Increased Mutagen Sensitivity in Patients With Head and Neck Cancer Is Less Pronounced in Patients With Nasopharyngeal Carcinoma

Jenq-Yuh Ko, MD; Louis Tak Lui, MD; Tzung-Shiahn Sheen, MD; Pei-Jen Lou, MD; Mow-Ming Hsu, MD

Background: Mutagen sensitivity tested with bleomycin sulfate can determine a susceptible phenotype, which is relevant only in organs and tissues that have direct contact with the external environment. Patients with head and neck cancers have more mutagen sensitivity than control subjects without cancer, and the hypersensitive phenotype has a risk for the development of a second primary cancer. Head and neck cancers, however, represent a heterogeneous group of neoplasms. The biological behavior of nasopharyngeal carcinoma (NPC) and other head and neck cancers differs.

Objective: To evaluate the difference in mutagen sensitivity among patients without cancer, patients with NPC, patients with oral or oropharyngeal cancer (ORC), and patients with laryngeal or hypopharyngeal cancer (LHC).

Design: Peripheral blood was cultured at 37°C, using 5% carbon dioxide, for 72 hours. After 67 hours of incubation, bleomycin in a concentration of 30 IU/L was added to induce chromatid breaks. The number of chromatid breaks per cell was scored in 50 metaphases of cultured lymphocytes and compared in the 4 groups.

Subjects: Patients with histologically proven squamous cell carcinoma of the mucosa of the upper digestive tract, which included 3 groups: patients with NPC, patients with ORC, and those with LHC. Control subjects were hospital inpatients with no tumor history. There were 35 patients in each group.

Results: The mean (±SD) number of breaks per cell in the control group and in the groups with NPC, ORC, and LHC were 0.80 (±0.32), 1.03 (±0.45), 1.30 (±0.44), and 1.35 (±0.46), respectively. All the cancer groups had significantly higher mean breaks per cell and a higher prevalence of hypersensitivity than the control group. Patients with NPC had a significantly lower mean number of breaks per cell than the group with ORC or that with LHC.

Conclusions: Patients with NPC had less mutagen sensitivity than those with ORC or LHC. Our results support the clinical and epidemiological findings of a difference between NPC and other head and neck cancers. Environmental factors might play a less pronounced role in the carcinogenesis of NPC.

Cigarette smoking and alcohol consumption are the major risk factors of head and neck squamous cell carcinoma (HNSCC). Cancers develop in only a fraction of exposed persons, however, an intrinsic susceptibility to environmental genotoxic insults has been suggested as having a role in carcinogenesis. In the general population, there may exist varying degrees of the capacity to repair DNA. To investigate this hypothesis, Hsu et al developed an in vitro assay in which the number of chromatid breaks was scored in metaphases of cultured lymphocytes challenged with bleomycin sulfate in the G2 phase of the cell cycle. It has now been shown that mutagen sensitivity tested with bleomycin is a biomarker of cancer susceptibility and a risk factor for the development of HNSCC and a second primary cancer.

Head and neck cancers, however, represent a heterogeneous group of neoplasms. Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in Taiwan and has the highest rate of distant metastasis among head and neck cancers. The association of Epstein-Barr virus with NPC is unique. The effect of cigarette smoking on NPC is modified by age. The older a person is, the more striking is the dose-response relation between cigarette smoking and NPC. There is no significant association between alcohol consumption and NPC. A hereditary component explains more than 50% of the NPC risk among Chinese people in Taiwan. The carcinogenesis of NPC may be different from that of other head and neck cancers. To evaluate the difference of mutagen sensitivity among patients without cancer and those with NPC and other head and neck cancers, we used...
PATIENTS AND METHODS

Our study consisted of patients with histologically proven squamous cell carcinoma of the mucosa of the upper digestive tract and included 3 groups: patients with NPC, patients with oropharyngeal cancer (ORC), and patients with laryngeal or hypopharyngeal cancer (LHC). They were untreated previously. Control patients were hospital inpatients with no tumor history. Each group comprised 35 patients.

Venous blood specimens were collected in heparinized tubes from each patient of each group before treatment. The standard blood culture procedure was performed at 37°C, using 5% carbon dioxide, for 72 hours. Heparinized whole blood (0.5 mL) was incubated with 4.5-mL RPMI-1640 culture medium with leovaglutamide (Atlanta Biologicals Inc, Norcross, Ga) supplemented with 20% fetal calf serum (Tissue Culture Biologicals, Tulare, Calif), 2.5% phytohemagglutinin (Murex Diagnostics, Dartford, England), and 1.5% penicillin G sodium (10 000 IU/mL), streptomycin sulfate (10 mg/mL), and amphotericin B (0.025 mg/mL) (Biological Industries, Beth Haemek, Israel). Culture of the specimen of whole blood lymphocytes was started the same day they were obtained, or the specimen was stored at 4°C for a maximum of 2 days before culture. After 67 hours of incubation, bleomycin sulfate (Nippon Kayaku Co, Tokyo, Japan) in a concentration of 30 mIU/mL was added to induce the DNA damage in the late S and G2 phases of the cell cycle. During the last hour, the culture was treated with 100 µL of colchicine (Colcemid) in a concentration of 10 µg/mL (GIBCO BRL, Gaithersburg, Md) to induce mitotic arrest at the metaphase stage before harvest. The metaphases were harvested by centrifugation (300 × g) for 5 minutes and treated with a hypotonic solution (0.075-mol/L potassium chloride) for 20 minutes, fixed and washed with Carnoy fixative (methanol-glacial acetic acid, 3:1), then air-dried on wet slides. The slides were stained with 5% Giemsa solution (Diagnostica Merck, Darmstadt, Germany), coded, and scored by one of us (J.-Y.K.) using a light microscope with a magnification ×1000.

Fifty well-spread metaphases were examined for the presence of chromatid breaks, and the mean number of breaks per cell was taken as the measure of mutagen sensitivity. Metaphases with chromatid breaks are the lymphocytes damaged by bleomycin in the G2 phase of the cell cycle. The Student t test was used to compare the mean number of breaks per cell among the groups. This mutagen sensitivity assay can be subject to variability depending on the definition of the chromatid break. In this study, the guidelines of Hsu et al were used, which proved to generate reliable data. In brief, an achromatic lesion whose length is smaller than the diameter of the chromatid is classified as a chromatid gap, which is omitted, and a lesion whose length is equal to or longer than the diameter of the chromatid is regarded as a chromatid break. When the axis of a fragment is not parallel to the chromatid arm, it is a frank break (Figure).

RESULTS

Table 1 presents the results of bleomycin-induced chromatid breaks per cell among control patients and those in the NPC, ORC, and LHC groups. Table 2 shows the mean (±SD) number of breaks per cell in the 4 groups. Patients with ORC or LHC had significantly higher numbers of bleomycin-induced chromatid breaks per cell than the NPC group and those in the control group. Whereas the number of bleomycin-induced chromatid breaks per cell of the group with NPC was higher compared with the control group, it was lower than that of patients with ORC or LHC. In Table 3, the level of more than 1.10 chromatid breaks per cell (mean value +1 SD of control values) was defined as “hypersensitive phenotype to bleomycin.” This level was chosen because the mutagen sensitivity among control patients except identical twins is heterogeneous and because only 16.5% of the observations lie above the value of 1.10 (mean +1 SD of controls). The results showed that all the cancer groups had a higher prevalence of the hypersensitive phenotype than the control group, and the prevalence of the hypersensitive phenotype in the group with NPC was significantly lower than that in the group with LHC.

COMMENT

For the induction of DNA damage, bleomycin was chosen for its clastogenic properties and for its mechanism of action, which resembles that of an environmental car-
somes. This finding has stimulated further research to
dom but to occur at specific sites of specific chromo-
years of tobacco consumption (drinking habits. No correlation was found between the
HNSCC was not related to known cancer risk factors such
study the molecular mechanisms underlying mutagen sen-
which patients have the highest risk of a second
tivity assay for patients with HNSCC is that it may pre-
may reflect a susceptibility to cancer in exposed per-
sensitivity to cigarette smoke carcinogens, they will ac-
assay developed by Hsu et al can reflect mutagen sen-
togenesis of the digestive tract. Patients with NPC had
3.6. This is a clear indication that mutagen sensitivity did
nogenesis at the
play a role in the carcinogenesis of NPC, although less

**Table 1. Distribution of Bleomycin-Induced Chromatid Breaks/Cell Among 4 Groups of Patients**

<table>
<thead>
<tr>
<th>Chromatid Breaks/Cell</th>
<th>Control (n = 35)</th>
<th>NPC (n = 35)</th>
<th>ORC (n = 35)</th>
<th>LHC (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31-0.50</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.51-0.70</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>0.71-0.90</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>0.91-1.10</td>
<td>5</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>1.11-1.30</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>1.31-1.50</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>1.51-1.70</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1.71-1.90</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.91-2.10</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2.11-2.30</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2.31-2.60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*NPC indicates nasopharyngeal cancer; ORC, oral or oropharyngeal cancer; and LHC, laryngeal or hypopharyngeal cancer.

**Table 2. Mean (±SD) Number of Bleomycin-Induced Chromatid Breaks/Cell* Among 4 Groups of Patients**

<table>
<thead>
<tr>
<th>Chromatid Breaks/Cell</th>
<th>Control (n = 35)</th>
<th>NPC (n = 35)</th>
<th>ORC (n = 35)</th>
<th>LHC (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>0.80 (0.32)</td>
<td>1.03 (0.45)</td>
<td>1.30 (0.44)</td>
<td>1.35 (0.44)</td>
</tr>
</tbody>
</table>

*Student t test: Control group vs NPC group, P = .02; vs ORC group, P < .001; and vs LHC group, P < .001. NPC group vs ORC group, P = .09; vs LHC group, P = .03. ORC group vs LHC group, P = .62. See the footnote to Table 1 for an explanation of the abbreviations.

**Table 3. Prevalence of Subjects With Hypersensitive Phenotype to Bleomycin (Defined as >1.10 Chromatid Breaks/Cell*) Among 4 Groups of Patients**

<table>
<thead>
<tr>
<th>Chromatid Breaks/Cell</th>
<th>Control (n = 35)</th>
<th>NPC (n = 35)</th>
<th>ORC (n = 35)</th>
<th>LHC (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1.10</td>
<td>29 (83)</td>
<td>20 (57)</td>
<td>13 (37)</td>
<td>11 (31)</td>
</tr>
<tr>
<td>&gt;1.10</td>
<td>6 (17)</td>
<td>15 (43)</td>
<td>22 (63)</td>
<td>24 (69)</td>
</tr>
<tr>
<td>Odds ratio</td>
<td>1</td>
<td>3.6</td>
<td>8.2</td>
<td>10.5</td>
</tr>
<tr>
<td>(95% Confidence interval)</td>
<td>...</td>
<td>(1.1-12.8)</td>
<td>(2.4-29.5)</td>
<td>(3.0-39.0)</td>
</tr>
</tbody>
</table>

*χ² test: Control group vs NPC group, P = .02; vs ORC group, P < .001; and vs LHC group, P = .001. NPC group vs ORC group, P = .09; vs LHC group, P = .03. ORC group vs LHC group, P = .62. See the footnote to Table 1 for an explanation of the abbreviations.

The bleomycin assay developed by Hsu et al can reflect mutagen sensitivity, which is relevant only in organs and tissues that have direct contact with the external environment. Mutagen sensitivity is a model system that measures the response to genotoxic insults and may reflect a susceptibility to cancer in exposed persons. An important implication of this mutagen sensitivity assay for patients with HNSCC is that it may predict which patients have the highest risk of a second primary cancer developing. In addition to a high number of chromatid breaks in patients with lung cancer, the induced chromatid breaks have been found not to be random but to occur at specific sites of specific chromosomes. This finding has stimulated further research to study the molecular mechanisms underlying mutagen sensitivity.

Although the mean age of the group with LHC in this study was higher than that of the other 3 groups (P < .001), previous reports showed that the increase in mutagen sensitivity in control persons or patients with HNSCC was not related to known cancer risk factors such as age or lifestyle factors such as smoking and alcohol drinking habits. No correlation was found between the break-per-cell value and age (r = 0.25; P = .12), pack-years of tobacco consumption (r = 0.22; P = .17), or unit-years of alcohol drinking (r = 0.23; P = .16). To assess the reproducibility of the assay, Cloos et al tested multiple specimens from the same person using various time intervals (1-12 weeks) in 5 patients with HNSCC and 4 patients without cancer. Reproducibility was high, reflected by a coefficient of variation of 8.9%. Our results showed that all the cancer groups had significantly higher mean values of breaks per cell and a higher prevalence of hypersensitivity than the control group. These results show that all the cancer groups, especially those with ORC and with LHC, had higher mutagen sensitivity than patients without cancer and that this might contribute to the carcinogenesis of head and neck cancers. A recent study shows that cigarette smoke carcinogens, such as benzpyrene, can make strong and selective adduct formation at the p53 gene corresponding to the major mutations in human lung cancers. If persons have a higher sensitivity to cigarette smoke carcinogens, they will accumulate more genetic damage in the upper digestive tract, increasing the chance of carcinogenesis. The bleomycin assay developed by Hsu et al can reflect mutagen sensitivity, which is relevant only in organs and tissues that have direct contact with the external environment, such as the colon, the upper digestive tract, and the lungs, but not the breasts. The genotoxic effect of bleomycin in lymphocytes of blood cultures can mimic the direct carcinogenic effect of toxins such as benzpyrene to the upper digestive tract. In this study, the groups with ORC and LHC showed increased chromatid breaks per lymphocyte. This finding is consistent with the finding that external carcinogens play a significant role in the carcinogenesis of the digestive tract. Patients with NPC had a significantly lower mean number of breaks per cell than the ORC or the LHC group. The odds ratio of patients with the hypersensitive phenotype to bleomycin in the group with NPC compared with the control group was 3.6. This is a clear indication that mutagen sensitivity did play a role in the carcinogenesis of NPC, although less so than for the other cancers.

Nasopharyngeal carcinoma is one of the most common head and neck cancers in Chinese patients and is thought to have a multifactorial cause. Both environmental and genetic factors have been implicated in the tumor formation of NPC. Among environmental factors, the consumption of Cantonese salted food and infections with the Epstein-Barr virus are most commonly documented. By in situ hybridization and using bio-

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tin- and radioisotope-labeled probes, Epstein-Barr virus DNA is detected in 81.7% and 100% of NPC tissues, respectively. Previous studies also have indicated that a familial clustering of NPC is not uncommon. Indeed, Chinese patients with NPC have higher frequencies of HLA antigens A2 and BW46. A linkage study of affected sibling pairs suggested that a gene closely linked to the HLA locus confers a greatly increased risk of NPC. Previous cytogenetic studies of NPC biopsy specimens and cell lines have detected deletions of the short arms in chromosomes 3 and 9. A loss of heterozygosity was found by restriction fragment length polymorphism on chromosome arms 3p, 9p, and 11q in patients with NPC and more recently by the use of microsatellite markers. The biological behavior of NPC differs from that of the other head and neck cancers. Nasopharyngeal cancer and regional neck metastasis are radiosensitive with a high rate of successful treatment by radiotherapy only, which is not the case for other HNSCCs. The serum titer of antibody to the Epstein-Barr virus is high in patients with NPC but low in patients with other HNSCCs. Distant metastases occur more commonly from NPC than from other cancers that originate in the head and neck. Nasopharyngeal cancer has a strong tendency of familial clustering. Environmental factors such as cigarette smoking and alcohol abuse are the most dependent risk factors in the carcinogenesis of oral, oropharyngeal, hypopharyngeal, and laryngeal cancers. Although alcohol consumption and cigarette smoking are associated with NPC in a dose-response relation, the odds ratios are not statistically significant. Neither alcohol consumption nor cigarette smoking has a significant effect on the risk of NPC. Furthermore, the consumption of Cantonese salted food was not popular in other Chinese areas outside of Canton and Hong Kong. The above evidence shows that environmental carcinogens might be less involved in the carcinogenesis of NPC. Our results showing that patients with NPC had less mutagen sensitivity than those with other head and neck cancers support the different clinical and epidemiological findings between NPC and other HNSCCs. The role of genetic factors on the carcinogenesis of NPC needs to be investigated further.

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