Human Cancer Biology

Decreased NK Cells in Patients with Head and Neck Cancer Determined in Archival DNA

William P. Accomando1, John K. Wiencke3, E. Andres Houseman4, Rondi A. Butler1, Shichun Zheng3, Heather H. Nelson5,6, and Karl T. Kelsey1,2

Abstract

Purpose: Natural killer (NK) cells are a key element of the innate immune system implicated in human cancer. To examine NK cell levels in archived bloods from a study of human head and neck squamous cell carcinoma (HNSCC), a new DNA-based quantification method was developed.

Experimental Design: NK cell-specific DNA methylation was identified by analyzing DNA methylation and mRNA array data from purified blood leukocyte subtypes (NK, T, B, monocytes, granulocytes), and confirmed via pyrosequencing and quantitative methylation specific PCR (qMSP). NK cell levels in archived whole blood DNA from 122 HNSCC patients and 122 controls were assessed by qMSP.

Results: Pyrosequencing and qMSP confirmed that a demethylated DNA region in NKP46 distinguishes NK cells from other leukocytes, and serves as a quantitative NK cell marker. Demethylation of NKP46 was significantly lower in HNSCC patient bloods compared with controls (P < 0.001). Individuals in the lowest NK tertile had over 5-fold risk of being a HNSCC case, controlling for age, gender, HPV16 status, cigarette smoking, alcohol consumption, and BMI (OR = 5.6, 95% CI, 2.0 to 17.4). Cases did not show differences in NKP46 demethylation based on tumor site or stage.

Conclusions: The results of this study indicate a significant depression in NK cells in HNSCC patients that is unrelated to exposures associated with the disease. DNA methylation biomarkers of NK cells represent an alternative to conventional flow cytometry that can be applied in a wide variety of clinical and epidemiologic settings including archival blood specimens. Clin Cancer Res; 18(22); 6147–54. ©2012 AACR.

Introduction

Genetics alone cannot account for lineage commitment during cellular differentiation, because terminally differentiated cells possess identical genomes but exhibit a wide array of cellular phenotypes. Epigenetics, or the heritable and stable regulation of gene expression beyond the DNA sequence, plays an essential role in determining cellular fates during developmental progression from stem cells to differentiated cells (1–3). As a result, epigenetic marks can serve as reliable indicators of cell lineages, and subsequently as biomarkers for specific cell types and subtypes (4). The archetypical epigenetic mark is DNA methylation occurring on cytosine residues in the context of cytosine-guanine dinucleotide (CpG) sites; it is both easy to measure and critical to the maintenance of cellular identity (5, 6). De novo DNA methylation of somatic cell lineages occurs during differentiation and is followed by maintenance methylation (7), suggesting that DNA methylation profiles are less prone to transient variation than RNA expression profiles and may be more reliable markers of cellular phenotype than gene expression products such as surface proteins and secreted factors.

Human leukocytes can be quantified in cell mixtures by DNA-based assays that target cell lineage specific differentially methylated regions (DMR). For example, demethylation of FOXP3 is a specific marker of stable CD4+ CD25+FOXP3+ Regulatory T cells (Tregs) that can be measured by quantitative methylation specific PCR (qMSP) to enable highly sensitive and accurate counts of Tregs in blood and tissues (8–10). This type of DNA methylation-based cell quantification assay offers several advantages over traditional protein based methods, such as fluorescence-activated cell sorting (FACS). First, patterns of DNA methylation can offer superior resolution in distinguishing certain cell subtypes. Activated T cells are very difficult to
Research aimed at further understanding immune cell level alterations associated with cancer and other diseases has, until now, been restricted by the limitations of immunodiagnostic methods. Here we describe a new method for measuring natural killer (NK) cell levels in human blood and tissue based on cell-lineage specific DNA methylation that can be applied to samples regardless of handling and storage procedures. This represents a step forward in immune cell detection and quantification that could potentially be applied to many types of clinical samples. Applying the method to a case-control study of human head and neck squamous cell carcinoma (HNSCC) revealed a case-associated decrease in circulating NK cells that is independent of known risk factors and treatments. This suggests that it is important to monitor NK cell levels in patients with HNSCC, and that it may be worthwhile to pursue future immune therapies aimed at restoring circulating NK cells in patients with HNSCC.

In recent years, the role of natural killer (NK) cells in the immune system has received much attention. NK cells are a type of lymphocyte that can function as both a component of the innate immune system and as an effector of the adaptive immune system. They are able to recognize and destroy precancerous and malignant cells, and are able to recognize and destroy virally infected cells. NK cells are also able to kill tumor cells that have escaped the surveillance of the adaptive immune system.

NK cells are of particular interest in the context of HNSCC and other cancers, because they are able to recognize and destroy precancerous and malignant cells. In fact, NK cells have been associated with improved survival in studies of many cancers and exposures.

Materials and Methods

Sorted leukocyte samples

Normal human peripheral blood leukocytes were isolated by magnetic-activated cell sorting (MACS; Miltenyi Biotech Inc.) and purity was confirmed by FACS. The major cell types obtained included NK cells (n = 9), B cells (n = 5), T cells (n = 16), monocytes (n = 5), and granulocytes (n = 8).

Archival blood samples

Details of this study population have been previously described (25). In brief, peripheral blood from 122 control donors and 122 HNSCC patients was collected between December 1999 and December 2003 in the greater Boston area. Patient bloods were drawn at or as soon as possible after disease diagnosis. Population based control subjects with no prior history of cancer were from the same region as cases, and were frequency matched on age and gender. Study approval was obtained from the Brown University Institutional Review Board. All subjects provided written informed consent for participation in this study. Venous anticoagulated whole blood was drawn into sodium citrate and stored at −20°C before DNA isolation.

Nucleic acid extraction and bisulfite conversion

DNA and RNA were co-extracted from MACS sorted leukocytes using AllPrep DNA/RNA mini kit (Qiagen Inc.). DNA from archived blood was extracted with DNeasy Blood & Tissue kit (Qiagen Inc.). DNA was sodium bisulfite treated with the EZ DNA Methylation Kit (Zymo Research Corporation).

DNA methylation microarray

The Infinium HumanMethylation27 Beadchip Microarray (Illumina Inc.) quantifies the methylation status of 27,578 CpG loci from 14,495 genes, with a redundancy of 15- to 18-fold. The ratio of fluorescent signals was computed from both alleles using the following equation: 
$$β = (max(M,0))/(|U| + |M|) + 100.$$ 

The resultant β value is a continuous variable ranging from 0 (unmethylated) to 1 (completely methylated) that represents the methylation at each CpG site and is used in subsequent statistical analyses. Data were assembled with the methylation module of GenomeStudio software (Illumina, Inc.; ref. 30).

We have identified a novel DMR that distinguishes NK cells from other leukocytes to facilitate the quantification of overall NK cell numbers in archived blood samples from a case-control study of HNSCC. Many chemical exposures, such as tobacco and alcohol, as well as viral factors, such as human papilloma virus (HPV), are known or suspected to be causal factors in HNSCC (25, 26) and may independently affect immune profiles (27–29). Hence unlike previous studies, this is among the initial attempts to evaluate the effects of these factors on the depression in NK immune profile. Here we evaluate patient risk factors and disease characteristics (e.g., tumor location) in relationship to NK cells to determine the independent associations of HNSCC with innate immune parameters.

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**Bisulfite pyrosequencing**

Pyrosequencing assays to validate microarray results were designed using Pyromark Assay Design 2.0 (Qiagen Inc.), and carried out on a Pyromark MD pyrosequencer running Pyromark qCpG 1.1.11 software (Qiagen Inc.). Oligonucleotide primers were obtained from Life Technologies.

**RNA expression microarray**

The Whole-Genome DASL HT Assay Kit (Illumina Inc.) simultaneously profiles of over 29,000 mRNA transcripts. Data were assembled with the expression module of GenomeStudio software (Illumina Inc.).

**qMSP**

Primers and TaqMan major groove binding (MGB) probes with 5’ 6-FAM and 3’ nonfluorescent quencher (NFQ) as well as TaqMan 1,000 RXN Gold with Buffer A Pack were obtained from Life Technologies. Oligonucleotide sequences are shown in Supplementary Table S1. Solutions and conditions for qMSP were as described previously (31) with some modifications. The 10× TaqMan Stabilizer containing 0.1% Tween-20, 0.5% gelatin was prepared weekly. Each reaction of 20 μl contained 5 μl DNA, 11.9 μl preMix, 3 μl oligoMix, and 0.1 μl Taq DNA polymerase. Cycling was carried out using a 7900HT Fast Real-Time PCR System (Applied Biosystems); 50 cycles at 95°C for 15 seconds and 60°C for 1 minute after 1 minute at 95°C preheat. All samples were run in triplicate using the absolute quantification method.

Quantification of total bisulfite converted DNA copies was determined by reference to the C-less PCR assay as described previously (31, 32). In brief, C-less primers and probes recognize a DNA sequence without cytosines; hence, the assay amplifies the total amount of DNA in a PCR regardless of bisulfite conversion or methylation status. A conversion factor of 1 diploid human cell equal to 6.6 picograms (pg) of DNA (thus 3.3 pg per copy) was used to calculate copy number.

Normal human blood DNA quantified by UV absorption (Nanodrop, Inc.) was used to generate a 4 point standard curve with 30,000 copies, 3,000 copies, 300 copies, and 30 copies of genomic DNA. This standard curve was run on each sample plate enabling quantification of DNA from Ct values. As C-less primers hybridize to both strands of the standard DNA (nonbisulfite converted) whereas bisulfite converted samples allow for only single strand hybridization during the first cycle, the resultant copy number in bisulfite samples was multiplied by 2. Bisulfite converted, universal methylated DNA standard (Zymo Research Corporation) and bisulfite converted, isolated NK cell DNA were quantified at the same time using the C-less assay. Resultant copy number measurements were used to create a calibration curve for the NKp46 demethylation assay. Known NK cell DNA copy numbers were spiked into universal methylated DNA in ratios that maintained a constant total number of DNA copies (10,000 copies) in each reaction across the dilution scheme. This mimics the conditions of detection that exist in differentiating different relative numbers of NK cells within a complex mixture of cells in a biologic sample. For absolute quantification of NKp46 demethylation, the 4-point standard curve used 10,000 copies, 1,000 copies, 100 copies, and 10 copies of bisulfite converted NK cell DNA.

**FACS**

Venous whole blood samples were collected in citrate EDTA and processed using a lysis no wash protocol (Invitrogen, Cat. No. GAS-010). Cells were labeled by direct staining with the appropriate fluorochrome-conjugated antibodies for the detection of CD45”CD16“CD3” CD56dim NK cells and CD45”CD16“CD3” CD56bright NK cells (eBioscience Inc.), and incubated for 20 minutes in the dark at 4°C; anti-human CD16 PE-Cy7 (Cat. No. 25-0168-41), anti-human CD56 PE (Cat. No. 12-0567-41), anti-human CD56 PerCP-Cy5.5 (Cat. No. 45-0459-41), and anti-human CD3-fluorescein isothiocyanate (FITC, Cat. No. 11-0038-41). Isotype control mAbs were used as negative controls. Accucheck counting beads (Invitrogen, Cat. No. PCB100) were used for quantifying leukocyte numbers. Acquisition was carried out within 12 hours of blood draw on the FACS Aria III flow cytometer (Becton Dickinson) using FACS Diva software (Becton Dickinson). Absolute counts (number of cells per μl) were obtained by taking the number of cells counted, divided by total number of beads counted, and multiplied by the known concentration of beads. Flowjo software (TreeStar Inc.) was used for data analysis.

**Statistical methods**

**Modeling the DNA methylation microarray data.** A linear mixed effects model was applied to the Illumina Infinium® HumanMethylation27 data using SAS (SAS Institute Inc., Cary, NC). Cell type was designated as the fixed effect whereas beadchip plate was the random effect. For this study, the fixed effect groups were NK cells and non-NK cells, which included pan T-lymphocytes, CD4+ T-lymphocytes, Tregs, CD8+ T-lymphocytes, B-lymphocytes, granulocytes and monocytes. This generated coefficients that estimated differential methylation such that, for any particular locus, a negative coefficient indicated less methylation in NK cells than in the other cell types. Resultant p-values were adjusted for multiple comparisons using the “qvalue” package in R (http://www.r-project.org/).

**Modeling the RNA expression microarray.** Linear models were applied to the Illumina Whole-Genome DASL HT using the “limma” package in R (http://www.r-project.org/). RNA expression for MACS isolated NK cells was compared with each of the following MACS isolated leukocytes: pan T-lymphocytes, CD4+ T-lymphocytes, Tregs, CD8+ T-lymphocytes, B-lymphocytes, granulocytes, and monocytes. This generated estimates for log-fold changes in RNA expression between NK cells and each of the aforementioned cell types, where a positive value indicated...
higher RNA expression in NK cells compared with a particular cell type. Resultant P values were adjusted for multiple comparisons using the "qvalue" package in R (http://www.r-project.org/). NK cell specific differential RNA expression was considered significant only when all 7 q values were less than 0.1.

**Results**

**NKp46 demethylation is a biomarker of NK cells**

An unbiased, bioinformatics-based approach was implemented to select candidate NK cell-specific DMRs, and these candidates were then validated by pyrosequencing and qMSP. Statistical analyses of DNA methylation and RNA expression microarray data from MACS isolated human leukocytes (FACS validated) normal human leukocyte subtypes were integrated to identify many putative DMRs that could potentially serve as reliable epigenetic biomarkers of NK cells. This list was then narrowed to CpG loci that were significantly demethylated in NK cells \( (q < 0.1, \text{ coefficient } < 0) \) and within genes whose RNA expression was significantly elevated in NK cells \( (q < 0.1, \log \text{ fold-change } > 1) \) relative to other leukocyte subtypes (Fig. 1). Pyrosequencing and qMSP of bisulfite converted DNA from the MACS purified leukocytes confirmed that a region near the promoter of NKp46 is demethylated in NK cells, but methylated in T cells, B cells, granulocytes, and monocytes (Fig. 2 and Supplementary Fig. S1). Furthermore, the CD56\(^{dim}\) subset of NK cells showed complete demethylation in the NKp46 region, whereas CD56\(^{bright}\) NK cells seem to exhibit only partial demethylation in the region as measured by qMSP. The NKp46 qMSP assay was optimized to fit a log-linear relationship between lower Ct values (more demethylated copies of NKp46) and increased NK cell DNA content (Pearson \( R = -0.996, P < 2.2 \times 10^{-16}; \) Fig. S3). Fresh blood samples collected from 5 individuals were analyzed using standard flow cytometry for CD56\(^{dim}\) and CD56\(^{dim}\) NK cells, and DNA extracted from the same 5 samples was subjected to the NKp46 demethylation assay to compare this new method of assessing NK cells levels to an established, protein based method. The results revealed a significant linear relationship between the 2 methods \( (P = 0.034) \) with a high correlation (Pearson \( R = 0.91; \) Supplementary Fig. S2).

**Diminished circulating NK cell levels in HNSCC**

The calibrated NKp46 qMSP assay was used to measure the level of circulating NK cells in the peripheral blood of patients with HNSCC and cancer free controls. The demographics of the study population are shown in Table 1. Univariate analysis revealed that significantly fewer demethylated copies of NKp46 were detected in HNSCC bloods than in control bloods \( (P < 0.0001, \text{ Supplementary Fig. S3}), \) indicative of a diminished NK cell compartment in the peripheral blood of HNSCC patients. In all cases and controls, there was no significant univariate association between the measured number of demethylated NKp46 copies and age, gender, HPV16 (E6 and/or E7) serology, cigarette smoking, alcohol consumption, or body mass index (BMI). In HNSCC case bloods, there were no significant differences in the number of demethylated NKp46 copies detected in patients with oral, pharyngeal, and laryngeal tumors (Supplementary Fig. S4), nor were there
significant differences in NKp46 demethylation between individuals with stage I, II, III, and IV disease (Supplementary Fig. S5). To determine whether the observed association between NK cells and case status was attributable to systemic chemotherapy or other treatments, we compared the number of demethylated NKp46 copies detected in case bloods drawn within 1 month of diagnosis to those drawn more than 1 month after diagnosis and found no significant difference (Supplementary Fig. S6).

The NKp46 qMSP measurements from cancer-free control bloods were used to determine cutoffs for NKp46 demethylation tertiles. The proportion of total HNSCC cases decreased significantly with increasing demethylation tertile (P < 0.001, Fig. 4), indicating that HNSCC patients are more likely to have depressed levels of NK cells in their peripheral blood. The trend held true even when cases were stratified by HPV16 (E6 and/or E7) serology, and when cases were stratified by whether or not blood was drawn within a month of diagnosis. Multivariate logistic regression controlling for age, gender, cigarette smoking, alcohol consumption, BMI, and HPV16 (E6 and/or E7) serology confirmed increased HNSCC risk for individuals in the lower 2 normal NKp46 demethylation tertiles (Table 2), indicating that lower levels of NK cells in the peripheral blood are significantly associated with HNSCC.

Discussion

The present study advances our understanding of immune factors in HNSCC in 2 ways: first we developed a novel and sensitive biomarker of NK cells that is highly useful in archival blood specimens, and second we assessed this new NK biomarker in relationship to risk factors and clinical prognostic factors in HNSCC. Applying this new method to archived blood samples from a case-control study of HNSCC revealed a decreased NK cell fraction in the peripheral blood of HNSCC patients compared with cancer free controls. Past studies have indicated that NK cell suppression is a feature of HNSCC (15), but none have conclusively shown a disease-associated decrease in circulating NK cells relative to other leukocytes in the peripheral blood. One study did find decreased levels of the more regulatory CD56bright NK cell subset in the peripheral blood of patients with HNSCC compared with controls (14). It is likely that the HNSCC-associated circulating NK cell decrease we have observed in this study is primarily the result of a diminished CD56dim NK cell compartment in the peripheral blood of HNSCC patients compared with cancer free controls. Past studies have indicated that NK cell suppression is a feature of HNSCC (15), but none have conclusively shown a disease-associated decrease in circulating NK cells relative to other leukocytes in the peripheral blood. The trend held true even when cases were stratified by HPV16 (E6 and/or E7) serology, and when cases were stratified by whether or not blood was drawn within a month of diagnosis. Multivariate logistic regression controlling for age, gender, cigarette smoking, alcohol consumption, BMI, and HPV16 (E6 and/or E7) serology confirmed increased HNSCC risk for individuals in the lower 2 normal NKp46 demethylation tertiles (Table 2), indicating that lower levels of NK cells in the peripheral blood are significantly associated with HNSCC.

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Table 1. Demographic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 122)</th>
<th>HNSCC (n = 122)</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>62 (12)</td>
<td>61 (12)</td>
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<td>L1+, no. (%)</td>
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<td>6 (5%)</td>
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<td>Cigarette smoking status</td>
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<td>Never, no. (%)</td>
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Figure 4. Prevalence of HNSCC by normal NKp46 demethylation tertile. Normal NKp46 demethylation tertile cutoffs were determined from control bloods only. Higher tertiles indicate higher NK cell levels. HNSCC prevalence refers to the percentage of total cases from this study whose NKp46 demethylation measurements fell within the control derived tertile range. Displayed P value is from a χ² test for trend in proportions.

Previous work has shown that NK cell activity has prognostic significance (34–36). Our strategy for developing a DMR that would identify NK cells was aimed at assessing NK cell numbers, and not to specifically inform us about the functional activities of NK cells that are present. However, future work could also define NK cell DMRs associated with activation. Comparing patterns of DNA methylation in isolated NK cells exhibiting different types of cytotoxic activation using the same methodology used in this work might reveal important biomarkers. NK cell activity-associated DMR assays could then be run in tandem with the NKp46 demethylation assay to assess both NK cell numbers and activities.

Other studies have indicated that cigarette smoking can attenuate the cytotoxic activity of NK cells. However, this is not accompanied by a difference in the number of NK cells in the blood or lung tissue of smokers compared with nonsmokers (27, 37). Consistent with these findings, our data confirmed that peripheral blood NK cell levels are not associated with cigarette smoking. In our data, peripheral blood NK cell levels were not associated with age, gender, HPV16 (E6 and/or E7) serology, alcohol consumption or BMI. We investigated primarily men with lean BMIs (<30), limiting our power to detect differences in NK cell levels associated with gender and BMI. In addition, because blood samples were collected as close to the diagnosis date as possible, it was not possible to use these data to analyze the longer-term effects of chemotherapy or radiation treatment on circulating NK cell levels. However, this study design allowed us to rule out oncologic treatment as the underlying cause of the observed decrease in NK cells. There were also no differences in peripheral blood NK cell levels between HNSCC patients with oral, laryngeal and pharyngeal...
HNSCC could also shed light on the relationship between the underlying cause of the disease that is independent from other risk factors. Individuals with lower levels of NK cells could be predisposed to developing cancers, such as HNSCC, due to reduced antitumor immune surveillance, because NK cells play a prominent role in the recognition and elimination of incipient cancer cells. The underlying cause of the disease may also be systemic inflammation, which causes the peripheral blood to be flooded with granulocytes hereby causing an apparent decrease in the proportion of NK cells in a blood sample. Alternatively, the lower numbers of circulating NK cells in the peripheral blood of patients with HNSCC could be a result of the disease itself. Tumors manipulate their microenvironment by secreting chemokines that induce suppression and apoptosis of cytotoxic lymphocytes (38), particularly the CD56dim NK cell subset (39). The induction of NK cell death by secreted chemical signals emanating from immunoedited tumor cells may occur to such an extent that systemic alterations in immune cell levels can be detected in the peripheral blood. Likewise, the tumor may stimulate systemic inflammation by secreting proinflammatory cytokines. This could potentially increase the amount of granulocytes in the blood, causing an apparent decrease in the proportion of NK cells.

Prospective studies would provide the best evidence to determine if individuals with lower NK cell numbers are predisposed to developing cancer, and whether or not NK cells levels are altered by presence of head and neck cancer. This type of study is more feasible than even before, because our novel DNA methylation-based approach can assess NK cells levels in almost any blood sample, regardless of freshness or special handling regimes. Prospective studies of HNSCC could also shed light on the relationship between circulating NK cell levels and disease outcome. It is possible that extreme changes in the balance of circulating blood cells could compromise the body’s ability to monitor for malignant cells outside the tumor microenvironment, providing favorable conditions for local invasion and metastasis. In addition, immune status may be associated with effectiveness of any treatment, as a proper balance of immune cells could contribute to recovery.

Disclosure of Potential Conflicts of Interest

W.P. Accomando, J.K. Wiencke, R.A. Butler, H.H. Nelson, K.T. Kelsey share a relevant patent pending. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.P. Accomando, K.T. Kelsey
Writing, review, and/or revision of the manuscript: W.P. Accomando, J.K. Wiencke, R.A. Butler, H.H. Nelson, K.T. Kelsey
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.P. Accomando, R.A. Butler, K.T. Kelsey
Study supervision: K.T. Kelsey.

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Table 2. Logistic regression of HNSCC risk

<table>
<thead>
<tr>
<th>NKp46 demethylation tertile</th>
<th>Crude OR (95% CI)</th>
<th>P value</th>
<th>Adjusted OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st (lowest)</td>
<td>4.3 (2.2, 9.0)</td>
<td>5.0 × 10^{-5}</td>
<td>5.6 (2.0, 17.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>2nd (middle)</td>
<td>2.8 (1.4, 6.0)</td>
<td>0.006</td>
<td>4.9 (1.8, 16.1)</td>
<td>0.004</td>
</tr>
<tr>
<td>3rd (highest)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
</tbody>
</table>

*Unconditional multivariate model controlling for age, gender, smoking, drinking, BMI, and HPV16 (E6 and/or E7) serology.
Clinical Cancer Research

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