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Advances in Brief

c-Ha-*ras* Containing 8-Hydroxyguanine at Codon 12 Induces Point Mutations at the Modified and Adjacent Positions[1]

Hiroyuki Kamiya, Kazunobu Miura,[2] Hiroyuki Ishikawa, Hideo Inoue, Susumu Nishimura, and Eiko Ohtsuka[3]

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-ku, Sapporo 060, Japan [H. K., K. M., H. Is., H. In., E. O.], and Biology Division, National Cancer Center Research Institute, 5-5-1 Tsukiji, Chuo-ku, Tokyo 104, Japan [S. N.]

Abstract

To determine the type of mutation induced by 8-hydroxyguanine in a mammalian system, we examined the mutations induced by a synthetic c-Ha-*ras* protooncogene containing 8-hydroxyguanine in the second position of codon 12 (GGC) in NIH3T3 cells. Transfection of this gene significantly increased the number of transformed foci. The c-Ha-*ras* gene present in these foci was analyzed by the polymerase chain reaction-restriction enzyme method. Interestingly, sequence analysis revealed random mutations at the modified site (G→T, G→A, and G→C) as well as mutations of the adjacent G on the 5′-side of 8-hydroxyguanine (G→A and G→T).

Introduction

oh[8]Gua[4] is one of the DNA lesions produced by oxygen-free radicals generated during cellular respiration, cell injury, and exposure to environmental oxygen radical-forming agents (1). Formation of oh[8]Gua in DNA is likely to be involved in mutagenesis and consequently carcinogenesis (2). An oh[8]Gua residue in DNA was found to induce exclusively G→T transversion *in vitro* in the polymerase reaction (3) and in a prokaryotic system using phage or plasmid with oh[8]Gua at a specific position (4–6). The DNA sequence of the *Escherichia coli mutM* mutator locus that generates G→T transversion was recently shown to be the same as that of the formamidopyrimidine glycosylase (oh[8]Gua endonuclease) gene (7–9). To determine the type of mutation induced by oh[8]Gua in a mammalian system, we examined the mutations induced by a synthetic c-Ha-*ras* protooncogene containing oh[8]Gua in the second position of codon 12 (GGC) in NIH3T3 cells. We found random mutations at the modified site (G→T, G→A, and G→C) and mutations of the adjacent G on the 5′-side of oh[8]Gua (G→A and G→T).

Materials and Methods

Enzymes. *Taq* DNA polymerase was obtained from Perkin Elmer. *Bss*HII and *Aat*II were purchased from Toyobo. *Msc*I and proteinase K were purchased from New England Biolabs. and Boehringer Mannheim, respectively. Other enzymes were obtained from Takara.

Oligonucleotides. The oligonucleotide containing oh[8]Gua was synthesized by the phosphoramidite method in an Applied Biosystems Model 380A DNA synthesizer. The procedures for phosphitylation of N^2-acetyl-8-methoxy-5′-*O*-monomethoxytrityl-2′-deoxyguanosine and purification and deprotection of the oligonucleotide will be reported elsewhere.[5] The other oligonucleotides were synthesized and purified as reported previously (10).

Construction of c-Ha-*ras* Vectors. Construction of the DNA cassette and its insertion into the vector using *Cla*I and *Bss*HII sites (indicated by shading) were carried out as described previously (10), except that a new plasmid pCB (11) was used instead of pRSV-rg12 (12).

DNA Transfection. The vectors were transfected into NIH3T3 cells by the calcium phosphate procedure as described previously (12, 13). Fifty, 150, or 500 ng of the vector DNA and 30 μg of genomic DNA isolated from NIH3T3 cells were used for each transfection assay.

Sequence Analysis of Synthetic c-Ha-*ras* Genes Present in the Transformed NIH3T3 Cells. The sequence in the region of codon 12 of the c-Ha-*ras* gene present in the transformants was analyzed by the polymerase chain reaction-restriction enzyme method described previously (10). The mutagenic primers for *Aat*II, *Sal*I, and *Bbe*I were described previously (10). In this experiment, *Bbe*I was used instead of *Nar*I. The sequence of the mutagenic primer used for *Stu*I, *Msc*I, and *Apa*I was 5′ AAGCTGGTGGTGGTGGN*G*GCC 3′, which corresponds to the human c-Ha-*ras* sequence from codon 5 to codon 11 except for the italicized *G* and bold N; N was A (*Stu*I cleavage), T (*Msc*I cleavage), or G (*Apa*I cleavage).

Results and Discussion

Fig. 1 shows a DNA cassette containing oh[8]Gua in the second position of codon 12 of the synthetic c-Ha-*ras* gene. The nucleotide sequence of this DNA cassette is according to that of a human c-Ha-*ras*-1 gene. The cassette was joined with a c-Ha-*ras*-expressing vector after cleavage with *Cla*I and *Bss*HII according to the procedure described previously (10), except for use of plasmid pCB (11) instead of pRSV-rg12 (12) to reduce the background focus formation induced by a normal *ras* gene. Control vectors containing GGC at codon 12 (normal) and GAC at codon 12 (activated) were also prepared by the same procedure. These vectors expressing c-Ha-*ras* were transfected into NIH3T3 cells by the calcium phosphate method (12, 13) for analysis of their focus-forming ability. The c-Ha-*ras* gene containing oh[8]Gua in the second position of codon 12 induced significantly more foci than the normal c-Ha-*ras* gene but about 1 to 2% of the number induced by the activated c-Ha-*ras* gene (GAC at codon 12, Asp-12) (Table 1). An enzyme responsible for removal of oh[8]Gua from DNA has been found in mammalian cells (14) as well as in *E. coli* (7), so the relatively low efficiency of transformation induced by the c-Ha-*ras* gene containing oh[8]Gua may be partly due to a repair reaction. Another reason for the low transformation efficiency is that the oh[8]Gua residue partly recognizes C during replication (3).

The sequence in the region of codon 12 of the c-Ha-*ras* gene present in the transformants was analyzed by the polymerase chain reaction-restriction enzyme method described previously (10, 15). After the polymerase chain reaction (16) using each mutagenic primer, the amplified DNA was treated with an appropriate restriction enzyme such as *Aat*II, *Sal*I, *Bbe*I, *Stu*I,

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[2] Present address: KRI International, Shimogyo-ku, Kyoto 600, Japan.

[3] To whom requests for reprints should be addressed.

[4] The abbreviation used is: oh[8]Gua, 8-hydroxyguanine.

[5] Inoue *et al.*, manuscript in preparation.

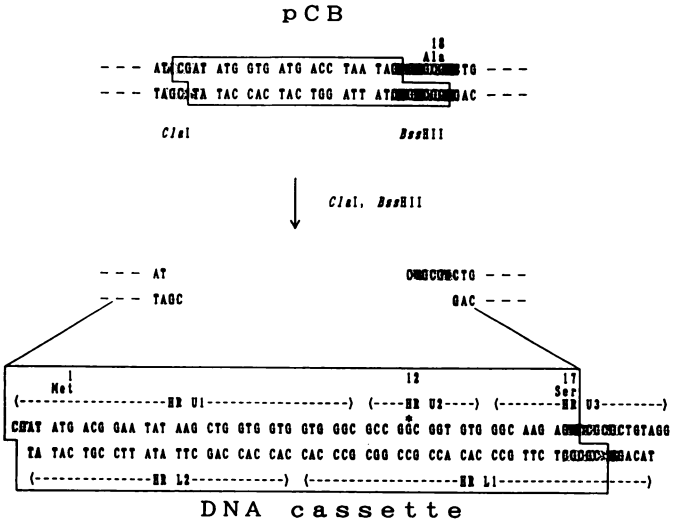

Fig. 1. Generation of a synthetic c-Ha-*ras* gene carrying oh^{8}Gua in the second position of codon 12. The oligonucleotide HRU2 which contains oh^{8}Gua (specified as G with * above) was synthesized by the phosphoramidite method in an Applied Biosystems Model 380A DNA synthesizer. Construction of the DNA cassette and its insertion into the vector using *Cla*I and *Bss*HII sites (indicated by *shading*) were carried out as described previously (10), except that a new plasmid pCB (11) was used instead of pRSV-rg12 (12).

*Msc*I, or *Apa*I. The three former enzymes were used to detect mutations to T, A, and C in the second position of codon 12, and the three latter to detect mutations to T, A, and C in the first position of codon 12. The third position (on the 3′-side of oh^{8}Gua) was not analyzed because no mutation in this position would alter the 12th amino acid. The cleavage was examined by polyacrylamide gel electrophoresis. Fig. 2*A* shows the results for the clone obtained with c-Ha-*ras* containing oh^{8}Gua in Experiment 3 in Table 1. Cleavage was detected when the amplified DNA obtained with the mutagenic primer was digested with *Aat*II (*Lane 1*), indicating that dAMP was incorporated into the site opposite the oh^{8}Gua residue, resulting in G→T transversion. Clones containing a c-Ha-*ras* gene with A or C in the modified position were also detected (Fig. 2, *B* and *C*). More strikingly, DNA from the clone obtained with c-Ha-*ras* containing oh^{8}Gua in Experiment 1 in Table 1 was cleaved by *Stu*I, indicating that dAMP was incorporated into the site opposite the flanking G, next to the oh^{8}Gua residue, resulting in G→T transversion (Fig. 2*D*, *Lane 4*). A clone with A in the flanking position was also detected (Fig. 2*E*). Of 18 clones analyzed, 14 clones were found to have a point mutation. Of these 14 clones, 12 clones had a mutation at the modified site (8 G→T transversions, 3 G→A transitions, and a single G→C transversion), and 2 clones had a mutation in the adjacent position (a single G→A transition and a single G→T transversion). These results were confirmed by nucleotide sequencing of the amplified DNA by Maxam-Gilbert analysis (17) (Fig. 3). The results confirmed the presence of point mutations in the position adjacent to oh^{8}Gua as well as in the position of oh^{8}Gua. Four of the 18 clones analyzed did not have a mutation at codon 12 of the human c-Ha-*ras*. This conclusion was supported by the finding that the polymerase chain reaction products were cleaved by *Hap*II or *Nae*I. No mutated gene was detected in 3 cases on amplification of DNA from foci induced by transfection of a normal c-Ha-*ras* gene (Fig. 2*F*).

Mutation assay after transfection of c-Ha-*ras* containing oh^{8}Gua into NIH3T3 cells clearly demonstrated that oh^{8}Gua in DNA induced G→T, A and C mutations in the position of the oh^{8}Gua residue, and also G→T and G→A mutations at the G residue next to oh^{8}Gua. This is in contrast to the findings in *in vitro* and *in vivo* systems of only G→T transversion in the position of oh^{8}Gua in *E. coli* (3–6). It should, however, be noted that the most frequent mutation in the mammalian system was also G→T transversion in the position of the oh^{8}Gua residue. *E. coli* oh^{8}Gua endonuclease (formamidopyrimidine glycosylase) can remove an oh^{8}Gua residue paired with C, T, or G (9). We recently found that mammalian oh^{8}Gua endonuclease can remove an oh^{8}Gua residue from duplex DNA (18). In contrast to prokaryotic cells, in mammalian cells an oh^{8}Gua:C pair is most preferentially recognized. This may be one reason why only G→T transversion was found in the position of oh^{8}Gua in DNA in *E. coli* systems.

We previously reported that oh^{8}Gua not only had the poten-

Table 1 *Number of foci induced by c-Ha-ras genes*

	Experiment 1[a]	Experiment 2[b]	Experiment 3[b]	Experiment 4[a]	Experiment 5[b]	Experiment 6[c]
Gly-12[d] (normal)	0	0	0	0	1	5
oh^8-Gua	2	4	1	2	3	18
Asp-12 (activated)	92	303	97	162	304	672

[a] Fifty ng of DNA were used.
[b] One-hundred fifty ng of DNA were used.
[c] Five hundred ng of DNA were used.
[d] Gly-12, C-Ha-*ras* gene have GGC at codon 12; oh^{8}Gua, C-Ha-*ras* gene having G (oh^{8}Gua) C at codon 12; Asp-12, C-Ha-*ras* gene having GAC at codon 12.

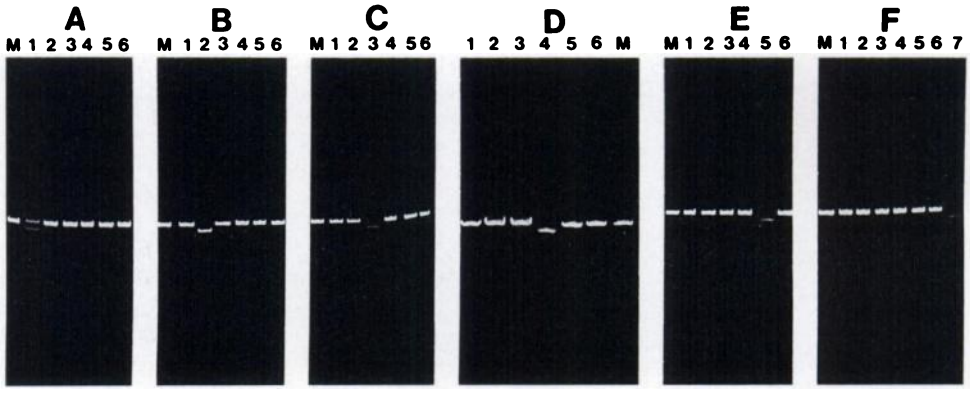

Fig. 2. Sequence analysis of synthetic c-Ha-*ras* genes present in the transformed NIH3T3 cells. *A* to *C*, analysis of clones with a mutation at the oh^{8}Gua site; *D* and *E*, analysis of clones with a mutation in the position adjacent to oh^{8}Gua; *F*, analysis of a clone obtained by transfection of a normal c-Ha-*ras* gene. *Lanes 1* to *3*, analysis of the second position of codon 12, *Aat*II for T (*Lane 1*), *Sal*I for A (*Lane 2*), and *Bbe*I for C (*Lane 3*). *Lanes 4* to *6*, analysis of the first position of codon 12, *Stu*I for T (*Lane 4*), *Msc*I for A (*Lane 5*), and *Apa*I for C (*Lane 6*). *Lane 7*, *Hap*II digestion for detection of a normal c-Ha-*ras* gene. *M* corresponds to the polymerase chain reaction product without treatment with the restriction enzymes.

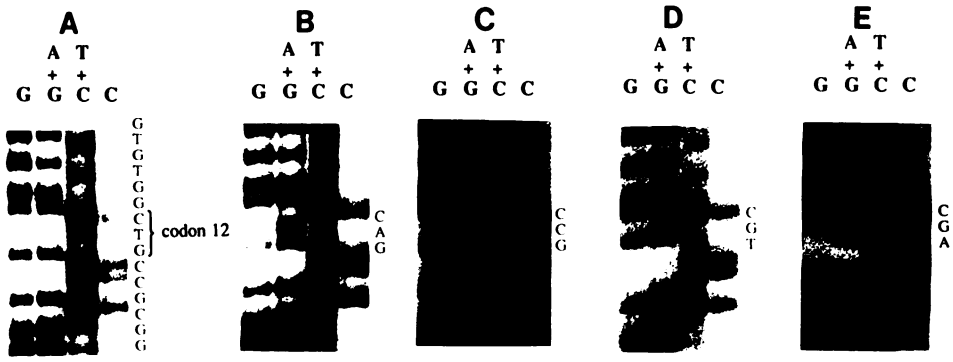

Fig. 3. Maxam-Gilbert sequencing of the polymerase chain reaction products. *A* to *E* correspond to the mutated c-Ha-*ras* genes found to have GTC, GAC, GCC, TGC, and AGC, respectively, at codon 12 by the polymerase chain reaction-restriction enzyme method.

tial to pair with any base, but also caused misinsertion at adjacent pyrimidines when DNA containing oh^{8}Gua was used as template for *in vitro* DNA synthesis with Klenow enzyme (19). This observation was somewhat analogous to the present findings. On the contrary, Shibutani *et al.* (3) recently reported selective incorporation of dAMP and dCMP into the site opposite oh^{8}Gua and no misincorporation at the neighboring site with Klenow enzyme or polymerase α. This discrepancy between our previous findings and those of Shibutani *et al.* may be due to the use of Klenow fragment and dideoxynucleotides. Exonuclease activity (proofreading activity) of the Klenow fragment may bring misreading oh^{8}Gua and the adjacent positions in the presence of a dideoxynucleotide (3).

The molecular mechanism of random incorporation of deoxynucleoside triphosphates into the site opposite to oh^{8}Gua and the incorporations of dAMP and dTMP at the G residue adjacent to oh^{8}Gua are not fully understood. The base pairings between oh^{8}Gua and C and between oh^{8}Gua and A were elucidated in nuclear magnetic resonance studies (20, 21). It is interesting that oh^{8}Gua can take either an *anti-* or *syn*-conformation depending upon the base in the complementary strand. These observations suggest that the conformation of oh^{8}Gua in DNA is energetically more mobile than that of guanine. In addition, the electron negativity of oh^{8}Gua was found to be quite different from that of normal guanine (22). Possibly the local structure of the DNA near oh^{8}Gua is modulated by the difference in mobility and electrostatic charge of oh^{8}Gua. The region around codon 12 of the c-Ha-*ras* gene is highly GC rich. Modification of a guanine base in a GC-rich sequence might induce local structure alteration such as B→Z transition (23). The present finding may reflect a mutation spectrum of oh^{8}Gua in DNA in a non-B-type structure to some extent. Various DNA polymerases and protein components involved in DNA replication in mammalian systems may also influence the fidelity of replication at the site opposite oh^{8}Gua *in vivo*, unlike in an *in vitro* system with purified polymerase α (3).

Further study would reveal the type of mutation induced by oh^{8}Gua in the second position of codon 12 of human c-Ha-*ras* more precisely. In addition, mutation analysis using human c-Ha-*ras* with oh^{8}Gua in the first position of codon 12 or in the first position of codon 61 in the antisense strand would provide more insight into the mechanism of mutagenesis by oh^{8}Gua in mammalian systems. Studies along these lines are in progress.

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