DNA Repair in Lymphoblastoid Cell Lines From Patients With Head and Neck Cancer

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Objective: To analyze and compare components of the 3 primary DNA repair pathways of Epstein-Barr virus–transformed lymphocyte (lymphoblastoid) cell lines derived from 9 patients with squamous cell carcinoma of the head and neck and 11 cancer-free controls. These cell lines were previously characterized by using an established cytogenetic marker of cancer susceptibility (mutagen sensitivity assay).

Design: To evaluate nucleotide excision repair (NER), we measured the reactivation level of a tobacco carcinogen–damaged plasmid containing a bacterial reporter gene transfected into these cells. To assess mismatch repair (MMR) and recombinational repair, selected gene transcript levels were quantified by using a multiplex reverse transcriptase–polymerase chain reaction assay. The results of these DNA repair assays were correlated with the previously measured mutagen sensitivity values.

Results: The NER capacities of the 2 groups were similar: 25.1% (range, 14.3%-33.3%) for the patient cell lines and 26.0% (range, 9.4%-47.7%) for the control lines. Transcriptase levels for 6 MMR genes (hMSH3, hMSH2, hPMS2, GTBP, hMLH1, and hPMS1) did not differ in the 2 groups. Transcript levels for 4 of 6 recombinational repair genes (XRCC7, XRCC6, XRCC1, and RAD51) were higher in the patient cell lines, though this difference was significant only for XRCC7 (P = .003). The mutagen sensitivity values correlated with the NER capacity (P = .05) and the expression of XRCC4 (P = .01) and RAD51 (P = .06) genes.

Conclusions: As revealed by the above-named assays, these lymphoblastoid cell lines derived from patients with head and neck cancer had minor alterations in DNA repair function. However, these differences in DNA repair do appear to affect the cytogenetic marker of cancer susceptibility, mutagen sensitivity.


Approximately 46 million adult Americans smoke, yet only a small fraction (41 000) develop squamous cell carcinoma of the head and neck (SCCHN) annually. One would presume that differences in susceptibility to tobacco-induced carcinogenesis partially underlie the disparity between those exposed and those who ultimately develop cancer. Others have suggested that cancer susceptibility has an etiologic role in the development of SCCHN at a young age and in patients without tobacco or alcohol exposure.

A genetic source for these differences in susceptibility has been implied in family studies of SCCHN. These studies have demonstrated a significantly increased relative risk (3.0- to 3.8-fold) for developing an SCCHN in persons having a first-degree relative with SCCHN. The field of ecogenetics encompasses such genetic variations (polymorphisms) in response to carcinogen exposure, and differences in carcinogen metabolism and DNA repair ability have been suggested to underlie such genetic susceptibility. While several case-control studies have examined the risk of SCCHN in individuals with genetic variants of the phase I and phase II carcinogen-metabolizing genes, only minor increases in risk have been demonstrated. Differences in DNA repair ability may provide a better risk estimate.

Bleomycin-induced mutagen sensitivity, an in vitro phenotypic marker of cancer susceptibility, is believed to reflect genetically determined differences in DNA repair ability. In this assay, peripheral blood lymphocytes grown in culture are exposed to the mutagen, bleomycin. Then, cytogenetic analysis is performed to quantify induced chromatic break. Multiple case-control studies have been performed in patients with lung cancer and SCCHN and have demonstrated a consistently higher number of
MATERIALS AND METHODS

CELL LINES

Twenty lymphoblastoid cell lines were obtained from T. C. Hsu, PhD (Department of Cell Biology, M. D. Anderson Cancer Center, Houston, Tex). The establishment and characterization of these cell lines has been described elsewhere.36 Briefly, these cell lines were established by Epstein-Barr virus transformation of peripheral blood lymphocytes obtained from 9 patients with SCCCHN and 11 cancer-free donors. The bleomycin mutagen sensitivity assay, a cytogenetic marker of cancer susceptibility, had been performed previously 1 time on each original blood sample and 4 times on each transformed cell line. As the values obtained from the cell lines correlated with those of the original blood samples (r 0.70), these cell lines were believed by Hsu et al to phenotypically represent the original subjects. Three additional Epstein-Barr virus–immortalized human lymphoblastoid cell lines were used for controls: the normal cell line GM00131A and 2 xeroderma pigmentosum (XP) cell lines, GM02345B (XP-A) and GM02246B (XP-C) (Human Genetic Mutant Cell Repository, Camden, NJ). Two colon cancer lines that have deficient mismatch repair were also used for controls: LoVo (229-CCL), which has mutations in the hMSH2 gene, and SW48 (231-CCL), which has mutations in the hMLH1 gene (American Type Culture Collection, Rockville, Md). The lymphoblastoid cell lines were maintained in RPMI 1640 medium with 15% fetal bovine serum, 1% l-glutamine, and 1% penicillin/streptomycin combination. The LoVo was maintained in Ham F 12 medium with 20% fetal bovine serum and 1% penicillin/streptomycin combination. The SW48 was maintained in Liebovitz L15 medium with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C in a 5% carbon dioxide atmosphere except for SW48, which was maintained without carbon dioxide.

HOST CELL REACTIVATION ASSAY

This assay has been described in detail elsewhere12,17,18 and was used as a measure of nucleotide excision repair (NER). The plasmid (500 μg/mL pCMVβ-gal, a gift from Lawrence Grossman, PhD, The Johns Hopkins University, Baltimore, Md) was treated as previously described18 with the 0 or 60 μM of activated tobacco carcinogen benzo[a]pyrene diol epoxide (BPDE) per liter for 3 hours in a dark room. Then, the plasmids were precipitated 3 times in 70% ethanol, dissolved in Tris-EDTA buffer to yield a final 50-μg/mL concentration, and checked for conformational changes on a 0.8% agarose gel. The BPDE forms adducts with the plasmid, preventing chloramphenicol acetyltransferase gene transcription, and these adducts are removed by the NER pathway.17,19 Aliquots of treated plasmid were stored at −80°C until used, and all plasmids used in this study were from a single treatment.

For transfection, 2 × 10^6 cells from an early passage of each cell line growing approximately the same density and in the same medium conditions were washed in Tris-EDTA–buffered saline solution with calcium (0.7 μM/L) and magnesium (0.5 μM/L) and transfected with treated and untreated plasmid by using the diethylaminoethyl-

dextran (Pharmacia Biotech Inc, Piscataway, NJ) method.10 Triplicate transfections were performed with both treated and untreated plasmid. Chloramphenicol acetyltransferase gene expression (and thus NER capacity) was measured 40 hours after transfection as described elsewhere.17 The assay was performed in triplicate or quadruplicate for each cell line. The normal cell line GM00131A was used as a negative (NER-proficient) control, and the 2 (XP) cell lines GM02345B (XP-A) and GM02246B (XP-C) were used as positive (NER-deficient) controls.

MULTIPLEX REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT-PCR)

As described elsewhere20,21 total RNA was isolated from 5 × 10^6 cells by using Tri-Reagent, an RNA-DNA-protein isolation reagent (Molecular Research Center Inc, Cincinnati, Ohio), according to the manufacturer’s protocol. The RNA was quantified by spectrophotometry. Complementary DNA was synthesized by reverse transcription from 5 μg of RNA by using random primers. Multiplex PCR was performed in triplicate using 5 μL of the complementary DNA product and specific primers for 6 mismatch repair (MMR) genes (hMSH3, hMSH2, hPMS2, GTBP, hMLH1, and hPMS1) and β-actin. The β-actin was used as a loading control and internal standard for transcript quantification. After gel electrophoresis separation of the PCR products, computer densitometry was used to calculate the relative level of each band on the gel by comparison with that of the ubiquitous β-actin. These RNA samples were then analyzed using a multiplex-PCR system designed to measure the transcripts of 5 genes involved in recombinational repair (XRCC7, XRCC6, XRCC1, RAD51, and XRCC4) and the ATM gene transcript.

Initially, as previously20,21 the primer cocktails used in both of these PCR assays were fully characterized (Figure 1). After 3 dose-curve analyses of various amounts of pooled RNA, 5 μg of RNA was chosen for complementary DNA synthesis because it ensured maximum amplification and quality of RT-PCR products (data not shown). Three dose-curve analyses were also performed by using increasing amounts of pooled complementary DNA product, 5 μL of which was subsequently used for all multiplex PCRs, again owing to the consistent quality and amplification of the products (data not shown). Nuclease-free water, a normal cell line (GM00131A), and 2 colon cancer lines (LoVo, which has mutations in the hMSH2 gene, and SW48, which has mutations in the hMLH1 gene) were used for negative, normal (proficient), and positive (deficient) controls, respectively.

STATISTICAL ANALYSIS

The measurements for NER capacity, MMR transcript levels, and recombinational repair transcript levels were analyzed as continuous variables. The Student t test was used to compare values for the group of cell lines derived from patients and those derived from cancer-free donors. Correlation analyses were performed between values of each of the 3 DNA repair assays and the mutagen sensitivity values. For the MMR and recombinational repair assays, the correlation analyses were performed first with each individual gene transcript level and then with the average transcript level of all 6 repair genes in the panel.
chromatid breaks per cell in cases than in controls.\textsuperscript{11,13-15} While bleomycin-induced mutagen sensitivity has an intriguing role as a cancer risk factor, it provides only indirect evidence for a role for altered DNA repair ability in cancer susceptibility. In fact, laboratory evidence linking mutagen sensitivity and DNA repair ability is scant.\textsuperscript{12}

Because there is only indirect evidence of reduced DNA repair in patients with SCCHN, we undertook a pilot project to examine components of 3 DNA repair pathways in lymphoblastoid cell lines derived from patients with SCCHN and normal donors. We also correlated the mutagen sensitivity profile of these cell lines with the values obtained from 3 DNA repair assays to further examine the mechanisms underlying mutagen sensitivity.

## RESULTS

### NUCLEOTIDE EXCISION REPAIR

The host cell reactivation assay was used to assess NER. The mean (± SD) NER capacity was 25.1\% ± 6.1\% (range, 14.3\%-33.3\%) for the lymphoblastoid cell lines derived from patients with SCCHN and 26.0\% ± 10.8\% (range, 9.4\%-47.7\%) for those cell lines derived from cancer-free donors (Figure 2). These differences were not statistically significant ($P = .83$). The 2 positive control XP-C and XP-A cell lines demonstrated deficient NER capacity (4.2\% and 1.0\%, respectively), as expected, and the normal control cell line expressed 29\% NER capacity (Figure 2). When the NER capacity value for each cell line was compared with its mutagen sensitivity value, there was a trend toward an inverse correlation ($r = −0.44$, $P = .05$) (Figure 3). In other words, the more mutagen sensitive the cell line, the less NER capacity it expressed.

### MISMATCH REPAIR

A multiplex RT-PCR assay was used to measure the relative expression level of 6 MMR genes as a partial assessment of MMR. There was no difference in the expression level of these genes between the 2 groups (Figure 4). Furthermore, all the cell lines expressed all the genes measured by this assay. As expected, the positive (deficient) control cell lines LoVo and SW48 consistently did not
express the hMSH2 and hMLH1 gene transcripts, respectively, whereas the water and normal control (GM00131A) were appropriately negative and normal, respectively (Figure 5). There was no correlation between the average or individual MMR gene transcript level and the mutagen sensitivity value for these cell lines.

RECOMBINATIONAL REPAIR

A multiplex RT-PCR assay was used to assess, in part, recombinational repair. In the cell lines derived from patients, there was elevated expression of 4 (XRCC7, XRCC6, XRCC1, and RAD51) of the 6 genes examined from this pathway (Figure 6), though the increase was statistically significant only for the XRCC7 gene (P = .003). All cell lines expressed all of these genes as measured by this assay. There was no correlation between the average transcript level of the genes involved in recombinational repair and the mutagen sensitivity value for each cell line. However, increasing XRCC4 gene levels correlated with increasing mutagen sensitivity (r = 0.56, P = .01), and a similar trend existed for RAD51 gene (r = 0.43, P = .06) (Figure 7).

In this study, we explored the susceptibility to cancer (specifically, genetically determined DNA repair function) of patients with SCCHN by examining DNA repair pathways in 20 lymphoblastoid cell lines previously characterized by a cytogenetic assay of cancer susceptibility. While there was no difference in the repair ability between the 2 groups (Figure 2), there was a wide range of NER capacity, consistent with a broad spectrum of susceptibility in the population. Additionally, there was no obvious absence or reduction in expression of either the MMR or recombinational repair gene transcripts in the patient cell lines (Figures 4 and 6). In fact, there appeared to be higher expression of 4 recombinational repair genes in the cell lines derived from patients. There are at least 2 mechanistic explanations for this observation of higher recombinational repair activity in the patient cell lines. It could either be a response to greater levels of intrinsic DNA damage or an attempt to compensate for a decrease in recombinational repair protein function. Further studies will be needed to compare differences in spontaneous and induced damage and to functionally assess the recombinational repair pathway in these 2 groups.

However, this study has limitations. One is that it examined a small number of cell lines (but inclusive of the entire group previously reported). Also, viral transformation of an individual’s lymphocytes may reduce phenotypic differences in susceptibility. In fact, the difference in mutagen sensitivity between patients and cancer-free donors dropped by a factor of 2.6 after the transformation process. Therefore, the utility of cell lines for evaluating cancer susceptibility may be limited, and larger patient-based studies of fresh lymphocytes will be needed to explore actual differences in cancer susceptibility.

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NER capacity also appeared to correlate with the mutagen sensitivity value (Figure 3). Although this seems contradictory, one must keep in mind that there is overlap between the repair pathways both in their target lesions and in their constituent components. In fact, the ERCC1 and ERCC4 genes appear to have roles in both NER and recombinational repair. Consequently, mutations in or polymorphisms of these 2 genes could affect both pathways and so lead to such a correlation between bleomycin-induced mutagen sensitivity (hypothetically due to recombinational repair) and BPDE-damaged plasmid reactivation (an NER process). However, given this rather limited correlation, we can surmise that factors in addition to DNA repair ability also contribute to a cell line's mutagen sensitivity phenotype.

In conclusion, although there was no significant decrease in NER capacity or MMR transcript levels in lymphoblastoid cell lines derived from patients with SCCHN compared with those from cancer-free individuals, there was a trend toward an increase in recombinational repair transcript levels. This result may reflect limitations in the number of cell lines available or in the nature of the transformed cell line, and consequently we have recently employed these assays in much larger case-control studies using fresh lymphocytes. In contrast to our results from transformed cell lines, studies of fresh lymphocytes revealed differences between control subjects and those with cancer and suggest that such assays can be used as phenotypic markers of cancer susceptibility. Secondly, the well-established mutagen sensitivity assay partially measures differences in NER capacity and recombinational repair transcript levels, but obviously other factors requiring further study contribute to this phenotypic marker of cancer susceptibility.

Accepted for publication August 25, 1998.

This work was supported in part by National Institutes of Health (Bethesda, Md) grants for Training of the Academic Head and Neck Surgical Oncologist T32 CA 60374 (Dr Clayman), R03 CA 70242, and R01 CA 74851 (Dr Wei).


From the University of Texas, Houston, we wish to thank T. C. Hsu, PhD (Department of Cell Biology), for kindly providing the lymphoblastoid cell lines used in this study and Maureen Goode, PhD (Department of Scientific Publications), for editing of the manuscript.

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