p53 and Ha-ras mutations in chemically induced hamster buccal pouch carcinomas

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Well-differentiated squamous cell carcinomas were induced in hamster buccal pouch epithelium by twice weekly topical applications of N-methyl-N-benzyl nitrosamine (MBN) or 7,12-dimethylbenz[a]anthracene (DMBA) over a period of 15 weeks. Each of the 22 tumors induced (14 MBN and eight DMBA) were evaluated by single-strand conformation polymorphism and DNA sequencing to identify mutations in conserved exons (E5–E8) of the p53 tumor suppressor gene and codons 12/13 and 61 of Ha-ras. In addition, Northern blot analysis of 10 MBN tumors and five DMBA tumors was performed to determine whether the mdm-2 gene was overexpressed. p53 mutations were detected in five of 14 (35%) MBN-induced carcinomas and in two of eight (25%) DMBA-induced carcinomas. Ha-ras mutations were detected in three of 14 (21%) MBN-induced carcinomas and in three of eight (37%) DMBA-induced carcinomas. One MBN-induced carcinoma exhibited a mutation in both the p53 and Ha-ras genes. The majority (five of seven) of p53/Ha-ras mutations induced by MBN were G→A transitions and two of these occurred at hamster p53 codon 248, which corresponds to human p53 codon 245, a known functional tumor ‘hot spot’. A→T transversion at Ha-ras codon 61 accounted for three of five (60%) DMBA-induced mutations. There was no evidence of mdm-2 overexpression in any of the tumors evaluated. Overall, the results provide additional support for the validity of the hamster buccal pouch model of oral carcinogenesis, as applied to sequential cellular and molecular analysis and cancer chemoprevention studies.

Introduction

The hamster buccal pouch (HBP*) model of oral carcinogenesis has been used extensively for the study of premalignant processes (1–8), identification of putative intermediate markers of carcinoma development (1–10) and evaluation of potential cancer chemopreventive agents (11–14). This model recapitulates many of the features observed during oral carcinogenesis in man (15–17). For example, many of the structural alterations observed in carcinogen-treated hamster pouch mucosa, both grossly and at the light microscopic level, closely resemble those observed during the course of human oral cancer development (15,16,18). Some of the specific molecular changes which are likely to have mechanistic relevance to oral carcinoma development in man have also been identified and linked to temporal stages in carcinogenesis in this model. Biswas and colleagues have demonstrated that Ha-ras mutations occur in the very early pre-dysplastic phase of HBP carcinogenesis mediated by 7,12-dimethylbenz[a]anthracene (DMBA) and that late events include amplification and overexpression of c-erbB and its product, the epidermal growth factor receptor (19,20).

Alterations in the p53 tumor suppressor gene and its expression occur in a majority of head and neck carcinomas and their precursor lesions, including those of oral mucosa (21–25). Cell hybridization studies have provided evidence that loss of suppressor gene function may also play a role in cancer development in the HBP model (7). In addition, Conti and colleagues have recently obtained immunohistochemical evidence that endophytic DMBA-induced HBP carcinomas express an altered protein product resulting from mutation in the p53 tumor suppressor gene (26). In view of these observations, the present study was undertaken to identify and characterize p53 and Ha-ras mutations occurring in the HBP model of oral carcinogenesis. The results demonstrate the occurrence of p53 and Ha-ras mutations in HBP carcinomas induced with two different chemical carcinogens.

Materials and methods

Animals and carcinogen treatment

Six male Syrian golden hamsters, 3–5 weeks of age, were treated by painting both buccal pouches each Monday and Friday for 15 weeks with a 1% solution of W-methyl-N-benzylnitrosamine (MBN) dissolved in propylene glycol (18). Six hamsters were similarly treated twice weekly with a 0.5% solution of DMBA in mineral oil (4). Individual control hamsters were also treated in a similar manner with either solvent alone. Within 1 week after completing treatment the hamsters were sacrificed and gross tumors were excised and analyzed for the presence or mutations of p53 exons 5–8 and Ha-ras mutations at codons 12/13 and 61. A small section of each tumor was also fixed in formalin and processed for routine histological evaluation.

DNA and RNA isolation

Tumor DNA was isolated by conventional proteinase K/phenol/chloroform extraction (27). Tumors of sufficient size were used to extract RNA to make DNA. Polymorphic DNA was detected using the RNAzol (Tel-Test Inc., Friendswood, TX) method (28).

Polymerase chain reaction

Segments of the hamster p53 gene were amplified in vitro by PCR, as previously described (29), using the following oligonucleotide primers:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Hap53-14</td>
<td>5′-TCATCGCTCCAACCTGACCCTG-3′</td>
</tr>
<tr>
<td>Hap53-15A</td>
<td>5′-AAGAGCAATCAAAGACTCAACG-3′</td>
</tr>
<tr>
<td>Hap53-15</td>
<td>5′-CAATTAGAAATGCTTGCCTGGGG-3′</td>
</tr>
<tr>
<td>Hap53-15</td>
<td>5′-CCAGCTGACGGGCTAGTTCGCC-3′</td>
</tr>
<tr>
<td>Hap53-16</td>
<td>5′-AGCTTCTGATGACGAACTAAAC-3′</td>
</tr>
<tr>
<td>Hap53-17A</td>
<td>5′-AAACACGCAAAGCAGACAGCAA-3′</td>
</tr>
<tr>
<td>Hap53-17</td>
<td>5′-CTACTGCTGCTGCCCTCCTCCTC-3′</td>
</tr>
<tr>
<td>Hap53-18A</td>
<td>5′-TGAAGCTGACGGGCTAGTTCGCC-3′</td>
</tr>
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</table>

*Abbreviations: HBP, hamster buccal pouch; DMBA, 7,12-dimethylbenz[a]anthracene; MBN, N-methyl-N-benzyl nitrosamine; SSCP, single-strand conformation polymorphism; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
A hamster Ha-ras sequence of 68 bp, including codons 12 and 13 of exon 1, was amplified using the synthetic oligonucleotide rat primers 5'-AACCTGTGTTGTTGTGC-3' and 3'-TAGGTCAATGTTGCTTGTT-5' (30). A hamster Ha-ras sequence of 175 bp, including codon 61 of exon 2, was amplified using the mouse primers 5'-GACTCCCTACCGGAAACAGGT-3' and 3'-CTTCTGTAGTAGTCTCTGC-5' (20). Parameters for amplification were 1 min at 94°C, 1 min at 55-57°C and 1 min at 72°C for 35 cycles. All amplifications were performed in a TempCycler II (Grass Lake, MI).

Single-stranded conformation polymorphism (SSCP) analysis

A primary amplification was executed in duplicate as described above with unlabeled primers. The sense strand was analyzed in a second amplification in which the only sense oligonucleotide was end-labeled with [y-32P]ATP (DuPont/New England Nuclear, Wilmington, DE) using T4 polynucleotide kinase (Promega, Madison, WI). The amplified product with only one strand radioactively tagged was added to a formamide-based loading buffer, denatured at 95°C for 5 min, cooled on ice for 10 min and loaded onto a 1 X MDE gel matrix (AT Biochem, Malvern, PA) with 1 X Tris-borate, EDTA buffer (29). Electrophoreses were at 20 W constant power with two fans used to maintain a high stringency buffer. The amplified product was loaded on a high performance gel with an electrophoresis buffer containing 10% sucrose, 40% glycerol and a xylene cyanol dye marker (29). Electrophoreses were run at 40 W constant power with two fans used to maintain a high stringency buffer. The gels were transferred to Whatman 3MM paper and vacuum dried at 70°C. Autoradiography was at -70°C for 2-5 h with one intensifying screen (Hyperscreen; Amersham, Arlington Heights, IL) and Hyperfilm-MP (Amersham) (29).

Cloning and sequencing

Abnormally shifted bands revealed by SSCP were excised from each gel after electrophoreses were at 20 W constant power with two fans used to maintain a high stringency buffer. The gels were transferred to Whatman 3MM paper and vacuum dried at 70°C. Autoradiography was at -70°C for 2-5 h with one intensifying screen (Hyperscreen; Amersham, Arlington Heights, IL) and Hyperfilm-MP (Amersham) (29).

Table I. p53 and Ha-ras mutations in MBN and DMBA induced hamster buccal pouch carcinomas

<table>
<thead>
<tr>
<th>Hamster</th>
<th>Tumor</th>
<th>p53 exon</th>
<th>Ha-ras codon</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>E5</td>
<td>E7</td>
</tr>
<tr>
<td>MBN</td>
<td></td>
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<td></td>
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<tr>
<td>A</td>
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<td>B</td>
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<td>3</td>
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<tr>
<td>B</td>
<td></td>
<td>4</td>
<td>GGC→AGC</td>
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<tr>
<td>C</td>
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<td></td>
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<tr>
<td>C</td>
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<tr>
<td>C</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>9</td>
<td>CCC→TCC</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>10</td>
<td></td>
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<tr>
<td>D</td>
<td></td>
<td>11</td>
<td>GAG→GAA</td>
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<tr>
<td>E</td>
<td></td>
<td>12</td>
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<tr>
<td>E</td>
<td></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>14</td>
<td>GGC→GAC</td>
</tr>
<tr>
<td>DMBA</td>
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<tr>
<td>A</td>
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<td>1</td>
<td></td>
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<tr>
<td>A</td>
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<td>B</td>
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<tr>
<td>C</td>
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<td>Intron 5</td>
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<tr>
<td>D</td>
<td></td>
<td>6</td>
<td></td>
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<tr>
<td>D</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>8</td>
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</table>

*MBN indicates N-methyl-N-benzyl-N-nitrosamine-induced tumors, DMBA indicates 7,12-dimethylbenz[a]anthracene-induced tumors. Mutations resulting in altered protein sequences are shown in bold. The codon site exhibiting a mutation and the resulting amino acid change or the type of intronic mutation is shown in parentheses.

Results

Fourteen MBN-induced carcinomas and eight DMBA-induced carcinomas were obtained for evaluation by SSCP analysis and sequencing of their p53 and Ha-ras DNAs. Each of the tumors were well differentiated squamous cell carcinomas. No histological differences were observed between the carcinomas.
induced by MBN and those induced by DMBA. p53 mutations were detected in five of the 14 (35%) MBN-induced carcinomas and in two of the eight (25%) DMBA-induced carcinomas. Ha-ras mutations were detected in three (21%) of the MBN-induced carcinomas and in three (37%) of the DMBA-induced carcinomas. The results are summarized in Table I.

Seven of the 14 MBN-induced tumors showed altered band migrations of p53 or Ha-ras DNA by SSCP analysis. An example of the SSCP results is shown in Figure 1a. DNA sequencing of these bands confirmed five p53 point mutations involving exons 5 and 7 and intron 8 and three Ha-ras mutations involving codons 12 and 61 of exons 1 and 2 respectively. Examples of the sequencing results are shown in Figures 1b, 2b and 3. The most common mutations observed (63%) were G→A transitions. One tumor (tumor 14; Table I) exhibited a protein-altering mutation in both p53 and Ha-ras. Of the five tumors exhibiting a p53 mutation, three (tumors 4, 9 and 14; Table I) contained sequences coding for an altered protein product. MBN tumor 10 exhibited deletion of a G in p53 intron 8. MBN tumor 11, from the same hamster, exhibited a silent G→A transition at codon 183 of p53 exon 5 (Figure 1b), resulting in retention of a glutamate.

Five of eight DMBA-induced tumors exhibited altered band migrations of p53 or Ha-ras DNA sequences by SSCP analysis. An example is shown in Figure 2a. In each case the band shifts resulted from a single point mutation, as determined by DNA sequencing. An A→T transversion at Ha-ras codon 61 accounted for three (60%) of the observed DMBA-induced mutations. Only one protein-altering p53 mutation, a C→T transition at exon 8 codon 281, resulting in the replacement of a proline with a serine, was observed in this small group of DMBA-induced tumors.

Sufficient RNA was obtained from 10 MBN and five DMBA tumors for Northern blot analysis of mdm-2 gene expression. In each case the level of mdm-2 expression was similar to that of control hamster pouch tissue. Examples of mdm-2 expression levels in four MBN-induced tumors are shown in Figure 4.

Discussion

In this study nine of 22 (41%) chemically induced carcinomas in HBP epithelium exhibited a mutation in Ha-ras or the conserved exons of the p53 gene. Only one of the 22 tumors evaluated exhibited a mutation in both p53 and Ha-ras. Previous studies have suggested a role for Ha-ras overexpres-

![Fig. 1](image1.png)

**Fig. 1.** (a) SSCP analysis of a portion of exon 5 (E5b) of 22 buccal pouch carcinomas shows a band shift in MBN tumors 9 and 11. A band shift is also observed in lane 5S, using DNA derived from an incidental perioral skin carcinoma from a DMBA-treated hamster. (b) Sequencing demonstrates a C→T transition at codon 180 and a silent G→A transition at codon 183 in MBN tumors 9 and 11 respectively (see Table I).

![Fig. 2](image2.png)

**Fig. 2.** (a) SSCP analysis of the Ha-ras codon 61 region of eight DMBA-induced carcinomas exhibits equivalent band shifts for tumors 1, 3 and 8. (b) Sequencing of DNA derived from an MBN (1) and an DMBA (1) tumor demonstrates an A→T transversion in the second position of Ha-ras codon 61.

![Fig. 3](image3.png)

**Fig. 3.** Sequence of DNA derived from an MBN tumor (2) following SSCP analysis (data not shown) demonstrates a G→A transition in the second position of Ha-ras codon 12.
A study of MBN-induced rat esophageal papillomas, wherein the frequency of point mutations in the $p53$ gene in HBP carcinogenesis. The specific mutations observed in MBN-induced esophageal papillomas of the rat in the present study were three of 14 (21%) MBN-induced carcinomas exhibited $p53$ protein-altering point mutations. The majority of MBN-induced mutations were $G \rightarrow A$ transitions and it is intriguing to note that $G \rightarrow A$ transitions are the most common $p53$ mutations occurring in human head and neck cancer, including those of oral mucosa (34). It should also be noted that the two $p53$ protein-altering $G \rightarrow A$ transitions observed in MBN-induced carcinomas (tumors 4 and 14; Table 1) occurred at hamster codon 248, which corresponds to human $p53$ codon 245 (35), a known high frequency mutational 'hot spot' for human tumors (34).

It is well established that $p53$ dysfunction may also result from mechanisms other than mutation (36). For example, $p53$ functions may be altered when the wild-type $p53$ protein binds to specific viral proteins or to endogenous mdm-2 protein, the product of a gene which is overexpressed in a variety of neoplasms (36–39). However, in the present study there was no evidence of mdm-2 overexpression in any of the buccal pouch carcinomas examined.

The present study describes specific genetic changes in chemically induced HBP carcinomas which have frequently been observed in human oral carcinomas. The spectrum of molecular alterations identified in oral carcinomas exhibit wide global variations, which appear to be largely a function of indigenous chewing habits, smoking habits and alcoholic beverage consumption (21,22,34,40–46). For example, Ha-ras mutations are observed in oral carcinomas induced by constituents of the betel quid popular in many Asian cultures (40). These mutations are an infrequent occurrence among populations where smoking tobacco products and alcohol are the principal causes of oral cancer (41–44). Conversely, whereas the frequency of $p53$ mutations in oral carcinoma is high among patient populations in the West (21,22,34), $p53$ mutations are infrequent in specific Eastern populations, where chewing of betel quid, with or without tobacco, is most strongly implicated (45,46). As the present study demonstrates, both Ha-ras and $p53$ mutations may arise during HBP carcinogenesis. The apparent overlap in the spectrum of molecular lesions observed in oral and HBP carcinomas should provide further support for the analytical value of this animal model.

Several immunohistochemical studies have demonstrated $p53$ expression in pre-invasive oral lesions and thus it has been proposed that $p53$ mutation is a common early event in oral carcinogenesis (23–25). Expression of wild-type $p53$ is up-regulated when DNA is damaged and this leads to a block in cell replication which enables DNA repair processes to proceed with fidelity (47–49). In principle, loss of this normal function is likely to confer a selective growth advantage on mutant $p53$ pre-cancerous cell populations in the oral mucosa and contribute to their genetic instability and malignant progression in response to repeated exposures to tobacco-specific N-nitrosamines and other DNA damaging agents in tobacco products. Similar mechanisms are likely to foster clonal expansion of pre-cancerous lesions in the HBP model, wherein acute inhibition of cell replication results from carcinogen application (8,50). If $p53$ mutation is also a frequent early event in MBN-mediated HBP carcinogenesis, this model may prove valuable for a detailed analysis of the sequence of molecular events in carcinogen-exposed squamous mucosa beginning with early $p53$ mutant cell populations.

The HBP model has been used extensively for cancer chemoprevention studies, most notably in studies of retinoids and related compounds, which appear efficacious in retarding...
References

13. Schwartz, J. L., Tanaka, J., Khandekar, V., Herman, T. S., and Teicher, B. A. (1992) Beta-carotene and vitamin E as modulators of alkylating agents in development of tobacco smoke-induced lesions in humans (11–14, 51–53). The future identification of very early (e.g., pre-invasive or pre-dysplastic) carcinogen-induced mutant p53 lesions in hamster pouch epithelium could also provide a valuable new approach for evaluating presumptive cancer chemopreventive agents for oral mucosa and contiguous head and neck sites. If MBN-induced p53 mutant populations arise early during HBP carcinogenesis, in a manner similar to their early occurrence in human oral mucosa, the identification of early focal mutant p53 populations in buccal pouch epithelium may serve as a rapid quantitative index of ongoing carcinogenesis. Predictably, prior and concurrent administration of effective oral anticarcinogenic agents would impede or prevent the induction, clonal expansion, dysplastic conversion or malignant progression of these early mutant p53 lesions. The utility of HBP epithelium for enumeration of early carcinogen-induced intra-epithelial pre-cancerous lesions (4,18,54) and their numerical reduction by concurrent administration of anticarcinogen has been previously demonstrated (14,55).

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References


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