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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 smoke 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 applied a flow cytometry-based method to detect the DSB repair phenotype first in four EBV-immortalized human lymphoblastoid cell lines and then in human peripheral blood T-lymphocytes (PBTLs). 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 was significantly lower in cases (42.1%) than that in controls (54.4%) ($P < 0.001$). When we used the median DSB repair capacity level in the controls as the cut-off value for calculating the odds ratios (ORs) with adjustment for age, sex, smoking and drinking status, the cases were more likely than the controls to have a reduced DSB repair capacity (adjusted OR = 1.93; 95% confidence interval, CI = 1.04–3.56, $P = 0.037$), especially for those subjects who were ever drinkers (adjusted OR = 2.73; 95% CI = 1.17–6.35, $P = 0.020$) and had oropharyngeal tumors (adjusted OR = 2.17; 95% CI = 1.06–4.45, $P = 0.035$). In conclusion, these findings suggest that individuals with a reduced DSB repair capacity may be at an increased risk of developing SCCHN. Larger studies are warranted to confirm these preliminary findings.

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 contracting HPV-associated SCCHN. The fact that only a fraction of smokers, drinkers, or people exposed to HPV eventually 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 apoptosis to eliminate cells with overwhelming damage to DNA.

Smoking causes DNA damage, including double-strand DNA breaks (DSBs) that are one of the most serious forms of DNA damage to the cells. In eukaryotes, there are two major pathways for DSB repair: homologous recombianation (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 the DSB repair phenotype to assess individual susceptibility to SCCHN. Previous attempts to measure DNA DSB repair in patients undergoing

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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chemotherapy relied on the comet assay, an extremely sensitive method for measuring DNA damage, including basic sites and alkali sensitive sites, in which individual cells are molded into agarose on microscopic slides and exposed to an electrical field after the 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 in cancer research to monitor DSBs [12]. The phosphorylated form of H2AX was named γ-H2AX, because it was first observed in cells exposed to γ-rays. The γ-H2AX does not always indicate the presence of DSBs in normal conditions; however, with the treatment of Etoposide (ETOP, a topoisomerase II inhibitor), which could induce DSBs by inhibiting the ligation function of topoisomerase II (topo II) on cleaved DNA, changes of the γ-H2AX levels would specifically reflect the change of DSBs [12]. The formation of γ-H2AX is an early cellular response to DSBs, and thus, γ-H2AX is a universal biomarker for DSB induction [13]. It has been reported that the capability of γ-H2AX assay for detecting DSBs is 100-fold more sensitive by using the flow cytometry method than by the comet assay [14]. ETOP has been used as an anti-cancer drug that can induce 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 DSB levels before and after the treatment [16–18].

For the methodology, the foci counting for γ-H2AX is performed with 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 the difficulty of identifying suitable foci by eye, which is somewhat subjective, particularly when slides were not well coded [22]. Moreover, this method is difficult to adapt in epidemiology research and clinical practice. In contrast, the flow cytometry method, a high-throughput, statistically robust technique, allows simple detection and quantification of γ-H2AX in a large number of cells in a short time period [23], although the main disadvantage is of a 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]. Accordingly, when we used ETOP to induced DSBs, the flow-cytometry-based method was easy to detect the DSB repair phenotype in human peripheral blood T-lymphocytes (PBTLs). With this blood-based laboratory assay, therefore, we conducted a pilot case-control study with 100 SCCHN patients and 124 cancer-free controls to evaluate the effect 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 primaries (n = 3; 3%), who were recruited from The University of Texas M. D. Anderson Cancer Center between Jan 2012 and May 2013. Patients with second SCCHN primary tumors, primary tumors of the nasopharynx or sinonasal tract, or any histopathologic diagnosis other than SCCHN were excluded. Additional 124 cancer-free controls of non-Hispanic whites 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 any one included in this study. Having provided their written informed consent, each eligible subject provided additional information about risk factors, such as tobacco smoking and alcohol use in a form of life-style questionnaire and a one-time sample of 10 ml of blood for biomarker tests. The research protocol was approved by the institutional review board.

2.1.1. Cell lines, cell culture and ETOP treatment

In initial experiments, we used four EB virus (EBV)-immortalized human lymphoblastoid cell lines from the Human Genetic Mutant Cell Repositories (Camden, NJ): 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, with which we tested the sensitivity and specificity of cellular repair of ETOP-induced DSBs. All of the cells were cultured in 12-mm × 50-mm tubes at 37°C in 5% CO_2 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 that was completely dissolved in DMSO (Life Technologies, Inc., Grand Island, NY). The final concentration of DMSO was of 0.1% in the culture medium, which 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 μM. Each cultured sample (1×10^6 cells) were then aliquoted into four 12-mm × 50-mm tubes and cultured for 2 h with the ETOP-working solution added into the culture medium. Then the culture medium was replaced by the fresh medium, and the cells were allowed to be cultured for an additional period of up to 4 h, at the indicated time points. Finally, cells were harvested and fixed with 1% paraformaldehyde, washed with 1x PBS and stored in 70% ethanol at −20°C until used for the flow cytometry analysis of DSB repair.

2.2. Human peripheral blood T-lymphocyte (PBTL) culture and ETOP treatment

For the ETOP-induced DSB assay, we used phytohemagglutinin-stimulated PBTLs from the whole blood, because T-lymphocytes have a complete repair response to DNA damage. Each day, peripheral blood samples from the subjects were 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 h at 37°C in an incubator with 5% CO_2. Each cultured sample was then aliquoted (1×10^6 cells×4) into four 12-mm × 50-mm tubes (*i.e.*, one treated for 2 h compared with one untreated; one treated for 2 h and then medium replaced for repair for another 4 h compared with one untreated). 10-μM ETOP was chosen for the *in vitro* treatment based on prior determined doses. At the indicated time points, cells were harvested and fixed 1% paraformaldehyde, washed with 1x PBS and finally stored in 70% ethanol at −20°C until used for the flow cytometry analysis of DSB repair.

2.3. Cell viability assay

Cell viability was assessed by 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 seeded in triplicate onto a 96-well plate (1×10^5/well) in 100 μl of the complete RPMI 1640 medium, and each condition was performed in eight replicate wells.