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Reduced DNA double-strand break repair capacity and risk of squamous cell carcinoma of the head and neck – A case-control study

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Abstract

Tobacco and alcohol use play important roles in the etiology of squamous cell carcinoma of the head and neck (SCCHN). Smoking causes DNA damage, including double-strand DNA breaks (DSBs), that leads to carcinogenesis. To test the hypothesis that suboptimal DSB repair capacity is associated with risk of SCCHN, we established a flow cytometry-based method to detect the DSB repair phenotype in four EBV-immortalized human lymphoblastoid cell lines and then in human peripheral blood T-lymphocytes (PBTs). With this blood-based laboratory assay, we conducted a pilot case-control study of 100 patients with newly diagnosed, previously untreated SCCHN and 124 cancer-free controls of non-Hispanic whites. We found that the mean DSB repair capacity level (42.1) in cases was significantly lower than that in controls (54.4) ($P < 0.001$). When we used the median DSB repair capacity level in controls as the cutoff value for calculating the odds ratios (OR), after adjustment for age, sex, smoking and drinking status, the cases were more likely than the controls to have reduced DSB repair capacity (adjusted OR = 1.9; 95% confidence interval, CI = 1.0–3.6, $P = 0.037$), especially for cases who were ever drinkers (adjusted OR = 2.7; 95% CI = 1.2–6.4, $P = 0.020$) and had oropharyngeal tumors (adjusted OR = 2.2; 95% CI = 1.1–4.5, $P = 0.035$). In conclusion, these findings suggest that individuals with a reduced DSB repair capacity may be at an increased risk of developing SCCHN. Large studies are warranted to confirm these preliminary findings.

Keywords

biomarker; DNA damage; γ -H2AX; DNA double-strand breaks repair capacity; head and neck cancer

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1. Introduction

Squamous cell carcinoma of the head and neck (SCCHN), which includes cancers of the oral cavity, pharynx, and larynx, is one of the six most common cancers worldwide [1]. In the United States, approximately 60,000 new cases are diagnosed annually and 12,000 die of this disease each year [2]. Although tobacco smoking and alcohol use play a role in the etiology of SCCHN, the oropharynx is the most common site for HPV-associated SCCHN. The fact that only a fraction of smokers, drinkers, and people exposed to HPV develop SCCHN suggests that genetic factors also contribute to the disease [3–5]. Tobacco carcinogens cause different types of DNA damage in the target cells. To avoid uncontrolled cell growth due to mutations resulting from DNA damage, these cells must initiate cell cycle control mechanisms that allow for repair of DNA damage or initiation of the apoptosis mechanism to eliminate cells with overwhelming damage to DNA.

Smoking causes DNA damage, including double-strand DNA breaks (DSBs), which are one of the most serious forms of DNA damage to the cells. In eukaryotes, there are two major pathways for DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ), which differ in their requirement for a homologous template DNA and in the fidelity of DSB repair in mammalian cells [6, 7]. If DSBs are not repaired efficiently, they can cause genomic instability, ultimately leading to cancer [8, 9]. Hence, it is important to have a quantitative way of measuring DSB repair phenotype. Previous attempts to measure DNA DSB repair in patients undergoing chemotherapy have relied on the alkaline comet assay, an extremely sensitive method for measuring DNA damage, in which individual cells are molded into agarose on microscopic slides and exposed to an electrical field after alkaline-gel lysis. The electrical field forces cellular DNA containing strand breaks to migrate from the nucleus, generating a ‘comet tail’ that is proportional to the level of single strand breaks (SSB) and DSBs in the cell [10, 11].

In recent years, the phosphorylated histone H2AX has become a powerful tool to monitor DSBs in cancer research [12]. The phosphorylated form of H2AX was named γ -H2AX, because it was first observed in cells exposed to γ -rays. Although γ -H2AX is not specific to DSBs, the γ -H2AX focus remains the most sensitive way to detect a DSB [12]. The formation of γ -H2AX is an early cellular response to DSBs, and thus, γ -H2AX is a universal biomarker for DSBs induction [13]. It has been reported that the capability of γ -H2AX assay for detecting DNA damage is 100-fold more sensitive by flow cytometry than by comet assay [14]. Etoposide (ETOP, a topoisomerase II inhibitor) is used as anti-cancer drug and induces DSBs in genomic DNA and subsequent cell death in cycling cells [15]. Because DSBs induce γ -H2AX in the chromatin flanking the break site, an antibody directed against γ -H2AX can be employed to measure DNA damage levels before and after patient treatment [16–18]. For the methodology, these results have been shown using foci counting for γ -H2AX by a fluorescent microscopy. The local formation of γ -H2AX allows microscopical detection of distinct foci by fluorescent γ -H2AX-specific antibodies that most likely represent single DSBs [19, 20], and the potential to detect a single focus within the nucleus makes this the most sensitive and efficient method currently available for detecting DSBs in cells [21, 22], although the main disadvantage of the method is difficulty if done by

eye is somewhat subjective if slides are not coded [22]. However, this method is difficult to adapt in epidemiology research and clinical practice. In contrast, flow cytometry, a high-throughput, statistically robust technique, allows simple detection of quantify γ -H2AX in a large number of cells in a short time period [23], although the main disadvantage of flow cytometry is higher background in G2/S-phase cells, which is responsible for a two- to three-fold reduction in the sensitivity for detecting DSBs in cells [24], the sensitivity of γ -H2AX by flow cytometry analysis is not as good as by foci counting [22]. Accordingly, we used ETOP-induced DSB by flow-cytometry-based method to detect the DSB repair phenotype in human peripheral blood T-lymphocytes (PBTs). With this blood-based laboratory assay, we conducted a pilot case-control study with 100 SCCHN patients and 124 cancer-free controls to evaluate the influence of suboptimal DSB repair capacity on SCCHN risk.

2. Materials and Methods

2.1 Study subjects

The 100 cases included in this study were non-Hispanic whites with newly diagnosed, histopathologically confirmed, untreated primary cancers of the oral cavity ($n = 27$; 27%), oropharynx ($n = 59$; 59%), or larynx and hypopharynx ($n = 11$; 11%) and unknown primary ($n = 3$; 3%), who were recruited from The University of Texas M. D. Anderson Cancer Center in the period of Jan 2012 and May 2013. The 124 cancer-free controls were also non-Hispanic whites, who were randomly recruited from hospital visitors at M. D. Anderson Cancer Center in the same time period; they were biologically unrelated to the cases or to any one included in this study. Patients with second SCCHN primary tumors, primary tumors of the nasopharynx or sinonasal tract, or any histopathologic diagnosis other than SCCHN were excluded. Having provided their written informed consent, each eligible subject provided additional information about risk factors, such as tobacco smoking and alcohol use and a one-time sample of 10 ml of blood for biomarker tests. The research protocol was reviewed and approved by the institutional review board.

2.2 Cell lines, Cell culture and ETOP treatment

Four EB virus (EBV)-immortalized human lymphoblastoid cell lines from the Human Genetic Mutant Cell Repositories (Camden, NJ) were used: two apparently normal cell lines (GM00892B and GM3798) and two Human transformed lymphoid-cell lines of ataxia telangiectasia (GM1525/AT2BI/AT1, GM1526/AT8BI/AT2) with deficient DSB repair. We used these two types of cell lines to test the sensitivity and specificity of cellular repair of ETOP-induced DSB damage. All of the cells were cultured in 12-mm $\times$ 50-mm tubes at 37°C in 5% CO₂ atmosphere in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 15% fetal calf serum (GIBCO BRL). ETOP was purchased from Sigma (Sigma Chemical Co., St. Louis, MO) as a white powder and was completely dissolved in DMSO (Life Technologies, Inc., Grand Island, NY). The final concentration of DMSO, which did not exceed 0.1% in the culture medium, did not influence cell viability [25]. The ETOP working solution was added to the tubes to final concentrations of 0, 10, 25, 50, 100 and 150 μ M. Each cultured sample (1×10^6 cells) were then aliquoted into four 12-mm $\times$ 50-mm tubes and cultured for 2 hours with the ETOP-working solution added into the culture

medium. Then the medium was replaced and the cells cultured for an additional period of up to 4 hours, at the indicated time points, and cell samples were then harvested and fixed with 1% paraformaldehyde, washed with 1× PBS and finally stored in 70% ethanol at −20°C until used for the DSB repair analysis.

2.3 Human peripheral blood T-lymphocyte (PBTl) culture and ETOP treatment

For the ETOP-induced DSBs assay, we used phytohemagglutinin-stimulated PBTls from the human blood, because T-lymphocytes have a complete response to initial DNA damage and subsequent repair. Each day, peripheral blood samples from the subjects was used to obtain the cultured lymphoblast cells. Briefly, the lymphocytes were isolated from the whole blood by using Ficoll (Pharmacia Biotech Inc., Piscataway, NJ) gradient centrifugation, and then cultured in RPMI 1640 supplemented with 15% fetal calf serum (GIBCO BRL) and 56.25 µg/ml phytohemagglutinin (Murex Diagnostics, Norcross, GA) for 48 hours at 37°C in an incubator with 5% CO₂. Each cultured sample was then aliquoted (1×10^6 cells × 4) into four 12-mm × 50-mm tubes (i.e., 1 treated for 2 h compared with 1 untreated; 1 treated for 2 h and then medium replaced for repair for another 4 h compared with 1 untreated). 10-µM ETOP was chosen for the *in vitro* treatment based on prior determined doses. At the indicated time points, cell samples were harvested and fixed 1% paraformaldehyde, washed with 1× PBS and finally stored in 70% ethanol at −20°C until used for the DSBs repair analysis.

2.4 Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8, Dojindo Laboratories, Minato-ku, Japan) according to the manufacturer's instructions. Briefly, the cultured cell lines with/without ETOP for 2 or 4 h were seed in triplicate onto a 96-well plate (1×10^5 /well) in 100 µl of complete RPMI 1640 medium, and each condition was performed in eight replicate wells. Subsequently, 10 µl of CCK-8 solution was added to each well, which was incubated for another 2 h. The optical densities (OD) of the wells were measured at 450 nm on a 96-well multi-scanner autoreader (Thermo Electron Corp, Waltham, MA, USA). At least three independent repeats of this experiment were performed.

2.5 DNA DSBs analysis with flow cytometry

For the ETOP-induced DNA DSB repair assay, we used the γ-H2AX Phosphorylation Assay Kit, following the manufacturer's instructions (upstate, Inc., Temecula, CA), and the FITC-positive cells and the FITC-negative cells were counted by flow cytometry at our institutional core facilities. Nuclei were counted by using a Coulter EPICS-XL Flow Cytometer with the 488 nm line of a line of an argon laser and standard optical emission filters (Flow Cytometry Core Lab, Department of Immunology, M.D. Anderson Cancer Center). The ratio of DSBs cells ($\sim 1 \times 10^5$) was measured by flow cytometry according to the instructions of the manufacturer. The percentages of cells were estimated from the FITC-positive cells provided by the manufacturer.

2.6 Detection of ETOP-induced apoptosis in human lymphoblastoid cell lines

To measure the apoptosis levels after ETOP treatment, we used the Terminal Transferase dUTP Nick End Labeling (TUNEL) assay method as previously described.[26] Briefly, we detected apoptotic cells with an APO-BrdU kit (Phoenix Flow Systems), which contains apoptosis-positive and -negative cells as the assay controls and uses a two-color staining method for labeling DNA breaks and total cellular DNA. The kit includes washing, reaction, and rinsing buffers for processing each step of the assay; terminal deoxynucleotidyl transferase enzyme, bromodeoxyuridine triphosphate, and fluorescein-labeled anti-bromodeoxyuridine antibody for labeling DNA; and propidium iodide/RNase A solution for counterstaining the total DNA. The ratio of apoptotic cells ($\sim 1 \times 10^5$) was calculated based on the measurements by flow cytometry (Epics Profile II Flow Cytometer, Beckman Coulter, Inc.) according to the instructions of the manufacturer. Specific apoptosis (the apoptotic index) was determined by the formula $[100 \times (\text{experimental apoptosis} - \text{spontaneous apoptosis}) / 100 - \text{spontaneous apoptosis}]$ [26, 27].

2.7 Statistical analysis

The differences in selected demographic variables, smoking, and alcohol consumption between SCCHN cases and controls were evaluated by using the χ^2 -test. The Wilcoxon rank-sum test was conducted to test the difference between cases and controls for baseline, etoposide induced γ -H2AX level, and γ -H2AX ratio. The DSB index was indicated by the percent of remained DSB after ETOP treatment, which was calculated using the following formula: $\text{DSB index (\%)} = 100 \times (\gamma\text{-H2AX after treatment/baseline})$. The DSB repair capacity (DSBRC) indicated the percent of repaired DSB after removing ETOP for 4 hours, which was calculated with formula: $\text{DSBRC} = 100 \times [1 - (\gamma\text{-H2AX of 4 hours} - \text{baseline}) / (\gamma\text{-H2AX level of 2 hours} - \text{baseline})]$. The differences in means of DSBRC between cases and controls were assessed by Wilcoxon rank-sum test, Student *t* test and general linear regression analysis. We also dichotomized DSBRC based on the median of controls to evaluate its effect on the risk of SCCHN by computing odds ratios (OR) and their 95% confidence intervals (CIs) from both univariate and multivariate logistic regression models in the case-control analysis. These analyses were performed with or without adjustment for age (in years), sex, smoking and drinking status. Subjects who had smoked <100 cigarettes in their lifetime were defined as never smokers; all others were defined as ever smokers. Among ever smokers, those who had quit and had not smoked for >1 year before the interview were defined as former smokers and the others were defined as current smokers. Similarly, subjects who had reported drinking alcoholic beverages at least once a week and longer than 1 year prior to diagnosis or interview were defined as ever drinkers. Those who had quit drinking for longer than 1 year prior to diagnosis or interview were defined as former drinkers and the others as current drinkers. All tests were two sided, and $P < 0.05$ was considered significant. All statistical analyses were performed with the SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC).

3. RESULTS

3.1. DSB repair capacity in cell lines

In the assay development, we performed the experiments with different concentrations at different time points for ETOP treatment in EBV-immortalized human lymphoblastoid cell lines. Fig. 1 shows the kinetics of DSBs repair in the lymphoblastoid cell lines with/without ETOP treatment. We found that the DSB index increased between 1 and 2 hours after treatment and remained constant between 2 and 4 hours (DSB levels after ETOP exposure) (Fig. 1A), which indicated that 2 hours was the optimal treatment time point. We also tested the DSB index at three different time points (4h, 16h, and 24h) after removal of ETOP and found that the index dropped significantly during the first 4 hours (residual DSB levels after ETOP removed) (Fig. 1B), which indicated that the DSB damage was mainly repaired at the first four hours, which could be used as the optimal repair cutoff time point.

Although it is known that DSB is an early event and apoptosis is a late event, to exclude the possible influence of apoptosis induced by ETOP on the DSB index, we compared the apoptosis levels with different time points and different treatment concentration, and we found that there was no significant change or any trend in the measurements (Supplementary Fig. 1). Further cell viability analysis also indicated that the numbers of viable cells after ETOP treatment did not significantly change compared with those without ETOP treatment (Supplementary Fig. 2). The final protocol included normal lymphoblast cell lines (GM0892 and GM3798) and human ataxia telangiectasia lymphoblastoid cell lines (GM1525/AT2BI/AT1 and GM1526/AT8BI/AT2), which were treated with ETOP of different concentrations for 2 hours before the culture medium was replaced, and the cells were then cultured for additional 4 hours. The results showed that the dose-response curves of AT cells and normal cells were well separated for the doses of ETOP in the range of 25–150 μ M. More than 2-fold differences were found between AT (DSBR deficient) cell lines and normal cell lines (Fig. 1C). The Fig. 1D showed flow cytometry plot of the tested normal cell line (GM3798).

To confirm the results from the flow cytometry method, we also performed immunofluorescence staining in normal cells (GM3798), which were treated with ETOP (0, 25, 50 and 100 μ M) for 2 hours and then cultured for additional 4 hours after removal of ETOP. Cells at each of the time points were fixed and stained with an anti γ -H2AX antibody and DAPI. The subcellular localizations of γ -H2AX (red) and DAPI (blue) were examined in the same cells by conventional fluorescence microscopy. We found that the γ -H2AX foci intensity was higher in the 2-hour group than in the 4-hour group (Fig. 1E), suggesting that some damage loci was repaired later in the 4 hours.

3.2. Case-control analysis

Because the repair of ETOP-induced DSBs was different between cell lines and human PBTLs, the PBTLs from four cancer-free controls were treated by ETOP with different concentrations for 2 hours before the culture medium was replaced, and the cells were cultured for additional 4 hours after the removal of ETOP. The results showed that the DSBs began to increase at 5 μ M of ETOP and remained constant from 10 to 80 μ M

(Supplementary Fig. 3). Therefore, the best ETOP concentration was 10 μ M for PBTLs to be compared.

In the present study, we detected the DSB repair capacity of 100 SCCHN cases and 124 cancer-free controls by using the ETOP-induced DSB repair assay as summarized in Table 1. We did not match age and sex between cases and controls but controlled for their confounding effects in the final multivariate regression model. As shown in Table 1, there was no significant difference in baseline γ -H2AX levels between cases and controls (3.9 ± 0.9 vs. 4.0 ± 1.0 ; $P = 0.312$). However, the ETOP-induced γ -H2AX levels measured 2 hours after ETOP treatment were significantly lower in SCCHN cases than in controls (27.6 ± 18.0 vs. 40.0 ± 19.5 ; $P < 0.0001$). Likewise, the γ -H2AX ratio was significantly lower in cases than in controls (7.3 ± 4.7 vs. 10.4 ± 5.6 ; $P < 0.0001$). Whereas there was no significant difference in baseline and ETOP-induced γ -H2AX levels measured 4 hours after ETOP treatment. In all stratified subgroups, except for the never smokers and current smokers as well as former drinkers, cases had a significantly lower γ -H2AX ratio than controls (Table 2). Similarly, except for the never smokers and former smokers as well as never drinkers, cases had a significantly lower DSB repair capacity than controls (Table 3).

We further performed stratified analyses to evaluate the effects of DSB repair capacity on the risk of SCCHN by age, sex, smoking status and alcohol use using the median DSB repair capacity in controls as the cutoff point value for calculating the odds ratios. As shown in Table 4, cases were almost two times more likely than the controls to have reduced DSB repair capacity (adjusted OR = 1.9; 95% CI = 1.0–3.6, $P = 0.037$). Ever drinkers and oropharyngeal tumor were more likely than controls to have reduced DSB repair capacity (adjusted OR = 2.7; 95% CI = 1.2–6.4, $P = 0.020$ for ever drinkers; adjusted OR = 2.2; 95% CI = 1.1–4.5, $P = 0.035$ for oropharyngeal tumor).

4. Discussion

In the present study, we used a flow cytometry-based method to detect the DSB repair capacity, which could be used in population studies with large sample sizes. Moreover, we demonstrated that reduced DSB repair capacity was associated with an increased risk of SCCHN. Although the study size was relatively small, the data presented here provide evidence that cases with SCCHN are likely to have genetically determined low DSB repair capacity, compared to cancer-free controls.

Accumulating evidence has demonstrated that the DSBs are associated with cancer development, because they cause genomic instability and ultimately carcinogenesis. Previous attempts to measure DSBs in cancer patients have relied on the neutral elution, pulsed field electrophoresis, alkaline comet assay and immunohistochemical analysis of expression of various DSB repair enzymes and γ -H2AX [28–31]; however, no studies have reported a quantitative way of measuring DSB repair capacity for epidemiology studies. Recent studies have shown that ETOP elicits the DSB repair induction of γ -H2AX in foci that reflect the detection of DSBs by the cell prior to the onset of DNA replication [32, 33]. For example, Sunter *et al.* investigated that the TOP2 β wild-type and null MEF cell lines were exposed to different concentration of ETOP (0, 0.1, 1, 10 or 100 μ M) for 2 hours, then

the cells were placed in drug-free media for 20 minutes before being assayed for γ -H2AX foci. They observed that the foci numbers per nucleus increased in both cell lines with an increasing dose of drug; at 100 μ M, foci were no longer distinct and therefore uncountable [34]. Consistent with the results, we observed in the present study that the γ -H2AX was at the highest level with exposure to ETOP (100 μ M) at 2 hours and then dropped after 4 hours. Similarly, using the RABiT image analysis in 94 healthy adults, Sharma *et al.* reported that radiation induced γ -H2AX total fluorescent yields rapidly increased in 0.5 hours and peaked in 2 hours with a gradual decline over time reaching close to control levels in 24 hours post radiation, with almost 75% of foci disappeared in 7 hours [35]. In addition, we also observed that apoptosis levels did not significantly change thereafter, which supports the notion that DSBs are an early event, whereas apoptosis is a late event.

It has been demonstrated that the latent DNA damage can lead to genetic instability and subsequent development of cancer, and DNA repair plays a fundamental role in the maintenance of genomic integrity [36]. Exposure to environmental carcinogens, such as tobacco smoke, alcohol use and ultraviolet (UV) radiation, can result in various types of DNA damage [37, 38]. DNA damage in response to environmental carcinogens accumulates more rapidly in people with suboptimal DNA repair capacity. Therefore, interindividual differences in DNA repair capacity have been suggested to have a significant impact on cancer susceptibility in the general population.

In the present study, we did not find a significant difference in the baseline γ -H2AX levels between cases and controls, which is consistent with the findings from other previously published studies [39–41]; however, we found that, upon the ETOP treatment, cases exhibited significantly lower levels of γ -H2AX accumulation, which is conflicted with these previous studies [39–41]. For example, in a study of 174 bladder cancer cases and 174 cancer-free controls in a Caucasian population, using the laser scanning cytometer-based immunocytochemical method to measure ionizing radiation-induced γ -H2AX levels, Fernandez *et al.* observed a significantly higher level of γ -H2AX in the cases than in the controls [39]. There are several possible reasons for the apparent discrepancy between our results and those reported by others. One reason is that a different DSB inducer was used; the other is that a different method was used for measuring γ -H2AX accumulation; another explanation is that given the same level of DSBs induced at the same concentration of ETOP treatment, the formation of γ -H2AX foci was more effective in the controls than in cases, reflecting better repair activities in the controls. In addition, Brzozowska *et al.* also observed that the γ -H2AX levels did not increase in prostate cancer patients prior to γ -rays treatment, compared with controls [42]. It is well known that NHEJ repair occurs throughout the whole cell cycle, and HR is mainly active in the late S and G2 phase [43], γ -H2AX is dephosphorylated after DSBs are re-joined, and thus, the disappearance of the γ -H2AX foci is correlated to the active repair of DSBs [23, 44]., Based on the results of our pilot study and previously published study according to [34], we selected the concentration of 10 μ M ETOP to induce γ -H2AX, and we calculated the DSB repair capacity as recommended by previously published reports, including our own [45, 46]. We found that the mean DSB repair capacity levels were significantly lower in SCCHN patients than in cancer-free controls, which is consistent with our previously reported findings, demonstrating that DNA repair capacity (DRC) for nucleotide-excision repair (NER) was reduced in SCCHN patients

and associated with an increased risk of SCCHN [45, 47]. Our results are also consistent with another study of 18 SCCHN patients and 20 healthy Caucasians, in which Walczak et al. found that DSBs induced by gamma radiation were repaired slower in lymphocytes from SCCHN patients than in lymphocytes from healthy controls using the comet assay [48]. Taken together, our results suggest that reduced DSB repair capacity may play a role in the etiology of SCCHN. Once validated in a large study, this finding will advance our knowledge in the etiology of SCCHN. It is possible that this assay could be as an effective biomarker for risk assessment and the identification of at-risk individuals who can be targeted for primary prevention and early detection of SCCHN in the general population.

The present study has some limitations. First, because this study was a hospital-based case-control study and the controls were not selected from the same population from which the cases may occur, there are some inherited selection biases. Second, although sub-optimal DSB repair capacity has been found to be associated with increased SCCHN risk, we cannot exclude the influence of other potential confounder (e.g., HPV infection status) that were not controlled in this pilot study. Finally, the variation in γ -H2AX baseline activities may be due to difference in the interval between blood sample collection and the cell-based experiments. To avoid this, the interval time between blood draw and whole blood culture was minimized.

In summary, the present study shows the application of the flow cytometry-based method to measure the DSB repair capacity in human peripheral T-lymphocytes. Our preliminary data support the use of this assay in future case-control studies evaluating the role of DSB repair capacity in the etiology of cancer. The results of the present pilot case-control study support our hypothesis that suboptimal DSB repair capacity may increase the risk of SCCHN. A large study is underway to further validate the utility of this assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DSBs	DNA double-strand breaks
ETOP	etoposide
SCCHN	squamous cell carcinoma of the head and neck
OR	odds ratio

CI confidence interval

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A

B

C

D

Fig. 1.

The kinetics of DSBs repair (DSBR) after ETOP treatment and DDSBR capacity in EBV-immortalized human lymphoblastoid cell lines. Cell lines were first tested to determine the appropriate dose and time points of ETOP-induced DSBs damage. **(A)**. The normal cell line (GM3798) was treated by etoposide (ETOP) with different concentration and different times. The DSB index increased between 1 and 2 hours with after treatment and remained constant between 2 and 4 hours (DSB levels after ETOP exposure), which indicated that 2 hours was, is the optimal treatment time point. **(B)**. The DSB index of the normal cell line (GM3798) were at the highest with ETOP (100 μ M) treatment at 2hrs, after removal of ETOP, the DSB index dropped significantly during the first 4 hours but remained almost constant from 4 to 24 hours (residual DSB levels after ETOP removed), which indicated that the DSB damage was mainly repaired at the first four hours, which could be used as the optimal repair cutoff time point. **(C)**. repair response to ETOP-induced DSBs in normal and AT (DSBR deficient) cell lines. The dose-response curves of AT cells and normal cells are well separated for the doses of ETOP in the range of 50–150 μ M. **(D)**. DSBs assay by flow cytometry (GM3798 normal lymphoblast cell line). **(E)**. GM3798 normal lymphoblast cell line was treated by ETOP with different concentrations and time. The subcellular localization of γ H2AX in red

and DAPI (in blue, as control) was examined in the same cells by conventional fluorescence microscopy.

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Characteristics of squamous cell carcinoma of the head and neck (SCCHN) cases and cancer-free controls

Table 1

Variables	Cases (n = 100)		Controls (n = 124)		p ^a
	n	%	n	%	
Age (years)					0.030
<55	33	33.0	59	47.6	
55	67	67.0	65	52.4	
Sex					<0.001
Female	20	20.0	56	45.2	
Male	80	80.0	68	54.8	
Smoking status	99		118		<0.001
Never	26	26.3	8	6.8	
Former	35	35.3	42	35.6	
Current	38	38.4	68	57.6	
Alcohol use					0.412
Never	46	46.5	48	40.7	
Former	19	19.2	26	20.0	
Current	34	34.3	44	37.3	
Tumor site					
Oropharynx	59	59			
Non-oropharynx ^b	38	38			
Unknown primary	3	3			

^aTwo-sided χ^2 test.
^bIncluded oral cavity (n=27), larynx (n=10) and hypopharyngeal (n=1) cancer cases.
^cOriginal readings of the fluorescence signals by flow cytometry.
^dWilcoxon rank-sum test.

Table 2

DSB repair capacity (DSBRC) between squamous cell carcinoma of the head and neck (SCCHN) patients and healthy control subjects

Variable	Cases (DSBRC) ^a			Control (DSBRC) ^a			P ^c
	No.	Mean±SD	P ^b	No.	Mean±SD	P ^b	
Overall	100	42.1±28.3		124	54.4±22.2		<0.001
Age (years)							
<55	33	36.7±26.0		59	51.2±20.0		0.004
55	67	44.7±29.2	0.106	65	57.2±23.7	0.371	0.008
P _{trend}							
Sex							
Male	80	42.8±28.6		68	52.6±21.0		0.019
Female	20	39.0±27.8	0.831	56	56.5±23.5	0.151	0.008
Smoking status							
Never	26	40.2±25.5	ref.	8	44.1±29.9	ref.	0.720
Former	35	43.5±31.2	0.705	42	49.0±18.9	0.302	0.360
Current	38	41.9±28.4	0.859	68	59.2±22.9	0.091	<0.001
Ever	73	42.6±29.6	0.844	110	55.3±22.0	0.145	0.002
Alcohol status							
Never	46	47.3±28.5	ref.	48	51.7±22.3	ref.	0.399
Former	19	30.6±23.5	0.024	26	54.9±23.6	0.571	0.001
Current	34	41.2±29.6	0.247	44	56.9±22.5	0.313	0.010
Ever	53	37.4±27.8	0.361	70	56.2±22.8	0.180	<0.001
Tumor site							
Oropharynx	59	39.4±27.4					
Non-oro-pharynx	38	44.8±30.8	0.367				

^aDSBRC= $100 \times [1 - (\gamma\text{H2AX of 4 hours} - \text{baseline})/(\gamma\text{H2AX level of 2 hours} - \text{baseline})]$.

^bP-values for the differences between subgroups were determined by the Wilcoxon rank-sum test.

^cP-values for the difference between cases and controls.

Stratified analysis for association between DSB repair capacity (DSBRC) and risk of squamous cell carcinoma of the head and neck (SCCHN)

Table 3

Variable	Cases, N (100) ^a	Controls N (124) ^a	Crude OR (CI)	Adjusted OR (CI) ^b	P ^c
All subjects:	68/32	62/62	2.13 (1.23–3.68)	1.93 (1.04–3.56)	0.037
Age					
<55 (years)	26/7	32/27	3.13 (1.18–8.34)	2.59 (0.85–7.89)	0.093
55 (years)	42/25	30/35	1.96 (0.98–3.93)	1.59 (0.74–3.42)	0.232
Sex					
Male	54/26	38/30	1.64 (0.84–3.20)	1.73 (0.84–3.53)	0.136
Female	14/6	24/32	3.11 (1.04–9.28)	2.66 (0.74–9.59)	0.134
Smoking statuses					
Never	18/8	6/2	0.75 (0.12–4.56)	2.53 (0.24–26.40)	0.438
Ever	49/24	53/57	2.20 (1.19–4.06)	1.92 (0.99–3.71)	0.052
Alcohol statuses					
Never	29/17	28/20	1.22 (0.53–2.79)	1.24 (0.49–3.17)	0.652
Ever	38/15	32/38	3.01 (1.41–6.44)	2.73 (1.17–6.35)	0.020
Tumor site					
Oropharynx 1	40/19	62/62	2.25 (1.17–4.30)	2.17 (1.06–4.45)	0.035
Non-oro-pharynx	25/13	62/62	2.05 (0.96–4.37)	1.97 (0.83–4.67)	0.123

^aLow DSBRC group (<52.27)/high DSBRC group (≥ 52.27) using the median DSBRC in controls as the cutoff point value.

^bAdjusted for age, sex and smoking status in logistic regression models when appropriate.

^cObtained from the logistic regression analysis with adjustment for age, sex and smoking status.