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HLA class I antigen processing machinery (APM) component expression and PD-1:PD-L1 pathway activation in HIV-infected head and neck cancers

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Abstract

Human immunodeficiency virus (HIV)-infected individuals are at increased risk for developing several non-AIDS related malignancies and are often excluded from cancer immunotherapy regimens. To evaluate the immune competence of this cancer patient population, we evaluated HLA class I antigen presenting machinery (APM) component expression and PD-1:PD-L1 pathway upregulation in HIV(+) and HIV(−) head and neck cancers (HNCs). Sixty-two HIV(+) and 44 matched HIV(−) controls diagnosed with HNC between 1991–2011 from five tertiary care referral centers in the United States were identified. HLA class I APM component, PD-1, and PD-L1 expression were analyzed by immunohistochemical staining with monoclonal antibodies (mAbs). Clinical data was abstracted from the medical records. There was no significant
difference between the cases and controls in LMP2, TAP1, HLA-A and HLA-B/C, as well as PD-1 and PD-L1 expression. Overall, 62% of all subjects had high PD-1 expression and 82% of the subjects expressed PD-L1 within the tumor microenvironment. LMP2, HLA-A and HLA-B/C expression were significantly associated with moderate to high PD-1 expression in the HIV(+) HNC cases (p=0.004, p=0.026, and p=0.006, respectively) but not in the HIV(−) controls. In addition, HLA-A expression was significantly associated with PD-L1 expression in the HIV(+) HNC cases only (p=0.029). HIV-infected individuals diagnosed with HNC do not have any detectable defects in HLA class I APM component expression and in PD-1:PD-L1 pathway activation. Given the current successes of HAART therapy in maintaining immune cell counts, HIV(+) patients diagnosed with cancer may benefit from the recently FDA-approved immune checkpoint blockade therapy.

Keywords
Human Immunodeficiency virus; Human Papillomavirus; head and neck cancer; PD-1; PD-L1; immune checkpoint blockade; antigen processing machinery

Introduction
Recent research has found that human immunodeficiency virus (HIV)-infected individuals are at increased risk for developing several non-AIDS related malignancies, with a reported higher incidence of both tobacco-related and virus-related cancers (1–5). The higher incidence of tobacco-related cancers among HIV-infected individuals (6, 7) has been attributed to the significantly higher prevalence of cigarette smoking among HIV-infected compared with HIV-uninfected individuals (40–60% vs. 17%) (8, 9). However, several recent studies have suggested that HIV-induced inflammation or immunodeficiency may also play a role in the development of non-AIDS defining cancers (2, 10). This is highlighted in the documented increased incidence of malignancies derived from oncogenic viruses, including Epstein Barr Virus (lymphoma, nasopharyngeal cancer), Hepatitis B and C (hepatocellular carcinoma), Human Herpes Virus 8 (Kaposi Sarcoma), and Human Papillomavirus (oropharyngeal, cervical and anal cancer) (3, 6, 11). The latter data may reflect defects in this patient population ability to clear persistent viral infections.

Chronic exposure to viral antigens can result in T cell exhaustion by activating negative regulatory pathways. Programmed death-1 (PD-1) has been identified as a major regulator of T cell exhaustion during the progression of virus-associated head and neck cancer (HNC) (12) and may play a role in HIV infection and disease progression as well. Immunotherapy, specifically PD-1:PD-L1 immune checkpoint blockade, has yielded success in various cancer types, including HNC (13–14). Since these drugs are aimed to enhance existing host immune responses, an immunodeficient state or infection with HIV has conventionally excluded patient participation in clinical trials or receipt of these class of drugs. However, given the current successes of HAART therapy in maintaining CD4 T cell levels in HIV-infected patients and the role of the PD-1:PD-L1 axis in the development of virus specific T cell immune dysfunction, we were interested in assessing host immune competency in HIV-infected individuals diagnosed with HNC.
In this study, we performed a retrospective case-control study of HIV-infected and HIV non-infected individuals diagnosed with HNC identified from five tertiary care referral centers (15–16). We analyzed HIV (+) and matched HIV (−) HNCs for the expression of: i) HLA class I antigen presenting machinery (APM) components, since this machinery plays a crucial role in the generation of HLA class I antigen-peptide complexes which are critical in the ability to elicit tumor specific T cells and ii) the PD-1:PD-L1 axis, since this axis can lead to T cell anergy.

Materials and Methods

Patients

HIV-infected and non-HIV-infected patients diagnosed with HNC between 1991–2011 at one of five tertiary care referral centers (Emory University, Johns Hopkins University, M.D. Anderson Cancer Center, University of Michigan, and University of Pittsburgh) across the United States were identified. This study was part of a Head and Neck Cancer Specialized Programs of Research Excellence (HNC-SPORE) collaborative project funded through the Translational Research Program of the National Cancer Institute (NCI). HIV+ HNC cases were contributed by Emory University (n=23), University of Pittsburgh (n=10), MD Anderson Cancer Center (n=6), Johns Hopkins University (n=3), and University of Michigan (n=2). HIV (−) HNC controls were matched to the HIV (+) HNC cases by anatomic sub-site, gender, and age (within 10 years) and were contributed by University of Michigan (n=38), Johns Hopkins University (n=4), and Emory University (n=2). The Institutional Review Board (IRB) of each participating institution and IRB approval or exemption to share de-identified data with the study data center was obtained at each study site.

Medical Record Abstraction

Demographic, risk factor, and clinical-pathologic data were abstracted from the medical record and a sample of tumor tissue was obtained for biomarker testing. De-identified information was then submitted to the study data center. Quality assurance was performed on abstracted data including range and logic checks, with re-verification or correction of all atypical data reported by the sites, through a second medical record review.

Tumor HPV Testing

Formalin fixed, paraffin embedded (FFPE) tumor specimens were collected from each center, given a barcode, and tested in a single centralized laboratory (at the University of Michigan, led by TEC). Tissue microarrays (TMAs) were created from the tumor tissue when sufficient material was available, or tests were conducted on individual slides when the biopsy was too small. DNA was isolated from a single tumor tissue core or from tumor tissue micro-dissected from tissue sections for small samples. FFPE blocks were evaluated by a board certified head and neck pathologist (JBM) in the University of Michigan Pathology Laboratory for the presence of sufficient tumor for construction of a TMA and for DNA isolation. Tumors were tested for: 1) oncogenic HPV by in situ-hybridization (ISH), 2) oncogenic HPV DNA using PCR-MassArray, and 3) p16 expression by immunohistochemistry (IHC)(16). Cases that were p16 positive but HPV negative were re-
assessed with L1 consensus primers and sequenced to detect and identify other HPV types. ISH for HPV DNA using the INFORM HPV III assay (Ventana, AZ) designed to detect any of 12 high risk (oncogenic) HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66) was carried out according to the manufacturer’s protocol. ISH was scored for the presence of blue signals indicating the presence of HPV DNA in tumor cell nuclei as single punctate (integrated) or diffuse (episomal) signals. Expression of p16^INK4a was assessed by IHC using the CINtec p16INK4a Histology kit and protocol (mtm laboratories, Westborough, MA). These tests were carried out on the TMA or on individual slides from tissue samples too small to bearrayed. The p16 staining was scored for percentage of stained tumor cells and for staining intensity, each on a four-point scale. Percentage of stained tumor cells: 1: <5%, 2: 5–20%, 3: 21–50%, 4: 51–100%. Intensity of tumor cell staining: 1=no staining, 2=low, 3=moderate, and 4=high; IHC scores (percentage times intensity) from each 0.6 mm diameter core or tissue section were averaged for each patient and IHC scores of 12–16 were considered to be positive for p16. A pathologist JBM who was blinded to the origin of the individual samples scored both assays at 400X magnification.

High risk oncogenic HPV types were assessed in tumor DNA using PCR-MassArray designed to detect and identify 15 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73), using type-specific, multiplex, competitive PCR and single base extension followed by MALDI-TOF mass spectrometry analysis, as previously described (17). For any sample with adequate DNA that was positive by p16^INK4a but negative by HPV16 ISH the sample was re-examined by consensus PCR targeting the L1 region of the viral genome using PGMY primers (18) and sequencing of the PCR product to identify the unknown type. All specimens that were found to contain an identifiable high risk HPV type were scored as HPV positive (16).

**HLA class I APM Component Expression by Immunohistochemistry**

The mAb HCA-2 which recognizes an epitope expressed on β2m-free HLA-A (excluding -A24), -B7301, and -G heavy chains (19, 20), the mAb HC10, which recognizes an epitope expressed on all β2m-free HLA-B and C heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32 and -A33 heavy chains (19–21), the low-molecular-weight protein (LMP) 2–specific mAb SY-1 (22) and the TAP1-specific mAb NOB-1 (23) were developed and characterized as described. mAb were purified from ascitic fluid by affinity chromatography on Protein G columns. The purity and specific reactivity of mAb preparations were assessed by SDS-PAGE, binding assays and Western blotting, respectively.

A tissue microarray (TMA) was centrally constructed, distributed by the University of Michigan and utilized as a substrate in IHC reactions with mAbs. Slides were deparaffinized and rehydrated using a standard histology protocol. Antigen retrieval was performed using Diva Retrieval solution (Biocare Medical, Concord, CA) and a Decloaking chamber at 124°C, 3:00 minutes, and cooled for 10:00 minutes on the counter. The slides were placed on an Autostainer Plus (Dako, Carpenteria, CA) using a TBST rinse buffer (Dako) and stained using the following protocol: 3% H$_2$O$_2$ (ThermoFisher Scientific, Pittsburgh, PA) for 5 minutes, CAS Block (Invitrogen, Grand Island, NY) for 10 minutes, the HLA-A (HCA2
mAb) and HLA-B/C (HC-10 mAb) Ab were applied using a 1:6000 dilution for 30 minutes. The secondary consisted of Envision Dual Link + (Dako) polymer for 30 minutes, rinsed, then a TBST holding rinse was applied for 5 minutes. The substrate used was 3,3', Diaminobenzidine + (Dako) for 7 minutes and counterstained with hematoxylin. HLA-A and B/C, as well as LMP2 and TAP1, staining were quantified by positive pixel count v9 algorithm (Aperio). The protein expression level was represented by the score of intensity of staining multiplied by the positive area percentage. Immunohistochemistry analysis was performed by an independent observer (Dr. Lin Wang, a trained histopathologist) without prior knowledge of clinicopathological data.

PD-L1 and PD-1 Detection by Immunohistochemistry

Whole mount tissue sections were used for PD-1 and PD-L1 staining. PD-L1 expression was assessed using mAb 5H1 as previously described (12). Briefly, FFPE tissue sections were deparaffinized and dehydrated in xylene and graded ethanol solutions. Antigen retrieval was performed in Tris-EDTA buffer and the DAKO Catalyzed Signal Amplification System for mouse antibodies was used for staining and detection (DAKO, Carpinteria, CA). Mouse IgG1 isotype matched control antibody and secondary biotinylated anti-mouse IgG1 antibody were used (BD Biosciences). PD-L1 expression in deep crypts and germinal centers in non-tumor involved areas of lymphoid tissue served as an internal positive control. A 5% threshold of cell surface PD-L1 expression on tumor cells was defined as positive (12). The anti-PD-L1 mAb (clone 405.9A11) was previously validated by Dr Gordon J. Freeman (Dana Farber Cancer Institute, Boston, MA) (24).

For PD-1 immunostaining, the murine anti-human PD-1 mAb, clone M3, was used. Citrate buffer (pH 6.0) was used for antigen retrieval, and a CSA System (DAKO; Glostrup, Denmark) was used for signal amplification, followed by development with diaminobenzidine chromagen. PD-1 expression was scored as moderate/high or none/low based on a threshold positivity of greater than 25% or less than 25%. All slides were reviewed and interpreted independently by a head and neck pathologist (WHW).

Statistical Analysis

Descriptive statistics were computed to summarize the data distributions. In this case-control study, due to the relatively relaxed matching criteria, case-control pairs were not tightly corresponded. Chi-square test and Fisher’s exact test were applied as two-sample tests to compare the proportions of categorical variables between cases and controls using all available data. McNemar’s test was used as the matched pair analysis to compare the proportion of categorical variables between the case-control pairs. Pearson’s correlation coefficients were calculated to measure the association between continuous APM markers. Wilcoxon rank-sum test and Kruskall-Wallis tests were used to compare the distribution of continuous markers between two or more than two groups. A two-sided p-value of less than or equal to 5% is considered statistically significant.
Results

Demographic data

We identified a total of 62 HIV (+) and 44 HIV (−) patients diagnosed with HNC between 1991 to 2011. HIV (+) cases were matched to HIV (−) patients based on gender, age (<10 year difference), and anatomic sub-site (Table 1). There were 8 HPV (+) HNSCCs among 35 HIV (+) HNSCCs (23% of cases) and 8 HPV (+) HNSCCs among 43 HIV (−) HNSCCs (19% of controls).

HLA class I APM component expression

The initiating signal of T cell activation requires clonotypic T cell receptor triggering by its ligand, the HLA-peptide antigen complex, and its synthesis and expression requires an intact APM pathway. To assess the integrity of this pathway in our cohort of HIV (+)/(−) HNC, a TMA was used to stain for 4 HLA class I APM components. They included LMP2, TAP1, HLA-A heavy chain and HLA-B heavy chain, which were tested in 33, 33, 36 and 33 cases, respectively. All 4 markers were tested in the 42 HIV (−) HNC controls (Figure 1). Pooled two-sample analysis of the cases and controls demonstrated no difference in LMP2, TAP-1, HLA-A, and HLA-B/C expression between HIV (+) cases and HIV (−) control HNCs (p>0.05 for all markers). Similarly, a sub-analysis of 31 matched cases and controls which were stained for all immune markers, LMP2, TAP1, HLA-A, and HLA-B/C, also detected no difference in expression of these HLA class I APM components between HIV (+) and HIV (−) HNCs.

PD-L1 and PD-1 expression

Using whole tissue sections, both PD-1 and PD-L1 expression was evaluated in 32 cases and 34 controls; 29 of them were matched pairs. No statistically significant difference in the expression levels of PD-L1 or PD-1 between cases and controls were detected in both the pooled and matched paired analyses. Specifically, 85% of all HIV (−) HNCs expressed PD-L1 and 78% of HIV (+) HNCs expressed PD-L1 (p=0.45), yielding an overall frequency of 82% of HNCs expressing PD-L1 (Figure 2). In the matched pair analysis, we found 19 pairs were both PD-L1 (+), 6 pairs were PD-L1 (+) in the control cases but PD-L1 (−) in the cases, 3 pairs were PD-L1 (−) in the controls but PD-L1 (+) in the cases and 1 pair was PD-L1 (−) in both the control and case (McNemar’s test, p=0.51). Similarly, we did not find PD-1 expression to differ between cases and controls in the two-sample analysis (p=0.16) and in the matched pair analysis (p=0.53, McNemar’s test).

PD-1 and PD-L1 expression were positively associated with each other (p=0.018, Fisher’s exact test). Interestingly, this association was higher in the HIV (−) HNC patients (p=0.074) as compared to the HIV (+) HNC patients (p=0.31).

Association of HLA class I APM component expression with PD-1:PD-L1 expression

Next, we compared HLA class I APM component expression and PD-1:PD-L1 pathway upregulation. Taking APM expression as a continuous variable, the expression of LMP2, HLA-A, and HLA-B/C was significantly associated with moderate to high PD-1 expression levels (p=0.001, p=0.001, and p=0.011, respectively). The relationship of these same
markers to PD-1 expression was also significant in the HIV (+) HNC cases (LMP2 p=0.006; HLA-A p=0.004; and HLA-B/C p=0.026, respectively) but not significant in the HIV (−) HNC controls (Figure 3). No association was found between TAP1 and PD1 expression in the overall group, cases, and controls.

The presence of TAP1 and HLA-A expression was significantly associated with PD-L1 expression (p=0.013 and p=0.015, respectively) in the overall group. The association between HLA-A and PD-L1 was maintained when testing with a different anti-PD-L1 antibody (a gift of G. Freeman, DFHCC, Boston, MA) and in a separate laboratory (RLF) (p= 0.001). In the HIV (+) HNC cases, HLA-A expression was positively associated with PD-L1 expression (p=0.029). Whereas, in the HIV (−) HNC controls, TAP1 expression was positively associated with PD-L1 expression (p=0.006).

**Association of local PD-1 and PD-L1 expression in the tumor microenvironment with peripheral CD4 cell counts**

We then evaluated PD-L1 and PD-1 expression with systemic CD4 cell counts at the time of cancer diagnosis and at nadir during their documented HIV infection. We did not find any association between CD4 cell count at the time of cancer diagnosis or at the CD4 nadir in the course of HIV infection with local PD-L1 or PD-1 expression within the tumor microenvironment.

**Association of HPV status and PD-1:PD-L1 in HIV+− HNSCCs**

There were no significant associations found between HPV status and PD-L1 expression in the overall group and subset analysis based on HIV status (p=0.43 for overall; p=1.0 for HIV (−) patients, and p=0.63 for HIV (+) patients, Fisher’s exact test). However, HPV status was significantly associated with higher PD-1 expression in the overall group (p=0.05). The association held and approached significance in the HIV (+) cases (p=0.06) but did not hold in the HIV (−) controls (p=0.62).

**Discussion**

This study is, to our knowledge, one of the first translational immune biomarker studies of HIV-infected HNC and these findings help to characterize the baseline immune status of HIV-infected individuals with HNC, as a basis to include these patients in clinical trials or standard of care treatments with immunotherapeutic agents. We assessed two common immune escape mechanisms reported in HNC to determine whether these same pathways may contribute to the development of HNC in HIV-infected patients. HLA class I APM down-regulation is not a preferential mechanism of immune escape utilized in HIV (+) HNC patients as compared to matched HIV (−) HNC patients. Interestingly, we found that 78% of HIV (+) HNC patients express PD-L1 and 86% express PD-1 in their local tumor microenvironment. Furthermore, we found that expression of the HLA class I APM components (LMP2, HLA-A and HLA-B/C) by tumor cells was significantly associated with moderate to high PD-1 expression levels in the HIV (+) HNC patients, suggesting that tumor antigen specific immune responses are being elicited in the HIV (+) HNC patients.
However, local PD-L1 expression may be abrogating their cytotoxic function to facilitate cancer persistence and growth.

HIV-infected individuals have increased exposure to tobacco, alcohol, and HPV infection, the three primary HNC risk factors. 23% of the HIV (+) and 19% of the HIV (-) HNC were HPV (+) in this cohort. Anti-PD-1 monotherapy appears to be more effective in HPV (+) HNC patients (13–14), suggesting that our studies of PD-1:PD-L1 pathway activation and intact APM component expression, warrant treatment of HIV (+) HNC patients with the FDA approved PD-1 targeted immunotherapies as well as enrollment in clinical trials incorporating these agents into other therapeutic combinations. In addition, the lack of HPV infection in 42% of oropharyngeal cases and in 72% of HNC overall suggests that although HPV is a causal factor in a notable number of cases, tobacco use and other factors remain important contributors in the majority of HIV (+) HNC.

PD-L1 is currently the best single predictive biomarker of response to PD-1:PD-L1 pathway blockade. We report that HIV (+) HNC patients express PD-L1 at similar frequencies as HIV (-) HNC patients. We did not find any association between CD4 cell count at the time of cancer diagnosis or at the CD4 cell nadir in the course of HIV infection with local PD-L1 expression, suggesting that systemic CD4 cell counts may not be reflective of local immune responses within the tumor microenvironment. Currently, HIV status has precluded patient treatment with various immunotherapeutic agents and our data suggests that re-consideration of HIV status as an exclusionary criteria should be given for immunotherapy trials targeting the PD-1:PD-L1 network. Additional research is needed to validate our findings in a larger cohort of patients and to better understand whether HIV infected immune cells expressing PD-1 can be re-activated to a similar capacity as non-HIV infected immune cells. The latter can be achieved only through the enrollment and/or participation of the HIV-infected population in clinical trials administering these agents.

This study has several limitations as well as strengths. Tumor specimens were not available for all cases to permit analysis of all the biomarkers using IHC, as TMA “drop out” or whole sections limited the specimens available for analyses by IHC. Cases were not systematically sampled but represent a convenience sample of all HIV (+) HNC cases identified at participating centers. Each center searched extensively for cases at their institution including cross-references with the HIV clinics at their center. Strengths of this study included centralized validated tumor testing, analysis stratification by tumor site and tumor HPV status, and inclusion of a diverse group of U.S. clinical centers. Lastly, the analysis of immune biomarkers which regulate TCR (signal 1) and co-stimulatory (signal 2) ligands which regulate T lymphocyte recognition and activation in HIV (+) HNCs is a unique feature of this study and represents one of the first to evaluate immune evasion mechanisms as well as validate the immune competence of this historically viewed “immune-compromised” patient population.

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References


Highlights

- HIV status has precluded patient treatment with various immunotherapeutic agents, including immune checkpoint blockade.
- We report that HIV-infected individuals diagnosed with head and neck cancer (HNC) have intact expression of HLA Class I APM components and activation of the PD-1:PD-L1 axis.
- Given the current successes of HAART therapy in maintaining immune cell counts, our data suggests that re-consideration of HIV status as an exclusionary criteria should be given for immunotherapy trials targeting the PD-1:PD-L1 network in this historically viewed “immune-compromised” patient population.
Figure 1.
Representative staining of APM expression in HIV+ and HIV− patients. Tissue sections were stained using immunohistochemistry, as previously published (22–23) and briefly described in the Materials and Methods. Tissue microarray (TMA) slides were deparaffinized and rehydrated using a standard histology protocol. HLA-A and B/C, as well as LMP2 and TAP1, staining were quantified by positive pixel count v9 algorithm (Aperio). Protein expression level was represented by the score of intensity of staining multiplied by the positive area percentage. Immunohistochemistry analysis was performed by an independent observer without prior knowledge of clinicopathological data. Representative high and low staining examples are shown, which were aggregated and compared HIV+ or HIV− cohorts, showing no significant differences in median expression.
Figure 2.
Representative staining of PD-L1 in HIV+ and HIV− patients.
Tissue sections were stained using immunohistochemistry as previously published (12) and briefly described in the Materials and Methods. Both the HIV+ and HIV− cohorts expressed PD-L1 within the tumor microenvironment at a similar frequency. The representative HIV+ and HIV− cases demonstrate both tumor and stromal PD-L1 positivity. (A) and (C) are low magnification (50x) and (B) and (D) are high magnification (400x) images.
Table 1

Demographic and Baseline Characteristics (N=88)

<table>
<thead>
<tr>
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<th>Case N (%)</th>
<th>Control N (%)</th>
<th>All N (%)</th>
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<tbody>
<tr>
<td>All</td>
<td>44 (50%)</td>
<td>44 (50%)</td>
<td>88 (100%)</td>
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Gender  
- Male: 41 (93.2%), 42 (95.4%), 83 (94.3%)
- Female: 3 (6.8%), 2 (4.5%), 5 (5.7%)

Institutions
- University of Michigan: 2 (4.5%), 38 (86.4%), 40 (45.4%)
- Emory University: 23 (52.3%), 2 (4.5%), 25 (28.4%)
- University of Pittsburgh: 10 (22.7%), 10 (11.4%)
- Johns Hopkins University: 3 (6.8%), 4 (9.1%), 7 (8.0%)
- MD Anderson Cancer Center: 6 (13.6%), 6 (6.8%)

Disease Site
- Oral Cavity: 24 (54.5%), 24 (54.5%), 48 (54.5%)
- Oral Pharynx: 9 (20.5%), 10 (22.7%), 19 (21.6%)
- Larynx: 4 (9.1%), 6 (13.6%), 10 (11.4%)
- Laryngopharynx: 5 (11.4%), 2 (4.5%), 7 (8.0%)
- Other: 2 (4.5%), 2 (4.5%), 4 (4.5%)

Ethnicity
- White-Non-Hispanic: 19 (43.2%)
- Black-Non-Hispanic: 19 (43.2%)
- Hispanic: 1 (2.3%)
- Unknown: 5 (11.4%)

Smoking
- Current: 30 (68.2%)
- Former: 7 (15.9%)
- Never: 2 (4.5%)
- Unknown: 5 (11.4%)

Alcohol
- Current: 28 (63.6%)
- Former: 6 (13.6%)
- Never: 4 (9.1%)
- Unknown: 6 (13.6%)

Age
- 49.7±9.7
- 54.1±10.1
- 51.9±10.1

1. Row percentage. All other percentages are column percentages.

2. One case-control pair had a gender mismatch.

3. Based on best matches.
Only available for cases.

5 Mean ± standard deviation; median (minimum-maximum)