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Measurement of DNA damage in peripheral blood by the γ-H2AX assay as predictor of colorectal cancer risk

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ABSTRACT

The detection of γ-H2AX focus is one of the most sensitive ways to monitor DNA double-strand breaks (DSBs). Although changes in γ-H2AX activity have been studied in tumor cells in colorectal cancer (CRC), changes in peripheral blood lymphocytes (PBLs) have not been examined previously. We hypothesize that higher levels of irradiation-induced γ-H2AX in PBLs may be associated with an elevated risk of colorectal cancer (CRC). In a case-control study, the baseline and ionizing radiation (IR)-induced γ-H2AX levels in PBLs from frequency-matched 320 untreated CRC patients and 320 controls were detected by a laser scanning cytometer-based immunocytochemical method. We used unconditional multivariable logistic regression to evaluate CRC risk by using the ratio of IR-induced γ-H2AX to the baseline levels with adjustment of age, sex and smoking status. We found CRC cases had significantly higher γ-H2AX ratio (1.5 vs. 1.41, $P < 0.0001$) compared with controls. When using the median γ-H2AX ratio of controls as a cutoff point, we found higher γ-H2AX ratio was significantly associated with an increased risk of CRC (OR = 6.72, 95% CI = 4.54–9.94). Quartile analyses also showed significant dose–response relationship between higher γ-H2AX ratio and increased risk of CRC (P for trend < 0.0001). Age, sex, BMI and smoking status also influenced the association of γ-H2AX ratio with CRC risk; however, no interactions with γ-H2AX ratio were observed. These results support the premise that DSBs in peripheral blood as measured by γ-H2AX level might represent an intermediate phenotype to assess the risk of CRC. Future prospective studies are necessary to confirm our findings in independent populations.

1. Introduction

In the United States, about 132,700 new CRC cases are diagnosed annually, of which 93,090 are colon and 39,610 are rectal cancers. Annually, approximately 49,700 cases die of colon cancer, of which 26,100 are male and 23,600 are female [1]. Although many environmental risk factors are known to be associated with CRC risk and have altered recommendations for CRC screening to some extent [2], many cases occurred with no known risk factors, indicating inherited susceptibility and genetic factors might also increase the likelihood of developing CRC.

DNA double-strand breaks (DSBs) is one of the major and deleterious forms of DNA damage. The repair of DSBs is a major cellular function to maintain genomic stability and integrity. Susceptibility to DNA damage and any failure to repair DSBs might cause increased risk of cancer [3,4]. A key component of DNA repair is the appearance of serine 139 phosphorylated histone protein H2AX (γ-H2AX), which form rapidly in the chromatin surrounding a DSB site after DNA damage and has become a popular and promising biomarker to predict DNA DSB repair in cancer research and translational studies [5,6].

γ-H2AX could easily be detected by specific antibodies against the phosphorylated C-terminal peptide of H2AX, allowing quantification of DSBs by using only a small sample of biological material [5,6]. Peripheral blood lymphocytes (PBLs) are good models for γ-H2AX and we have developed a high-throughput, image-based phenotypic assay to detect ionizing radiation (IR)-induced DSBs in PBLs. Our previous studies also showed that ironizing radiation-induced gamma-H2AX activity in whole blood culture was strong predictor for the risk of different types of cancer [7–9].

Currently, there are no studies that examined the relationship between γ-H2AX activity and CRC risk. In this case-control study,

Abbreviations: DSBs, DNA double-strand breaks; PBLs, peripheral blood lymphocytes; CRC, colorectal cancer; IR, ionizing radiation; ORs, odds ratios; BMI, body mass index; HR, homologous recombination; NHEJ, non-homologous end joining.

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we utilized a high-throughput laser scanning cytometer based quantitative fluorescence assay to detect the γ-H2AX level of baseline as well as the H2AX ratio before and after gamma irradiation in PBLs. We hypothesized that a higher level of radiation-induced DSBs as represented by γ-H2AX ratio is associated with elevated risk of CRC.

2. Materials and methods

2.1. Study population and epidemiologic data

All CRC patients were newly diagnosed (within 1 year of diagnosis before enrollment in this study) and histologically confirmed colorectal cancer patients who were recruited from the University of Texas MD Anderson Cancer Center between June 2009 and December 2014. There were no age, gender, ethnicity, and clinical stage restrictions on recruitment. The healthy controls without any prior history of cancer (except non melanoma skin cancer) were recruited using the random digit dialing method from the Kelsey-Seybold Clinic in the Houston metropolitan area. Controls must have lived for more than 1 year in the same county or socioeconomically matched surrounding counties in Texas as the cases reside. Selected controls were frequency matched with the case patients in age (±5 years), gender, and ethnicity. Only non-Hispanic White individuals were included in this study in order to minimize the effects of population admixture. Epidemiologic data were collected from all participants by face-to-face interviews utilizing a structured questionnaire by trained stuff. The data collected included demographic characteristics, family history of cancer, tobacco use history, medical history, alcohol consumption history, and environmental dust exposures, etc. An individual who never smoked or smoked less than 100 cigarettes in his or her lifetime was considered to be a never smoker. An individual who had smoked more than 100 cigarettes in his or her lifetime was considered as an ever smoker. Ever smokers included both former smokers and current smokers. A 40-mL sample of peripheral blood was collected and delivered to the laboratory for analyses after interview. All study participants signed a written informed consent, and the study was approved by both the Institutional Review Boards of The University of Texas MD Anderson Cancer Center (Houston, TX) and the Kelsey-Seybold Clinics (Houston, TX).

2.2. Whole-blood culture and γ-H2AX assay

All procedures were performed in uniform manner by designated, highly trained laboratory staff without knowledge of the case-control status of each sample. A total of 0.4 ml of fresh whole blood from each subject was divided into two 0.2 ml aliquots equally and then plated in two 30 mm dishes with 1.8 ml of RPMI 1640 (JRM Biosciences, Lenexa, KS) supplemented with 15% fetal calf serum (Life Technologies) and 1.25% phytohemagglutinin (Remel, Lenexa, KS, USA). Samples were cultured for 72 h at 37 °C. Baseline DSB (untreated) was measured in one culture and IR-induced DSB (treated) measured in the other. For the γ-radiation treatment, cultured whole blood cells were exposed to 2.5 Gy γ-radiation from a cesium-137 source (cesium irradiator Mark 1, Model 30; J.L. Shepherd and Associates, Glendale, CA) at room temperature. Afterward, the cells were immediately placed in a 37 °C incubator for 1 h, based on the fact that the highest induced γ-H2AX levels were observed 1 h after IR during our assay development (Supplemental Table 1). H2AX phosphorylation for both untreated and IR-treated whole blood cultures was detected by using a modification of a previously described protocol [10]. Briefly, Triton X-100 [0.12% in phosphate buffered saline (PBS)] was added to blood cells to lyse the erythrocytes. The remaining leukocytes were fixed in 4% formaldehyde (Sigma) for 10 min, then washed and fixed in 50% cold methanol in PBS for 10 min. The leukocytes were spotted onto glass slides and blocked in PBS with 4% bovine serum albumin for 30 min, incubated with mouse monoclonal γ-H2AX antibody (BioLegend, San Diego, CA) for 30 min, washed in PBS and incubated with FITC-conjugated horse anti-mouse secondary antibody (Vector Laboratories) for 30 min. After complete washing, slides were mounted with propidium iodide counterstain (Abbott Molecular, Abbott Park, IL) and covered with cover slips. All experiments were conducted at room temperature except when specific temperature is mentioned. The above immunocytochemistry assay was conducted simultaneously for the paired untreated and treated blood samples. The fluorescence signals were measured by an iCys™ laser scanning cytometer (CompuCyte, Cambridge, MA). The 488 nm laser was used to scan slides and cell count data were obtained with a ×40 objective at 4 μ pixelation. The clustered cells and fragments of fluorescence were excluded to eliminate uncountable cells and nonspecific fluorescence signals. Counting was performed until 5000 dispersed and contoured cells were registered. The mean readings of fluorescent intensity per cell was calculated as baseline (un-treated) and IR-induced γ-H2AX level (treated), and the γ-H2AX ratio which was defined as the ratio of γ-H2AX level in treated cells to that at baseline in untreated cells was also calculated to predict CRC risk. To test intra-assay variability, we have performed triplicate assays of some fresh blood samples from study enrollments, and the results were highly consistent. The average coefficient of variation (CV) of these triplicates was 3.87% for the γ-H2AX ratio, 8.28% for the baseline and 9.69% for the induced γ-H2AX level, respectively (Supplemental Table 2).

2.3. Statistical analysis

Pearson X^2 was preformed to compare the distributions of different variables between cases and controls. Student *t* test was utilized to detect the difference among continuous variables. The Wilcoxon rank-sum test was performed to test the difference for baseline and radiation-induced γ-H2AX levels, and γ-H2AX ratio between cases and controls. γ-H2AX ratios were stratified by median, tertile, and quartile on the basis of their distribution in the control group. Unconditional multivariable logistic regression was performed to assess odds ratios (ORs) after adjusting for age, sex, smoking status where appropriate. All statistical tests were two-sided. Associations were considered statistically significant at $P < 0.05$. All statistical analyses were conducted using the STATA 10.1 software (Stata Corp, College Station, TX).

3. Results

3.1. Demographic characteristics and γ-H2AX level in cases and controls

The demographic characteristics and γ-H2AX levels for the 320 patients with CRC and 320 healthy controls were summarized in Table 1. Majority of CRC patients received chemotherapy (72%) with the remainder surgery only treatment. As a result of frequency matching, no statistically significant difference was observed between cases and controls regarding any of the epidemiological factors, including age, gender, smoker/smoking status, BMI (body mass index), diabetes, cancer history of family and CRC history of family. Three levels of γ-H2AX were measured, including baseline, IR-induced, and the ratio of these two levels. Photomicroscopy of γ-H2AX signals in PBLs derived from a representative sample before and after IR is shown in Fig. 1. We found cases had borderline significant higher baseline γ-H2AX levels (1236.9 vs. 1123.4, $P = 0.0747$), but significantly higher IR-induced γ-H2AX

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