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Molecular analysis of mutations induced by 2-chloroacetaldehyde, the ultimate carcinogenic form of vinyl chloride, in human cells using shuttle vectors

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Vinyl chloride (VC) is a carcinogen associated with human and animal cancers. The ultimate carcinogenic form of VC, 2-chloroacetaldehyde (CAA), has been suspected to be mutagenic and we confirmed the mutagenicity of CAA using a modified shuttle vector plasmid. Base sequence analyses of 109 mutant plasmids with mutations in the *supF* gene, which were treated with CAA and propagated in the cultured human cells, revealed that more than half of the single base substitutions were G:C to A:T transitions with eight hotspots. The majority of the mutations involving G:C base pairs were in 5′-AAGG-3′ or 5′-CCTT-3′ sequences suggesting that these sequences are the main targets of mutagenesis caused by CAA.

Introduction

Vinyl chloride (VC*) is one of the widely used raw materials in the polymer industries. Many epidemiological studies and case reports have demonstrated that VC is associated with cancers in liver, brain, lung and the haematolymphopoietic system. The IARC working group classified VC as belonging to Group 1, which represents the substances with carcinogenicity supported by sufficient evidence in humans and animals (1,2).

Vinyl chloride is metabolized by the cytochrome P450-dependent monooxygenases to 2-chloroethylene oxide and then rapidly converted to 2-chloroacetaldehyde (CAA) in mammalian livers (3). 2-Chloroacetaldehyde reacts with DNA bases *in vitro*, resulting in production of four known cyclic adducts: 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (N^2,3-εG) and 1,N^2-ethenoguanine (1,N^2-εG) (4). These cyclic adducts were detected in liver DNA in rats following exposure to VC (5,6) and have been shown to cause mutations (7–13).

Recently, mutations involving G:C to A:T transitions in the second nucleotide at codon 13 in the c-Ki-*ras*-2 gene were detected in liver angiosarcomas in VC plant workers (14). This type of mutation could be specific to VC since the same type of mutation was found in bacteria exposed to VC (9–13,15). We intend to confirm whether this VC specific mutation arises in human cells.

The shuttle vector plasmid, pZ189 (16) and its derivative pS189 (17) and pYZ289 (18) have been widely used in assessing the carcinogen-induced mutations in mammalian cells. Each type of the plasmid carries a bacterial suppressor tRNA gene, *supF*, as a target gene for mutagenesis. The *supF* gene is small enough (176 base pairs) to facilitate rapid sequence analysis in many samples. We constructed a new shuttle vector plasmid, pMY189, by modification of the plasmid pZ189 to make the plasmid sequence suitable for the fluorescence dye primer cycle sequencing method.

We treated the shuttle vector plasmid pMY189 with CAA, the ultimate carcinogenic form of VC and transfected the plasmids to human fibroblast cells. The plasmids containing mutations in the *supF* gene were detected with the indicator bacteria system developed by Akasaka *et al.* (19) and the mutations were analyzed by the automatic DNA sequencing.

Materials and methods

Chemicals

2-Chloroacetaldehyde, ampicillin, chloramphenicol, nalidixic acid, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Wako Chemicals (Osaka, Japan). Restriction endonucleases and other enzymes were obtained from Takara Shuzo (Kyoto, Japan). QIAGEN plasmid-kit and Wizard™ Minipreps DNA Purification Systems were purchased from QIAGEN Inc. (Chatsworth, CA) and Promega (Madison, WI) respectively.

Cells

SV40-transformed human fibroblast cell lines were used. A normal human cell line WI38-VA13 (20) was obtained from the American Type Culture Collection (Rockville, MD). DNA repair deficient XP2OS(SV) cells were previously established by us from a Japanese group A XP patient (21). All cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Plasmid construction

The shuttle vector plasmid pZ189 was modified to be directly applied to the dye primer cycle sequencing method followed by the automatic DNA sequencing by a 370A DNA sequencer (Applied Biosystems, Foster, CA). Two 42-mer oligonucleotides were synthesized by a 380B DNA synthesizer (Applied Biosystems). One contains the sequence of the −21M13 fluorescent dye-labelled universal primer (5′-TGTAAAACGACGGCCAGT-3′) which is commercially available (Applied Biosystems) (5′-GACGAATTCTGTAAAA-CGACGGCCAGTGAGCTCGAATTCTTG-3′). The other is its complementary sequence, (5′-CAAGAATTCGAGCTCACTGGCCGTCGTTTTACAGAAT-TCGTC-3′). Both oligomers contain *Eco*RI restriction sequences (5′-GAATTC-3′) at their 5′- and 3′- ends and a *Sac*I restriction sequence (5′-GAGCTC-3′). Twenty micrograms each of these oligomers were annealed by heating and cooling in a solution of 750 mM NaCl and 75 mM Na_3 citrate. After ethanol precipitation, the annealed fraction (double strand DNA) was digested with *Eco*RI and inserted into the *Eco*RI site of pZ189 with T4 DNA ligase. The direction of the inserted oligomer was confirmed by the *Sac*I digestion and the DNA sequencing. The modified pZ189 was named pMY189 (Figure 1).

Bacterial strains

The indicator *Escherichia coli* strain KS40/pKY241 (19) was kindly supplied from Dr S.Akasaka, Division of Industrial Health, Osaka Prefectural Institute of Public Health, Osaka, Japan. KS40 is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 [*lacZ* (am) *CA7070 lacY1 HsdR HsdM* Δ (*araABC-leu*)*7679 galU galK rpsL thi*] (22), which has been used for detection of the mutated pZ189. Plasmid pKY241 was constructed by Akasaka *et al.* (19) and contains a chloramphenicol resistant marker and a *gyrA* (amber) gene. *Escherichia coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid, whereas the cells carrying the mutated *supF* form colonies on plates containing nalidixic acid, chloramphenicol and ampicillin. To ensure the selection of the mutated *supF* gene, IPTG and X-gal were

*Abbreviations: VC, vinyl chloride; CAA, 2-chloroacetaldehyde; IPTG, isopropyl-β-D-thiogalactoside; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; PBS, phosphate buffered saline.

added to the selection plates. *Escherichia coli* cells containing the active *supF* gene produce blue colonies, whereas cells having the mutated *supF* gene produce white or light blue colonies.

Treatment of plasmids with CAA and transfection to human cells

Purified stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit. The plasmids (40 μg) were treated with various concentrations of CAA in 0.3 M sodium acetate in total volume of 0.5 ml. The reaction was allowed to proceed for 1 h at 37°C followed by ethanol precipitation of the plasmids to remove the nonreacted excess CAA and the plasmids were redissolved in 0.5 ml of TE buffer (pH 8).

The human cells, W138-VA13 or XP2OS (SV) were trypsinized, washed and suspended in Dulbecco's phosphate-buffered saline (PBS) solution (pH 7.5). Cells (2×10^7) plus 14.4 μg CAA-treated pMY189 in PBS solution (0.2 ml) were placed in an electroporation chamber (electrodes 0.3 cm apart) (PDS Inc. Madison, WI) and the cells were transfected with the plasmids by electric pulses (600 V, 5×). The cells were plated in five 10 cm dishes and incubated at 37°C for 72 h in a CO_2 incubator.

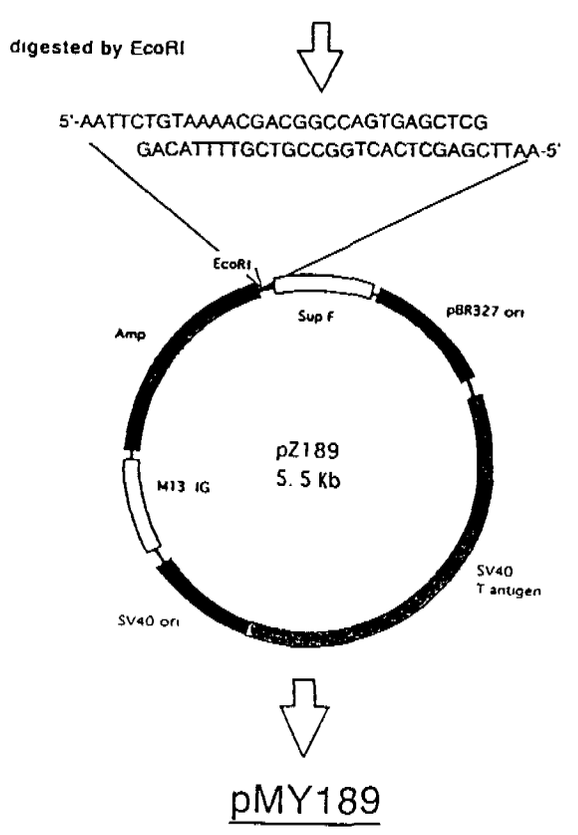

Fig. 1. Construction and the structure of the shuttle vector plasmid pMY189. Two 42 mer oligonucleotides, one of them containing a sequence of −21M13 5'-fluorescent dye-labelled universal primer for the automatic DNA sequencing, were annealed and digested by *Eco*RI, and ligated into the *Eco*RI site of the pZ189.

Plasmid recovery, selection of mutated supF and DNA sequencing

Plasmids were extracted from the cells using Wizard™ Minipreps DNA Purification Systems (Promega, Madison, WI). The purified plasmids were digested with the restriction endonuclease *Dpn*I (Boehringer-Yamanouchi, Tokyo, Japan) to eliminate the nonreplicated plasmids which retain the bacterial methylation pattern.

Plasmid DNA was introduced into the indicator bacteria KS40/pKY241 by the electroporation apparatus *E.coli* Pulser (Biorad, CA). The bacterial cells were plated in LB agar containing nalidixic acid, ampicillin, chloramphenicol at concentrations of 50, 150 and 30 μg/ml respectively, supplemented with IPTG and X-gal to select the plasmids containing the mutated *supF* genes. A part of the cells was plated on LB agar containing ampicillin and chloramphenicol to measure the total number of transformants. After 24 h incubation at 37°C, colonies were counted and mutation frequencies were calculated.

Mutated plasmids were extracted and purified from the overnight culture with the Wizard Minipreps Purification System (Promega) and the base sequences of the *supF* gene of the plasmids were determined with the −21M13 primer and Dye-Primer Cycle Sequencing Reagent Kit using a 370A automatic DNA sequencer (Applied Biosystems).

Results

Plasmid mutagenesis

2-Chloroacetaldehyde treatment of the pMY189 plasmids increased the frequency of mutation in the *supF* gene in both repair-proficient and repair-deficient cells (Table I). The background plasmid mutation frequency was 2.2 and 1.4×10^{-4} with VA13 and XP-A cells respectively. The mutation frequency increased similarly in both cell lines following treatment of the plasmid with CAA; and ~7- and 40-fold increases with 0.13 and 0.51 M CAA respectively, were observed.

Base sequence analysis

Analysis of 109 *supF* mutant plasmids transfected to the repair proficient cells was carried out by the nucleotide sequencing. Base sequence changes in plasmids were classified as single base substitutions, tandem base substitutions, multiple base substitutions (2 ≤ base substitutions more than three bases apart), frameshifts (single base insertions or deletions) and large deletions (Table II). Large deletions (average deleted sequence, 113 base pairs; range, 26–184 base pairs) were found in 10% of the plasmids and 14% of the plasmids contained the multiple base substitutions. The tandem base substitutions and the frameshift mutations were scarce. The rest (72%) contained the single base substitutions.

Among the mutant plasmids with single base substitutions, 90% were the substitutions of G:C base pair (Table III). The most frequent type of the base substitution mutations was G:C to A:T transition (54%). Other types of the base substitutions

Table I. Mutation frequency of CAA-treated plasmids pMY189 propagated in normal or xeroderma pigmentosum fibroblasts

Concentration of CAA (M)	W138-VA13			XP2OS(SV)		
	No. of colonies			No. of colonies		
	Mutant	Total (× 10^4)	Mutation frequency (× 10^{-4})	Mutant	Total (× 10^4)	Mutation frequency (× 10^{-4})
0	85 ± 7.1	38.0	2.2 ± 0.2	2.3 ± 1.9	1.6	1.4 ± 1.2
0.13	151.3 ± 11.9	10.2	14.9 ± 1.2	160.7 ± 18.3	12.1	13.3 ± 1.5
0.26				241.0 ± 23.8	7.7	31.4 ± 3.1
0.51	989.0 ± 61.7	16.7	59.2 ± 3.7	154.0 ± 4.5	2.8	55.3 ± 1.6

Average colony numbers from three independent experiments are given with the standard error of the mean.

were G:C to T:A transversion (30%), G:C to C:G transversion (6%), A:T to G:C transition (9%) and A:T to T:A transversion (1%). No single base A:T to C:G transversion was detected. Figure 2A shows the targets of the single base substitutions in the *supF* gene, compiled from the previous reports (16,17,23–39). Among the reported 93 target sites (* in Figure 2A), 54 sites are present in G:C base pairs and 39 sites are present in A:T base pairs. The proportion of the base substitution at G:C base pairs is higher than that at A:T base pairs, even when the difference of the number of target sites between G:C and A:T base pairs is adjusted (Table III).

Mutation spectra

Distribution of the single base substitutions and tandem or multiple base substitutions in the *supF* gene with the repair proficient cell is also shown in Figure 2A. Eight sites (123,133,134,156,159,160,168 and 169) had four or more single base substitutions. Most of the single base substitutions (91%) were produced at these hotspots. Seven of the eight hotspots were located at G:C base pairs. Five G:C to A:T or G:C to T:A hotspots (123,159,160,168 and 169) are located at G:C base pairs in 5′-AAGG-3′ sequences.

All multiple base substitutions were found in separate positions, except one tandem base substitution (Figure 2B). There are 15 two-base substitutions and one three-base substitutions.

Discussion

Plasmids pMY189 treated with CAA yielded the same frequency of mutations when they were propagated in XP-A and normal cells, suggesting that the DNA damage induced by CAA is not repaired by the nucleotide excision repair pathway in human cells. This was supported by the preliminary experiment indicating that the survival of the CAA-treated pMY189 plasmids introduced in *E.coli* MBM7070 (wild type) and KY46 (uvrA) were the same (data not shown).

CAA-induced DNA adducts were reported to be repaired by a human 3-methyladenine DNA glycosylase (40,41), suggesting that DNA repair other than the nucleotide excision type may be involved.

In mutation spectra of the single base substitutions, 72 out of 78 (92%) were located in the eight mutational hotspots among 93 mutation target sites in the *supF* gene. There are four 5′-AAGG-3′ or 5′-CCTT-3′ sequences in the *supF* gene (positions 68–71, 120–123, 157–160 and 168–171). Fifty-seven out of 78 (73%) single base substitutions were located at G:C pairs in these 5′-AAGG-3′ or 5′-CCTT-3′ sequences. This suggests that G:C pairs in the 5′-AAGG-3′ or 5′-CCTT-3′ sequences are the major mutational targets by CAA. The sequence 5′-AAGG-3′ on position 68–71 was not mutated. However, no mutation at position 70 and only two mutations at position 71 have been reported previously, suggesting that these sites are phenotypically silent or may be protected from attack by the chemical caused by the secondary structure of the gene. The possibility that we isolated sibling mutants is unlikely, because we took 109 colonies out of about 3000 mutant colonies and no identical mutation was found in the tandem and multiple base substitutions, frameshifts and deletion mutations. We do not know why such a high sequence specificity is achieved. DNA damage may be induced or DNA repair may be blocked selectively on this sequence by unknown mechanisms.

G:C to A:T transition mutations are predominant (53.8%), supporting the previous findings that chloroethylene oxide-treated *E.coli* (15) and CAA-treated gapped duplex M13 DNA (13) had mainly G:C to A:T transitions. The adducts which can cause mutations in G:C base pairs could be the 3,N^4 ethenocytosine (εC) and the N^2,3-ethenoguanine (N^2,3-εG). The N^2,3-εG:T mismatch caused G:C to A:T transitions in an *in vitro* DNA replication experiment (8). Site specifically incorporated N^2,3-εG in the M13 double strand DNA resulted in G to A transitions (11) and site specifically incorporated εC in the M13 single strand DNA (9) or gapped duplex DNA (10,12) led mainly to C to T transitions. These reports and our experiment suggest that the major contributors to the G:C to A:T transitions induced by CAA in human cells are εC and N^2,3-εG.

The previously published data of the mutations in G:C base pairs induced by CAA or its related cyclic adducts are summarized in Table IV. Compared with other studies using M13 phage, our results show relatively high frequency of G:C to T:A transversion mutations. It may reflect the different mechanisms of the DNA repair or the mutation fixation in *E.coli* and human cells, since the similar difference was observed in UV-induced mutations in M13 and human cells (33,42).

The published data of the mutations in A:T base pairs induced by CAA or its related cyclic adducts are summarized in Table V. The predominance of A:T to G:C transition mutations in our data supported the previous findings that site-specifically incorporated εA and 4-amino-5-(imidazole-2-yl)imidazole (β) in single strand M13 DNA induced mainly A:T to G:C transitions (9) in *E.coli*. The data of mutations in the CAA-treated M13 gapped duplex DNA replicated in *E.coli*, however, showed the predominance of A:T to T:A transversions (13). In addition to the difference in the mechanism of the

Table II. Types of mutations in the *supF* gene in CAA-treated shuttle vector plasmids pMY189 propagated in human fibroblast cells WI38-VA13

Type of mutation	No.	Percentage of total
Base substitution		
Single base substitution	78	71.6
Tandem base substitution	1	0.9
Multiple base substitution	15	13.8
Frameshift		
Single base insertion	0	0.0
Single base deletion	1	0.9
Deletion over 3 base pair	12	10.1
Others	2	1.8
Total	109	100

Table III. Types of CAA-induced single base substitutions in the *supF* gene in shuttle vector plasmids pMY189 propagated in human fibroblast cells WI38-VA13

Type of mutation	No. (%) of mutants	No. of mutants/target site[a] (%)
G:C to A:T	42 (53.8)	0.778 (51.8)
G:C to T:A	23 (29.5)	0.426 (28.4)
G:C to C:G	5 (6.4)	0.093 (6.2)
A:T to G:C	7 (9.0)	0.179 (11.9)
A:T to T:A	1 (1.4)	0.026 (1.7)
A:T to C:G	0 (0.0)	0 (0.0)
Total	78 (100)	1.502 (100)

[a]Numbers of targets of the single base substitution mutations in the *supF* gene are 54 at G:C pairs and 39 at A:T pairs.

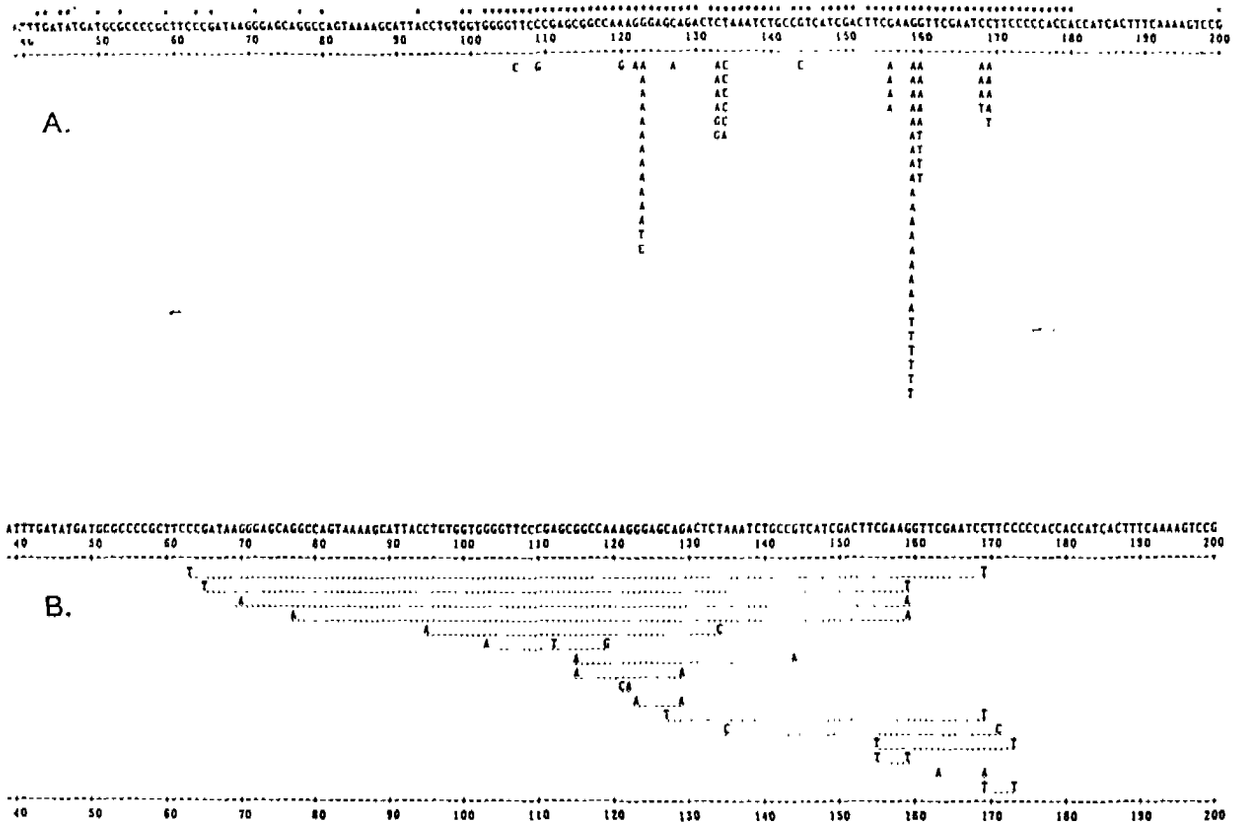

Fig. 2. Locations of the single (A) and tandem and multiple (B) base substitution mutations found in the *supF* suppressor tRNA gene in CAA-treated plasmids pMY189 propagated in WI38-VA13 cells. Each letter under the sequence represents a single base substitution mutation found in an independent plasmid (A). Multiple base substitutions are shown in (B). Positions of the targets of the single base substitutions which inactivate the suppressor function are as follows [Position (Ref.)]: 42(16), 43(16,39), 45(16), 46(16), 50(16, 38), 53(28), 59(39), 62(39), 63(16), 65(16,24,26), 71(16,26), 77(16,31), 80(24), 93(24,26), 99(24,26,34), 100(16), 102(16,17,25), 103(16,17,37), 104(16,17,33,37), 105(16,17,37,33,38), 106(16,34,38), 107(37), 108(16,23,26,31,36–39), 109(16,17,24–26,29,31,33,34,37), 100(16,25,30,31,36), 111(16,23,26,30,37), 112(16,17,29,30,33,34,37,38), 113(16,17,23,25,26,31,34,37,38), 114(16,26), 115(16,17,25,29,37,38), 116(16,17,25,26,29,31), 117(16,36), 118(16,24,34), 119(16,34), 120(16,24,31,33–35,37,38), 121(27,37), 122(16,25,31,34,36,37), 123(16,17,23–26,32–34,37–39), 124(16,17,24,33,34,36,38,39), 125(16,37), 126(16,37), 127(16,17,25,29,33,34,37,38), 128(30), 129(16,17,24–26,31,32,34,35), 130(37), 132(16,34,37), 133(16,17,24–27,29,31–34,38), 134(16,27,31,33,34,37–39), 135(16,27,31,33–35,38,39), 136(16,17,24,26,27,29,31,33–37,39), 137(16,17,31,33,34), 138(37), 139(16,17,24–26,29,33–35,37,38), 140(16,24,25,30,34), 141(16,25,33,34,37,38), 143(16,25), 144(16,17,24,26,29,33,34,37), 145(37), 147(35), 148(Akasaka *et al.*, in press), 149(26,33,35), 150(16,31,37), 151(37), 153(37,39), 154(16,31,35,37), 155(16,17,23,25,26,29,31–37,39), 156(16,17,23–26,31–39), 157(37), 158(16,31,34,37), 159(16,17,23–26,29,31–39), 160(16,17,23–27,29,30,31,33,34,38), 161(16,27,33,34,37), 162(16,25,27,34,36,37), 163(16,31,32,37), 164(16,17,24–26,29,31,33,34,36,37,39), 165(16,25,31,33,34,36,37), 166(27,37), 167(16,37), 168(16,17,23–27, 29–39), 169(16,17,23–26,29–31,33–39), 170(37), 171(16,34,37,39), 172(16,17,24–26,29,33,34,37,38), 173(16,34,39), 174(16,17,25,37), 175(16,31,33,37), 176(17), 177(16,17,34,35), 178(17,25), 179(16,34), 180(37), 200(16).

Table IV. Types of G:C base substitution mutations induced by 2-chloroacetaldehyde and its related cyclic adducts

Type of mutation	No. (%) of mutants				
	εG[a] M13 dsDNA in *E.coli*	εC[b] M13 ssDNA in *E.coli*	εC[c] M13 gdDNA in *E.coli*	CAA treated[d] M13 gdDNA in *E.coli*	Our data[e]
G:C to A:T	134 (99.3)	31 (56.4)	(64.0)	80 (72.1)	63 (64.9)
G:C to T:A	0 (0)	12 (21.8)	(10.9)	20 (18.0)	29 (29.9)
G:C to C:G	1 (0.7)	0 (0)	(0)	11 (9.9)	5 (5.2)
1 base deletion	0 (0)	12 (21.8)	(25.0)	0 (0)	0 (0)
Total	135 (100%)	55 (100%)	(100%)	111 (100%)	97(100%)

[a]Site-specifically incorporated N^2,3-ethenoguanine (5'-GG(εG)AAA-3') in double strand M13DNA was replicated in *E.coli* cells (11).
[b]Site-specifically incorporated 3,N^4-ethenocytosine (5'-TAG(εC)GGG-3') in single strand M13DNA was replicated in *E.coli* cells (9).
[c]Site-specifically incorporated 3,N^4-ethenocytosine (5'-TT(εC)TT-3') in gapped duplex M13DNA was replicated in *E.coli* cells (10). Mutation was analyzed by the multiplex sequence analysis.
[d]The CAA treated gapped duplex M13DNA was replicated in UV-irradiated *E.coli* cells (13).
[e]Data contain all single, tandem and multiple base substitutions

Table V. Types of A:T base substitution mutations induced by 2-chloroacetaldehyde (CAA) and its related cyclic adducts

Type of mutation	No. (%) of mutants			
	εA[a] M13 ssDNA in *E.coli*	β[b] M13 ssDNA in *E.coli*	CAA treated[c] M13 gdDNA in *E.coli*	Our data[d]
A:T to G:C	17 (56.7)	11 (68.8)	4 (17.4)	10 (76.9)
A:T to T:A	5 (16.7)	2 (12.5)	18 (78.3)	2 (15.4)
A:T to C:G	8 (26.7)	3 (18.8)	1 (4.3)	1 (7.7)
Total	30 (100%)	16 (100%)	23 (100%)	13 (100%)

[a]Site-specifically incorporated 1,N^6-ethenoadenine (5'-GCT(εA)GC-3') in single strand M13DNA was replicated in *E.coli* cells (9).
[b]Site-specifically incorporated 4-amino-5-(imidazol-2-yl)imidazole (β) [5'-GCT(β)GC-3'] in single strand M13DNA was replicated in *E.coli* cells (9).
[c]The CAA treated gapped duplex M13DNA was replicated in UV-irradiated *E.coli* cells (13).
[d]Data contain all single, tandem and multiple base substitutions.

mutation fixation in *E.coli* and human cells, the contradicting results with M13 may be due to the difference in the sites of the adducts produced in DNA as the adducts are located in the single stranded region of the gap in the gapped duplex DNA (13).

The shuttle vector plasmid pMY189 designed to be applied to the fluorescent dye primer cycle sequencing by an automatic DNA sequencer, made the analysis of the nucleotide sequences of the mutant *supF* genes easier and faster than the conventional radioisotope labelling and autoradiography method. This method also made the analysis more accurate than the dye deoxy terminator cycle sequencing method using the automatic DNA sequencer we previously used (33).

In conclusion, we found that about half of the CAA-induced mutations were G:C to A:T transitions and that most of the remaining mutations were G:C to T:A transversions and A:T to G:C transitions in the shuttle vector plasmid pMY189 propagated in human cells. G:C pairs in 5'-AAGG-3' or 5'-CCTT-3' sequences are the major target of mutations, mainly, G:C to A:T transitions. This sequence specificity can be extrapolated to the estimation of contribution of VC to carcinogenesis in human beings.

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