C*	X70218	0.385	0.039
17	*TNFAIP3*	M59465	0.384	0.023
18	*EEF1D*	Z21507	0.384	0.023
19	*PFKP*	D25328	0.365	0.028
20	*ENTPD2*	U91510	0.365	0.037
21	*CCL5*	M21121	0.358	0.035
22	*ACAT1*	D90228	0.352	0.048
23	*IQGAP1*	L33075	0.351	0.042
24	*PAX5*	M96944	0.342	0.038
25	*NRGN*	Y09689	0.336	0.042
26	*K-α-1*	K00558	0.328	0.048
27	*NDUFB7*	M33374	0.321	0.049
Resistant				
1	*HOXB1*	X16666	−0.600	0.000
2	*F10*	K03194	−0.514	0.002
3	*GPX2*	X53463	−0.509	0.002
4	*NR1I2*	AF061056	−0.498	0.002
5	*ANXA4*	M19383	−0.481	0.005
6	*PDLIM1*	U90878	−0.465	0.006
7	*LIPC*	X07228	−0.464	0.004
8	*SERPINF2*	D00174	−0.447	0.004
9	*HSD17B1*	M36263	−0.443	0.014
10	*MAN2B1*	U60266	−0.440	0.008
11	*LSS*	D63807	−0.430	0.014
12	*PIK3CG*	X83368	−0.415	0.010
13	*DBN1*	U00802	−0.414	0.017
14	*NDUFA4*	U94586	−0.410	0.038
15	*BDH*	M93107	−0.399	0.024
16	*BCL2L1*	Z23115	−0.385	0.039
17	*EEF1B2*	X60656	−0.383	0.030
18	*F2*	V00595	−0.382	0.026
19	*RARA*	X06614	−0.369	0.029
20	*ITGB4*	X53587	−0.367	0.042
21	*IMPA1*	X66922	−0.367	0.042
22	*PACE*	X17094	−0.367	0.042
23	*AGA*	M64073	−0.361	0.042
24	*MVD*	U49260	−0.353	0.038
25	*EHHADH*	L07077	−0.346	0.039
26	*TFPI2*	D29992	−0.343	0.035
27	*MARCKS*	M68956	−0.342	0.045
28	*FGB*	J00129	−0.334	0.035
29	*GPD1*	L34041	−0.322	0.049
C. Paclitaxel				
Sensitive				
1	*ADH6*	M68895	0.513	0.002
2	*RAB28*	X94703	0.480	0.007
3	*U2AF1*	M96982	0.441	0.017
4	*GPC1*	X54232	0.440	0.013
5	*HK1*	M75126	0.439	0.020
6	*CARS*	L06845	0.436	0.006
7	*TNFAIP3*	M59465	0.433	0.009
8	*K-α-1*	K00558	0.418	0.010
9	*PFKP*	D25328	0.416	0.012
10	*GDI2*	D13988	0.411	0.033
11	*VIL2*	X51521	0.410	0.034
12	*RUNX2*	AF001450	0.409	0.038
13	*NME2*	L16785	0.407	0.015
14	*CDC20*	U05340	0.395	0.025
15	*GNAI2*	X04828	0.391	0.033

NOTE: Column 2 shows the name of the gene according to HUGO database. Column 4 shows Pearson correlation coefficient between chemosensitivity to drugs and gene expression. "Sensitive" indicates candidate genes sensitive to each drug. "Resistant" indicates genes resistant to each drug.

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Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines (Cont'd)

Rank	Gene	Genbank ID	*r*	*P*
16	*ARHA*	L25080	0.381	0.041
17	*CNR2*	X74328	0.378	0.030
18	*PPP2R2B*	M64930	0.376	0.026
19	*SLC6A8*	L31409	0.374	0.046
20	*DDX9*	L13848	0.374	0.042
21	*ACAT1*	D90228	0.369	0.038
22	*PI3*	Z18538	0.329	0.047
Resistant				
1	*NAP1L1*	M86667	−0.530	0.004
2	*HOXB1*	X16666	−0.516	0.004
3	*PACE*	X17094	−0.507	0.004
4	*MAN2B1*	U60266	−0.486	0.003
5	*GPX2*	X53463	−0.480	0.004
6	*DBN1*	U00802	−0.469	0.006
7	*ANXA4*	M19383	−0.468	0.007
8	*SERPINF2*	D00174	−0.463	0.003
9	*AGA*	M64073	−0.444	0.011
10	*BCL2L1*	Z23115	−0.428	0.021
11	*LIPC*	X07228	−0.401	0.015
12	*BDH*	M93107	−0.393	0.026
13	*LSS*	D63807	−0.384	0.030
14	*PDLIM1*	U90878	−0.372	0.033
15	*ZNF161*	D28118	−0.368	0.038
16	*UBE2E1*	X92963	−0.363	0.032
17	*TLE1*	M99435	−0.360	0.039
18	*RARA*	X06614	−0.359	0.034
19	*PTPRN*	L18983	−0.357	0.035
20	*APOE*	M12529	−0.353	0.048
21	*F10*	K03194	−0.348	0.040
22	*NR1I2*	AF061056	−0.342	0.041
23	*UBE2L3*	X92962	−0.332	0.045
24	*FGB*	J00129	−0.313	0.049
D. SN-38				
Sensitive				
1	*EMS1*	M98343	0.573	0.001
2	*JUN*	J04111	0.564	0.003
3	*IL-6*	X04602	0.514	0.003
4	*RPL23*	X52839	0.495	0.004
5	*CDKN3*	L25876	0.455	0.017
6	*RPL3*	X73460	0.445	0.011
7	*TFPI*	J03225	0.442	0.009
8	*MRPL3*	X06323	0.437	0.009
9	*HLA-C*	M11886	0.424	0.014
10	*AARS*	D32050	0.419	0.012
11	*ARHGDIA*	X69550	0.416	0.031
12	*NOL1*	X55504	0.406	0.029
13	*SF1*	D26121	0.394	0.031
14	*SOD1*	M13267	0.389	0.037
15	*VEGF*	M32977	0.384	0.043
16	*EIF2S1*	J02645	0.382	0.034
17	*CDH5*	X79981	0.372	0.030
18	*FOSL1*	X16707	0.371	0.047
19	*IDS*	M58342	0.366	0.047
20	*PMVK*	L77213	0.364	0.044
21	*PPP2CB*	X12656	0.364	0.041
22	*NMBR*	M73482	0.362	0.035
23	*RPL26*	X69392	0.358	0.035
24	*PELP1*	U88153	0.356	0.042
25	*MC3R*	L06155	0.356	0.042
26	*RPS8*	X67247	0.355	0.036
Resistant				
1	*CAPN1*	X04366	−0.496	0.010
2	*MEL*	X56741	−0.478	0.010
3	*PACE*	X17094	−0.443	0.012
4	*TIMP2*	J05593	−0.433	0.019
5	*AOP2*	D14662	−0.422	0.025
6	*ZNF174*	U31248	−0.402	0.018
7	*ID3*	X69111	−0.393	0.038
8	*KLF5*	D14520	−0.384	0.036
9	*CALD1*	M64110	−0.382	0.031
10	*LOC54543*	AJ011007	−0.368	0.021
11	*PTPN3*	M64572	−0.363	0.038
12	*ACTB*	X00351	−0.362	0.025
13	*LY6E*	U42376	−0.360	0.037
14	*ID1*	D13889	−0.343	0.044

(Continued)

Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines (Cont'd)

was >90% as evaluated by transfection of a plasmid expressing the enhanced green fluorescent protein (data not shown). To validate this screening system, we examined the effect of *NQO1* gene, coding DT-diaphorase that increases cellular sensitivity to MMC (12). As shown in Fig. 3B, cells transfected with NQO1 significantly enhanced growth inhibition by MMC compared with the mock-transfected and LacZ-transfected cells. We confirmed the cellular expression of the *NQO1* gene product by immunoblot (Fig. 3C). Thus, this screening system can be used to detect changes in chemosensitivity in HT1080 cells. Using this screening system, we examined whether the 19 genes, which were extracted in Tables 2 and 3, altered sensitivity to drug. Notably, the *HSPA1A* gene coding 70-kDa heat shock protein, whose expression was correlated with MMC sensitivity in the breast and liver cancer cell lines, significantly enhanced the MMC sensitivity in HSPA1A-transfected HT1080 cells (Fig. 3B). Similarly, the *JUN* gene encoding c-JUN, whose expression was correlated with MMC sensitivity, also enhanced the MMC sensitivity in JUN-transfected HT1080 cells (Fig. 3B). The expression of *myc*-tagged LacZ, 70-kDa heat shock protein, and JUN in the transfected cells was confirmed by immunoblotting with anti-*myc* antibody (Fig. 3C). Transfection with 17 other genes did not alter the MMC sensitivity. For example, transfection with the *IL-18* gene did not affect MMC sensitivity (Fig. 3B).

Discussion

The assessment system for determining pharmacologic properties of chemicals by a panel of cancer cell lines was first developed in the National Cancer Institute (33–35). We established a similar assessment system (JFCR-39;

Table 3. Genes related to MMC sensitivity in breast, liver, and stomach cancer cell lines

Rank	Gene	Genbank ID	*r*	*P*
A. Breast cancer				
Sensitive				
1	*INHBB*	M31682	0.972	0.000
2	*NK4*	M59807	0.838	0.018
3	*HSPA1A*	M11717	0.751	0.050
4	*LOC54557*	AF075050	0.735	0.024
5	*CD47*	Y00815	0.717	0.045
Resistant				
1	*RPN2*	Y00282	−0.882	0.009
2	*ATP5O*	X83218	−0.842	0.017
3	*CAST*	D50827	−0.815	0.025
4	*HPCA*	D16593	−0.776	0.024
5	*ZNF9*	M28372	−0.774	0.024
6	*A2LP*	U70671	−0.772	0.042
7	*IL-18*	D49950	−0.747	0.033
8	*NRGN*	Y09689	−0.727	0.041
B. Liver cancer				
Sensitive				
1	*EB1*	U24166	0.872	0.002
2	*JUN*	J04111	0.813	0.008
3	*EIF3S8*	U46025	0.772	0.015
4	*CTSD*	M11233	0.753	0.012
5	*SCYA5*	M21121	0.741	0.022
6	*PHB*	S85655	0.739	0.023
7	*HSPA1A*	M11717	0.729	0.026
8	*SPP1*	X13694	0.723	0.018
9	*TAB7*	X93499	0.712	0.021
10	*ACTN1*	X15804	0.692	0.039
11	*RXRB*	M84820	0.678	0.045
12	*PSME2*	D45248	0.673	0.047
13	*HLA-C*	M11886	0.647	0.043
14	*RPL19*	X63527	0.643	0.033
Resistant				
1	*MAPK6*	X80692	−0.862	0.003
2	*GCSH*	M69175	−0.793	0.006
3	*G22P1*	M32865	−0.727	0.017
4	*USP11*	U44839	−0.725	0.027
5	*ACTB*	X00351	−0.715	0.020
6	*YWHAZ*	M86400	−0.706	0.022
7	*IL-10*	M57627	−0.694	0.018
8	*RFC4*	M87339	−0.677	0.016
9	*CRLF1*	AF059293	−0.644	0.033
10	*RPS6*	M20020	−0.619	0.042
11	*EMX1*	X68879	−0.618	0.043
12	*TK2*	U77088	−0.607	0.047
C. Stomach cancer				
Sensitive				
1	*TEAD4*	U63824	0.803	0.001
2	*NR2C2*	U10990	0.713	0.001
3	*CSF1*	M37435	0.711	0.004
4	*RAB28*	X94703	0.695	0.008
5	*CBR3*	Ab004854	0.683	0.007
6	*NFYC*	Z74792	0.639	0.019
7	*PGF*	X54936	0.627	0.022
8	*ERG*	M21535	0.620	0.005
9	*MLLT1*	L04285	0.613	0.015
10	*FOS*	K00650	0.599	0.014
11	*TNFAIP3*	M59465	0.584	0.011
12	*CNR2*	X74328	0.581	0.009
13	*DRPLA*	D31840	0.577	0.024
14	*PSMB5*	D29011	0.572	0.026
15	*SLC6A8*	L31409	0.570	0.017
16	*SERPINB10*	U35459	0.570	0.013
17	*VAT1*	U18009	0.570	0.009
18	*TJP1*	L14837	0.562	0.029
19	*PELP1*	U88153	0.545	0.035
20	*C1QBP*	L04636	0.545	0.024
21	*CDK10*	L33264	0.543	0.045
22	*SERPINA6*	J02943	0.542	0.025
23	*ACTB*	X00351	0.538	0.021
24	*SFRP4*	AF026692	0.538	0.018
25	*EMX1*	X68879	0.535	0.018
26	*ACTB*	X00351	0.529	0.024
27	*RPS9*	U14971	0.528	0.043
28	*AMD1*	M21154	0.522	0.038
29	*RPL26*	X69392	0.522	0.038
30	*HNRPF*	L28010	0.520	0.047
31	*PTMS*	M24398	0.502	0.040
32	*STK12*	AF008552	0.498	0.050
33	*NR2F6*	X12794	0.491	0.046
34	*GBE1*	L07956	0.470	0.049
Resistant				
1	*PSMD8*	D38047	−0.747	0.002
2	*LAMP2*	J04183	−0.677	0.002
3	*CTSD*	M11233	−0.651	0.006
4	*ADORA2B*	M97759	−0.645	0.005
5	*ANXA4*	M19383	−0.639	0.008
6	*PTPRK*	Z70660	−0.638	0.003
7	*RAD23A*	D21235	−0.622	0.010
8	*SDHA*	D30648	−0.613	0.015
9	*PET112L*	AF026851	−0.598	0.024
10	*DAD1*	D15057	−0.593	0.025
11	*HSPB1*	X54079	−0.588	0.013
12	*PSMA6*	X61972	−0.586	0.036
13	*KDELR1*	X55885	−0.584	0.028
14	*B2M*	AB021288	−0.581	0.023
15	*M6PR*	M16985	−0.579	0.038
16	*GCLC*	M90656	−0.576	0.015
17	*SPTBN1*	M96803	−0.557	0.038
18	*PACE*	X17094	−0.547	0.019
19	*RPL24*	M94314	−0.539	0.017
20	*SPINT2*	U78095	−0.538	0.039
21	*STX4A*	U07158	−0.534	0.027
22	*SIAT8B*	U33551	−0.532	0.028
23	*CTSK*	U13665	−0.529	0.029
24	*DCI*	L24774	−0.525	0.044
25	*MEL*	X56741	−0.525	0.045
26	*PITPNB*	D30037	−0.523	0.038
27	*YY1*	M76541	−0.512	0.043
28	*RAB1*	M28209	−0.495	0.037
29	*UBE2L6*	AF031141	−0.492	0.045
30	*PSMB7*	D38048	−0.484	0.049

NOTE: Column 2 shows the name of the gene according to HUGO database. Column 4 shows Pearson correlation coefficient between chemosensitivity to drugs and gene expression. "Sensitive" indicates candidate genes sensitive to each drug. "Resistant" indicates genes resistant to each drug.

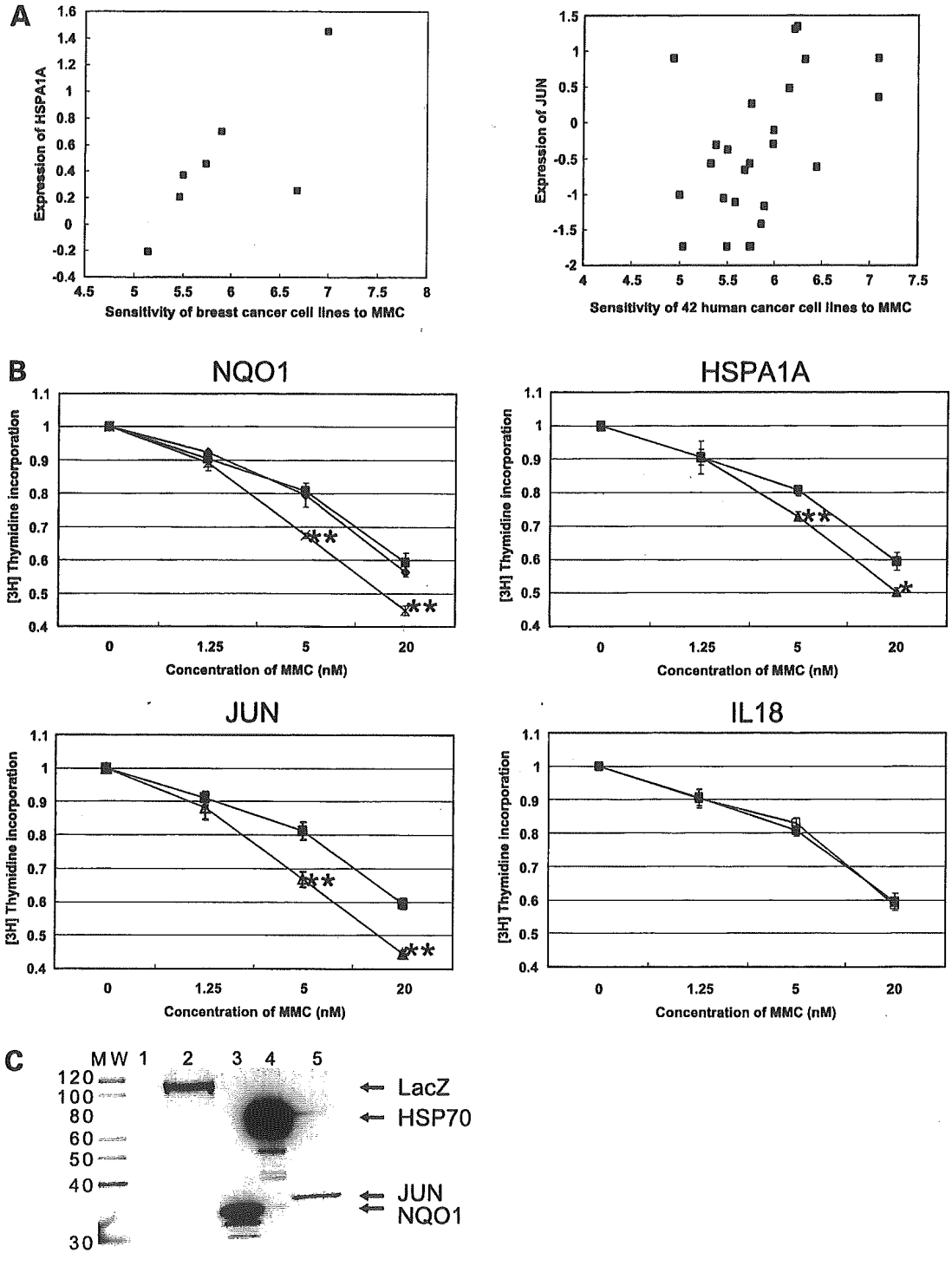

Figure 3. Relationships between MMC sensitivity and expression of HSPA1A in breast cancer cell lines (**A**, *left*) or JUN in 42 cell lines (**A**, *right*). Each symbol indicates one cell line. *X axis,* MMC sensitivity; *Y axis,* expression of HSPA1A or JUN. Pearson correlation coefficients between MMC sensitivity and expression of HSPA1A and JUN were 0.75 (P = 0.05) and 0.473 (P = 0.015), respectively. **B,** growth inhibition curves by MMC in mock (■), LacZ (♦), NQO1 (×), HSPA1A (▲), JUN (△), or IL-18 (□) transfected HT1080 cells. This growth inhibition by MMC was enhanced in HT1080 cells transfected with NQO1, HSPA1A, and JUN. *, $P < 0.002$; **, $P < 0.0001$, t test against mock-transfected cells. **C,** expressions of genes were certified by immunoblotting with anti-*myc* antibody: *myc*-tagged LacZ (*lane 2*), NQO1 (*lane 3*), 70-kDa heat shock protein (*HSP70*; *lane 4*), and JUN (*lane 5*).

ref. 32) and showed that drugs with similar modes of actions were classified into the same cluster by hierarchical clustering (19). In this study, we constructed a new panel of 45 human cancer cell lines (JFCR-45), comprising cancer cell lines derived from tumors from three different organ types: breast, liver, and stomach. In particular, the inclusion of cell lines derived from gastric and hepatic cancers is a major point of novelty. JFCR-45 can be used for analyzing both organ-specific differences in chemosensitivity and intraorgan heterogeneity of chemosensitivity. We examined 53 anticancer drugs for their activity against JFCR-45 and observed differential activity across the whole panel as well as within a single organ type (e.g., breast, liver, or stomach). Furthermore, as shown in Fig. 1, using JFCR-45, drugs with a similar mode of action (such as a tubulin binder or topo I inhibitor) were classified into the same cluster, which were the same as the clusters established for NCI-60 (35) and JFCR-39 (19). These results suggest that the cell line panel-based assessment system is generally effective for classifying anticancer drugs with the same modes of action into the same set of clusters.

In this study, we investigated the gene expression profiles of 42 cell lines of JFCR-45 using cDNA array consisting of 3,537 genes. Hierarchical clustering analysis of these gene expression profiles classified organ-specific cell lines mostly into the same cluster, suggesting that these cell lines maintained the genetic characteristics of the parent organ as far as the gene expression profiles were concerned.

We did a Pearson correlation analysis of the gene expression database and the drug sensitivity database. Consequently, many genes whose expressions were correlated with respect to the sensitivity of each drug were identified. For example, DNA alkylating agents and nucleic acid–related genes, including *SF1* encoding ZFM1, *c-JUN* oncogene, and *SFRS9* were extracted as the genes sensitive to MMC. The genes that were sensitive to paclitaxel included tubulin binder and cytoskeleton-related genes, such as *VIL2* encoding ezrin and *ACTB* encoding β-actin.

These results suggest that the extracted genes are the predictive markers of drug efficacy. We further applied Pearson correlation analysis to each type (i.e., breast, liver, or stomach cancer) of cell lines. There were two advantages in this type of analysis: one is that we could compare the cell lines having the same organ background and another is that organ-specific genes, which worked as the sensitive or resistant factors, could be extracted. For example, for MMC, several genes (such as *INHBB*, *NK4*, and *HSPA1A*) were newly extracted as candidate genes sensitive to MMC from the breast cancer cell lines. Surprisingly, compared with the breast and liver cancer cells, many new candidate genes were extracted from the stomach cancer cell lines. These extracted genes were considered as the candidates for organ-specific predictive markers of drug efficacy.

We hypothesized that some of the candidate sensitivity genes described above might causally affect the chemosensitivity of cancer cell lines. To validate this possibility, we selected 19 genes, including *HSPA1A*, *JUN*, and *IL-18*, and examined whether the expression of these candidate genes would affect the cellular sensitivity to anticancer drugs. Overexpression of 2 of the 19 genes, *HSPA1A* encoding 70-kDa heat shock protein and *JUN* encoding c-JUN, indeed enhanced cellular sensitivity to MMC in HT1080 cells (Fig. 3), suggesting that they function to mediate MMC sensitivity. This was an unexpected finding, because a direct relationship between these two genes and MMC sensitivity has not been reported previously, although a relationship between heat shock protein and cancer has been suggested previously (36, 37). How these two genes potentiate MMC sensitivity remains to be clarified. In this validation, we used the HT1080 cell line instead of those in JFCR-45 because of its high transfection efficiency. As the alteration of chemosensitivity following the overexpression of any particular gene may depend highly on the genotypic/phenotypic background of the transfected HT1080 cells, further validation using cell lines within JFCR-45 will be required. In addition to the overexpression experiments, validation by silencing chemosensitivity-related genes using small interfering RNA will be required.

Pioneering attempts to discover new leads and targets and to investigate new aspects of the molecular pharmacology of anticancer drugs by mining the NCI-60 database have been done (31, 33-35). Recently, Szakacs et al. (38) have identified interesting compounds whose activity is potentiated by the MDR1 multidrug transporter. Our previous studies using JFCR-39 (19, 20, 31) and the present study using JFCR-45 also indicate that a comprehensive analysis of chemosensitivity and gene expression data followed by experimental validation leads to the identification of genes that determine drug sensitivity.

In conclusion, we established a sensitivity database for JFCR-45, which focused on organ origin, to 53 anticancer drugs. Using JFCR-45, anticancer drugs were classified according to their modes of action. Moreover, we established a database of the gene expression profiles in 42 cell lines of JFCR-45. Using these two databases, we have identified several genes that may predict chemosensitivity of cancer. Among these candidate genes, we identified two genes, *HSPA1A* and *JUN*, which determined sensitivity to MMC. Thus, this approach is useful not only to discover predictive markers for the efficacy of anticancer drugs but also to discover genes that determine chemosensitivity.

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Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at ≥0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.

Keywords: Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

2. Materials and methods

2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions (5.0×10^5 cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% CO_2 in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then refed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

3. Results

3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

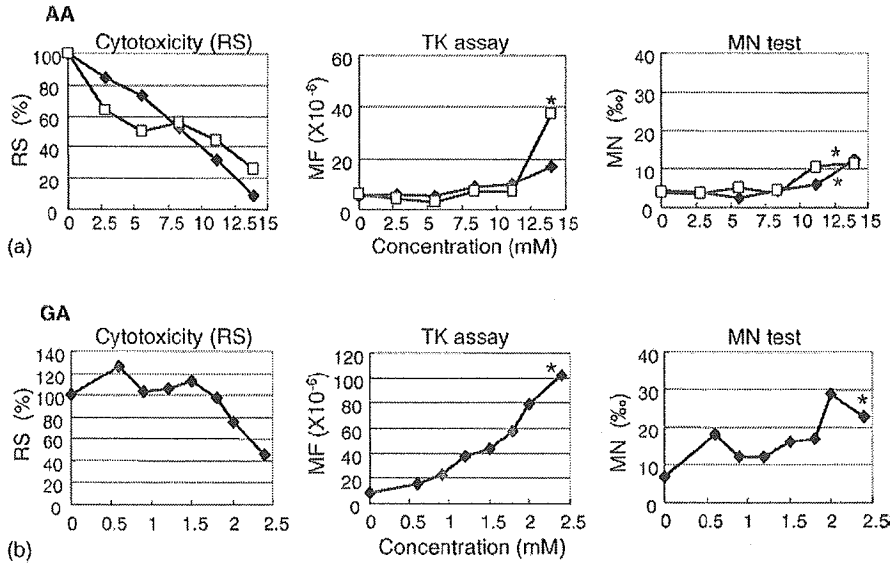

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11(50)	11(50)	0(0)
SG mutants				26	0(0)	13(50)	13(50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26(93)	2(7)	0(0)
SG mutants				16	0(0)	6(38)	10(62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n=37$), GA-induced ($n=17$) and spontaneous ($n=29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

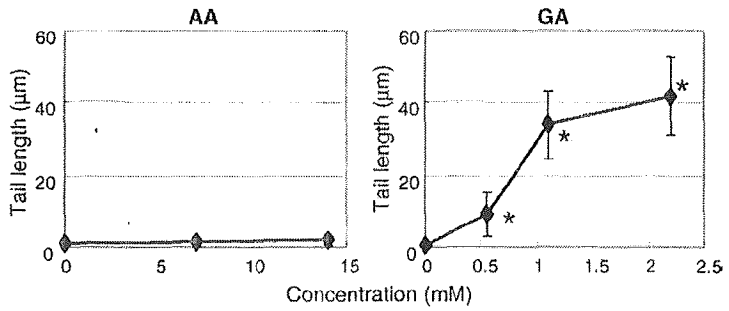

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P<0.05$).

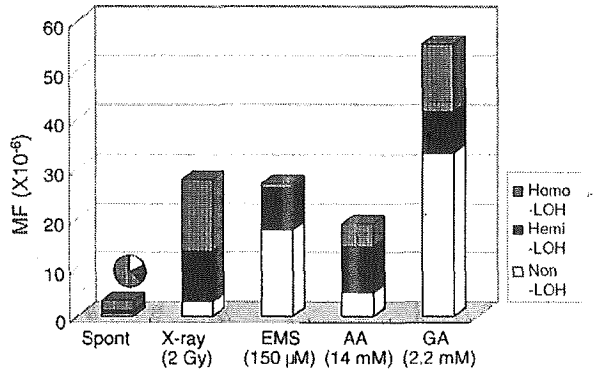

Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

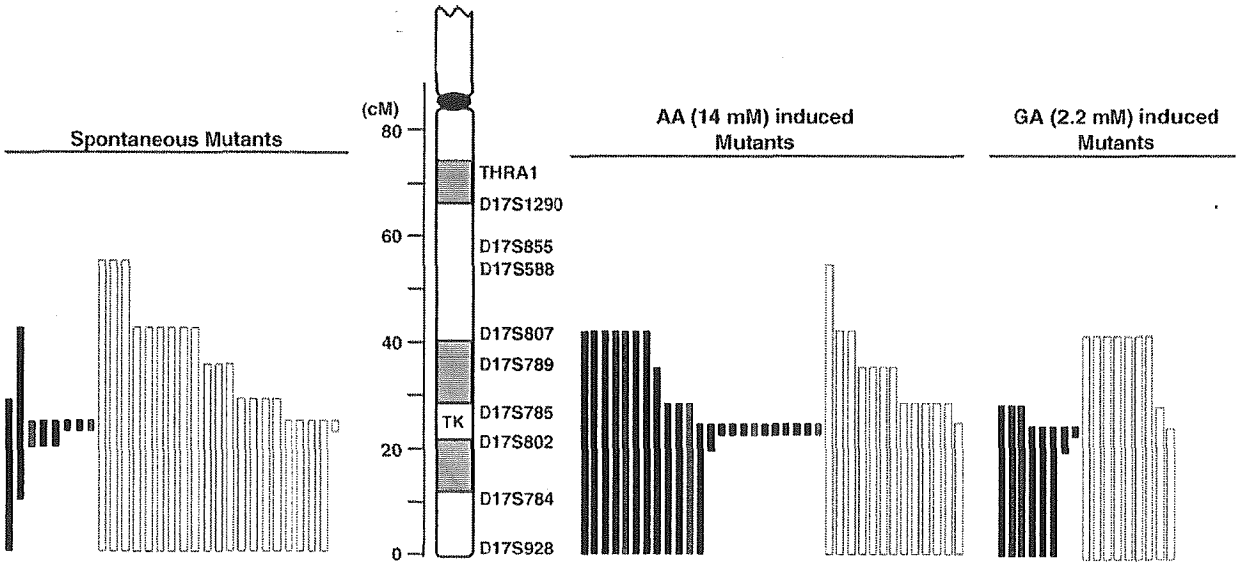

Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].

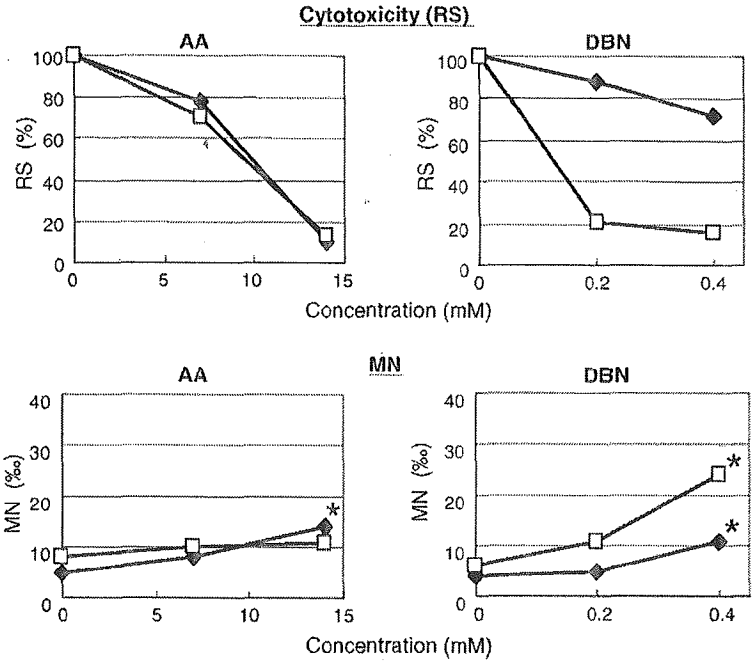

Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homlogous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homlogous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethyl) guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihidroxy propionamide [34,35]. Other in vitro studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in in vivo studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue™ mice that AA as well as GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.

This result may indicate that AA is metabolized to GA in vivo [38]. Tests that use rat liver S9 for metabolic activation may not be appropriate for in vitro investigations of AA genotoxicity and metabolism. Transgenic cells expressing CYP2E1, however, would be useful for demonstrating the in vitro genotoxicity of AA [39].

In conclusion, AA is weakly genotoxic, causing chromosome aberrations and a type of genomic instability. GA, its epoxide metabolite, is highly reactive with DNA. GA is a strong mutagen, inducing predominantly point mutations, and it may contribute to human cancers.

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