Human Papilloma Virus Status in Upper Aero-digestive Squamous Cell Carcinoma using p16 Staining at Uganda Cancer Institute

Fiona Kabagenyi (kabagenyiatwooki6@gmail.com)
Makerere University College of Health Sciences https://orcid.org/0000-0002-2287-2809
Jeff Otiti
Peter MacCallum Cancer Centre
Justine Namwagala
Makerere University College of Health Sciences
Adriane Kamulegeya
Makerere University College of Health Sciences
Sam Kalungi
Makerere University College of Health Sciences

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Authors:

1. Fiona Kabagenyi, M.Med. Department of Ear, Nose and Throat, College of Health Sciences Makerere University.
2. Jeff Otiti, M.Med. Head and Neck Surgeon. Department of Surgery, Uganda Heart Institute.
3. Justine Namwagala, M.Med. Department of Ear, Nose and Throat, College of Health Sciences Makerere University.
4. Adriane Kamulegeya, M.Med, Department of Oro-maxillofacial Surgery, College of Health Sciences Makerere University.
5. Sam Kalungi, M.Med, PhD, Department of Pathology, College of Health Sciences Makerere University.

Corresponding author: Fiona Kabagenyi

Mailing address: Department of Ear Nose and Throat, College of Health Sciences, Makerere University, P.O. Box 7072, Kampala, Uganda.

Phone numbers: +256774150102, +27651215887

Email address: kabagenyiatwooki6@gmail.com

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Author's contributions:

Fiona Kabagenyi the principal investigator designed the research, sought ethical approval, trained the data collectors and data entrants and wrote the report. Jeff Otiti, Justine Namwagala, Adriane Kamulegeya and Sam Kalungi helped in designing the study and obtaining ethical approval. Sam Kalungi also did the histology for all the tissue samples that were obtained. All the authors helped with writing the final report.

ABSTRACT

Introduction

Cancer burden is increasing in sub-Saharan Africa, where one-third of cancers are estimated to be caused by infectious agents. Head and neck squamous cell cancer (HNSCC) is the sixth most common malignancy in Sub-Saharan Africa(SSA), including tumours in the oral cavity (OC), oropharynx(OP), hypopharynx and larynx. Tobacco and alcohol exposure are established risk factors but the role of Human Papilloma Virus (HPV) has gained recent recognition. The HPV related HNC is seen predominantly in the oropharynx, presents at a younger age and has a better prognosis. With a rapidly increasing incidence of these cancers in the developed world, it was important to study HPV in HNC in Uganda. The HPV can easily be detected using P16 immunohistochemistry (P16 IHC) as a surrogate marker thus making it suitable for screening. The study aimed at establishing the presence of HPV and the sites it commonly affects in upper aero-digestive tract squamous cell carcinoma (UADT SCC) at Uganda Cancer Institute (UCI) using P16 IHC.

Methodology

This was a cross sectional study in which 59 patients with histologically proven squamous cell carcinoma from the oral cavity, oropharynx, larynx and hypopharynx at UCI were recruited. These patients' demographics and clinical data were collected. Tissue sections from retrieved histology samples were stained by Haematoxylin and Eosin to reconfirm SCC. Subsequently, P16 expression was determined using P16 immunohistochemistry.

Results

71 patients were enrolled and 59 patients with confirmed SCC of the sites of interest were analysed. The majority (79.7%) of the participants were male and over 50 years. 59.3% were tobacco smokers, 66.1% used alcohol, 52.2% used both. Only 27.1% used none of the substances. Only 27.1% of the participants were HIV positive. Most of the tumors were in the larynx (37.3%) and 64.4% were overall TNM stage 4. The overall prevalence of HPV in UADT SCC at UCI was 20.3%, 95%CI 10.9-32.8. The oropharynx had the highest prevalence (30.8%) closely followed by the oral cavity (29.4%).

Conclusion

The contribution of HPV in UADT SCC at UCI using P16 IHC is significant at 20.3 %. The oropharynx is the most affected site and is closely followed by the oral cavity.

Key words: Human Papilloma Virus, upper aero-digestive squamous cell carcinoma, Uganda Cancer Institute, p16 immunochemistry.

KEY POINTS

Question:

What is the overall prevalence of HPV in UADT SCC at UCI?

What UADT sites are commonly affected by HPV?

Findings:

The overall prevalence of HPV in UADT SCC at UCI was 20.3%.

The oropharynx had the highest prevalence closely followed by the oral cavity.

Meaning:

We should sensitize both medical care providers and the public about the risk factors associated with UADT SCC and HPV.

INTRODUCTION

Head and neck carcinoma (HNC) ranks sixth among the most common cancers seen worldwide(1). The commonest histological type is squamous cell carcinoma (SCC) accounting for more than 90% of HNC (2). Globally and locally, more than half of these cancers arise from the oral cavity/ oropharynx (1,3).

The upper aerodigestive tract (UADT) for this study comprises of the oral cavity, oropharynx, hypopharynx and larynx, sites of head and neck region that are exposed to the same risk factors(4). The etiology of UADT SCC has mainly been attributed to tobacco and alcohol consumption (5). The increasing incidence of head and neck squamous cell carcinomas(HNSCC) seen in the developed world however has been attributed to the Human Papilloma Virus (HPV) (6).HPV is the causative agent of cervical cancer, for which burden is extremely high throughout sub-Saharan Africa.

High risk HPV causes dysregulation of the cell cycle at the molecular level. The HPV produces onco-proteins that affect three tumor suppressor genes (p53, Rb and p16) in the host cell. When the HPV onco-protein E7 binds to the retinoblastoma gene in the host cell, it consequently releases the inhibition of P16 gene. This in turn causes an increased expression of p16 protein which can be detected by immunohistochemistry. P16 is currently used as a surrogate marker for HPV(7,8).

However, the contribution of HPV to HNSCC in Uganda is largely unknown. Twenty five percent of all HNSCC are HPV positive ((9). The commonest site affected is the oropharynx with frequencies of 39- 56% in the developed world and 13% in the rest of the world (8). Other sites involved are the oral cavity, hypopharynx and larynx(1,10,11).

Prior studies have used varying methodologies to detect HPV [immunohistochemistry (IHC), polymerase chain reaction (PCR), or a combination], often without detailed characterisation of anatomic site or simultaneous evaluation of other risk factors including HIV infection. We therefore sought to determine the presence of HPV using p16 IHC among histologically confirmed HNSCC in cases at Uganda Cancer Institute.

METHODS

This study was approved by Makerere University's Institutional Review Board and ethics committee of Uganda Cancer Institute. Written informed consent was obtained from all subjects participating in the study. Good Clinical Practice guidelines were followed. This was a cross sectional study conducted from October 2018 and May 2019.

The study was conducted at Uganda Cancer Institute (UCI), a public, tertiary care center for cancer treatment, research and training. It was also recently designated an excellence center for Oncology in Africa. It receives about 400 patients with HNC per year on referral basis with their histologically confirmed biopsies (from multiple laboratories both institutional and private) for staging and treatment planning in a tumor board setting. Prior to treatment, baseline investigations are done.

During the study period, patients with result slips showing histologically confirmed UADT SCC were interviewed. Their tissue blocks were retrieved from accessible pathology labs. The tissue blocks were then collected at the UCI pathology laboratory for histological re-confirmation followed by p16 IHC. It accorded credible supervision and facilities with p16 immunohistochemistry to this study.

We recruited all patients with histologically confirmed diagnosis of SCC involving the oral cavity, oropharynx, hypopharynx and larynx that consented to participating in the study. Exclusion criteria included patients with history of prior radiotherapy, those whose tissue blocks could not be accessed from the pathology labs and those whose retrieved blocks showed no malignancy or had insufficient tissue for histological analysis.

Sample size estimation

Using the Kish Leslie (1965) formula for sample size estimation [using the study in Sudan by Ahmed et al, Ahmed et al., 2012)], and adjusting to our local context using the finite correction factor, we aimed at having a minimum 70 patients.

Sampling method

Consecutive sampling was used to attain the sample size. The researcher assigned a unique identification number to the patient that was also used on the biopsy specimen. Histology lab

numbers on the histology report forms were used to trace the patients' biopsy block from the pathology labs.

Independent variables

These included age, gender, education level, occupation, socioeconomic status, history of smoking and alcohol ingestion, HIV status (documented evidence), sexual history and the Tumor, Node, Metastasis (TNM) stage of the patient (American Joint Committee on Cancer AJCC 7th edition).

Dependent variable

The p16 expression (positive or negative) showed whether there was HPV or not.

Study procedure

- The point of entry into the study was Uganda Cancer Institute.
- New patients with histologically confirmed UADT SCC at UCI were recruited.
- Data was collected by the researcher using the data collection form attached (Appendix I). Each form had a unique identification number. Proper history and examination of the patients was done by the investigator collaborated by the specialists as is routinely done in the head and neck tumor board setting.
- Follow up and retrieval of tissue blocks was done using the histology laboratory numbers on the histology report form. The retrieved blocks were kept at the pathology lab of UCI.
- Haematoxylin and Eosin (H&E) staining as well as IHC prepared slides were prepared and reported by the technician and confirmed by two independent pathologists. Findings were entered into the data collection form only when the two pathologists were in complete agreement. (Appendices I &II)
- All filled data collection tools were checked for completion.
- Data was entered using into an excel sheet.

Tissue processing

Retrieved Formalin Fixed Paraffin Embedded tissues were trimmed into 3-5 microns' thickness and prepared serially for routine H&E staining method by use of standard operating procedure (SOP) (Appendix II) by the technician. Consequently, tissue slides for IHC were

prepared serially by first trimming tissue into 5 microns thick following the SOP as is outlined in the protocol (Appendix III) for demonstration of p16 protein status.

Staining methods

Haematoxylin and Eosin method

The sections were stained routinely using H&E staining method by the technician. (Appendix II).

Immunohistochemistry (IHC)

The protocol as provided by the Ventana Benchmark XT machine was used for immune staining of histological sections for p16 expression. Both positive and negative controls were stained in parallel(12).

Reporting of H&E stained slides

The H&E tissue slides were reviewed by technician, investigator and results confirmed with the assistance of two pathologists and entered into the respective data collection form.

Scoring of p16 gene expression immuno-staining.

P16 protein expression was considered positive when tumor cells stain brown with different colour intensity (Appendix III). Positive and negative controls were used to aid the scoring. Slides were reviewed by the technician and by two pathologists. The results were then entered into the respective data collection form when at least two pathologists were in agreement (Appendix III). Positive results were reported with regard to site of staining, intensity of staining (>70%) and percentage of tumor cells staining (Appendix IV).

Quality control

- Clinical examination with TNM staging (Appendix V) was done by the Principal Investigator (PI). Staging was agreed upon in the HN tumor board as is routinely done.
- All procedures on the histology specimen were performed as per Standard Operating Procedure guidelines (Appendix II and III).
- Each case was assigned a unique identification number to ensure confidentiality.
- Laboratory work was performed by a qualified and competent technician.
- Reagents were freshly prepared.
- Test battery approach of staining control tissues before proceeding to main series including different antibody dilutions, with different incubation periods and different buffers were used to standardize IHC staining.

- Samples for IHC were run with positive (known HPV positive carcinoma cervix) and negative controls (omission of antibody during the staining).
- The same microscope was used to report all slides and was cleaned on regular basis.
- Diagnosis was confirmed by qualified pathologists (two independent pathologists).
- Clean tissue slides were used to prepare tissue sections.

Data collection

Data on participant's demographics, history, physical examination and tissue specimen was collected using interviewer administered questionnaires (Appendix I).

Data management and safety

The questionnaires were coded and the data entered into an excel sheet. Each record was assigned a unique identifier to maintain patient confidentiality. Verification of data was done using the double entry procedure.

Data backup drive was created in the PI's computer, an external hard drive and Google drive accounts. Completed questionnaires were stored in a cabinet under lock and key at the study site. Back up was performed on a daily basis. The data stored in the computer was secured with a password only known to the PI and statistician.

Data analysis

Participant characteristics were expressed as categorical and/or continuous variables. Continuous variables were expressed as means and standard deviations while categorical data was expressed as frequencies with their respective proportions. The main outcome of this study was prevalence of HPV, which was presented as frequencies and proportions.

The data was exported from the excel sheet to STATA 13.0 where data analysis was done.

- To determine the overall prevalence of HPV in UADT SCC at UCI, the number of participants that tested positive for HPV was divided by the total number of participants in the study.
- For site-specific prevalence of HPV in UADT SCC at UCI was determined by dividing the number of HPV positive by the total number of that site.

A p-value of 0.05 or less was considered statistically significant. The analysis was done by the study biostatistician.

RESULTS

A total of 79 patients were screened and 71 were enrolled for the study. Fifty nine patients were finally analysed as shown in figure 1.

Regarding participant's socio demographic characteristics, the majority of the participants were male (47/59) (Table 1). The median age was 54 +/- 12 with the youngest being 15years and the oldest being 81 years. The most common category was 51- 60 years as shown in figure 2.

Most of the participants used tobacco (59.3%), 66.1% used alcohol and 52.5 % used both tobacco and alcohol. The HIV positive participants constituted 27.1% (figure 3, Table 1)

For participants' tumor characteristics, most of the tumors were in the larynx (37.3%) followed by the oral cavity as shown in figure 4. In relation to the TNM staging, 61% were T stage 4, 49.1% had N0 stage while 50.9% had positive nodal stage with only 5.1% having distant metastases. The commonest overall stage was stage 4 (64.4%) as shown in figure 5.

The overall prevalence of HPV in UADT SCC at UCI using P16 IHC was 20.3 %, 95% Confidence interval (CI) of 10.9-32.8.

The site specific prevalence of HPV in UADT at UCI; the oropharynx had the highest prevalence (30.8%), followed by the oral cavity (29.4%) as summarized in the table 2.

DISCUSSION

Overall Prevalence of HPV in UADT SCC

The prevalence of HPV using P16 IHC in UADT SCC in this study was 20.3% which is lower than the global incidence of 25%(9). The findings of our study are in agreement with SSA studies like Ahmed's in Sudan at 20.7% and Faggon's in Malawi at 17%(12,13). The similarity of our results may be due to similar methodology and populations. However, a study by Ndiaye in Senegal reported a prevalence of 3.4% using PCR which is much lower than our findings(14). Sekee et al in South Africa found the HPV prevalence of 19.6% using P16 IHC that dropped to 6.3% using PCR(15). The variation seen with the different methods of HPV testing may suggest a possibly low prevalence if we subject our study to PCR. Our study also contrasts the findings in the developed countries where the prevalence ranged

between 39-56%(8). This notable variance may probably be due to differences in socioeconomic status, sexual habits and methodology(6,9,16).

Prevalence of HPV in SCC of the tumor sites of UADT.

The sites of interest in this study were the oral cavity, oropharynx, larynx and hypopharynx. The oropharynx had the highest HPV prevalence (30.8%) followed closely by the oral cavity (29.4%). This contrasts the intercontinental study where oral cavity was at a much lower percentage(17).

The oropharynx (30.8%) was the leading site in our study agreeing with findings in both the developed and developing world (8,9). Our study shows almost double the proportion of HPV in the oropharynx seen in Herero's intercontinental study where the HPV contribution was 18.3%. They recruited participants over a long period of time, accumulated great numbers using a case control design and used PCR (17). This may explain the difference seen with our study that had small numbers and used P16 IHC. Sekee found a prevalence of 25% that is almost similar to our findings. This may be probably due to similar methodology and population(15). We cannot comment further on other studies from the region which aggregated the prevalence in the oropharynx with other sites- oral cavity/ hypopharynx(12,13). This proportion does not however reach the percentages in the developed world (39-56%) probably due to the differences in the socioeconomic status, substance use and sexual patterns as earlier alluded to.

The prevalence of HPV in the oral cavity (29.4%) in our study was slightly higher than the observation by Ahmed in Sudan. He found that HPV contributed 22% to the oral cavity cancers(12). These similar occurrences may be explained by the proximity of the geographical locations of Uganda and Sudan. The prevalence of HPV in the oral cavity was eight times higher in our study when compared to Herrero's study(3.9%)(17). This discrepancy may also be attributed to the large numbers used in this multisite study and the differences in methodology. Several studies grouped oral cavity with oropharyngeal cancers making direct comparison difficult.

Our study shows a lower proportion of HPV in laryngeal carcinoma (9.1%) compared to other studies. The observation of the study done Hernandez et al on only laryngeal cancers in

USA had a 12.5 % HPV positivity(10). We found almost double and triple the proportions in the Sudan by Ahmed and Malawi by Faggons whose percentages were 26% and 33% respectively(12,18). Sekee in South Africa found a prevalence of 13.9%by P16 IHC and 5.06% using PCR(15). This may explain how different methods of detecting HPV can give varying results. Laryngeal cancers are mostly attributed to tobacco use in a dose- dependent manner(5). More than half of our participants smoked. In addition, little evidence exists for high risk HPV types involving the laryngeal region(10).

Our study showed a lower percentage of HPV in hypopharyngeal carcinoma (14.3%) in comparison with the China study by Yang et al that showed a prevalence of 26.1%using P16 IHC(11). This higher proportion may be due to research on only hypopharyngeal tumors increasing their sample size. Sekee found a prevalence of 20% positivity in South Africa with a strong agreement between PCR and p16 IHC for the hypopharyngeal carcinomas.(15). This would suggest that our findings of HPV in hypopharyngeal SCC using P16 IHC may be true representation. Their study findings agree with our observation probably due to geographical similarities.

Faggons in Malawi found no HPV in hypopharyngeal cancers (13). Ahmed aggregates the oropharynx and hypopharynx into pharynx limiting our use of their findings(12).

This study was done at Uganda Cancer Institute which receives patient referrals from across the country so the findings of this study may be generalizable to Uganda, we were able to get histology samples within a year of diagnosis increasing our chances of viability in the tissues and the slides were read by two consultant pathologists.

Limitation of the study is that P16 is a surrogate marker and the findings of this study were not confirmed by PCR or ISH.

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Figures

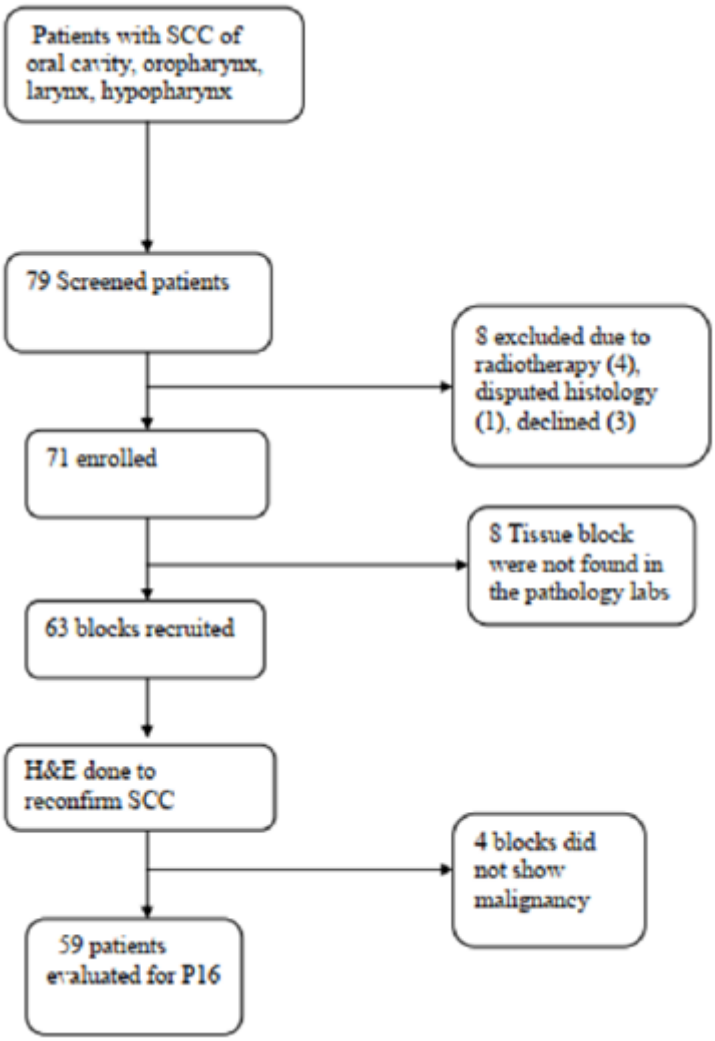


Figure 1

Study flow

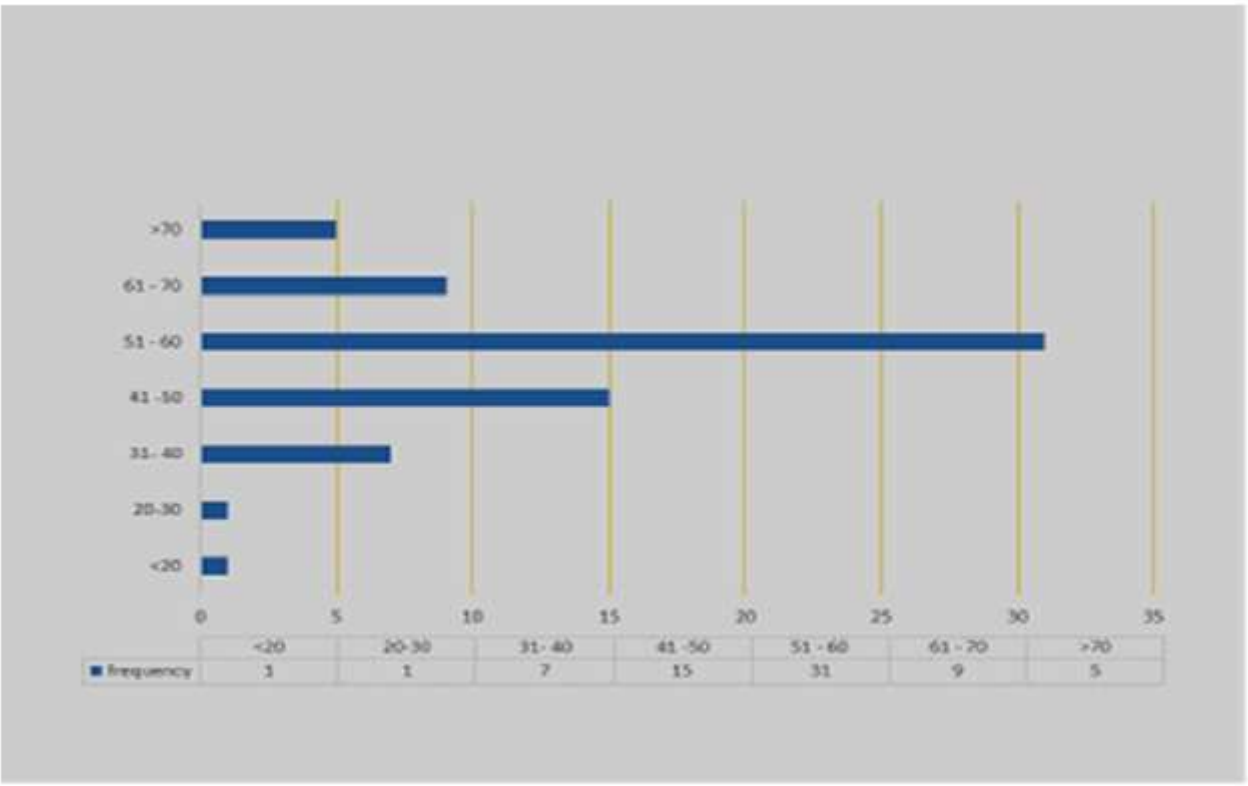


Figure 2

A bar chart showing participants' age categories.

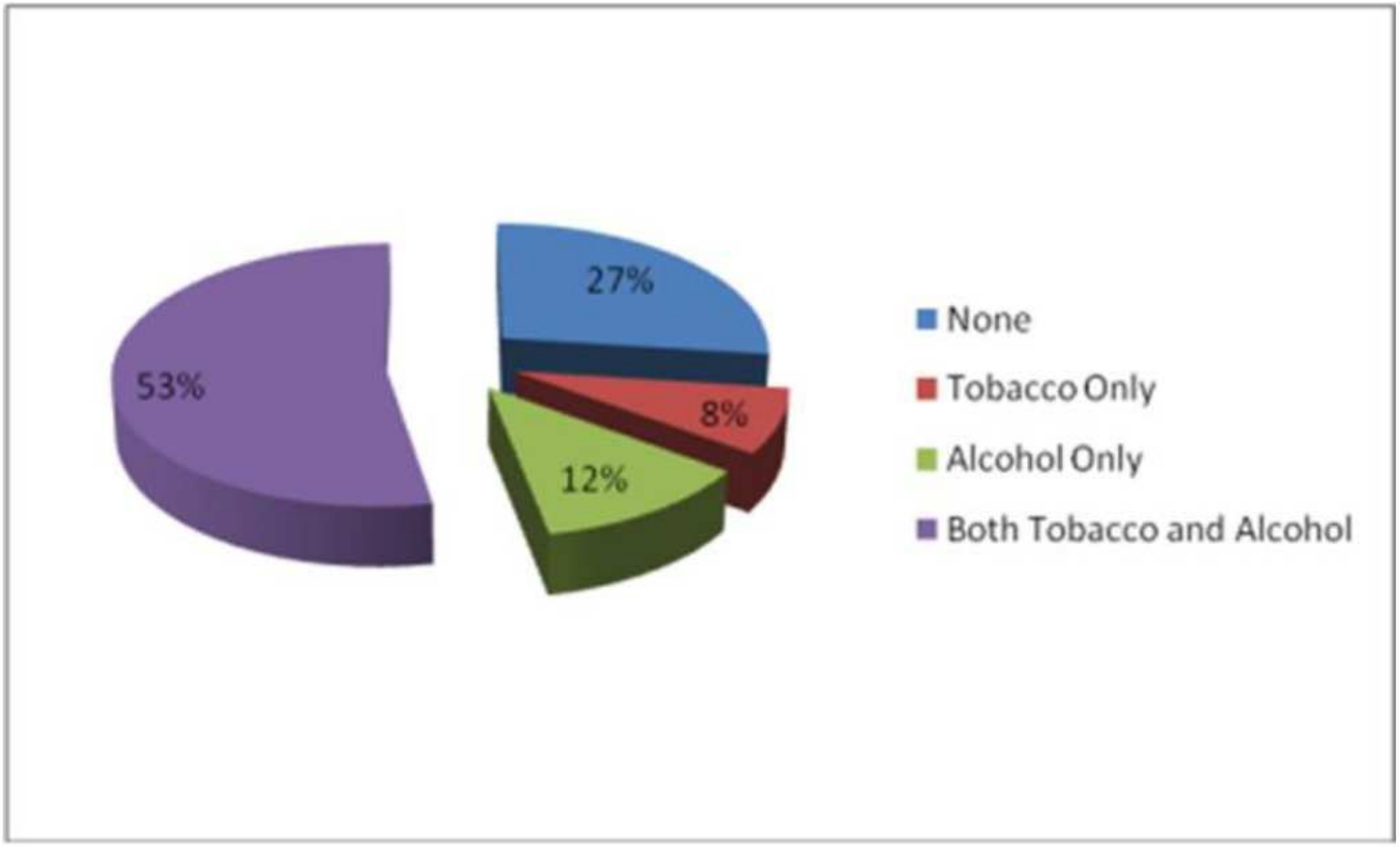


Figure 3

A pie chart showing participants' substance use habits.

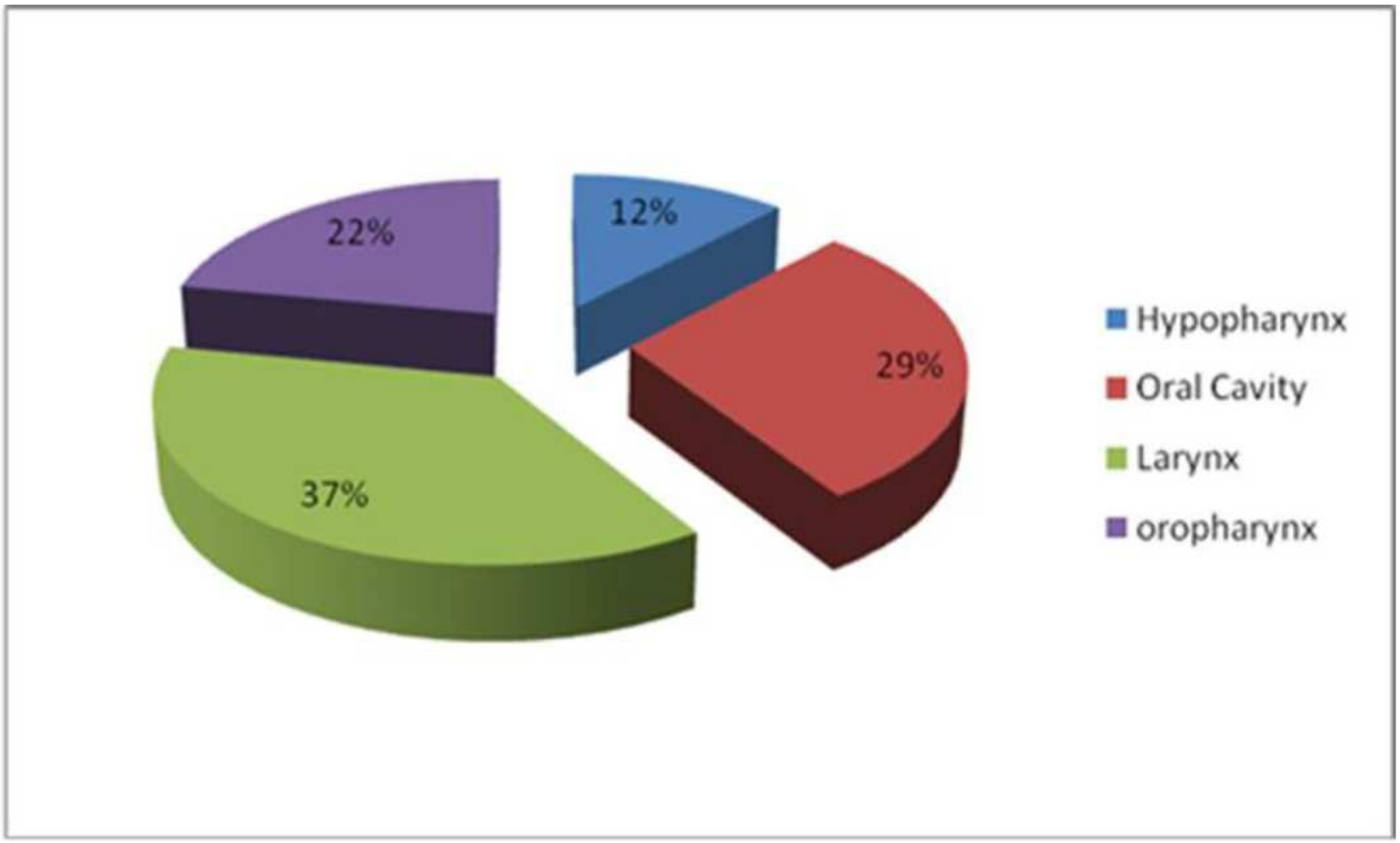
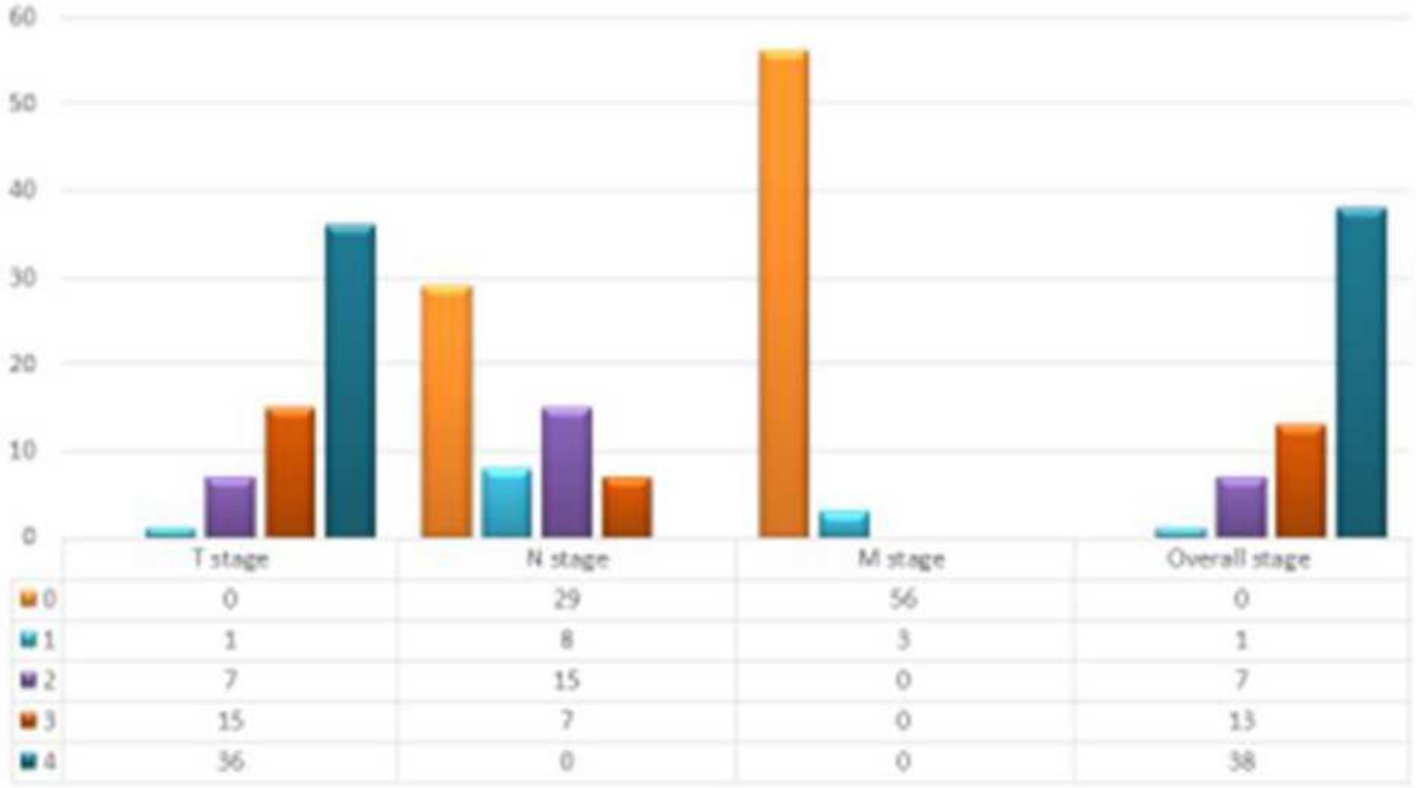


Figure 4

A pie chart showing the participants' tumor location.



	T stage	N stage	M stage	Overall stage
0	0	29	56	0
1	1	8	3	1
2	7	15	0	7
3	15	7	0	13
4	36	0	0	38

Figure 5

Bar charts showing the participants' TNM stage.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AppendixVFKabagenyi.pdf
- AppendixIIFKabagenyi.pdf
- AppendixIVFkabagenyi.pdf
- AppendixIIIFkabagenyi.pdf
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