



Genotoxicity of formaldehyde: molecular basis of DNA damage and mutation

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Formaldehyde is commonly used in the chemical industry and is present in the environment, such as vehicle emissions, some building materials, food, and tobacco smoke. It also occurs as a natural product in most organisms, the sources of which include a number of metabolic processes. It causes various acute and chronic adverse effects in humans if they inhale its fumes. Among the chronic effects on human health, we summarize data on genotoxicity and carcinogenicity in this review, and we particularly focus on the molecular mechanisms involved in the formaldehyde mutagenesis. Formaldehyde mainly induces *N*-hydroxymethyl mono-adducts on guanine, adenine and cytosine, and *N*-methylene crosslinks between adjacent purines in DNA. These crosslinks are types of DNA damage potentially fatal for cell survival if they are not removed by the nucleotide excision repair pathway. In the previous studies, we showed evidence that formaldehyde causes intra-strand crosslinks between purines in DNA using a unique method (Matsuda et al., 1998). Using shuttle vector plasmids, we also showed that formaldehyde as well as acetaldehyde induces tandem base substitutions, mainly at 5'-GG and 5'-GA sequences, which would arise from the intra-strand crosslinks. These mutation features are different from those of other aldehydes such as crotonaldehyde, acrolein, glyoxal, and methylglyoxal. These findings provide molecular clues to improve our understanding of the genotoxicity and carcinogenicity of formaldehyde.

Keywords: genotoxicity, mutation spectrum, *supF*, DNA intra-strand crosslink, DNA damage

INTRODUCTION

Formaldehyde (Methanal, CAS No.: 50-00-0) is abundantly produced in the chemical industry, and its annual global production is about 20 million tons (IARC, 2012). It is used in industrial and consumer products, and is also generated as a byproduct of biomass and petroleum burning, cigarette smoke and automobile exhaust. Cigarette smoke contains 12–106 μg of formaldehyde per cigarette (IARC, 2012). In medical and biological fields, it has been widely used as a fixative or preservative for pathological specimens. It also occurs as a natural product in most organisms and in the environment. There are several metabolic pathways in humans leading to formaldehyde synthesis, in particular, amino acid and methanol metabolisms, lipid peroxidation, and P450-dependent demethylation (Swenberg et al., 2013).

The major sources of formaldehyde exposure can be roughly classified into three categories: fumes generated from industry, those released in residential housing and those in various occupational indoor settings (Ma and Harris, 1988). Non-occupational exposure in the household is mainly due to the fumes released from formaldehyde-based resin for binding of plywood, particle board, paint, and textile fibers. Formaldehyde fumes have caused health problems across the country. People inhaling formaldehyde fumes indoors often show symptoms such as headache,

dizziness, wheezing, runny nose, and nausea. In severe cases, they have sore throat, burning eyes, and skin irritation. These symptoms are called “sick house syndrome” or “sick building syndrome” (Norbäck, 2009). The levels of formaldehyde in the air inside houses are typically 0.02–0.06 mg/m^3 , but these levels have been declining since the late 1980s as a result of standards for building materials (IARC, 2012). The World Health Organization now recommends a formaldehyde level of 0.1 mg/m^3 in indoor air (Kaden et al., 2010).

Formaldehyde also has activities that cause chronic adverse effects, such as genotoxicity, carcinogenicity, and teratogenicity. The health effects have been extensively studied for a long time and reviewed in many articles (Ma and Harris, 1988; Conaway et al., 1996; Swenberg et al., 2013). In this review, we focused on molecular basis of DNA damage and mutations induced by formaldehyde exposure. In this sense, our original data on the mutation spectrum of formaldehyde in human cells, have been clearly showed further details concerning the mechanism of formaldehyde carcinogenesis.

CARCINOGENICITY

In animal experiments using mice and rats, chronic inhalation of formaldehyde at high concentrations caused cancers, particularly,

squamous cell carcinomas of the nasal cavities (IARC, 2006). In other studies, the incidences of lymphoma and leukemia were also increased. In animals administered formaldehyde in drinking water, increased incidences of forestomach papillomas, leukemias, and gastrointestinal tract tumors were reported, but their frequencies varied. These studies have been summarized in IARC Monograph (IARC, 2006).

In the human body, many endogenous and exogenous compounds release formaldehyde through catabolic reactions. The primary metabolic system for formaldehyde involves an initial spontaneous reaction with glutathione to form S-hydroxymethylglutathione, followed by reaction facilitated by alcohol dehydrogenase-3 to convert the intermediate to S-formylglutathione. This intermediate is then further metabolized by S-formylglutathione hydrolase to yield nontoxic formate, which is excreted in the urine (Hedberg et al., 2002). There have been numerous cohort and case-control studies on formaldehyde exposure and an increase in nasopharynx cancer or leukemia in humans (IARC, 2012). The IARC (2006) judged that there was sufficient evidence for the carcinogenicity of formaldehyde, based primarily on its association with nasopharyngeal cancer, and that there was strong but not sufficient evidence for the leukemogenic effects of formaldehyde. Owing to advancements via recent epidemiological studies (Coggon et al., 2003; Hauptmann et al., 2004, 2009; Pinkerton et al., 2004; Marsh and Youk, 2005; Marsh et al., 2007a,b; Beane Freeman et al., 2009) and meta-analyses (Bosetti et al., 2008; Zhang et al., 2009; Bachand et al., 2010), the IARC (2012) concluded that occupational exposure to formaldehyde causes nasopharyngeal cancer and leukemia, and classified formaldehyde as a human carcinogen Group 1.

GENOTOXICITY

The genotoxicity of formaldehyde has been examined by various methods. In most reports using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 (Ames test), formaldehyde induced both base change and frame-shift mutations without metabolic activation, but its mutagenicity is not as strong as that of typical environmental mutagens such as 4-nitroquinoline-1-oxide and *N*-methyl-2-nitro-*N'*-nitrosoguanidine (Ma and Harris, 1988; Conaway et al., 1996). Formaldehyde also shows mutagenicity in assays using *Escherichia coli* WP2 (Takahashi et al., 1985; O'Donovan and Mee, 1993).

Formaldehyde induced chromosome aberrations (CA) and sister chromatid exchanges (SCE) in cultured Chinese hamster (Natarajan et al., 1983; Basler et al., 1985; Galloway et al., 1985; Merk and Speit, 1998, 1999) and human lymphocyte cells (Kreiger and Garry, 1983). In rats treated with formaldehyde through inhalation (0.5–12 ppm), no increase in the frequencies of CA and SCE was observed in lymphocytes (Kligerman et al., 1984). In rats inhaling 15 ppm formaldehyde, CA was induced in pulmonary lavage cells but not in bone marrow cells (Dallas et al., 1992). In mice treated with formaldehyde by oral administration, no CA or micronucleus (MN) formation was detected in bone marrow and spleen cells (Gocke et al., 1981; Natarajan et al., 1983). In rats administered a single dose of 200 mg/kg formaldehyde by gavage, gastrointestinal organs exhibited local

irritation and their cells formed MN (Migliore et al., 1989). In people occupationally exposed to formaldehyde, increases in the frequencies of MN and SCE in the peripheral lymphocytes were reported (Costa et al., 2008), but no statistically significant increases were also reported elsewhere (Ye et al., 2005; Pala et al., 2008).

Formaldehyde induces mutations in human cultured lymphoblastoid TK6 cells and Chinese hamster ovary (CHO) cells, which were detected by resistance to toxic nucleoside analogs, trifluorothymidine (Goldmacher and Thilly, 1983), 8-azaguanine and 6-thioguanine (Liber et al., 1989; Graves et al., 1996; Speit and Merk, 2002).

DNA DAMAGE AND REPAIR

Formaldehyde reacts immediately with primary and secondary amines, thiols, hydroxyls, and amides to form methylol derivatives. It acts as an electrophile and can react with macromolecules such as DNA, RNA, and protein to form adducts and cross-links. Guanine, adenine and cytosine have amino group in their molecules. When deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine were reacted with formaldehyde, the major products determined by high-performance liquid chromatography and electrospray mass spectrometry (LC-MS/MS) were *N*⁶-hydroxymethyldeoxyadenosine, *N*⁴-hydroxymethyldeoxycytidine and *N*²-hydroxymethyldeoxyguanosine (Beland et al., 1984; Cheng et al., 2003). In human and Chinese hamster cells treated with formaldehyde, *N*⁶-hydroxymethyldeoxyadenosine and *N*²-hydroxymethyldeoxyguanosine were detected in their genome (Beland et al., 1984; Zhong and Que Hee, 2004). *N*⁶-hydroxymethyldeoxyadenosine was detected in leukocyte DNA from 29 of 32 heavy smokers (Wang et al., 2009). The reaction of formaldehyde with deoxyadenosine and deoxyguanosine or DNA also produces three major methylene crosslinks: di-(*N*⁶-deoxyadenosyl)methane, (*N*⁶-deoxyadenosyl-*N*²-deoxyguanosyl)methane and di-(*N*²-deoxyguanosyl)methane (Huang et al., 1992; Huang and Hopkins, 1993; Cheng et al., 2003). In a study of the *in vitro* reactions between formaldehyde and all amino acids, deoxyguanosine crosslinks with cysteine were abundant and stable, while those with lysine were readily formed but rapidly disintegrated (Lu et al., 2010). There are many reports showing that formaldehyde-protein crosslinks were detected in cells treated with formaldehyde (IARC, 2012), but their molecular structures have not been fully clarified. The DNA-protein crosslinks with protein sizes less than 12–14 kDa can be removed by the nucleotide excision repair pathway, whereas oversized DNA-protein crosslinks are processed exclusively by RecBCD-dependent homologous recombination in bacteria (Nakano et al., 2007). In mammalian cells, the upper size limit of crosslinked proteins amenable to the nucleotide excision repair is relatively small (8–10 kDa), therefore, homologous recombination would be the major pathway in tolerance of chromosomal DNA-protein crosslinks (Nakano et al., 2009).

We attempted to determine the likelihood of formaldehyde-induced intra-strand crosslink formation between four kinds of nucleotide in DNA using the unique method developed by Kawanishi (1995) and Matsuda et al. (1998). Single-strand DNA (175 base pairs) and two 20-mer complementary

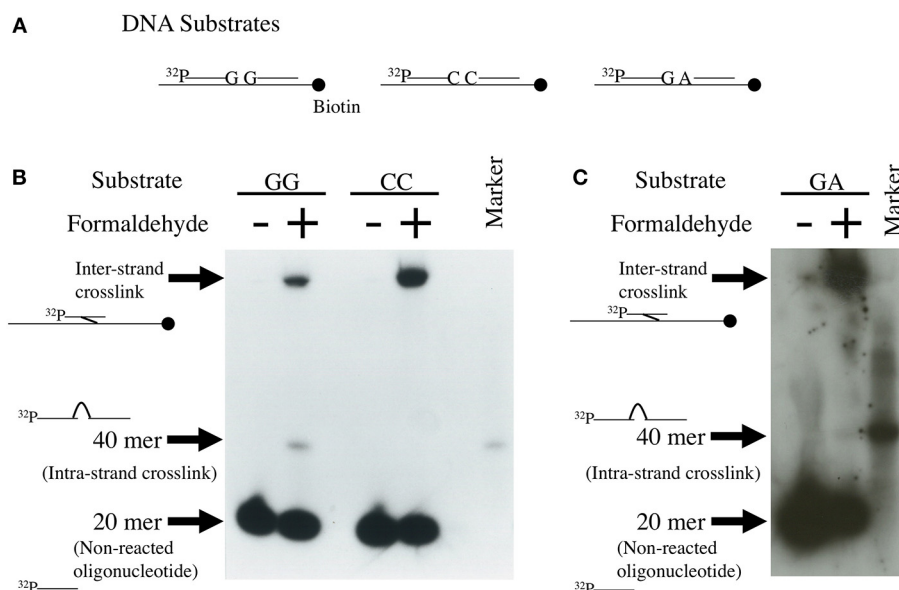


FIGURE 1 | Detection of formaldehyde-induced intra-strand crosslinks.

Single-strand 40 mer DNA bands were formed by binding between the 3'-end of ^{32}P -labeled 20-mer DNA and the 5'-end of non-labeled 20-mer DNA by formaldehyde crosslinks. *Method:* The experimental protocol has been described in Matsuda et al. (1998). Briefly, the 175 base-pair region of pBluescript KS(-) was amplified by polymerase chain reaction (PCR) using 5'-biotinylated and non-biotinylated primers. The biotinylated 175-mer DNA was immobilized to Dynabeads M-280 streptavidin. Four kinds of 20-mer complementary DNA, of which the 5'-ends were labeled with ^{32}P and the 3'-ends had G, A, or C, were prepared. Four kinds of

unlabeled 20-mer complementary DNA, the sequence of which followed the 3'-end of the ^{32}P -labeled 20-mer DNA and had G, A, T, or C at the 5'-end, were also prepared. The biotinylated 175-mer DNA, ^{32}P -labeled complementary 20-mer DNA and the following non-labeled complementary 20-mer DNA were annealed by heating and cooling for detecting GG, CC, and GA-intrastrand crosslinks, as shown in (A). The annealed DNA was treated with 270 mM formaldehyde for 24 h at 4°C. After collecting DNA using a magnet, DNA was purified and subjected to electrophoresis on denatured polyacrylamide gel. Autoradiography images of the gel are shown in (B,C).

oligonucleotides: one a 5' [^{32}P]-labeled sequence and the other an unlabeled sequence following the [^{32}P]-labeled sequence, were annealed as shown in **Figure 1A**. If the oligonucleotides are bound between the 3'-end base of the [^{32}P]-labeled oligonucleotide and the adjacent 5'-end base of the unlabeled oligonucleotide by a formaldehyde crosslink, a 40-mer single-strand oligonucleotide band should appear on the denatured polyacrylamide gel. As shown in **Figures 1B,C**, formaldehyde formed crosslinks between deoxyguanosines (5'-GG) of the ends of the oligonucleotides. Fewer crosslinks were formed between deoxyguanosine and deoxyadenosine (5'-GA), but no crosslinks were formed between deoxycytidines (5'-CC). **Figures 1B,C** also shows formaldehyde-produced inter-strand crosslinks. The repair mechanism of the formaldehyde-induced DNA inter-strand crosslinks has not been reported so far, but the inter-strand crosslinks might be removed by the Fanconi anemia (FA) pathway and homologous recombination, which is shown in the studies of mitomycin C-induced inter-strand crosslinks (Kottemann and Smogorzewska, 2013; Unno et al., 2014).

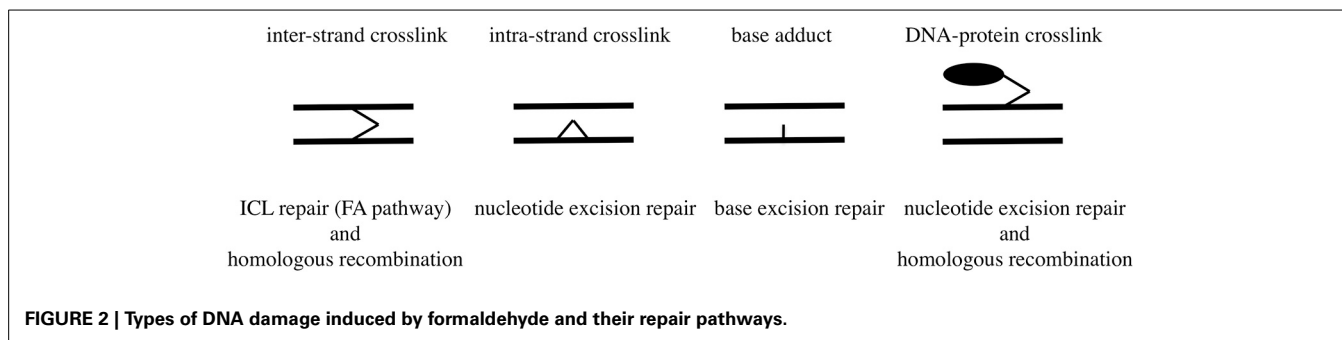
Human and Chinese hamster mutant cells deficient in *XPF* or *ERCC1* are hypersensitive to formaldehyde (Kumari et al., 2012), and *E. coli uvrA* mutant is more sensitive to formaldehyde than wild-type *E. coli* (Takahashi et al., 1985). These lines of evidence indicate that intra-strand crosslinks constitute a type of damage that could be fatal to cell survival

and are removed by the nucleotide excision repair pathway; whereas mono-adducts, hydroxymethyldeoxyadenosines and hydroxymethyldeoxyguanosines, would be removed by the base excision repair pathway. Types of DNA damage induced by formaldehyde and their repair pathways are summarized in **Figure 2**.

MUTATION SPECTRUM

In *E. coli*, mutations induced by formaldehyde in the xanthine guanine phosphoribosyl transferase (*gpt*) gene were characterized (Crosby et al., 1988). Exposure of *E. coli* to 4 mM formaldehyde induced insertions (41%), deletions (18%), and point mutations (41%). DNA sequencing revealed that most of the point mutations were transversions at the G:C base pair in the gene. In contrast, exposure of *E. coli* to 40 mM formaldehyde produced point mutations (92%), 62% of which were transitions at the single A:T base-pair in the gene, indicating that there were concentration-dependent variations in mutation spectra. When naked pSV2*gpt* plasmid was exposed to 3.3 or 10 mM formaldehyde and transfected into *E. coli*, most mutations were frameshifts, which also suggests the presence of various mechanisms in formaldehyde-induced mutation.

In human lymphoblastoid TK6 cells treated with 150 μM formaldehyde, 30 *hprt* mutant colonies were characterized by Southern blot analysis (Liber et al., 1989). Fourteen (47%) of these mutants had large deletions, while the other 16 mutants



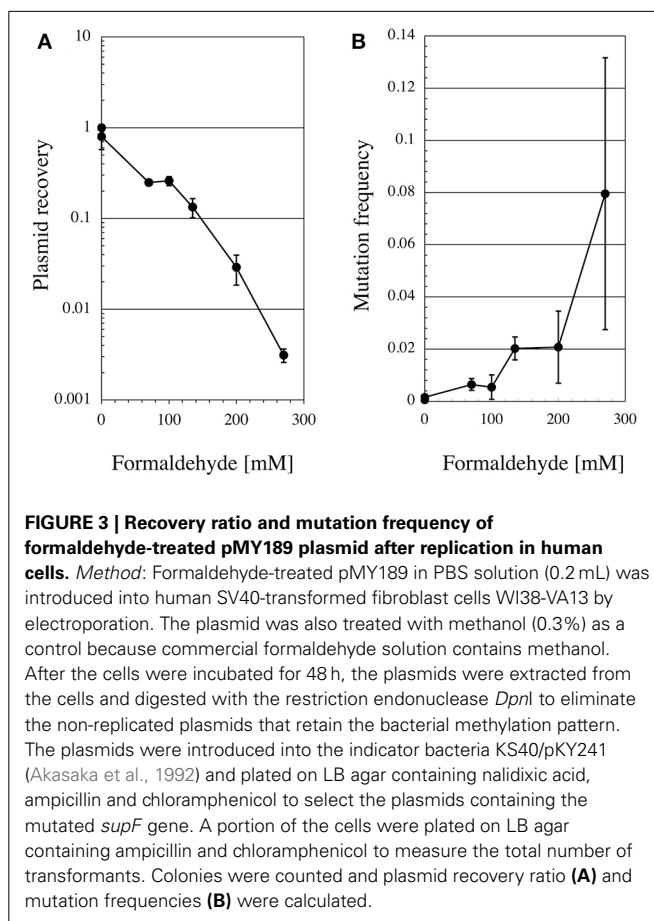
(53%) showed normal restriction patterns. Of these, 12 mutants produced *hprt* mRNA and 3 mutants produced no detectable mRNA. Sequence analyses of the mRNA showed that 6 mutants had base substitutions occurring at the A:T base pair. Four mutants had A:T to C:G transversions at a specific site that appears to be a mutation hot spot. The remaining 2 mutants lost exon 8 of the *hprt* gene.

CHO cells treated with 1 mM formaldehyde increased the *hprt* mutation frequency to 4.7-fold higher than the spontaneous mutation frequency, and mutations of 6 mutant clones were identified. All mutations were single base transversions with 3 A:T to T:A, 2 A:T to C:G, and 1 G:C to T:A (Graves et al., 1996).

Mutations in the *p53* gene from rat primary nasal squamous cell carcinomas induced by formaldehyde inhalation (15 ppm up to 2 years) were analyzed (Recio et al., 1992). Five tumors had point mutations, namely, 3 G:C to T:A transversions, 1 G:C to A:T transition, and 1 G:C to C:G transversion.

SHUTTLE VECTOR MUTAGENESIS

Since no previous reports had described that formaldehyde induces tandem base substitutions that would arise from intra-strand crosslinks, we adopted the shuttle vector plasmid pMY189 (Matsuda et al., 1995; Yagi, 2013) that can be treated with a high concentration of formaldehyde. The plasmid pMY189 can replicate in both human and *E. coli* cells, and has an ampicillin-resistant marker gene and a mutation marker gene *supF* that can be selected as colored colonies on bacterial agar plates. The result was published by Kawanishi (1995), and we describe its outline below. The formaldehyde-treated pMY189 propagated in human fibroblast cells caused reduction of the number of ampicillin-resistant bacterial colonies and enhanced the mutation frequency of the *supF* gene (Figure 3). The mutation frequency of the plasmid was increased 4- to 50-fold following treatment of the plasmid with 70–270 mM formaldehyde. Nucleotide sequences of the *supF* mutation marker gene of 94 plasmids were determined. Mutated plasmids induced by 200–270 mM formaldehyde were classified and are shown in the third column of Table 1. About half (49%) of the plasmids had tandem base substitutions. Among the mutant plasmids with single base substitutions, 89% were substitutions of G:C base pairs (Table 1). The most common type of single base substitution mutation was G:C to T:A transversions (52%). Types of tandem base substitution are shown in Figure 4A. Thirty-nine percent were 5'-GG to 5'-TT (5'-CC to 5'-AA), and 15% were 5'-GA to 5'-AT (5'-TC to 5'-AT), and 5'-GA



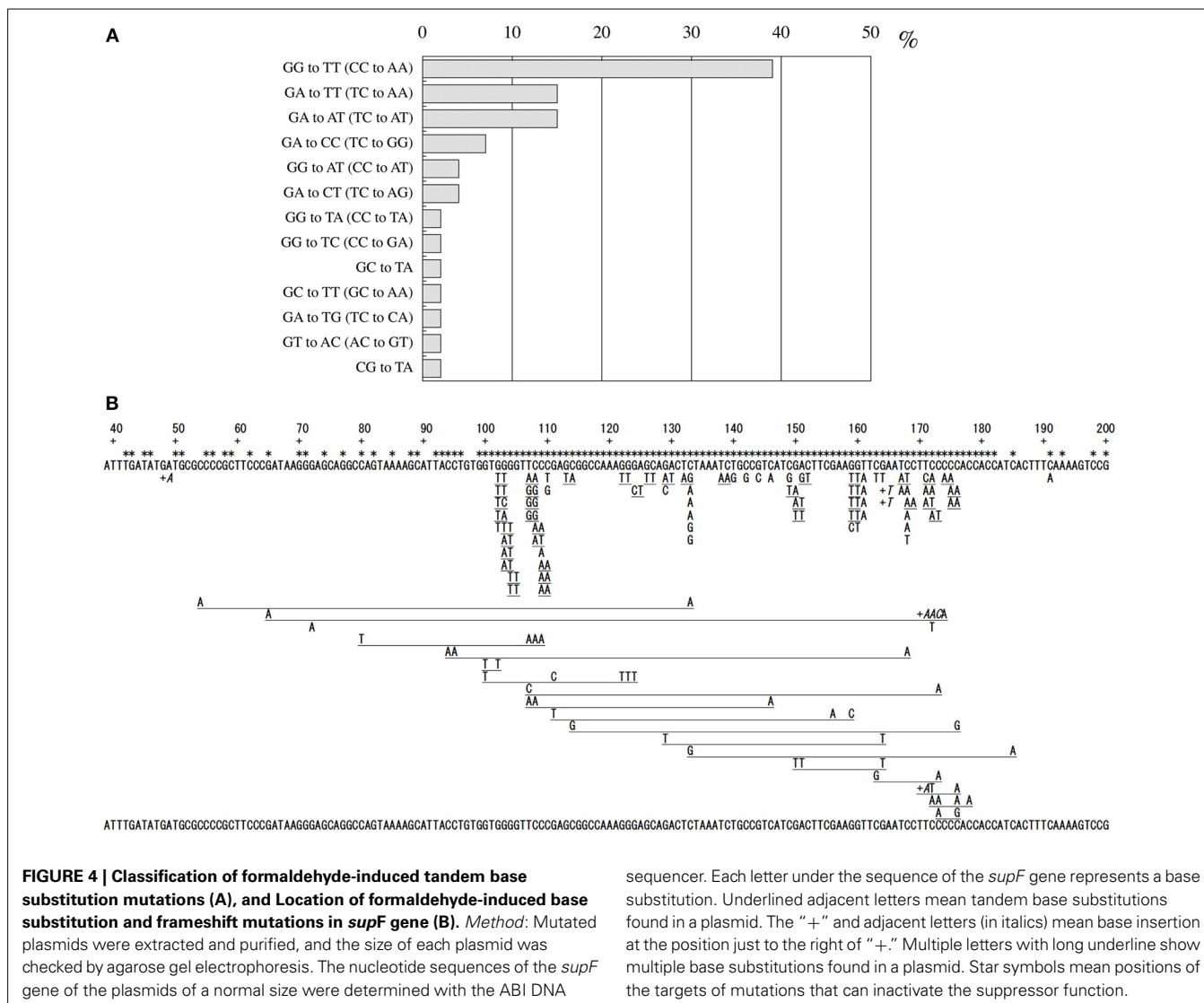
to 5'-TT (5'-TC to 5'-TT) substitutions. About half (47%) of them occurred at 5'-GG (5'-CC) sequences and 44% occurred at 5'-GA (5'-TC) sequences. The distribution of the formaldehyde-induced base substitutions in the *supF* gene is shown in Figure 4B. Underlined letters indicate that these base changes were found in the same mutated plasmid. The “d” or “del” means a deletion. The tandem base substitutions were not randomly distributed in the *supF* gene, but appeared preferentially at certain sites. About half of the tandem base substitutions occurred at 4 5'-GG (5'-CC) sequences (at base pairs 102–103, 103–104, 109–110, and 159–160) and 2 5'-GA (5'-TC) sequences (107–108 and 171–172).

Table 1 | Types of mutations induced by various aldehydes in supF shuttle vector plasmids propagated in mammalian cells.

Mutation type	References	Proportion of mutation type (%)								
		Spontaneous	Formaldehyde	Acetaldehyde	2-Chloro-acetaldehyde	Crotonaldehyde	Acrolein	Glyoxal	Methylglyoxal	
	Kawanishi et al., 1998b; Nakayama et al., 2004		Kawanishi, 1995	Matsuda et al., 1998	Matsuda et al., 1995	Kawanishi et al., 1998a	Kawanishi et al., 1998b	Murata-Kamiya et al., 1997	Murata-Kamiya et al., 2000	
BASE SUBSTITUTION										
Single	35	33	24	72	47	46	48	35 [#]		
Tandem	2	49	62	1	12	12	4	8		
Multiple	36	2	9	14	26	18	11	-		
Frameshift	7	3	0	1	6	0	4	4		
Others*	20	12	5	12	11	21	32	54		
Total	100	100	100	100	100	100	100	100	100	
MUTATION AT G:C										
G:C → A:T	34	14	8	54	23	24	13	17		
G:C → T:A	41	52	38	30	50	44	48	33		
G:C → C:G	23	23	54	6	13	12	23	39		
MUTATION AT A:T										
A:T → G:C	0	0	0	9	9	4	0	11		
A:T → T:A	0	8	0	1	4	12	17	0		
A:T → C:G	3	3	0	0	2	5	0	0		
Total	100	100	100	100	100	100	100	100	100	

*Others include deletion, insertion, and complex mutations.

[#]This number includes single and multiple mutations.



COMPARISON OF MUTATION SPECTRA WITH OTHER ALDEHYDES

Mutagenesis of various aldehydes such as acetaldehyde, 2-chloroacetaldehyde, crotonaldehyde, acrolein, glyoxal, and methylglyoxal has been examined using *supF* shuttle vector plasmids (Matsuda et al., 1995, 1998; Murata-Kamiya et al., 1997, 2000; Kawanishi et al., 1998a,b). These aldehydes react with DNA and form exocyclic guanines, ethenoguanine, and propanoguanine, as major adducts (Minko et al., 2009; Voulgaridou et al., 2011). These DNA lesions generate mutations mainly at G:C base pairs, but the spectra of base substitutions differ among aldehydes with different chemical structures. **Table 1** summarizes mutation spectra of various aldehydes, which are induced in the shuttle vector plasmids propagated in mammalian cells. All aldehydes induced base substitutions mainly at G:C base pairs. It should be noted that acetaldehyde induced tandem base substitutions at 5'-GG sequences like formaldehyde, suggesting that acetaldehyde forms intra-strand crosslinks between adjacent guanines, probably through propanoguanine formation (Matsuda et al.,

1998). Glyoxal is an endogenous mutagen produced abundantly in the process of DNA oxidation by reactive oxygen free radicals that have been thought to contribute to the pathogenesis of many age-related human diseases including cancer. Features of glyoxal-induced mutations are similar to those of spontaneous mutations (**Table 1**), which may suggest that glyoxal is a cause of spontaneous mutations.

CONCLUSION

Formaldehyde is one of the most abundantly exposed environmental mutagens for humans, and shown to be carcinogenic in experimental animals. Formaldehyde causes MN, SCE, and CA in mammalian cells and induces gene mutations in mammalian and bacterial cells. Formaldehyde acts as an electrophile to react mostly with guanine and adenine in DNA, and forms various kinds of DNA lesions. The DNA lesions are classified into DNA adducts, DNA intra-strand crosslinks, DNA inter-strand crosslinks and DNA-protein crosslinks. Organisms have mechanisms to remove these DNA lesions, which are specific to

each type of DNA lesions. If these DNA lesions are not repaired, mutations are induced during DNA replication. Formaldehyde-induced mutations in human cells were analyzed using shuttle vector plasmids (Kawanishi, 1995). Interestingly, about half of the formaldehyde-induced mutations were tandem base substitutions, and most of them occurred at 5'-GG (or 5'-CC) and 5'-GA (or 5'-TC) sequences. These mutation features agree with the predominance of formaldehyde-induced 5'-GG and 5'-GA crosslinks, indicating that the intra-strand crosslinks lead to tandem base substitutions. The prevalence of N^2 -hydroxymethyl adduct to guanine would lead to the G to T transversion that is the major single base substitution mutation. In early studies using bacteria, cultured cells and animals, exposure concentrations of formaldehyde would not have been sufficiently high to manifest the features of the mutation spectrum because formaldehyde mutagenicity is relatively weak and its acute cytotoxic effect is strong compared with those of other environmental mutagens. Our findings as well as the accumulation of molecular data provide clues to promote our understanding of the mechanisms behind the genotoxicity and carcinogenicity of formaldehyde.

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