Different genetic alterations in rat forestomach tumors induced by genotoxic and non-genotoxic carcinogens

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Human beings are exposed to a multitude of carcinogens in their environment, and most cancers are considered to be chemically induced. Here we examined differences in genetic alterations in rat forestomach tumors induced by repeated exposure to a genotoxic carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methylnitrosourea (MNUR), and chronic treatment with a non-genotoxic carcinogen, butylated hydroxyanisole (BHA) or caffeic acid (CA). A total of 132, 6-week-old male F344 rats were employed. Forty rats were treated with MNNG by intragastric administration at a dose of 20 mg/kg body wt once a week for 32 weeks, and 20 rats received 20 p.p.m. MNUR in their drinking water for 48 weeks. Further groups of 20 animals were administered 2% BHA or 2% CA in the diet for 104 weeks. The remaining rats were maintained without any supplement as controls. Multiple forestomach tumors were observed in all rats of the MNNG-, MNUR-, BHA- and CA-treated groups. Histopathologically, MNUR- and CA-treated groups showed almost the same pattern. On polymerase chain reaction–single strand conformation polymorphism analysis, H-ras and p53 gene mutations were observed at high and relatively low frequencies, respectively, in forestomach tumors induced by MNNG and MNUR. Most H-ras gene mutations were G→A transitions in codons 7 and 12 of exon 1. On the other hand, forestomach tumors due to the non-genotoxic carcinogens, BHA and CA, had almost no mutations of the H-ras and p53 genes. Moreover, relative overexpression of cyclin D1 and p53 was detected in forestomach tumors induced by the genotoxic carcinogens, while their non-genotoxic counterparts had a tendency to show low expression of those molecules. Mutations of the β-catenin gene were not detected in any group. The present study demonstrates that rat forestomach tumors induced by genotoxic and non-genotoxic carcinogens have different underlying genetic alterations, even if their pathological features are similar.

Introduction

Carcinogenesis in man is believed to be a multi-step process, involving a progressive accumulation of genetic alterations (1,2). Human beings are exposed to numerous environmental carcinogens, and most cancers are considered to relate to these (3,4). While a number of genetic alterations have been associated with carcinogenesis in the human case, it is difficult to demonstrate clear relationships with specific environmental carcinogen exposure. In contrast, with experimental protocols in rodents, because only one carcinogen is employed to induce cancers, links can more readily be clarified.

Mutagenicity tests *in vitro*, such as the Ames test, are very important to detect genotoxicity of new chemicals for human risk assessment (5) and carcinogens are grouped into two classes, genotoxic and non-genotoxic agents (6). Genotoxic carcinogens, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methylnitrosourea (MNUR) and N-methyl-N-nitrosourea (MNU) are considered to interact with DNA in their target organ cells, for example in the forestomach, by alkylating DNA bases or forming DNA adducts, resulting in DNA damage and finally causing genetic alterations which are considered to be irreversible (7–10). For example, malignant forestomach tumors induced by MNU exhibit H-ras and p53 gene mutations, generally leading to G:C to A:T transitions related to alkylation (11). It has also been reported that in rat esophageal papillomas induced by N-nitrosomethylbenzylamine (NMBA), another genotoxic carcinogen, H-ras and p53 gene mutations are similarly present, with a high prevalence of G→A transitions in H-ras codon 12 (12,13).

Various phenolic compounds, which are non-genotoxic, such as butylated hydroxyanisole (BHA), caffeic acid (CA), sesamol and catechol, have been shown to have carcinogenic potential in the rat forestomach (14–18). While the forestomach hyperplasias and papillomas induced in the early stages readily regress after cessation of carcinogen administration (19–22), with long-term chronic application and maximum tolerated dosing regimens, non-genotoxic carcinogens will yield tumors, including carcinomas (14,15,18,23). However, it is still unclear if the molecular mechanisms of non-genotoxic agents induced forestomach carcinogenesis. It has been indicated that non-genotoxic carcinogens induced inflammation leading to carcinogenesis (24–27), but there have been no clear data. Moreover, the significance of genetic alterations and changes in protein expression in such tumors remains to be clarified, the question of whether variation exists from the situation with lesions induced by genotoxic carcinogens *in vivo* has received but little attention.

The most common gene alterations known in human and rodent tumors that appear to contribute to processes of carcinogenesis are point mutations in the p53 tumor suppressor gene (28,29) and in *ras* oncogenes (30,31). Cyclin D1 plays an important role as a main regulatory protein controlling the normal progression of cells through G1 (32,33) and cyclin D1 gene amplification and overexpression has been reported in...
human and rodent tumors of esophagus, breast, liver and colon (34–38). β-catenin plays an important role in the cadherin-mediated cell–cell adhesion system, and a high frequency of mutations in its gene has been reported in human and rodent tumors of colon, liver and lung (39–43).

In the present study, genetic alterations of H-ras, p53 and β-catenin genes in rat forestomach tumors induced by repeated exposure to single genotoxic carcinogens, MNNG and MNUR, and chronic treatment with a non-genotoxic carcinogen, BHA or CA, were examined using polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP). In addition, expression of p53, cyclin D1 and β-catenin protein was examined immunohistochemically.

Materials and methods

Animals
A total of 132, 6-week-old, male F344/DuCrj rats (Charles River Japan, Hino, Shiga, Japan) were randomly divided into six groups, housed four per a plastic cage with a 12 h light/dark cycle at a temperature of 22±2°C and 55±5% humidity, and weighted once a month. They were maintained on Oriental MF powdered basal diet (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. Carcinogens

The genotoxic carcinogens, MNNG, (purity >95%) and MNUR (purity >90%) were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan, and the non-genotoxic carcinogens, BHA (purity >98%) and CA (purity >98%) from Wako Pure Chemical Industries, Osaka, Japan.

Experimental design
The experimental design is shown in Figure 1. Forty rats were treated with an i.g. administration of MNNG at a dose of 20 mg/kg body wt once a week until week 32 (group 1). Twenty rats received MNUR at a dose of 20 p.p.m. in the drinking water for the 48 weeks (group 2). Further groups of 20 rats were given 2% BHA in Oriental MF diet (group 4) or 2% CA in MF diet for 104 weeks (group 5). The remainder received MF diet and tap water for 48 (group 3 and 104 weeks (group 6) as controls. The doses and methods for giving these four chemicals and the tumor induction time were in line with previously described results (14,15,19). All rats were killed under ether anesthesia at the end of the treatment schedule, or when they began to show moribund condition, and the stomach, liver and kidneys were immediately removed, the latter two being weighed. Large forestomach tumors were cut in two, one half frozen in liquid nitrogen immediately for DNA extraction, and the other, with the remaining tumors, was fixed in 10% phosphate-buffered formalin, embedded in paraffin and sectioned for pathological analysis.

Histopathology and immunohistochemistry

Sections (4 µm thick) of forestomach specimens were stained with hematoxylin and eosin for histopathological examination. Immunohistochemical staining was performed using the standard avidin–biotin peroxidase complex method with a Vectorstain ABC Elite kit (Vector Laboratories, Burlingame, CA). Serial sections were deparaffinized and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 5 min. Then sections were microwaved in citrate buffer (pH 6.1) for 30 min for antigen retrieval. Non-specific binding was blocked with 5% normal horse serum in PBS buffer in room temperature for 30 min. Incubation was performed with rabbit polyclonal p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution, rabbit polyclonal cyclin D1 antibody (Upstate Biotechnology, Lake Placid, NY) at 1:1500 dilution and mouse monoclonal β-catenin antibody (Transduction Laboratories, Lexington, KY) at 1:100 dilution, overnight at 4°C. Color was developed using 3,3′-diaminobenzidine and counterstaining was performed with Mayer’s hematoxylin. p53 and cyclin D1 overexpression was concluded when over 10% of nuclei were positive throughout a tumor.

PCR–SSCP analysis and direct sequencing

DNA was extracted using a Sepagene Kit (Sankyo Junyaku Co., Tokyo, Japan). For mutational analysis of exons 1 and 2 of the H-ras gene, exons 5–9 of the p53 gene and the β-catenin gene, non-radioisotopic SSCP analysis was performed as described previously (44,45) with minor modifications. The oligonucleotide primers used are listed in Table I. They were designed based on published sequences (39,46) and were labeled at 5′ terminus with Rhodamine for H-ras, β-catenin primers and FITC for H-ras and β-catenin primers (Takara Shuzo Co., Otsu, Japan). Most primers included both exon and intron portions to avoid amplification of pseudogenes. Hot-start PCR was carried out in a 5 µl reaction volume using AmpliTaq Gold (Perkin-Elmer Cetus Instruments, Norwalk, CT) under the conditions listed below; initial preheating at 94°C for 9 min to achieve enzymatic activity followed by 38 cycles (94°C for 30 s, annealing temperature 55°C for 40 s and 72°C for 30 s) and final elongation (72°C for 10 min). Five microliters of each reaction mixture was mixed with 145 µl of stop solution (95% formamide, 20 mM EDTA). Samples were heated for 3 min at 95°C and immediately chilled on ice for 10 min and loaded into 0.5× MDE gels both with and without 5% glycerol. Gels were run at 40 W for 3–5 h or 4 W for 16–18 h at room temperature. After electrophoresis, gels were visualized using an FMBIO II Multi-View fluorescent image analyzer (Takara Shuzo Co., Japan). DNAs from abnormally shifted bands on SSCP gels were cut out, dissolved in 15 µl distilled water and amplified by PCR using AmpliTaq Gold (Perkin-Elmer Cetus Instruments, Norwalk, CT). PCR products were subcloned using a TaKaRa BKL Kit (Takara Shuzo Co.) and recombinant colonies were picked up and amplified in 3 ml of LB culture medium. Plasmids were isolated using Wizard Plus SV Miniprep DNA Purification Systems (Promega Co., Madison, WI), Direct sequencing was performed using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA) and analyzed on a DNA Sequencing System (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) according to the manufacturer’s instructions.

Statistical analysis

Statistical comparisons between the different groups were completed with Stat View J-5.0 software (Abacus Concepts, Berkeley, CA), and significant differences were determined by the Fisher’s exact probability or χ² probability tests.

Results

Final body and relative organ weights
Final body weights in the MNUR (334 ± 22 g)- and BHA (359 ± 19 g)-treated groups were significantly decreased as compared with the respective control values (352 ± 17 and 438 ± 37 g, respectively) (P < 0.001). No differences were
Table II. Incidences of forestomach tumors and incidences of p53 and cyclin D1 overexpression in immunohistochemistry

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Effective number</th>
<th>Histology</th>
<th>Immunohistochemistry overexpression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Papillma</td>
<td>SCC</td>
</tr>
<tr>
<td>1</td>
<td>MNNG</td>
<td>30</td>
<td>9 (30)</td>
<td>21 (70)</td>
</tr>
<tr>
<td>2</td>
<td>MNUR</td>
<td>18</td>
<td>10 (56)</td>
<td>8 (44)</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>14</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4</td>
<td>BHA</td>
<td>18</td>
<td>16 (89)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>5</td>
<td>CA</td>
<td>18</td>
<td>12 (67)</td>
<td>6 (33)</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>16</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Significantly different from groups 1 and 4 (P < 0.0001) and groups 5 (P < 0.001); (–, not examined).

Fig. 2. Immunohistochemical analysis of p53 and cyclin D1 expression. Expression of p53-positive cells are demonstrated in a cluster pattern and distributed in both epithelium (A) and invasive area (B) in tumors of MNNG-treated group. These were demonstrated in mainly epithelium in CA-treated group (C). Cyclin D1-positive cells are demonstrated in a cluster pattern and distributed in tumor cells of MNUR-treated group (D). The microscopic magnifications are: (A) and (D), ×200; (B) and (C) ×400.

observed for the MNNG (355 ± 28 g)- and CA (421 ± 25 g)-treated groups. Relative liver weights of the MNUR-treated group (2.6%) were significantly decreased while in the BHA-treated group (3.4%) a significant increase was noted as compared with the control values (3.0 and 2.9%, respectively) (P < 0.001).

Macroscopic and microscopic findings

Macroscopically, multiple polypoid or califlower-shaped tumors were observed at an incidence of 100% in the MNNG-, MNUR-, BHA- and CA-treated groups. Histopathologically, the incidences of squamous cell carcinoma (SCC) were 70% in group 1, 44% in group 2, 11% in group 4 and 33% in group 5 (Table II). The other tumors were squamous cell papillomas with downward growth and basal cell hyperplasia with mild atypia. No tumors were observed in control groups 3 and 6.

Immunohistochemistry

Findings of the immunohistochemical analyses are summarized in Table II. p53-positive cells demonstrated cluster pattern and distributed both within tumors (Figure 2A) and in invasive areas (Figure 2B) with lesions of the MNNG- and MNUR-treated groups. Seven tumors (23%) (six cases of SCC and one papilloma) in the MNNG-treated group and four (22%) (three SCCs and one papilloma) in the MNUR-treated group demonstrated p53 overexpression. On the other hand, this was the case for only one tumor (6%) (a papilloma) in the CA-treated group (Figure 2C) and no overexpression was found.
Table III. Summary of incidences of H-ras, p53 and β-catenin mutations in forestomach tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of samples</th>
<th>H-ras mutation</th>
<th>p53 mutation</th>
<th>β-catenin mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNNG</td>
<td>30</td>
<td>20 (67%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 (13%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>MNUR</td>
<td>18</td>
<td>10 (56%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 (17%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>4</td>
<td>BHA</td>
<td>18</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>5</td>
<td>CA</td>
<td>18</td>
<td>1 (6%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly different from groups 4 and 5 (P < 0.0001).  
<sup>b</sup>Significantly different from groups 4 and 5 (P < 0.001).

Table IV. Summary of H-ras and p53 gene mutations spectra

<table>
<thead>
<tr>
<th>H-ras mutations</th>
<th>p53 mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codon 7 GTG → GTA</td>
<td>Codon 12 GGA → GAA</td>
</tr>
<tr>
<td>1 20 13 14 0 2</td>
<td>1 6 209 ACT → TCT</td>
</tr>
<tr>
<td>2 10 9 8 0 0</td>
<td>6 231 CAC → CAT</td>
</tr>
<tr>
<td>4 1 0 0 1 2</td>
<td>6 238 AGC → GCC</td>
</tr>
<tr>
<td>5 1 0 0 1 2</td>
<td>7 276 CCT → TCT</td>
</tr>
<tr>
<td>6 9 2 0 1 2</td>
<td>6 190 CAA → CAC</td>
</tr>
<tr>
<td>7 186 GAG → GGG</td>
<td></td>
</tr>
<tr>
<td>8 306 CTG → CTA</td>
<td></td>
</tr>
</tbody>
</table>
genotoxic and non-genotoxic carcinogens are similar, marked differences exist in alteration of genetic pathways or protein expression.

Thus, H-ras mutations, G→A transitions in codons 7 or 12 were observed at high frequency in forestomach tumors induced by the genotoxic carcinogens, MNNG and MNU. On the other hand, those induced by non-genotoxic carcinogens, BHA and CA, had no H-ras mutations, the single exception being a CA-induced lesion with a H-ras G→C transition in codon 13. The present data showed that the incidence of SCC was high in the MNNG-treated group and low in BHA-treated group, but values for the MNU and CA-treated groups were both moderate. Moreover, four (20%) papillomas in the MNNG-treated group and seven (70%) papillomas in the MNU-treated group were shown to have H-ras mutations. The data demonstrate that the different frequency of these mutations between tumors induced by genotoxic and non-genotoxic carcinogens is present without relation to the histological pattern.

Our present study indicated that characteristic H-ras G→A transitions at codons 7 and 12 play important roles in forestomach carcinogenesis by genotoxic agents. It has been reported that the presence of θ-methylguanine causes misrepairing with deoxothymidine during DNA replication, leading to base substitutions from G to A (47–50). Thus, it is possible that θ-methylguanine caused the G→A transitions in the forestomach tumors induced by genotoxic carcinogens in the present study.

Our findings further indicate that p53 point mutations are present at relatively low frequencies in forestomach tumors of rats treated with the genotoxic carcinogens, MNNG and MNU. Whereas characteristic H-ras G→A transitions were observed with high frequency, no mutational patterns were apparent within the p53 gene. Various p53 mutations have been reported in rat carcinomas induced by genotoxic carcinogens, such as esophageus, liver and nose, but most of them demonstrated only low incidence and lacking mutational hot spots (12,51,52). Our present data are thus in line with the literature, except for the MNU, which p53 G→A transitions were detected in four (80%) of five forestomach tumors (11). This apparent carcinogen dependence warrants further study and p53 mutations may make some contribution to forestomach carcinogenesis induced by genotoxic agents. Again, this appears not to be the case with lesions induced by non-genotoxic carcinogens. However, in the present study, only a mutation analysis was performed in the section for a p53 gene, and epigenetic changes such as methylation of tumor suppressers is known to occur, so that examination of this point is also necessary in the future.

It is reported that G→A point mutations at H-ras codon 12 are frequent in esophageal papillomas induced by NMBA, and G:C→A:T transitions in the p53 gene have been found in ~40% of esophageal papillomas (12,13). Therefore, it might be concluded that these transitions are important genetic alterations in carcinogenesis in squamous epithelium.

However, to our knowledge, there have been few reports about the molecular mechanisms of rodent forestomach tumorigenses induced by non-genotoxic carcinogens. BHA, CA and 4-methoxyphenol are known to cause overexpression of the protooncogenes, c-myc and c-fos only 15 min after oral treatment, associated with increase in anti-bromodeoxyuridine immunohistochemical staining of squamous epithelial cells (24,25). In our recent study, proliferating cell nuclear antigen-positive indices and cyclooxygenase-2 overexpression were found to be elevated in hyperplastic epithelial lesions of the rat forestomach induced by BHA or CA treatment for 8 weeks (unpublished data). Hirose et al. (25,26) suggested that non-genotoxic carcinogens were characterized by induction of severe cell proliferation as well as toxicity-dependent changes such as inflammation, erosion and ulceration in the forestomach during chemical treatment (24,27). Therefore, it may be that continuous stimulation of cell proliferation caused by regeneration leads to development of tumors.

Cyclin D1 plays an important role as a main regulatory protein controlling the normal progression of cells through G1 (32,33). In the present study, relative expression of cyclin D1 in forestomach tumors induced by genotoxic carcinogens had a tendency to show higher than their non-genotoxic counterparts. However, concerning the non-genotoxic carcinogen-treated groups, 28% of CA-induced forestomach tumors showed cyclin D1 overexpression immunohistochemically, while only low expression was evident in BHA-induced lesions. Although it is reported that non-genotoxic carcinogens are characterized by induction of marked cell proliferation (24–27), there has been no reports, to our knowledge, about cyclin D1 expression in non-genotoxic agent-induced forestomach tumorigenesis. Our present data indicate that difference in cyclin D1 regulation of cell proliferation may exist between non-genotoxic carcinogen. Also, concerning genotoxic carcinogens, although almost all MNU-induced tumors showed cyclin D1 overexpression, this was not the case with their counterparts in MNNG-treated animals.

While β-catenin mutations or aberrant protein expression have been detected in several tumors of rats (39–43), they were lacking in lesions induced by both genotoxic and non-genotoxic carcinogens in the present study, so that the Wnt-β-catenin signaling pathway may not have significance for forestomach carcinogenesis.

In conclusion, our present data demonstrate clear differences in frequencies of alterations in genes such as H-ras and p53 in tumors induced by genotoxic or non-genotoxic carcinogens, even if their pathological features are similar, providing support for the conclusion that genetic mechanisms for rat forestomach carcinogenesis are different between genotoxic carcinogen and non-genotoxic carcinogen. They further point to the importance of in vitro mutagenicity tests to detect genotoxicity of new chemicals for human cancer risk assessment. Clearly, different factors other than those examined genetic alterations also may have impact on genotoxic and non-genotoxic forestomach carcinogenesis. Recently, cDNA microarrays have been used to search for global patterns of gene expression, and this approach should similarly be applied in further study.

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References


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