Nasopharyngeal Carcinoma in Scotland: A Pilot Study on the Role of Human Papillomaviruses and Genetic Markers

Perla Steinsdóttir

Thesis for the degree of Bachelor of Science
University of Iceland
Faculty of Medicine
School of Health of Science

UNIVERSITY OF ICELAND

Nasopharyngeal Carcinoma in Scotland: A Pilot Study on the Role of Human Papillomaviruses and Genetic Markers

Perla Steinsdóttir

Thesis for the degree of Bachelor of Science

Supervisor: Dr Ingólfur Johannessen

Co-supervisor: Dr Kate Cuschieri

Faculty of Medicine

School of Health Sciences

June 2014

Table of Contents

ABSTRACT 5

ABBREVIATIONS 6

1. INTRODUCTION 7

1.1 CANCERS IN SCOTLAND 7

1.1.1 ALL CANCERS 7

1.1.2 HEAD AND NECK CANCERS 7

1.2 NASOPHARYNGEAL CARCINOMA 7

1.2.1 EPIDEMIOLOGY 7

1.2.2 RISK FACTORS AND PATHOGENESIS 8

1.2.3 CLASSIFICATION 8

1.2.4 STAGING 9

1.2.5 CLINICAL FEATURES 10

1.2.6 MANAGEMENT 10

1.3 EPSTEIN-BARR VIRUS 11

1.3.1 EBV MALIGNANCIES 11

1.3.2 EBV LATENCY 11

1.3.3 EBV AND NPC PATHOGENESIS 12

1.4 HUMAN PAPILLOMAVIRUS 13

1.4.1 PAPILLOMAVIRUSES 13

1.4.2 HUMAN PAPILLOMAVIRUS 13

1.4.3 HPV LIFE CYCLE 13

1.4.4 CLINICAL MANIFESTATIONS OF HPV INFECTIONS 14

1.5 GENETIC SUSCEPTIBILITY 14

1.6 STUDY AIM 15

2. MATERIAL AND METHODS 16

2.1 CAPTURE OF NATIONAL STATISTICS ON NPC 16

2.2 STUDY POPULATION 16

2.3 DNA EXTRACTION 17

2.4 HPV DETECTION 17

2.5 SNP GENOTYPING 17

2.6 MICROSATELLITE MARKER GENOTYPING 18

2.7 ANALYSIS 18

2.8 GOVERNANCE 18

3. RESULTS 19

3.1 NPC INCIDENCE IN SCOTLAND AND NORDIC COUNTRIES 19

3.2 PATIENT CHARACTERISTICS 20

3.3 IMPACT OF HPV 22

3.4 GENOTYPE DISTRIBUTION OF SNPS 23

3.5 MICROSATELLITE MARKERS 25

4. DISCUSSION 26

5. ACKNOWLEDGMENTS 29

6. REFERENCES 30

APPENDIX 40

Nasopharyngeal Carcinoma in Scotland:
A Pilot Study on the Role of Human Papillomaviruses and Genetic Markers

ABSTRACT

Perla Steinsdóttir[1], Kate Cuschieri[2], Ingólfur Johannessen[2]

[1]Faculty of Medicine, the University of Iceland and [2] Scottish HPV Reference Laboratory, the Royal Infirmary of Edinburgh

Background: The aetiology of nasopharyngeal carcinoma (NPC), a common cancer in certain parts of the world (in particular, SE-Asia), remains unclear although Epstein-Barr virus (EBV) is considered an essential co-factor. This study aimed to delineate the possible role of human papillomaviruses (HPV) in NPC development in a Scottish cohort and assess correlation with single nucleotide polymorphisms (SNPs) and microsatellite markers known to be associated with human malignancies.

Method: Using data from the Scottish and Nordic Cancer Registries, incidence rates were investigated for a median of 23 years up until 2011. Clinical data was collated for 58 patients treated for NPC at the Edinburgh Cancer Centre (NHS Lothian) during the period 2000-2013 and biopsy samples from a subset of 32 patients analysed for HPV infection and SNP/microsatellite markers by molecular methods.

Results: Over a median period of 23 years (up until 2011), NPC incidence has not risen significantly in Scotland or the Nordic countries, which is in contrast to the observed rise in (HPV-associated) oropharyngeal cancer in Scotland. Of the biopsy sample panel examined, 10 out of 32 cases (31%) were HPV+ve (8 HPV16; 2 HPV18/82). All 10 HPV+ve samples, and 19 out of 22 (86.4%) HPV-ve biopsies, comprised of non-keratinising carcinomas (NKC) whilst 3 out of 22 (13.6%) HPV-ve samples were keratinising carcinomas. Patients with HPV+ve NPC tended to be younger at time of diagnosis (mean age 53.4 *vs* 57.5 years; p=0.405) and had a lower male-to-female ratio (1.5 *vs* 6.3; p=0.1655) than patients suffering from HPV-ve lesions. No HPV+ve cases were diagnosed in patients of Asian background whereas 3 out of 22 (13.5%) HPV-ve cases were Scottish Asians (p=0.0694). SNP/microsatellite marker analyses did not show any significant correlation with NPC when compared with controls. However, the rs6904029 GG genotype was common in HPV+ve cases (80% *vs* 55%) and patients of Caucasian background (75.0% *vs* 28.6%).

Conclusions: The study suggests a possible role for HPV (in particular, HPV16 and 18) and the rs6904029 GG genotype in the development of a subset of NPC, which merits further investigation of a larger sample panel.

Abbreviations

BLCL: B lymphoblastoid cell lines

CIS: Carcinoma in situ

CT: Computerized tomography

CyPA: Cyclophilin A

EBNA: Epstein-Barr nuclear antigen

EBV: Epstein-Barr virus

FFPE: Formalin fixed paraffin embedded

HLA: Human leukocyte antigen

HPV: Human papillomavirus

IARC: International Agency for Research on Cancer

IL28B: Interleukin-28B

IM: Infectious mononucleosis

IMRT: Intensity-modulated radiotherapy

LMP: Latent membrane protein

MRI: Magnetic resonance imaging

NPC: Nasopharyngeal Carcinoma

OPSCC: Oropharyngeal squamous cell carcinoma

PTLD: Post-transplant lymphoproliferative disease

PV: Papillomavirus

SNP: Single-nucleotide polymorphism

VCA: Viral capsid antigen

VL: Viral load

WASR: World age-standardised rate

WHO: The World Health Organisation

1. Introduction

1.1 Cancers in Scotland

1.1.1 All cancers

In 2011 approximately 14,500 males and 15,600 females were diagnosed with cancer in Scotland (excluding non-melanoma skin cancers). The most commonly diagnosed cancers in all persons were cancers of the trachea, bronchus and lung followed by breast, colorectal and prostate cancers. Head and neck cancers were the 6th most common overall and when stratified by gender they ranked 4th in males and 9th in females with 821 and 365 diagnosed in 2011, respectively (1).

1.1.2 Head and neck cancers

Head and neck cancers are commonly divided into seven groups of which cancer of the lip, oral cavity and pharynx is one. In 2011, about 550 males and 300 females were diagnosed in Scotland with cancer of the lip, oral cavity and pharynx. Recently, incidences have been rising in that group of cancer. The incidence rate for all persons has risen from 10.1 in 1987 to 16.2 in 2011 (2). This has among other things been attributed to the rise of oropharyngeal squamous cell carcinoma (OPSCC). A growing proportion of OPSCC are associated with human-papillomavirus (HPV). HPV has both been found to be an independent risk factor for OPSCC and indicator of better prognosis when compared with the HPV-ve lesions (3).

1.2 Nasopharyngeal carcinoma

1.2.1 Epidemiology

Nasopharyngeal carcinoma (NPC) is a rare cancer in developed countries with an overall age-adjusted incidence of 0.6 in males and 0.2 in females. The incidence rate is higher in males with the male-to-female rate ratio often ranging between 2 and 3 males per female. Although the overall age-adjusted incidence rate for developing countries is only slightly higher, 2.0 and 0.8 in males and females, respectively, certain geographical regions and countries have considerably higher incidence. Overall, 82% of NPC worldwide occur in Asia (4). The highest incidence is reported in SE-Asia with NPC being endemic in Malaysia, Indonesia and Singapore. Other observed high-incidence areas include Hong Kong, Guangdong province in China and

Southern Asia as well as the Inuit populations of Alaska, Greenland and Northern Canada (5, 6). Among Chinese individuals living in the US, the rates remain higher than in Caucasians but the rates are still considerably lower than that found among Chinese populations living in SE China (7). Reported rates among Scots are consistent with developed countries with the overall age-adjusted incidence rate of 0.7 and 0.2 in males and females, respectively. However, recent evidence suggests that incidence may be rising in Scotland (8).

1.2.2 Risk factors and pathogenesis

The pathogenesis of NPC is still not fully understood despite considerable research although epidemiological studies suggest that environmental, genetic and ethnic factors play a role in the aetiology of the lesions. Environmental risk factors include Epstein-Barr virus (EBV), formaldehyde, Cantonese salted fish, tobacco smoking and wood dust, and the International Agency for Research on Cancer (IARC) has classified these environmental factors as Group 1 human carcinogens for NPC (9). Importantly, EBV is found in the majority of NPC (see section 1.3 below) (10). However, EBV is ubiquitous and >90% of the world population will have acquired the virus by adulthood (11), yet NPC is relatively rare which leads to the observation that the above-mentioned risk factors may play a role in pathogenesis but are on their own not sufficient to cause NPC. Three separate groups have performed genome-wide linkage analysis on families with strong history of NPC and have identified susceptibility loci on chromosome 4p, 5p13 and 3p21 (12, 13). However, these results lack consistency and more studies on genetic predisposition are needed. A postulation is that EBV is a co-factor that acts in the context of host human leukocyte antigen (HLA) alleles in NPC tumorigenesis (14).

1.2.3 Classification

NPC comprises of squamous carcinoma cells and a non-neoplastic lymphocytic infiltrate. The World Health Organisation (WHO) classifies NPC according to histological type into keratinising squamous cell carcinoma (KSCC; WHO-I), non-keratinising carcinoma (NKC; WHO-II/III) and basaloid squamous cell carcinoma. NKC can be further classified into differentiated (WHO-II) and undifferentiated (WHO-III) lesions although the clinical usefulness of this subdivision is debated (15). Overall, NKC is more prevalent than KSCC but KSCC is increasingly represented in

non-endemic NPC regions. Of southern Chinese NPC patients, 98%[1] were diagnosed with NKC and 2% with KSCC. In North America, the distribution was 75% [2] and 25%, respectively (16). Recent studies have found KSCC to constitute 8.9-15.5% of NPC in the UK population (17, 18).

1.2.4 Staging

The two most commonly used tumour-node-metastasis (TNM) staging systems are the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) system (favoured in Europe and North America) and The Ho system (favoured in Asia) (19). Table 1 shows AJCC/UICC 7th (and most recent) edition of NPC – TNM Classification of malignant tumour (20, 21). Imaging studies are a crucial tool in staging of NPC. Ideally, both magnetic resonance imaging (MRI) and computerized tomography (CT) should be used since they complement each other. MRI is better suited than CT to imaging soft tissue and differentiating tumour from healthy soft tissue (22). However, when it comes to assessing the tumour growth into the skull and the erosion of bone, CT is the appropriate approach (23). With a higher TNM stage comes the need to assess possible metastasis with distant imaging such as chest X-ray, bone scan and liver ultrasonography (24). At time of diagnosis, NPC is most often at TNM stage III or IV (25).

Table 1 AJCC/UICC 7th TNM staging system (21)

T-primary tumour	
T1	Nasopharynx, oropharynx or nasal cavity extension
T2	Parapharyngeal extension
T3	Bony structure of skull base and/or paranasal sinuses
T4	Intracranial, cranial nerves, hypopharynx, orbit, infratemporal fossa/masticator space
N-regional lymph nodes	
N0	No regional lymph node metastases
N1	Unilateral metastasis in cervical lymph node(s), uni/bilateral retropharyngel lymph nodes, ≤ 6 cm in greatest dimension, above the supraclavicular fossa involvement
N2	Bilateral metasis in cervical lymph nodes(s), ≤ 6 cm in greatest dimension, above the supraclavicular fossa
N3a	> 6cm
N3b	Extension to the supraclavicular fossa

M-distant metastasis	
M0	No distant metastasis
M1	Distant metastasis
Stage grouping	
I	T1+N0+M0
II	T1+N1+M0, T2+N0-1+M0
III	T1-2+N2+M0, T3+N0-2+M0
IVa	T4+N0-2+M0
IVb	Any T+N3+M0
IVc	Any T+Any N+M1

[1] 3% WHO-II and 95% WHO-III
[2] 12% WHO-II and 63% WHO-III

1.2.5 Clinical features

NPC is commonly at an advanced stage when diagnosed (see 1.2.4 above). This can be attributed to minimal symptoms for carcinoma in situ (CIS) and difficulties examining the nasopharynx, which is done endoscopically. A retrospective study of 4,768 patients in Hong Kong identified a neck mass as the most common presentation. The neck mass was most often due to enlarged upper cervical lymph nodes. At diagnosis, 74.5% patients had metastasis in cervical lymph nodes, which was bilateral in 50.4% of patients. The second most common complaint was nasal symptoms including discharge, bleeding and obstruction. Rarer complaints included otological symptoms and symptoms due to cranial nerve involvement. Although these symptoms were not a common source of presenting complaints, patients often developed them by the time they were diagnosed. The same study reported that most patients (81.9%) presented with multiple symptoms/signs, 17.8% presented with a single complaint and the rest were asymptomatic (25).

1.2.6 Management

Management of NPC has come a long way and is considered to be one of the success stories in oncology. The average 5-year survival rate reached 70% in the 1990s having increased more than 2-fold since the 1960s (26). The increased survival rate is attributed to better understanding of NPC pathogenesis, advancement of radiotherapy and the use of combined chemo- and radiotherapy. A 2006 meta-analysis found chemo-radiotherapy to be beneficial compared to radiotherapy on its own in both overall and event-free survival. As a result, concurrent chemo-radiotherapy is now considered to be best practice (27). However, surrounding the tumours in the base of the skull are dose-limiting vital organs that affect radiotherapy and can lead to dangerous complications if damaged. Intensity-modulated radiotherapy (IMRT) lessens the frequency of complications by reducing normal tissue doses and providing more homogenous target tissue doses (28). IMRT has been found to have great loco-regional impact on NPC but a recent study suggests the value of exploring alternative chemotherapy to the current cisplatin-based one, which is not effective enough against distant metastasis (29).

1.3 Epstein-Barr Virus

1.3.1 EBV malignancies

EBV is a ubiquitous human herpesvirus that establishes a latent infection in host B cells and persists for life. The virus was first discovered in Burkitt's lymphoma (BL) in 1964 by Epstein et al. (30) and was later classified as a herpesvirus, more specifically γ-herpesvirus of the Lymphocryptogenus. In 1968, primary EBV infection was linked to infectious mononucleosis (IM) (31). EBV's oncogenic potential became even more evident when it's relationship with NPC was discovered in 1970 (32). Since then, EBV has been associated with Hodgkin's lymphoma (HL), post-transplant lymphoproliferative diseases, NK/T-cell lymphomas and gastric carcinomas (11).

1.3.2 EBV latency

Three different latency types in EBV malignancies have been identified *in vitro* based on promoter activity and latent protein expression. Type I is characterised by Burkitt's lymphoma; type II by NPC, gastric carcinoma and HL and type III is characterised by *in vitro* EBV-immortalised B lymphoblastoid cell lines (BLCLs) and post-transplant lymphoproliferative disease (PTLD) (33, 34). Latent EBV proteins include six EB nuclear antigens (EBNAs) and three latent membrane proteins (LMPs). The EBNAs are expressed by all EBV-infected cells and include EBNA 1, 2, 3A, 3B, 3C and Leader Protein (LP) that can influence both viral and cellular transcription. LMPs include LMPs 1, 2A and 2B (11). EBNA2 and LMP1 are considered to be EBV oncogenes, and the presence of LMP1 in NPC tumour biopsies suggests that it might play a role in the lesions tumorigenesis (35). A study that found 71% of NPC patients with serum LMP1 antibodies compared to 5% of healthy EBV seropositives further emphasises the importance of LMP1 in EBV-infected NPC lesions (36). So-called BART transcripts and small EBV-encoded RNAs, EBER1 and EBER 2, are detected in EBV malignancies of all latency types. However, EBERs functions are unclear as they are non-polyadenylated, hence non-coding (37). Table 2 summarises EBV latency proteins and associated diseases in each EBV latency programmes.

Table 2 EBV latency programmes and human diseases (34)

Latency Programme	EBV gene expressed	Dieseases associated
Type I	EBNA1, EBERs, BART	BL
Type II	EBNA1, LMP1, LMP2A, LMP2B, EBERs, BART	NPC, HL, gastric carcinoma
Type III	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B, EBERs, BART	BLCL, PTLD

1.3.3 EBV and NPC pathogenesis

EBV's role in NPC pathogenesis is still not fully understood despite vast research and it being 44 years now since the connection was made. EBV DNA has been detected in NKC in both endemic and non-endemic areas (32, 38). The connection between KSCC and EBV isn't as established, suggesting that there may be other aspects involved in the pathogenesis of KSCC than in NKC (39). However, when EBV is found in NPC, the viral genome is clonal (from a single progenitor cell). Therefore, the virus infects the epithelial cell before clonal expansion of the malignant cell and may play an early role in pathogenesis (40, 41). Even though EBV infection is an early event, the virus is thought to behave as a tumour-promoting agent (co-factor) rather than an initiating factor. The proposed initiating factors are carcinogens (e.g. salted fish, tobacco smoke) that lead to genetic changes in nasopharyngeal epithelial tissue and eventually predispose the epithelium to EBV infection (42-44). Studies have identified important diagnostic and prognosis factors such as raised serum EBV capsid antigen (VCA) IgA levels in endemic areas, which can be used successfully as a screening method in such areas (45). Furthermore, for EBV+ve NPCs, pre- and post-treatment plasma EBV DNA viral load (VL) is strongly indicative of treatment response, risk of relapse and overall survival. Patients with higher pre-treatment EBV VL are more likely to have events post treatment and survive shorter than those with lower VL (46). Post-treatment EBV VL reflects residual tumour and higher VL indicates shorter overall survival (47).

1.4 Human papillomavirus

1.4.1 Papillomaviruses

Papillomaviruses (PV) are small double-stranded DNA viruses that infect various animal species and draw their name from the animal they infect (e.g. bovine papillomavirus, canine papillomavirus, etc.). The origin of PVs can be traced back 350 million years to the origin of amniotes (48). However, they are species-specific viruses meaning that bovine papillomavirus can only infect cows and no other species. PVs preferentially infect mucosal or cutaneous epithelium and are classified into genera based on genome sequencing. The genome is divided into early (E1-E7) and late region (L1-L2) and a new variant is classified as such if >10% of the L1 open reading frame (ORF) differs from all known PVs (49).

1.4.2 Human papillomavirus

Human papillomaviruses (HPV) are divided into five genera. The largest is the alpha genus, which contains both mucosal and cutaneous HPVs (50). Furthermore, HPVs are stratified into high- and low-risk according to their tumorigenic potential. The mucosal types are either high- or low-risk. WHO has identified twelve high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) of which HPV16 is considered to be the most oncogenic due to its association with multiple cancer sites (51, 52). The genes E6 and E7 code for the the primary oncoproteins of HPV (53) and their expression levels are directly related to the severity of the neoplasia (54).

1.4.3 HPV life cycle

HPV-infection occurs through a break in the epithelium, a so-called microwound, which allows the virus to access the basal lamina where it can infect basal stem cells (55). A key factor in persistent HPV lesions is thought to be the virus' entry into basal stem cells since the stem cells are long-lived. High-risk HPVs are tenacious and can persist for years while pushing the basal cell layers of their infection site into proliferation (56, 57). Regardless of the cell proliferation, the viral genome is initially amplified in the lower epithelial layers. A low number of genome copies, thought to be 200, are then maintained in the basal layer. Viral expression can lead to genome amplification in the upper epithelial layer where viral particles are packaged and released or the expression is deregulated which can lead to dysplasia and neoplasia.

Most HPV infections are cleared whilst some become latent and play an etiological role in some diseases (58).

1.4.4 Clinical manifestations of HPV infections

Diseases caused by HPVs include cutaneous warts, anogenital warts and neoplasia (59-61). Worldwide, the most common HPV-related cancer is cervical cancer and in 2012 there were more than 520.000 newly diagnosed cases and over 260.000 deaths associated with the malignancy (4). Of the high-risk types, HPV16 has the strongest association with cervical cancer, and together with HPV18, accounts for 65% of cervical cancer cases (62). HPV has also been found in other anogenital carcinomas such as that of the vulva, vagina, penis and anus (60, 63). Increasingly, HPV is found in head and neck cancers such as OPSCC (64). Since such HPV+ve tend to have better survival than HPV-ve lesions, the HPV status of OPSCC is used as a prognostic tool (65). This has spiked interest in possible association with NPC and recent studies have detected HPV in 5-50% of NPC cases. HPV+ve cases have mainly been KSCC histological type but the inconsistency of study findings necessitates further studies (66). Furthermore, meta-analyses have found HPV16 and HPV18 to be the most commonly found types in oropharyngeal, oral cavity and oesophagus cancers (67, 68). The enormous global burden of disease stemming from HPV is clear but to fully understand the impact of HPV on cancer, further studies are required.

1.5 Genetic susceptibility

Both EBV and HPV are ubiquitous viruses and both have been associated with NPC although the evidence for EBV is stronger than for HPV. However, NPC still remains a rare malignancy in developed countries and additional/unknown factors play a role in the pathogenesis. An important factor might be the individual's immune response to viral infections perhaps dictating whether the virus is able to develop a latent infection or not. A single-nucleotide polymorphism (SNP) rs12979860 in interleukin-28B (IL28B) has been associated with outcome of hepatitis C virus (HCV) infection. A CC genotype is linked both to a sustained virological response (SVR) to interferon-α/ribavirin treatment of HCV and spontaneous clearance of the infection (69, 70). Numerous studies have been done on genetic risk factors for EBV-related malignancies. In 1974, an NPC-susceptibility locus associated with the HLA region was identified in high-risk population of Singapore and has since been one focus of

investigation (71). A further study narrowed the region down between microsatellite markers D6S510 and D6S211 of the HLA-A locus (72). However, genome-wide association study (GWAS) in Taiwan identified multiple loci within the HLA region at chromosome 6p21.3 connected to NPC (73). Studies on other EBV-associated diseases seem to have obtained more specific results. For example, alleles of microsatellite markers D6S510 and D6S265 within the HLA class I region have been associated with development of IM and higher EBV VL in IM patients (74). GWAS on HL identified five loci (rs6904029, rs2734986, rs6903608, rs2395185 and rs2248462) in the HLA region on chromosome 6p21.3 associated with the disease (75).

1.6 Study aim

The study aimed to address epidemiological questions and use molecular techniques to identify the HPV status of Scottish NPC patients and distribution of polymorphism in SNPs and microsatellite markers. Firstly, we aimed to see whether NPC incidence has risen significantly in Scotland and compare the incidence to Nordic Countries (Denmark, Norway, Sweden, Iceland and Finland). We then hoped to identify the prevalence of HPV in Scottish NPC. Thirdly, we looked at specific SNPs and microsatellite markers to assess whether they are associated with NPC or subgroups of NPC patients.

2. Material and methods

2.1 Capture of national statistics on NPC

Information on NPC registration, survival and incidences in the Scottish population was acquired from the Information Services Division (ISD) of Health Protection Scotland (HPS; Glasgow) for the period 1975-2011 (8). ISD collate national health care statistics for Scotland including cancer registrations. All Nordic countries NPC incidence rate adjusted to world standardised incidence rate was accessed via each country's cancer registry. The Swedish Cancer registry had information on registration and incidences available on their website from 1992 to 2011 (76). Icelandic, Finnish, Norwegian and Danish cancer registries were contacted via e-mail to acquire data on registration and incidences from 1992 to 2011 (77-79).

2.2 Study population

Initially 62 patients with primary NPC treated in a single centre in Edinburgh were identified. The patients were diagnosed between 2000 and 2013. Three patients with adenoid cystic carcinoma and one with unknown primary were excluded. Notes of the remaining 58 patients were reviewed. Demographics and clinical variables were collated and included information on age, gender, ethnicity, tobacco use, alcohol consumption, TNM-classification, EBV status, tumour histology and survival. Other variables such as first symptoms/signs and treatment options were also collated for further studies. Formalin fixed paraffin wax-embedded (FFPE) samples were available for 32/58 patients and a single pathologist reviewed and confirmed diagnosis of NPC. The remaining 26 patients resided outside the Lothian (greater Edinburgh) area and associated histology blocks could not be accessed due to permission restrictions between health-boards during the project. Three sample sets were used as controls for SNPs and microsatellite markers. Firstly, OPSCC samples from patients diagnosed 2011-2012 with the median age 61 and of which 48.3% were HPV positive. Secondly, archived cytology-negative samples originally derived from the Scottish cervical cancer screening program of which 56.0% were HPV positive. Finally, EBV-seropositive health students at the University of Edinburgh. Available nucleic acid extractions from OPSCC samples were genotyped for all SNPs and microsatellite markers and compared to NPC samples (see below). Information on IL28B genotype was available for the cervical cancer screening samples and on

rs6904029, rs2734986, rs6903608, rs2395185, rs2248462, D6S510 and D6S265 genotype for EBV-seropositive healthy individuals.

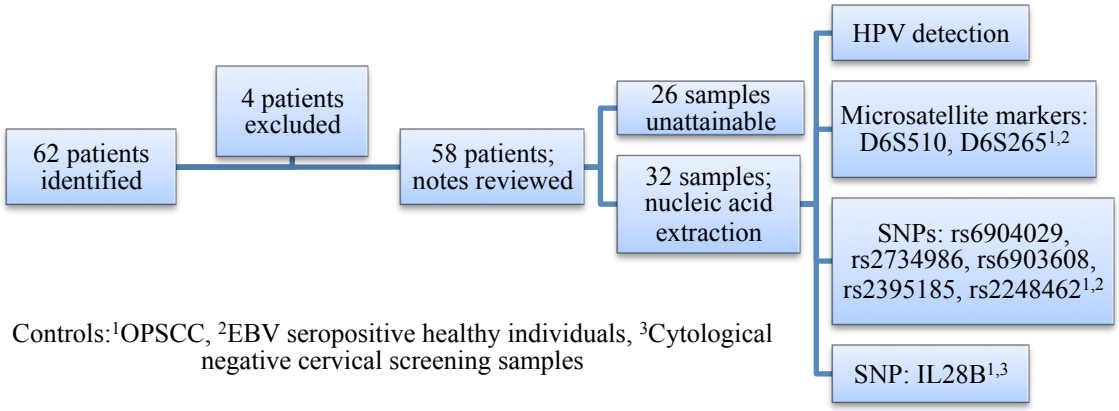

Figure 1 Research approach

2.3 DNA extraction

DNA was extracted from FFPE samples using QIAmp DNA Mini Kit (Qiagen, Crawley, UK) using an adaptation for tissue samples originally described by Steinau et al. (80). This involved overnight digestion of 10 µm sections of the relevant FFPE block with proteinase K prior to ethanol precipitation and purification of nucleic acid via silica spin columns. Quantity and purity of nucleic acid was assessed with a nanodrop spectrophotometer (Supplementary table 1).

2.4 HPV detection

Genotyping was done at the Scottish HPV Reference Laboratory (SHPVRL) at the Royal Infirmary of Edinburgh (RIE) and was performed using the Multimetrix HPV Genotyping Kit using the method previously described by Schmitt et al. (81, 82). Briefly, this involves broad-spectrum amplification of L1 DNA sequence using the Qiagen Multiplex PCR kit (Qiagen, Crawley, UK). Amplicons are subject to genotyping using Luminex 200 Analyzer. The assay detects all carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), 7 types considered probably/possibly carcinogenic (26, 53, 66, 68, 70, 73 and 82) and 5 low-risk types (6, 11, 42, 43 and 44) (83).

2.5 SNP genotyping

Genotyping of SNPs rs6904029, rs2734985, rs6903608, rs2395185 and rs2248462 was carried out at the Centre for Virus Research, University of Glasgow. The reaction

volume of 10 µl contained 1 x TaqMan Genotyping Master Mix (Applied Biosystems, Paisley, UK), 20 x SNP genotyping primer set and 20 ng extracted nucleic acid. PCR and allelic discrimination were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Paisley, UK) using standard protocols. IL28B genotyping was performed at University of Edinburgh using the Stratagene MxPro3000 instrument and software (Agilent Technologies, Craven Arms, UK). The reaction volume of 10 µl was prepared using LightMix Kit IL28B (TIB MOLBIOL, Berlin, Germany) and contained Roche FastStart and 20 ng of nucleic acid.

2.6 Microsatellite Marker genotyping

Microsatellite markers D6S265 and D6S510 from the HLA class I locus were amplified in a total reaction volume of 15 µl that contained 1x HotStar Taq Plus master mix (Qiagen, Crawley, UK), 2.5 µM of forward and reverse primers (74, 84) and 20 ng of nucleic acid. Amplified product was diluted 1:10 with nuclease free water and then further diluted (1:10) in Hi-Di formamide containing GeneScan 350Rox size standard (Applied Biosystems, Paisley, UK). For samples, when the analysis didn't yield results, the PCR product was used neat. The final product was run on 3130 Genetic Analyzer (Applied Biosystems, Paisley, UK) and analysed on Peak Scanner v. 2.0 (Applied Biosystems, Paisley, UK).

2.7 Analysis

Categorical data was cross-tabulated with Fishers exact test on R. Survival analysis was performed using Kaplan-Meier survival curves and presented with median survival times and log-rank statistics. Survival statistical analysis was performed on JMP.

2.8 Governance

Permission for use of the samples/associated information was provided by the South East of Scotland Bioresource – Project ID SR308. Activity related to the project was also performed in the context of an honorary research assistant contract with NHS Lothian.

3. Results

3.1 NPC Incidence in Scotland and Nordic Countries

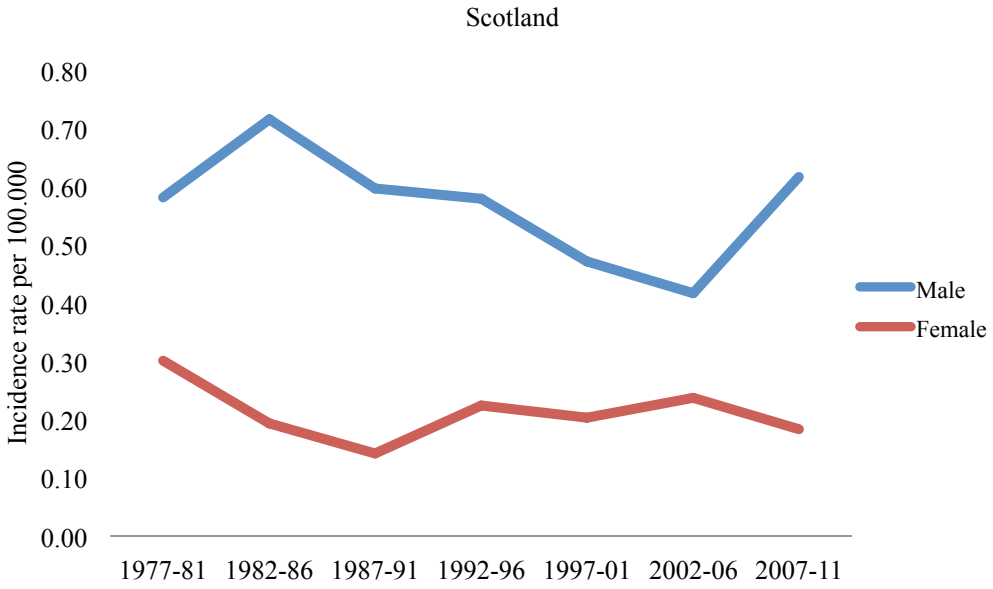

Figure 2 NPC in Scotland

For the period 1977 to 2011 incidence rates age adjusted to European standard population show no increase in the Scottish population (Figure 2). Furthermore, for the period 1992 to 2011 no increase in incidence was observed in Iceland, Finland, Sweden or Norway (Figure 3-4). At the time of analysis, Denmark's statistics weren't available. World standard population was used for Icelandic, Finnish, Swedish and Norwegian incidence rate and, thus, cannot be compared directly with the Scottish data. Due to similarity in incidence rate both between countries and time periods no statistical analysis was performed.

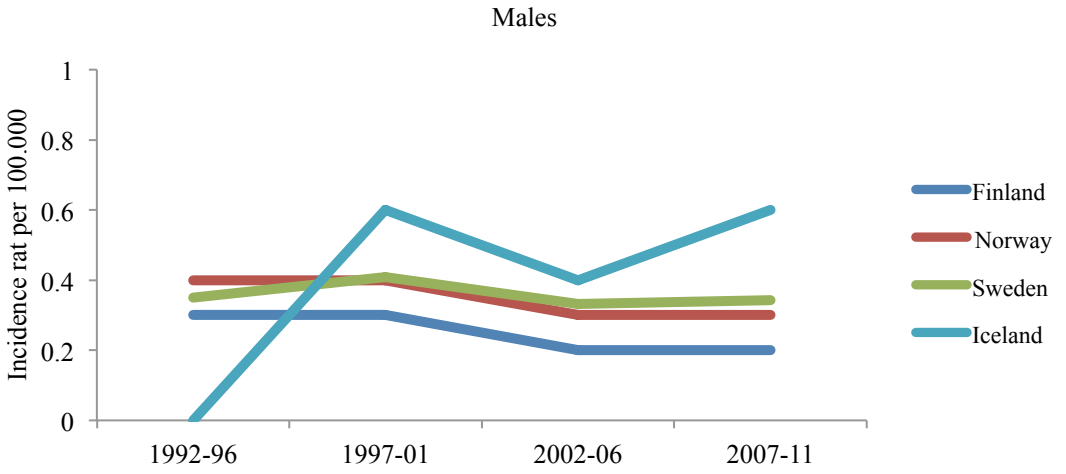

Figure 3 NPC in Nordic males

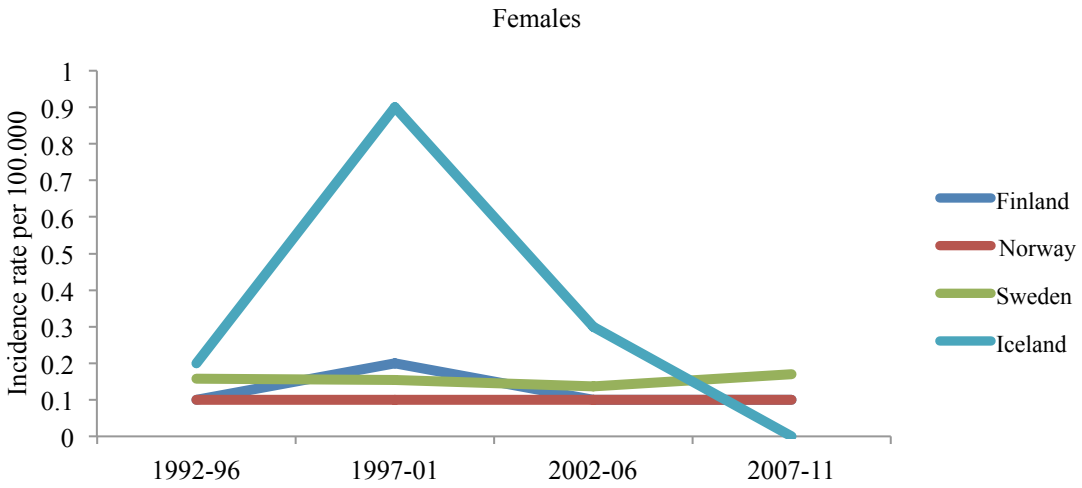

Figure 4 NPC in Nordic females

3.2 Patient Characteristics

Characteristics of all patients, including stratified by viral status, can be found in Table 3. Overall, the mean age at diagnosis was 55.2 years, majority of them were male (75.9%) and Caucasian (81.0%). Patients of Asian origin were mostly from S-China (10/11, 90.9%) with one from Thailand. Most patients were smokers or ex-smokers (62.0%) and 12 (20.7%) had a history of abnormal alcohol use. Forty-eight patients (82.8%) had metastasis in the regional lymph nodes at diagnosis. Thus, 50 (86.2%) patients have an advanced stage of III or IV. Unfortunately histological classification, KSCC vs. NKC, was not provided in the notes of all patients so information is lacking on histology type for most patients with unattainable samples.

Table 3 Baseline characteristics of study patients

Characteristics	All patients (n=58)	HPV-negative (n=22)	HPV-positive (n=10)	p-value*
Age, y				0.4050
Mean	55.2	57.5	53.4	
Standard deviation	15.5	14.2	11.9	
Sex, n (%)				0.1655
Female	14 (24.1)	3 (13.6)	4 (40.0)	
Male	44 (75.9)	19 (86.4)	6 (60.0)	
Ethnicity, n (%)				0.0694
Caucasian	47 (81.0)	15 (68.2)	10 (100.0)	
Asian	11 (19.0)	7 (31.8)	0	
Tobacco use, n (%)				0.1391
Ever	36 (62.1)	14 (63.6)	4 (40.0)	
Never	20 (34.5)	6 (27.3)	6 (60.0)	
Unknown	2 (3.4)	2 (9.1)	0	
Alcohol use, n (%)				1.0000
Heavy	7 (12.1)	3 (13.6)	1 (10.0)	
Ex-heavy	5 (8.6)	2 (9.1)	1 (10.0)	
Occasional	26 (44.8)	9 (40.9)	5 (50.0)	
Teetotaller	15 (25.9)	4 (18.2)	2 (20.0)	
Unknown	5 (8.6)	4 (18.2)	1 (10.0)	
Tumour classification, n (%)				0.5808
T1	16 (27.6)	7 (31.8)	2 (20.0)	
T2	8 (13.8)	2 (9.1)	0	
T3	9 (15.5)	3 (13.6)	3 (30.0)	
T4	20 (34.5)	8 (36.4)	4 (40.0)	
Unknown	5 (8.6)	2 (9.1)	1 (10.0)	
Nodal classification, n (%)				0.1171
N0	6 (10.3)	3 (13.6)	1 (10.0)	
N1	11 (19.0)	3 (13.6)	4 (40.0)	
N2	29 (50.0)	13 (59.1)	2 (20.0)	
N3	8 (13.8)	2 (9.1)	2 (20.0)	
Unknown	4 (6.9)	1 (4.5)	1 (10.0)	
AJCC stage, n (%)				0.6115
I	1 (1.7)	0	1 (10.0)	
II	3 (5.2)	2 (9.1)	0	
III	20 (34.5)	6 (27.3)	3 (30.0)	
IV	30 (51.7)	13 (27.3)	5 (50.0)	
Unknown	4 (6.9)	1 (4.5)	1 (10.0)	
Histology, n (%)				0.5343
NKC	35 (60.3)	19 (86.4)	10 (100.0)	
KSCC	3 (5.2)	3 (13.6)	0	
Unknown	20 (34.5)	0	0	

*P-values were calculated between patients group stratified by viral status using Fischer-exact test, two-sample T-test was used for age comparisons
Unknowns were excluded when applicable

3.3 Impact of HPV

All 32 samples analysed contained amplifiable β-globin and were, thus, considered to be of adequate quality for further assessments. HPV was found in 10 samples (10/32, 31.3%) of which 8 harboured HPV16 and 2 contained HPV18 together with HPV82. None of the HPV+ve patients were of Asian origin compared to 31.8% of the HPV-ve patients and a higher female to male ratio was observed in the HPV+ve group (40.0% vs. 13.6%). Furthermore, the HPV+ve patients had a higher ratio of individuals who never smoked (60.0% vs. 27.3%). The TNM stage at diagnosis between the two groups was similar although HPV-ve patients tended to have a higher nodal class (68.2% vs. 40.0%). However, upon formal statistical analysis, none of these associations were found to be significant.

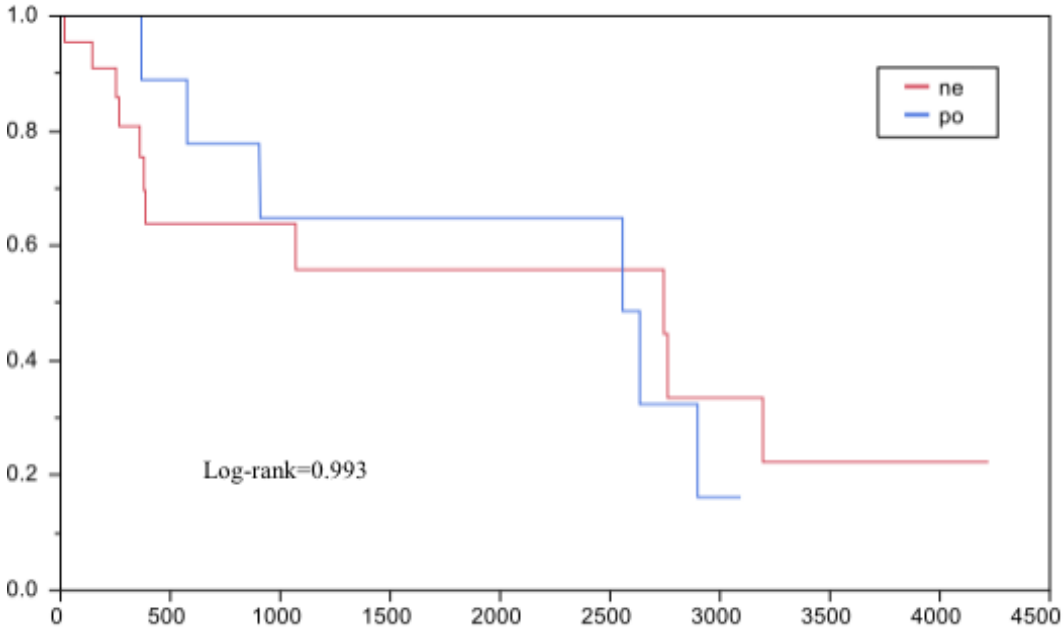

Figure 5 HPV status and survival, ne=HPV- and po=HPV+

The mean days of survival for all patients were 1900.88 (Std error: 254.64). When stratified by HPV status the HPV+ve patients had slightly higher mean of survival 2003.19 (Std error: 386.488) than the mean of HPV-ve patients, 1868.5 (Std error: 333.012). However, this was statistically insignificant and HPV status showed no evidence of having an impact on overall survival (Figure 5). Days survived were calculated from the date the biopsy was taken to date of death or date last seen by a health-care professional.

3.4 Genotype distribution of SNPs

Table 4 SNP genotype distribution

SNP	Genotype	NPC[§,⋆] (n=32) Frequency	OPSCC[⋆] (n=59) Frequency	Controls[§,⋆] (n=50, n=68[1]) Frequency	p[⋆]	p[§]
rs6904029	GG	62.5%	44.8%	47.1%	0.5806	0.3700
	AG	31.2%	46.6%	45.6%		
	AA	6.2%	8.6%	7.4%		
rs2734986	GG	6.2%	5.2%	4.4%	0.8255	0.6204
	AG	43.8%	32.8%	36.8%		
	AA	50.0%	62.1%	58.8%		
rs6903608	TT	50.0%	62.1%	54.4%	0.8144	0.9486
	CT	40.6%	29.3%	36.8%		
	CC	9.4%	8.6%	8.8%		
rs2395185	GG	34.4%	31.6%	47.1%	0.3710	0.5270
	GT	50.0%	47.4%	41.2%		
	TT	15.6%	21.1%	11.8%		
rs2248462[1]	GG	64.3%	63.8%	58.8%	0.2336	0.0707
	AG	25.0%	31.0%	39.7%		
	AA	10.7%	5.2%	1.5%		
IL28B[2]	CC	46.9%	53.4%	40.0%	0.2327	0.7762
	CT	37.5%	43.1%	48.0%		
	TT	15.6%	3.4%	12.0%		

[§,⋆] Fisher's exact 2-sided P-value.[1]NPC (n=28)
Controls are EBV seropositive healthy individuals except [2]which are archived cytological samples from cervical screening samples

SNPs rs6904029 (HLA class I, *HCG9*) and rs2248462 (HLA class I, adjacent to *MICB*) had the largest difference in genotype proportions between NPC patients and control (healthy EBV-seropositive individuals) (Table 4). Proportion of rs6904029 GG genotype was 62.5% and 47.1% and rs2248462 AG genotype was 25.0% and 39.7% in NPC patients and control population, respectively. When NPC patients were stratified by HPV status, an interesting trend is observed in rs6904029. The vast majority of HPV+ve patients (80.0%) had GG genotype of rs6904029 compared to 54.5% of the HPV-ve patients (Table 5). A comparion between NPC patient and control samples (including healthy EBV-seropositive individuals, archived cytology-normal cervical screening samples and OPSCC patients) did not reveal any statistically significant results.

Table 5 SNP genotype distributions by HPV status

SNP	Genotype	HPV- (n=22) Frequency	HPV+ (n=10) Frequency	p*
rs6904029	GG	54.5%	80.0%	0.2279
	AG	40.9%	10.0%	
	AA	4.5%	10.0%	
rs2734986	GG	4.5%	10.0%	0.6046
	AG	40.9%	50.0%	
	AA	54.5%	40.0%	
rs6903608	TT	45.5%	60.0%	0.6393
	CT	40.9%	40.0%	
	CC	13.6%	0%	
rs2395185	GG	27.3%	50.0%	0.5128
	GT	54.5%	40.0%	
	TT	18.2%	10.0%	
rs2248462[1]	GG	66.7%	60.0%	0.6987
	AG	27.8%	20.0%	
	AA	5.6%	20.0%	
IL28B	CC	45.5%	50.0%	0.7801
	CT	40.9%	30.0%	
	TT	13.6%	20.0%	

* Fisher's exact 2-sided P-value, [1]HPV- (n=18)

3.5 Microsatellite markers

When comparing NPC patients and the control population (EBV-seropositive individuals) the most difference between them is observed for allele 3 (16.7% and 10.3%, respectively), allele 6 (24.1% and 18.4%, respectively) and allele 8 (11.1% and 23.5%, respectively) of marker D6S510. Furthermore, a difference between NPC patients and control group (EBV-seropositive individuals) was observed for allele 4 (17.7% and 6.6%, respectively) and allele 8 (8.1% and 14.0%, respectively) of marker DS265. A comparison of allele frequency between NPC and control samples (including healthy EBV-seropositive individuals, archived cytology-normal cervical screening samples and OPSCC patients) did not show any significant associations (Table 6).

Table 6 Microsatellite markers allele frequency

Locus	Allele	Base pair size	NPC (n=64 alleles) Frequency	OPSCC (n=118 alleles) Frequency	Controls (n=100 alleles) Frequency	*p
D6S510	Allele 1	284	35.2%	33.0%	33.8%	0.5262
	Allele 2	290	0.0%	0.0%	0.0%	
	Allele 3	292	16.7%	12.5%	10.3%	
	Allele 4	294	7.4%	8.0%	7.4%	
	Allele 5	296	3.7%	3.6%	3.7%	
	Allele 6	298	24.1%	18.8%	18.4%	
	Allele 7	300	0.0%	0.9%	1.5%	
	Allele 8	302	11.1%	23.2%	23.5%	
	Allele 9	304	1.9%	0.0%	0.7%	
	Allele 10	306	0.0%	0.0%	0.7%	
D6S265	Allele 1	122	9.7%	12.1%	8.8%	0.1825
	Allele 2	124	0.0%	1.7%	0.0%	
	Allele 3	126	38.7%	34.5%	40.4%	
	Allele 4	128	17.7%	8.6%	6.6%	
	Allele 5	130	24.2%	26.7%	27.9%	
	Allele 6	132	8.1%	13.8%	14.0%	
	Allele 7	134	0.0%	0.9%	1.5%	
	Allele 8	136	0.0%	0.0%	0.0%	
	Allele 9	138	0.0%	0.9%	0.0%	
	Allele 10	140	0.0%	0.0%	0.0%	
	Allele 11	142	1.6%	0.0%	0.0%	
	Allele 12	144	0.0%	0.9%	0.7%	
	Allele 13	146	0.0%	0.0%	0.0%	

*Fisher's exact 2-sided P-value comparing NPC and controls which are EBV seropositive healthy individuals

4. Discussion

The study aimed to ascertain the characteristics of NPC in a Scottish population with particular reference to a possible role for HPV and/or SNP/microsatellite markers in NPC development.

For the period assessed (1977-2011), the study demonstrates that there is no significant increase in NPC incidence in Scotland despite an impression of a slight upwards trend for the Scottish male population. However, at the time of data collection, ISD had only published data up to the year 2011 and it will be interesting to re-examine the situation as more recent data becomes available. Fluctuations in Icelandic incidence rates are due to its small population but Finland, Norway and Sweden have similar and constant incidence rates for NPC.

To the best of our knowledge, this is the first study assessing HPV in the Scottish NPC patient population. Patients' characteristics such as mean age at diagnosis (55.2), TNM stage (86.2 at stage III or IV) and gender distribution (male to female ratio 3:1), see Table 3, is similar to that described in studies in other non-endemic areas such as the USA (85-88). For example, whilst a 2013 UK study by Robinson et al. (17) had slightly younger patient group (mean age 48), the proportion of males was the same (73.1%) and the majority of patients (83.5%) were also diagnosed at stage III or IV.

In the current study, 31.3% of lesions were HPV+ve tumours, but the reported proportion of such lesions varies greatly between studies. For example, Robinson et al. (2013) found 11 out of 67 tumours (16.4%) of their study panel to consist of HPV+ve tumours that is lower than the 31.3% reported in the current analysis, analysis although not a significant difference when the two studies are compared ($p=0.1164$). Our results further emphasise that HPV16 and 18 appear to be the most oncogenic HPV types. This conforms to results from other studies (85-87) as well as that none of the HPV+ve cases from our research panel where of Asian origin. The difference in tobacco use between HPV+ (40.0%) and HPV- (63.6%) NPC patients, although not significant, implies that as with OPSCC HPV infection might be an independent risk factor.

Further studies are crucial on the impact of HPV infection in NPC patients, in particular regards to survival. Stenmark et al. (87) found HPV+ve NPC to have a worse prognosis but failed to further validate their results when adjusting for competing risk factors. Survival of the current patient panel stratified by HPV status was not significant. This could be expected both due to the limited number of patients in each group and the short follow-up period for some patients. Twelve out of 32 patients were diagnosed 2010 or later and a longer follow up is needed to fully test survival statistics.

Interstingly, the results for SNPs rs6904029 and rs2248462 show the largest difference between NPC patients and controls, which may indicate a possible role for these markers. In line with this is a study by Urayama et al. (75) who found rs2248462 to be associated with HL irrespective of EBV status. With the minor allele A being associated with a reduced risk of the disease. In contrast, the current study found that more NPC patients were homozygous for the A allele than in the control group, although there was not a significant association. However, the minor allele A of rs6904029 was associated with reduced risk of EBV+ve HL which is in line with the current finding that more patients in the control group were homo- or heterozygous for allele A (53.0%) than in the NPC patiens (37.4%)- although (again) the association was not significant. Interestingly, studies have suggested that EBV and HPV infection are mutually exclusive in NPC (66). If that were correct it is plausible that more of our HPV- then HPV+ samples are EBV+. Therefore, genotype distribution of rs6904029 in patients stratified by HPV status is opposite to what we would expect. HPV- group had a higher proportion of homo- or heterozygous patients for allele A (45.4%) than the HPV+ group (20.0%). This is entirely speculative and EBV status of the samples is needed for further evaluation.

In order to ensure diagnostic consistency, the same histopathologist reviewed the current case series. Equally, the same researcher carried out all laboratory work. However, the biggest limitation of this research is the amount of samples that were unattainable for the time frame of the project. This influenced the power and unfortunately none of our findings were significant ($p<0.05$).

In conclusion, HPV is likely to have an aetiological part in NPC in the Scottish population. Patients of Asian origin are less likely to have HPV+ve lesions. GG genotype of rs6904029 and AA genotype of rs2248462 might be associated with NPC and further studies are warranted.

In order to build on, and expand, the findings outlined above, work is under way in SHPVRL to assess an extended NPC sample panel and to delineate EBV status as well as EBV and HPV expression profiles of the lesions with a view to gain insight into the possible contribution of these viruses to NPC development. The findings will inform future steps towards deepening our understanding of NPC tumorigenesis and translating that knowledge into the clinical setting in terms of identifying suitable biomarkers for improved diagnosis and therapeutic intervention.

5. Acknowledgments

First and foremost I would like to thank my instructors Dr Ingólfur Johannessen and Dr Kate Cuschieri for their invaluably advice and great patience. I would also like to thank everybody at the SHPVRL – in particular, Catherine Moore - for their help with the molecular work. Also, I would like to thank Dr Karen McAulay for welcoming me into her laboratory at the University of Glasgow. Dr Brendan Conn was extremely helpful and assessed and classified all histology slides. I would like to thank Dr Elizabeth Junor for collating the original patient list and her guidance. Dr Adonye Banigo's help was appreciated in collating demographics and clinical variables for all patients. I would also like to thank Dr Juergen Haas, Dr Samantha J. Griffiths and Cory Dunnigan for their help with genotyping of IL28B and welcoming me into their lab. Last but not least I would like to thank the Lyons family for welcoming me into their family and home whilst staying in Edinburgh.

6. References

1. Cancer in Scotland [Internet]. 2013 [cited 3/3/2014]. Available from: isdscotland.org/Health-Topics/Cancer/Publications/2013-11-26/Cancer_in_Scotland_summary_m.pdf.

2. [cited 07/05/2014]. Available from: http://isdscotland.org/Health-Topics/Cancer/Publications/2014-04-29/i_cancer_head_neck.xls.

3. Panwar A, Batra R, Lydiatt WM, Ganti AK. Human papilloma virus positive oropharyngeal squamous cell carcinoma: a growing epidemic. Cancer treatment reviews. 2014 Mar;40(2):215-9. PubMed PMID: 24080155. Epub 2013/10/02. eng.

4. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v 1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: Internation Agency for Research on Cancer. 2013 Available from: http://globocan.iarc.fr, accessed on 4/3/14.

5. Friborg JT, Melbye M. Cancer patterns in Inuit populations. The lancet oncology. 2008 Sep;9(9):892-900. PubMed PMID: 18760245. Epub 2008/09/02. eng.

6. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011 Mar-Apr;61(2):69-90. PubMed PMID: 21296855. Epub 2011/02/08. eng.

7. Sun LM, Epplein M, Li CI, Vaughan TL, Weiss NS. Trends in the incidence rates of nasopharyngeal carcinoma among Chinese Americans living in Los Angeles County and the San Francisco metropolitan area, 1992-2002. American journal of epidemiology. 2005 Dec 15;162(12):1174-8. PubMed PMID: 16282240. Epub 2005/11/12. eng.

8. 2013 [cited 01/03/2014]. Available from: http://www.isdscotland.org/Health-Topics/Cancer/Publications/2013-04-30/cancer_incandmort_summary.xls.

9. Cogliano VJ, Baan R, Straif K, Grosse Y, Lauby-Secretan B, El Ghissassi F, et al. Preventable exposures associated with human cancers. Journal of the National Cancer Institute. 2011 Dec 21;103(24):1827-39. PubMed PMID: 22158127. Pubmed Central PMCID: PMC3243677. Epub 2011/12/14. eng.

10. Raab-Traub N, Flynn K, Pearson G, Huang A, Levine P, Lanier A, et al. The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. International journal of cancer Journal international du cancer. 1987 Jan 15;39(1):25-9. PubMed PMID: 3025109. Epub 1987/01/15. eng.

11. Longnecker RM, Kieff E, Cohen JI. Epstein-Barr Virus. In: Knipe DM, Howley P, editors. Fields Virology. 2. 6th ed: Lippincott Williams and Wilkins; 2013. p. 1898-954.
12. Feng BJ, Huang W, Shugart YY, Lee MK, Zhang F, Xia JC, et al. Genome-wide scan for familial nasopharyngeal carcinoma reveals evidence of linkage to chromosome 4. Nature genetics. 2002 Aug;31(4):395-9. PubMed PMID: 12118254. Epub 2002/07/16. eng.
13. Xiong W, Zeng ZY, Xia JH, Xia K, Shen SR, Li XL, et al. A susceptibility locus at chromosome 3p21 linked to familial nasopharyngeal carcinoma. Cancer research. 2004 Mar 15;64(6):1972-4. PubMed PMID: 15026332. Epub 2004/03/18. eng.
14. Busson P, Keryer C, Ooka T, Corbex M. EBV-associated nasopharyngeal carcinomas: from epidemiology to virus-targeting strategies. Trends in microbiology. 2004 Aug;12(8):356-60. PubMed PMID: 15276610. Epub 2004/07/28. eng.
15. Barnes L, Eveson JW, Reichart P, Sidransky D. Pathology and Genetics of Head and Neck Tumours. World Health Organization Classification of Tumours. 2005;IARC Press: Lyon.
16. Nicholls JM. Nasopharyngeal Carcinoma: Classification and Histologic Appearances. Advances in Anatomic Pathology. 1997;4(2):71-84.
17. Robinson M, Suh YE, Paleri V, Devlin D, Ayaz B, Pertl L, et al. Oncogenic human papillomavirus-associated nasopharyngeal carcinoma: an observational study of correlation with ethnicity, histological subtype and outcome in a UK population. Infectious agents and cancer. 2013;8(1):30. PubMed PMID: 23938045. Pubmed Central PMCID: PMC3751535. Epub 2013/08/14. eng.
18. Colaco RJ, Betts G, Donne A, Swindell R, Yap BK, Sykes AJ, et al. Nasopharyngeal carcinoma: a retrospective review of demographics, treatment and patient outcome in a single centre. Clinical oncology (Royal College of Radiologists (Great Britain)). 2013 Mar;25(3):171-7. PubMed PMID: 23337060. Epub 2013/01/23. eng.
19. Wei WI, Sham JS. Nasopharyngeal carcinoma. Lancet. 2005 Jun 11-17;365(9476):2041-54. PubMed PMID: 15950718. Epub 2005/06/14. eng.
20. Pan J, Xu Y, Qiu S, Zong J, Guo Q, Zhang Y, et al. A Comparison Between the Chinese 2008 and the 7th Edition AJCC Staging Systems for Nasopharyngeal

Carcinoma. American journal of clinical oncology. 2013 Apr 19. PubMed PMID: 23608833. Epub 2013/04/24. Eng.
21. Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC Cancer Staging Manual. Philadelphia: Springer; 7th edition 2009. 649 p.
22. WP D, CM M, B K, J D, M. B-Z. Magnetic resonance imaging of the nasopharynx. Radiology. 1984;152:731-38.
23. Olmi P, Fallai C, Colagrande S, Giannardi G. Staging and follow-up of nasopharyngeal carcinoma: magnetic resonance imaging versus computerized tomography. International journal of radiation oncology, biology, physics. 1995 Jun 15;32(3):795-800. PubMed PMID: 7790266. Epub 1995/06/15. eng.
24. Kumar MB, Lu JJ, Loh KS, Chong LM, Soo R, Goh BC, et al. Tailoring distant metastatic imaging for patients with clinically localized undifferentiated nasopharyngeal carcinoma. International journal of radiation oncology, biology, physics. 2004 Mar 1;58(3):688-93. PubMed PMID: 14967421. Epub 2004/02/18. eng.
25. Lee AW, Foo W, Law SC, Poon YF, Sze WM, O SK, et al. Nasopharyngeal carcinoma: presenting symptoms and duration before diagnosis. Hong Kong medical journal = Xianggang yi xue za zhi / Hong Kong Academy of Medicine. 1997 Dec;3(4):355-61. PubMed PMID: 11847385. Epub 2002/02/16. Eng.
26. Lee AWM, Ng WT, Chan YH, Sze H, Chan C, Lam TH. The battle against nasopharyngeal cancer. Radiotherapy and Oncology. 2012 9//;104(3):272-8.
27. Baujat B, Audry H, Bourhis J, Chan AT, Onat H, Chua DT, et al. Chemotherapy in locally advanced nasopharyngeal carcinoma: an individual patient data meta-analysis of eight randomized trials and 1753 patients. International journal of radiation oncology, biology, physics. 2006 Jan 1;64(1):47-56. PubMed PMID: 16377415. Epub 2005/12/27. eng.
28. Hsiung CY, Yorke ED, Chui CS, Hunt MA, Ling CC, Huang EY, et al. Intensity-modulated radiotherapy versus conventional three-dimensional conformal radiotherapy for boost or salvage treatment of nasopharyngeal carcinoma. International journal of radiation oncology, biology, physics. 2002 Jul 1;53(3):638-47. PubMed PMID: 12062607. Epub 2002/06/14. eng.
29. Kong F, Ying H, Huang S, Du C, Zhou J, Hu C. Preliminary results of nasopharyngeal carcinoma treated with intensity-modulated radiotherapy: a retrospective study of 364 patients. European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies

(EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery. 2014 Feb 18. PubMed PMID: 24534894. Epub 2014/02/19. Eng.
30. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet. 1964 Mar 28;1(7335):702-3. PubMed PMID: 14107961. Epub 1964/03/28. eng.
31. Henle G, Henle W, Diehl V. Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. Proceedings of the National Academy of Sciences of the United States of America. 1968 Jan;59(1):94-101. PubMed PMID: 5242134. Pubmed Central PMCID: PMC286007. Epub 1968/01/01. eng.
32. Henle W, Henle G, Ho HC, Burtin P, Cachin Y, Clifford P, et al. Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms, and control groups. Journal of the National Cancer Institute. 1970 Jan;44(1):225-31. PubMed PMID: 11515035. Epub 1970/01/01. eng.
33. Shah KM, Young LS. Epstein–Barr virus and carcinogenesis: beyond Burkitt’s lymphoma. Clinical Microbiology and Infection. 2009;15(11):982-8.
34. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nature reviews Cancer. 2004 Oct;4(10):757-68. PubMed PMID: 15510157. Epub 2004/10/29. eng.
35. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell. 1985 Dec;43(3 Pt 2):831-40. PubMed PMID: 3000618. Epub 1985/12/01. eng.
36. Xu J, Ahmad A, D'Addario M, Knafo L, Jones JF, Prasad U, et al. Analysis and significance of anti-latent membrane protein-1 antibodies in the sera of patients with EBV-associated diseases. Journal of immunology (Baltimore, Md : 1950). 2000 Mar 1;164(5):2815-22. PubMed PMID: 10679125. Epub 2000/02/29. eng.
37. Lerner MR, Andrews NC, Miller G, Steitz JA. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. Proceedings of the National Academy of Sciences of the United States of America. 1981 Feb;78(2):805-9. PubMed PMID: 6262773. Pubmed Central PMCID: PMC319891. Epub 1981/02/01. eng.
38. Desgranges C, Wolf H, De-The G, Shanmugaratnam K, Cammoun N, Ellouz R, et al. Nasopharyngeal carcinoma. X. Presence of epstein-barr genomes in separated epithelial cells of tumours in patients from Singapore, Tunisia and Kenya. International journal of cancer Journal international du cancer. 1975 Jul 15;16(1):7-15. PubMed PMID: 170215. Epub 1975/07/15. eng.

39. Nicholls JM, Agathanggelou A, Fung K, Zeng X, Niedobitek G. The association of squamous cell carcinomas of the nasopharynx with Epstein-Barr virus shows geographical variation reminiscent of Burkitt's lymphoma. The Journal of pathology. 1997 Oct;183(2):164-8. PubMed PMID: 9390028. Epub 1997/12/09. eng.
40. Raab-Traub N, Flynn K. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell. 1986 Dec 26;47(6):883-9. PubMed PMID: 3022942. Epub 1986/12/26. eng.
41. Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. The New England journal of medicine. 1995 Sep 14;333(11):693-8. PubMed PMID: 7637746. Epub 1995/09/14. eng.
42. Lo KW, Huang DP. Genetic and epigenetic changes in nasopharyngeal carcinoma. Semin Cancer Biol. 2002 Dec;12(6):451-62. PubMed PMID: 12450731. Epub 2002/11/27. eng.
43. Chan AS, To KF, Lo KW, Ding M, Li X, Johnson P, et al. Frequent chromosome 9p losses in histologically normal nasopharyngeal epithelia from southern Chinese. International journal of cancer Journal international du cancer. 2002 Nov 20;102(3):300-3. PubMed PMID: 12397655. Epub 2002/10/25. eng.
44. Chan AS, To KF, Lo KW, Mak KF, Pak W, Chiu B, et al. High frequency of chromosome 3p deletion in histologically normal nasopharyngeal epithelia from southern Chinese. Cancer research. 2000 Oct 1;60(19):5365-70. PubMed PMID: 11034072. Epub 2000/10/18. eng.
45. Li S, Deng Y, Li X, Chen QP, Liao XC, Qin X. Diagnostic value of Epstein-Barr virus capsid antigen-IgA in nasopharyngeal carcinoma: a meta-analysis. Chinese medical journal. 2010 May 5;123(9):1201-5. PubMed PMID: 20529563. Epub 2010/06/10. eng.
46. Lo YM, Chan AT, Chan LY, Leung SF, Lam CW, Huang DP, et al. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. Cancer research. 2000 Dec 15;60(24):6878-81. PubMed PMID: 11156384. Epub 2001/01/13. eng.
47. Chan AT, Lo YM, Zee B, Chan LY, Ma BB, Leung SF, et al. Plasma Epstein-Barr virus DNA and residual disease after radiotherapy for undifferentiated nasopharyngeal carcinoma. Journal of the National Cancer Institute. 2002 Nov 6;94(21):1614-9. PubMed PMID: 12419787. Epub 2002/11/07. eng.

48. García-Vallvé S, Alonso Á, Bravo IG. Papillomaviruses: different genes have different histories. Trends in microbiology. 2005 11//;13(11):514-21.
49. de Villiers E-M, Fauquet C, Broker TR, Bernard H-U, zur Hausen H. Classification of papillomaviruses. Virology. 2004 6/20/;324(1):17-27.
50. Bernard H-U, Burk RD, Chen Z, van Doorslaer K, Hausen Hz, de Villiers E-M. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010 5/25/;401(1):70-9.
51. Muñoz N, Castellsagué X, de González AB, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine. 2006 8/21/;24, Supplement 3(0):S1-S10.
52. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, Ghissassi FE, et al. A review of human carcinogens—Part B: biological agents. The lancet oncology. 2009 4//;10(4):321-2.
53. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. Journal of virology. 1989 Oct;63(10):4417-21. PubMed PMID: 2476573. Pubmed Central PMCID: PMC251060. Epub 1989/10/01. eng.
54. Melsheimer P, Vinokurova S, Wentzensen N, Bastert G, von Knebel Doeberitz M. DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004 May 1;10(9):3059-63. PubMed PMID: 15131043. Epub 2004/05/08. eng.
55. Roberts JN, Buck CB, Thompson CD, Kines R, Bernardo M, Choyke PL, et al. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. Nature medicine. 2007 Jul;13(7):857-61. PubMed PMID: 17603495. Epub 2007/07/03. eng.
56. Barrow-Laing L, Chen W, Roman A. Low- and high-risk human papillomavirus E7 proteins regulate p130 differently. Virology. 2010 5/10/;400(2):233-9.
57. Zhang B, Chen W, Roman A. The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. Proceedings of the National Academy of Sciences of the United States of

America. 2006 Jan 10;103(2):437-42. PubMed PMID: 16381817. Pubmed Central PMCID: PMC1326189. Epub 2005/12/31. eng.

58. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The Biology and Life-Cycle of Human Papillomaviruses. Vaccine. 2012 11/20/;30, Supplement 5(0):F55-F70.

59. Jabłonska S, Majewski S, Obalek S, Orth G. Cutaneous warts. Clinics in Dermatology. 1997 5//;15(3):309-19.

60. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. The EMBO journal. 1984 May;3(5):1151-7. PubMed PMID: 6329740. Pubmed Central PMCID: PMC557488. Epub 1984/05/01. eng.

61. Krzyzek RA, Watts SL, Anderson DL, Faras AJ, Pass F. Anogenital warts contain several distinct species of human papillomavirus. Journal of virology. 1980 Oct;36(1):236-44. PubMed PMID: 6255208. Pubmed Central PMCID: PMC353634. Epub 1980/10/01. eng.

62. Guan P, Howell-Jones R, Li N, Bruni L, de Sanjose S, Franceschi S, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. International journal of cancer Journal international du cancer. 2012 Nov 15;131(10):2349-59. PubMed PMID: 22323075. Epub 2012/02/11. eng.

63. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. International journal of cancer Journal international du cancer. 2009 Apr 1;124(7):1626-36. PubMed PMID: 19115209. Epub 2008/12/31. eng.

64. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. The New England journal of medicine. 2007 May 10;356(19):1944-56. PubMed PMID: 17494927. Epub 2007/05/15. eng.

65. Petrelli F, Sarti E, Barni S. Predictive value of human papillomavirus in oropharyngeal carcinoma treated with radiotherapy: An updated systematic review and meta-analysis of 30 trials. Head & neck. 2014 May;36(5):750-9. PubMed PMID: 23606404. Epub 2013/04/23. eng.

66. Isayeva T, Li Y, Maswahu D, Brandwein-Gensler M. Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. Head and neck pathology. 2012 Jul;6 Suppl 1:S104-20. PubMed PMID: 22782230. Pubmed Central PMCID: PMC3394168. Epub 2012/07/13. eng.
67. Yong F, Xudong N, Lijie T. Human papillomavirus types 16 and 18 in esophagus squamous cell carcinoma: a meta-analysis. Annals of epidemiology. 2013 Nov;23(11):726-34. PubMed PMID: 23916383. Epub 2013/08/07. eng.
68. Jayaprakash V, Reid M, Hatton E, Merzianu M, Rigual N, Marshall J, et al. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985-2010. Oral oncology. 2011 Nov;47(11):1048-54. PubMed PMID: 21816661. Pubmed Central PMCID: PMC3640331. Epub 2011/08/06. eng.
69. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009 Sep 17;461(7262):399-401. PubMed PMID: 19684573. Epub 2009/08/18. eng.
70. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009 Oct 8;461(7265):798-801. PubMed PMID: 19759533. Pubmed Central PMCID: PMC3172006. Epub 2009/09/18. eng.
71. Simons MJ, Wee GB, Day NE, Morris PJ, Shanmugaratnam K, De-The GB. Immunogenetic aspects of nasopharyngeal carcinoma: I. Differences in HL-A antigen profiles between patients and control groups. International journal of cancer Journal international du cancer. 1974 Jan 15;13(1):122-34. PubMed PMID: 4131857. Epub 1974/01/15. eng.
72. Lu CC, Chen JC, Tsai ST, Jin YT, Tsai JC, Chan SH, et al. Nasopharyngeal carcinoma-susceptibility locus is localized to a 132 kb segment containing HLA-A using high-resolution microsatellite mapping. International journal of cancer Journal international du cancer. 2005 Jul 10;115(5):742-6. PubMed PMID: 15729690. Epub 2005/02/25. eng.
73. Tse KP, Su WH, Chang KP, Tsang NM, Yu CJ, Tang P, et al. Genome-wide association study reveals multiple nasopharyngeal carcinoma-associated loci within the HLA region at chromosome 6p21.3. American journal of human genetics. 2009

Aug;85(2):194-203. PubMed PMID: 19664746. Pubmed Central PMCID: PMC2725267. Epub 2009/08/12. eng.

74. McAulay KA, Higgins CD, Macsween KF, Lake A, Jarrett RF, Robertson FL, et al. HLA class I polymorphisms are associated with development of infectious mononucleosis upon primary EBV infection. The Journal of clinical investigation. 2007 Oct;117(10):3042-8. PubMed PMID: 17909631. Pubmed Central PMCID: PMC1994627. Epub 2007/10/03. eng.

75. Urayama KY, Jarrett RF, Hjalgrim H, Diepstra A, Kamatani Y, Chabrier A, et al. Genome-wide association study of classical Hodgkin lymphoma and Epstein-Barr virus status-defined subgroups. Journal of the National Cancer Institute. 2012 Feb 8;104(3):240-53. PubMed PMID: 22286212. Pubmed Central PMCID: PMC3274508. Epub 2012/01/31. eng.

76. Swedish Cancer Registry [Internet]. [cited 01/04/2014]. Available from: http://www.socialstyrelsen.se/statistik/statistikdatabas/cancer.

77. Finnish Cancer Registry [Internet]. Available from: http://www.cancer.fi/syoparekisteri/en/.

78. Krabbameinsskrá Íslands [Internet]. [cited 15.03.2014]. Available from: http://www.krabbameinsskra.is.

79. Norwegian Cancer Registry [Internet]. [cited 15/03/2014]. Available from: http://www.kreftregisteret.no/.

80. Steinau M, Patel SS, Unger ER. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. The Journal of molecular diagnostics : JMD. 2011 Jul;13(4):377-81. PubMed PMID: 21704270. Pubmed Central PMCID: PMC3123789. Epub 2011/06/28. eng.

81. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. Bead-based multiplex genotyping of human papillomaviruses. Journal of clinical microbiology. 2006 Feb;44(2):504-12. PubMed PMID: 16455905. Pubmed Central PMCID: PMC1392679. Epub 2006/02/04. eng.

82. Schmitt M, Dondog B, Waterboer T, Pawlita M. Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. Journal of clinical microbiology. 2008 Mar;46(3):1050-9. PubMed PMID: 18199790. Pubmed Central PMCID: PMC2268381. Epub 2008/01/18. eng.

83. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, Ghissassi FE, et al. A review of human carcinogens-Part B: biological agents. The lancet oncology. 2009;10(4):321-2.
84. Diepstra A, Niens M, Vellenga E, van Imhoff GW, Nolte IM, Schaapveld M, et al. Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin's lymphoma. Lancet. 2005 Jun 25-Jul 1;365(9478):2216-24. PubMed PMID: 15978930. Epub 2005/06/28. eng.
85. Lin Z, Khong B, Kwok S, Cao H, West RB, Le QT, et al. Human papillomavirus 16 detected in nasopharyngeal carcinomas in white Americans but not in endemic Southern Chinese patients. Head & neck. 2013 Apr 25. PubMed PMID: 23616441. Epub 2013/04/26. Eng.
86. Singhi AD, Califano J, Westra WH. High-risk human papillomavirus in nasopharyngeal carcinoma. Head & neck. 2012 Feb;34(2):213-8. PubMed PMID: 21484924. Epub 2011/04/13. eng.
87. Stenmark MH, McHugh JB, Schipper M, Walline HM, Komarck C, Feng FY, et al. Nonendemic HPV-Positive Nasopharyngeal Carcinoma: Association With Poor Prognosis. International journal of radiation oncology, biology, physics. 2014 Mar 1;88(3):580-8. PubMed PMID: 24521676. Epub 2014/02/14. eng.

Appendix

Supplementary table 1

Sample ID	ng/ul	260/280	260/230	Date extracted
CASKI POS 6/3	1.67	3.35	0.23	6.3.14
CASKI POS 7/3	1.67	1.71	0.17	7.3.14
NEG 6/3	2.32	1.91	0.23	6.3.14
NEG 7/3	5.02	1.34	0.31	7.3.14
NP1	11.46	1.92	1.14	6.3.14
NP2	9.57	1.86	0.84	6.3.14
NP3	37.61	1.86	1.70	6.3.14
NP4	10.73	1.79	0.81	6.3.14
NP5	14.30	2.52	1.01	6.3.14
NP6	1.25	2.44	0.22	6.3.14
NP7	17.18	1.86	1.03	6.3.14
NP8	72.96	1.94	1.83	6.3.14
NP9	81.25	1.95	2.09	6.3.14
NP10	25.56	1.98	1.63	6.3.14
NP11	48.78	2.12	1.97	6.3.14
NP12	42.06	2.10	1.51	6.3.14
NP14	11.25	2.11	0.70	7.3.14
NP15	229.45	2.02	2.12	7.3.14
NP16	3.84	1.31	0.65	7.3.14
NP17	4.41	3.80	0.46	7.3.14
NP18	1.29	1.03	0.32	7.3.14
NP19	33.58	1.88	1.85	7.3.14
NP20	103.92	1.99	2.04	7.3.14
NP21	1.60	0.76	0.29	7.3.14
NP22	14.04	1.71	0.72	7.3.14
NP23	44.73	2.05	2.02	7.3.14
NP24	3.12	2.18	0.13	7.3.14
NP25	84.20	2.00	2.26	7.3.14
NP26	12.54	1.98	1.29	7.3.14
NP27	13.03	1.66	1.40	7.3.14
NP28	2.34	2.83	0.32	7.3.14
NP29	92.27	2.02	2.02	7.3.14
NP30	2.24	2.07	0.20	7.3.14
NP31	30.29	1.91	1.59	7.3.14
NP32	29.23	1.66	0.45	7.3.14
NP33	15.97	1.91	1.40	7.3.14