Published OnlineFirst September 26, 2012; DOI: 10.1158/1078-0432.CCR-12-1008

Human Cancer Biology

Clinical Cancer Research

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

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## 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

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Microarray data: Microarray data has been deposited on the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) according to Minimum Information About a Microarray Experiment (MIAME) under accession number GSE39981.

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**doi:** 10.1158/1078-0432.CCR-12-1008

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## Translational Relevance

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.

differentiate from Tregs using FACS, whereas these 2 cell types are easily distinguished by assessing methylation in the Treg specific *FOXP3* DMR (4). Second, qMSP assays are generally less expensive than flow cytometry and can be carried out using any real-time PCR machine. In addition, measuring cell levels by FACS requires a fresh blood or tissue sample that has undergone special handling to ensure that cell surface proteins are not denatured or degraded. This precludes most archived samples from being characterized by FACS, as well as fresh samples that were not collected with the express intention of being subjected to FACS analysis. Assessing cell levels by measuring DNA methylation does not require fresh samples or any special handling requirements, and can therefore be applied to almost any biologic sample including older archival bloods and tissues. Thus, cell type specific DNA methylation assays are powerful new tools to further our understanding of immune cell level alterations associated with human diseases and exposures.

Recent studies indicate that head and neck squamous cell carcinoma (HNSCC) is strongly associated with alterations in the immune system, leading many to postulate that progression of HNSCC tumors is linked to immune evasion or failure of the immune system to fight the cancer (11–15). Natural killer (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 (16–20). NK cell infiltration into solid tumor tissue has been associated with improved survival in studies of many different types of cancer (21–23). Immune suppression is frequently seen in patients with head and neck cancer (11–15). Diminished NK cell and natural killer T (NKT) cell activity and number have been observed in the peripheral blood of patients with HNSCC in several studies (14, 24). 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.

## Materials and Methods

### Sorted leukocyte samples

Normal human peripheral blood leukocytes were isolated by magnetic-activated cell sorting (MACS; Miltenyi Biotec 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: $\beta = (\max(M,0))/(|U| + |M|) + 100$. The resultant $\beta$ 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).

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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 grove 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 10 minutes 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 qPCR 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 $CD45^{+}CD16^{+}CD3^{-}CD56^{dim}$ NK cells and $CD45^{+}CD16^{-}CD3^{-}CD56^{bright}$ 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 CD45-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 FACSAria III flow cytometer (Becton Dickinson) using FACSDiva 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, ganulocytes, 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

Published OnlineFirst September 26, 2012; DOI: 10.1158/1078-0432.CCR-12-1008



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.

***Analysis of qMSP data.*** Statistical analyses were carried out in R (http://www.r-project.org/). A generalized linear model and *F* test were carried out to determine log linear PCR kinetics for the NK cell standard curve. To test for univariate associations between continuous *NKp46* demethylation measurements and discrete variables, Wilcoxon rank sum tests (for dichotomous variables, such as case status) and Kruskal–Wallis one-way analysis of variance tests were used. To test for univariate associations between continuous *NKp46* demethylation and other continuous variables, we carried out linear regression, calculated Pearson product-moment correlations and carried out *F* tests. A $\chi^2$ test for trends in proportions was applied to identify trends in HNSCC prevalence by control-determined demethylation tertiles. Multivariate logistic regression analyses were carried out using the "glm" function with family set to binary.

## 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 purified (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 ($q < 0.1$, coefficient <0) and within genes whose RNA expression was significantly elevated in NK cells ($q < 0.1$, log 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. 3). Fresh blood samples collected from 5 individuals were analyzed using standard flow cytometry for $CD56^{bright}$ 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).

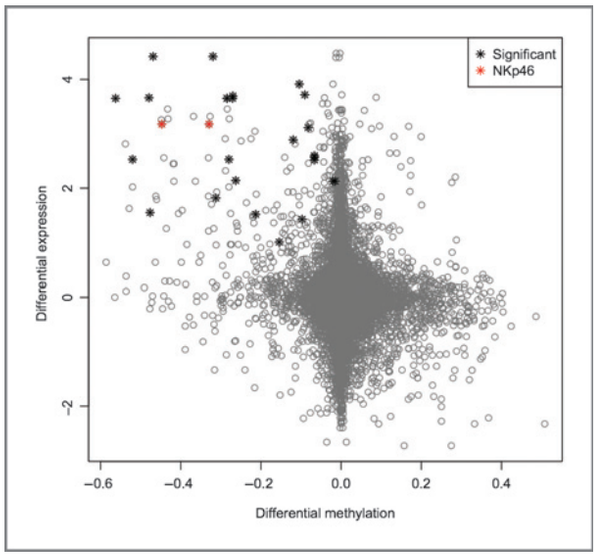


Figure 1. Loci in *NKp46* chosen from candidate NK cell-specific differential DNA methylation markers, selected by DNA methylation and mRNA expression criteria. Linear mixed effects modeling of DNA methylation microarray data from MACS isolated human leukocytes generated a coefficient estimating differential methylation in NK cells relative to other cell subtypes, shown on the *x*-axis. Linear modeling of mRNA microarray data from the same isolated cells determined log-fold change in expression between NK cells and each of the following subtypes: T cells, B cells, granulocytes, and monocytes. The average of these 4 log-fold change values is shown on the *y*-axis. Significance for a particular gene region was achieved when $q < 0.1$ for all 4 mRNA expression linear models as well as the DNA methylation mixed effects model. Candidates for NK cell-specific DNA methylation biomarkers were limited to significant gene loci exhibiting decreased methylation in NK cells (methylation estimate <0) and within genes that exhibited increased RNA expression (log fold change >1). These candidate loci are marked with asterisks in the top left quadrant, and *NKp46* loci are marked with red asterisks.

### 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 ($P < 0.0001$, 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

Published OnlineFirst September 26, 2012; DOI: 10.1158/1078-0432.CCR-12-1008



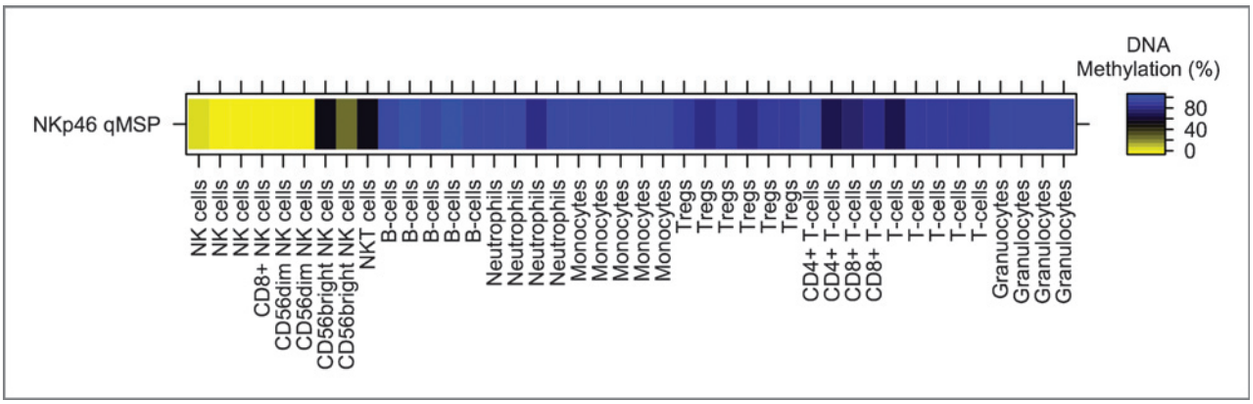


Figure 2. Demethylation status of *NKp46* determined by methylation specific quantitative PCR (qMSP) of isolated human leukocyte populations. Individual samples of MACS purified white blood cell subtypes were subjected to a qMSP assay that detects demethylated copies of *NKp46* DNA. Extent of *NKp46* methylation is illustrated in this heatmap where yellow indicates that all copies of DNA in particular sample were demethylated in the targeted region of *NKp46*, whereas blue indicates that all copies were methylated.

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.

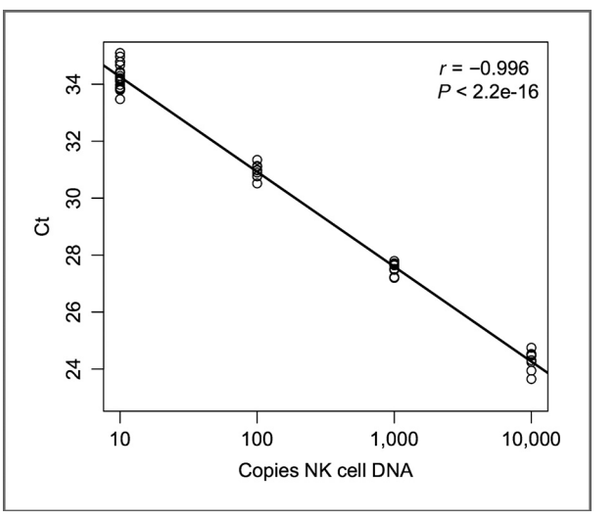


Figure 3. Linearity of *NKp46* qMSP calibration. Bisulfite converted universal methylated DNA was used to keep total amount of DNA in all samples constant. At least 3 replicates of each standard are plotted. Real-time PCR Ct values decrease linearly with 10-fold increase in bisulfite converted NK cell DNA concentration.

## 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 $CD56^{bright}$ 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 $CD56^{dim}$ NK cell compartment in the peripheral blood of patients with HNSCC, because the vast majority of NK cells circulating in the periphery belong to the $CD56^{dim}$ NK cell subset. In addition, our *NKp46* qMSP assay seems to be more specific to the detection of $CD56^{dim}$ NK cells than $CD56^{bright}$ NK cells based on measurements of MACS isolated human leukocytes. This may, in part, be an artifact of antibody-based method of cell isolation. The populations of $CD56^{dim}$ and $CD56^{bright}$ NK cells obtained by MACS may not be entirely distinct, but exist along a spectrum of NK cells with

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**Table 1.** Demographic characteristics

| Characteristic | Controls ($n = 122$) | HNSCC ($n = 122$) |
|---|---|---|
| Age | | |
| Mean (SD) | 62 (12) | 61 (12) |
| Median (range) | 60 (31–87) | 60 (29–86) |
| Gender | | |
| Male, no. (%) | 89 (73%) | 89 (73%) |
| Female, no. (%) | 33 (27%) | 33 (27%) |
| HPV 16 serology | | |
| L1+, no. (%) | 4 (3%) | 29 (24%) |
| E6+, no. (%) | 4 (3%) | 37 (30%) |
| E7+, no. (%) | 2 (2%) | 26 (21%) |
| E6+ and E7+, no. (%) | 0 (0%) | 25 (20%) |
| E6+ or E7+, no. (%) | 6 (5%) | 38 (31%) |
| Cigarette smoking status | | |
| Never, no. (%) | 41 (34%) | 24 (20%) |
| Former, no. (%) | 66 (54%) | 83 (68%) |
| Current, no. (%) | 15 (12%) | 15 (12%) |
| Cigarette pack-years | | |
| Mean (SD) | 17 (23) | 35 (32) |
| Median (range) | 7 (0–114) | 31 (0–116) |
| Alcohol drinks per week | | |
| Mean (SD) | 15 (27) | 21 (24) |
| Median (range) | 6 (0–199) | 14 (0–155) |
| Body mass index | | |
| Mean (SD) | 28 (5.9) | 25 (6.2) |
| Median (Range) | 27 (18–53) | 24 (17–68) |
| Tumor site | | |
| Oral, no. (%) | – | 43 (35%) |
| Pharyngeal, no. (%) | – | 53 (44%) |
| Laryngeal, no. (%) | – | 26 (21%) |
| Tumor stage | | |
| I, no. (%) | – | 14 (11%) |
| II, no. (%) | – | 16 (13%) |
| III, no. (%) | – | 25 (21%) |
| IV, no. (%) | – | 56 (46%) |
| Unknown, no. (%) | – | 11 (9%) |

varying cytotoxic activities. Recent evidence suggests that $CD56^{bright}$ NK cells can give rise to $CD56^{dim}$ NK cells through a differentiation process that results in decreased proliferative potential and increased cytolytic activity (33). It is possible that, as $CD56^{bright}$ NK cells proceed down a path of differentiation to become $CD56^{dim}$ NK cells, regions of cytotoxic genes undergo demethylation to prime them for expression in the effector cells. Thus, some of the NK cells that were classified as the more regulatory, $CD56^{bright}$ subset using antibody-based methods could be further along the spectrum towards $CD56^{dim}$ than others, and therefore exhibit *NKp46* demethylation. If there is a complex spectrum of NK cell subtypes that cannot be fully distinguished by traditional protein based methods of cell isolation and quantification, then careful examination of DNA methylation profiles could reveal hitherto unknown cell lineages.

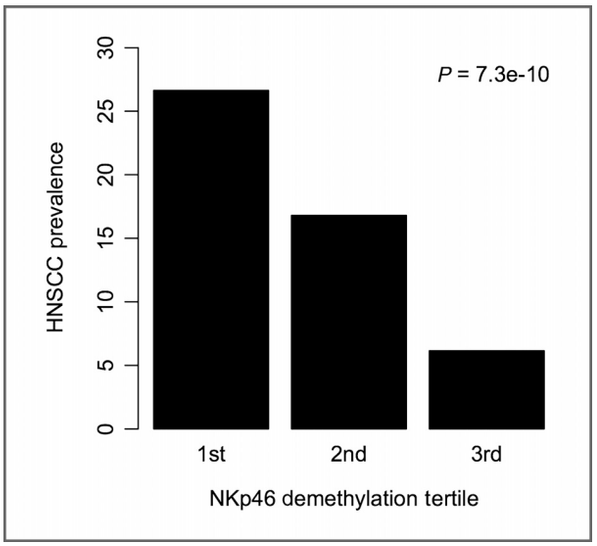


**Figure 4.** Prevalence of HNSCC by normal *NKp46* demethylation tertile. Normal *NKp46* demethylation tertile cutoffs were determined from control bloods only. Higher tertiles indicate higher NK cell levels. HNSCC prevalence refers to the percentage of total cases from this study whose *NKp46* demethylation measurements fell within the control derived tertile range. Displayed *P* value is from a $\chi^2$ 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

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**Table 2.** Logistic regression of HNSCC risk

| | Crude | | Adjusted[a] | |
|---|---|---|---|---|
| ***NKp46* demethylation tertile** | **OR (95% CI)** | ***P* value** | **OR (95% CI)** | ***P* value** |
| 1st (lowest) | 4.3 (2.2, 9.0) | $5.0 \times 10^{-5}$ | 5.6 (2.0,17.4) | 0.002 |
| 2nd (middle) | 2.8 (1.4, 6.0) | 0.006 | 4.9 (1.8, 16.1) | 0.004 |
| 3rd (highest) | Reference | | Reference | |

[a]Unconditional multivariate model controlling for age, gender, smoking, drinking, BMI, and HPV16 (E6 and/or E7) serology.

tumors. Thus, we have detected a HNSCC associated decrease in circulating NK cells that is independent of tumor site, known exposures and other putative risk factors and prognostic elements.

These data were not sufficient to determine whether the observed alterations in the balance of peripheral blood leukocytes associated with HNSCC occurred before or after onset of the disease. It is possible that the lower numbers of circulating NK cells in patients with HNSCC is an 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 anticancer 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 $CD56^{dim}$ 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 cell 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, E.A. Houseman, and K.T. Kelsey share a relevant patent pending. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** W.P. Accomando, K.T. Kelsey

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** W.P. Accomando, J.K. Wiencke, E.A. Houseman, H.H. Nelson, K.T. Kelsey

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## Acknowledgments

The authors wish to acknowledge the contributions of George Hsuang and Joseph Patoka from the Department of Neurological Surgery at University of California San Francisco for performing FACS to assess NK cell levels in whole blood samples.

## Grant Support

This work was supported by grants from the National Institute of Environmental Health Sciences (grant number ES06717) and the National Institutes of Health (grant numbers R01 CA52689, R01 CA126939, R01 CA121147, R01 CA100679, and R01 CA078609).

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Received April 11, 2012; revised September 12, 2012; accepted September 13, 2012; published OnlineFirst September 26, 2012.

## References

1. Ji H, Ehrlich LI, Seita J, Murakami P, Doi A, Lindau P, et al. Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature 467:338–42.

2. Hawkins RD, Hon GC, Lee LK, Ngo Q, Lister R, Pelizzola M, et al. Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell 2010;6:479–91.

Published OnlineFirst September 26, 2012; DOI: 10.1158/1078-0432.CCR-12-1008




3. Natoli G. Maintaining cell identity through global control of genomic organization. Immunity 33:12–24.
4. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res 2009;69:599–608.
5. Khavari DA, Sen GL, Rinn JL. DNA methylation and epigenetic control of cellular differentiation. Cell Cycle 2010;9:3880–3.
6. Zaidi SK, Young DW, Montecino M, van Wijnen AJ, Stein JL, Lian JB, et al. Bookmarking the genome: maintenance of epigenetic information. J Biol Chem 2011;286:18355–61.
7. Jaenisch R. DNA methylation and imprinting: why bother? Trends Genet 1997;13:323–9.
8. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur J Immunol 2007;37:2378–89.
9. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 2007;5:e38.
10. Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, Baron U, et al. DNA methylation controls Foxp3 gene expression. Eur J Immunol 2008;38:1654–63.
11. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. Clin Cancer Res 2004;10:3755–62.
12. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Imbalance in absolute counts of T lymphocyte subsets in patients with head and neck cancer and its relation to disease. Adv Otorhinolaryngol 2005;62:161–72.
13. Pries R, Wollenberg B. Cytokines in head and neck cancer. Cytokine Growth Factor Rev 2006;17:141–6.
14. Wulff S, Pries R, Borngen K, Trenkle T, Wollenberg B. Decreased levels of circulating regulatory NK cells in patients with head and neck cancer throughout all tumor stages. Anticancer Res 2009;29:3053–7.
15. Duray A, Demoulin S, Hubert P, Delvenne P, Saussez S. Immune suppression in head and neck cancers: a review. Clin Dev Immunol 2010;2010:701657.
16. Parham P. The Immune System. 2nd ed. New York, NY: Garland Science; 2005.
17. Whiteside TL. The role of immune cells in the tumor microenvironment. Cancer Treat Res 2006;130:103–24.
18. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007;121:1–14.
19. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev 2008;18:11–8.
20. Levy EM, Roberti MP, Mordoh J. Natural killer cells in human cancer: from biological functions to clinical applications. J Biomed Biotechnol 2011;2011:676198.
21. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer 2000;88:577–83.
22. Kondo E, Koda K, Takiguchi N, Oda K, Seike K, Ishizuka M, et al. Preoperative natural killer cell activity as a prognostic factor for distant metastasis following surgery for colon cancer. Dig Surg 2003;20:445–51.
23. Villegas FR, Coca S, Villarrubia VG, Jimenez R, Chillon MJ, Jareno J, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002;35:23–8.
24. Molling JW, Langius JA, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJ, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol 2007;25:862–8.
25. Applebaum KM, Furniss CS, Zeka A, Posner MR, Smith JF, Bryan J, et al. Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer. J Natl Cancer Inst 2007;99:1801–10.
26. Furniss CS, McClean MD, Smith JF, Bryan J, Applebaum KM, Nelson HH, et al. Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use. Ann Oncol 2009;20:534–41.
27. Mehta H, Nazzal K, Sadikot RT. Cigarette smoking and innate immunity. Inflamm Res 2008;57:497–503.
28. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. Arch Otolaryngol–Head Neck Surg 2010;136:1267–73.
29. Gao B, Seki E, Brenner DA, Friedman S, Cohen JI, Nagy L, et al. Innate immunity in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol 2011;300:G516–25.
30. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using Infinium® assay. Epigenomics 2009;1:177–200.
31. Campan M, Weisenberger DJ, Trinh B, Laird PW. MethyLight. Methods Mol Biol 2009;507:325–37.
32. Weisenberger DJ, Trinh BN, Campan M, Sharma S, Long TI, Ananthnarayan S, et al. DNA methylation analysis by digital bisulfite genomic sequencing and digital MethyLight. Nucleic Acids Res 2008;36:4689–98.
33. Moretta L. Dissecting CD56dim human NK cells. Blood 2010;116:3689–91.
34. Schantz SP, Campbell BH, Guillamondegui OM. Pharyngeal carcinoma and natural killer cell activity. Am J Surg 1986;152:467–74.
35. Schantz SP, Goepfert H. Multimodality therapy and distant metastases. The impact of natural killer cell activity. Arch Otolaryngol–Head Neck Surg 1987;113:1207–13.
36. Schantz SP, Shillitoe EJ, Brown B, Campbell B. Natural killer cell activity and head and neck cancer: a clinical assessment. J Natl Cancer Inst 1986;77:869–75.
37. Lu LM, Zavitz CC, Chen B, Kianpour S, Wan Y, Stampfli MR. Cigarette smoke impairs NK cell-dependent tumor immune surveillance. J Immunol 2007;178:936–43.
38. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;27:5904–12.
39. Bauernhofer T, Kuss I, Henderson B, Baum AS, Whiteside TL. Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer. Eur J Immunol 2003;33:119–24.

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*Clin Cancer Res* 2012;18:6147-6154. Published OnlineFirst September 26, 2012.