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Inverted Papilloma and Sinonasal Malignancies in Sweden

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Inverted Papilloma and Sinonasal Malignancies in Sweden

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Stockholm 2018

To my grandmother Ninni

I'm glad I did it, partly because it was worth it but mostly because I shall never have to do it again.
Mark Twain

ABSTRACT

Background: Sinonasal malignancies (SNMs) are rare and in Sweden they account for approximately 0,1% of all malignancies and 5% of all head and neck malignancies. Apart from sinonasal malignant melanoma (SNMM), their incidence has been reported to decrease since 1960 while the survival rates have remained rather stable during the same time-period.

Sinonasal inverted papilloma (IP) is a benign tumour with a high risk of local recurrence and a potential to malignify. The true incidence of IP is not yet known. From hospital based studies, its incidence has been estimated to approximately 0.5/100000 person years. Previous studies have reported a malignant transformation of IP in 1–53%. However, it is unknown to what extent IPs are associated with squamous cell carcinoma (SCC) on a population basis.

Also, the aetiology and prognostic factors for IP are mainly unknown. However, human papillomavirus (HPV) has previously been suggested as an aetiological factor by some authors. Moreover, p16INK4a (p16) overexpression, is often considered as a surrogate marker for high risk HPV in oropharyngeal carcinomas, but whether there is a correlation between p16 and HPV in IP and/or the prognosis of IP is uncertain. Similarly, a prognostic role of tumour infiltrating lymphocytes (TILs) has been observed in many head and neck tumours. However, their role in IP is sparsely investigated. Prognostic research on cell cycle related proteins such as oncoprotein 18, also called Stathmin, and epidermal growth factor receptor (EGFR) is relatively new regarding IP.

In summary, IP is a rare tumour and relevant knowledge regarding factors affecting recurrence, malignant transformation and prognosis is still limited.

Aims: The overall aim of this thesis was to investigate possible prognostic factors in IP, more specifically HPV, infiltration of TILs and expression of stathmin and EGFR. The aim was also to present epidemiological data on IP on a population basis and to describe how incidence and survival have changed for SNMs in the Swedish population.

Results: In total 3221 patients diagnosed with primary SNMs were identified in the Swedish Cancer Registry (SCR) from 1960-2010. Their incidence decreased during the study-period except for SNMM and adenoid cystic cancer. More than 50 % of the malignancies involved the nasal cavity. The five-year relative survival was highest for adenoid cystic cancer followed by adenocarcinoma. SNMM and undifferentiated carcinoma had the poorest prognosis.

In the SCR 814 patients with IP were identified. The incidence of IP increased from 1960 to 2010. Patients with IP had an overrepresentation of SCC when compared with the general population although this proportion was lower than previously reported.

Prognostic factors were analysed in tumours from 98 patients diagnosed with IP in Stockholm between 2000-2010. In total, 12.2% of the IPs were HPV positive and p16 overexpression was found in the only high-risk HPV positive tumour. Patients with HPV positive lesions were younger and tended to present with more dysplasia and to relapse less frequently. The tumours also had a higher proportion of EGFR expression compared to HPV negative tumours (91.7% and 52.3%, respectively). Stathmin was expressed by the tumours cells and not at all or weakly in the normal mucosa and more specimens with dysplasia were stathmin-positive than specimens without dysplasia (40.0% as compared to 12.6%). Stathmin positive IPs also tended to have earlier recurrences, although this difference was not statistically significant. No correlation was observed between TILs or EGFR and prognosis.

Conclusions: While the overall incidence of SNM showed a slight decrease, the incidence of IP has increased and SCC is less common among patients with IP than previously reported. The results suggest that patients with HPV positive and HPV negative IPs may have different clinical characteristics, possibly indicating two different disease entities. Stathmin is consistently expressed in inverted papilloma but not in the normal mucosa and stathmin positivity seems to be associated with dysplasia and possibly also with recurrence. Stathmin might therefore even more than EGFR be considered a future therapeutic target.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers which will be referred to in text by their numerals (I-IV).

I. Alexandra Elliot, Mattias Jangard, Linda Marklund, Niclas Håkansson, Paul Dickman, Lalle Hammarstedt-Nordenvall, Pär Stjärne
Sinonasal malignancies in Sweden 1960-2010; a nationwide study of the Swedish population.
Rhinology 53: 75-80, 2015

II. Alexandra Elliot, Linda Marklund, Niclas Håkansson, Huan Song, Weimin Ye, Pär Stjärne, Lalle Hammarstedt-Nordenvall
Incidence of IP and risk of malignant transformation in the Swedish population 1960–2010.
Eur Arch Otorhinolaryngol (2017) 274:1445–1448

III. Alexandra Elliot, Anders Näsman, Marit Westman, Linda Marklund, Pär Stjärne, Lalle Hammarstedt-Nordenvall
Human papillomavirus and infiltration of CD8 and Foxp3 positive immune cells in sinonasal inverted papillomas.
Submitted for publication

IV. Alexandra Elliot, Anders Näsman, Marit Westman, Lalle Hammarstedt-Nordenvall, Pär Stjärne, Linda Marklund
Stathmin and epidermal growth factor receptor (EGFR) expression in sinonasal inverted papillomas (IP) and its correlation to human papillomavirus (HPV) status and clinical outcome.
Manuscript

LIST OF ABBREVIATIONS

CD	cluster of differentiation
DNA	deoxyribonucleic acid
E	early region
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	high risk
IHC	immunohistochemistry
IP	inverted papilloma
ISH	in situ hybridization
OPSCC	oropharyngeal squamous cell carcinoma
PCR	polymerase chain reaction
RT	radiotherapy
SCC	squamous cell carcinoma
SCR	Swedish Cancer Registry
SIR	standardised incidence ratio
SNM	sinonasal malignancy
SNMM	sinonasal malignant melanoma
TGF	tumour growth factor
TIL	tumour infiltrating lymphocyte
Treg	regulatory T cell

CONTENTS

INTRODUCTION

SINONASAL MALIGNANCIES

Epidemiology

Malignancies evolving from the sinonasal mucosa covering the nasal cavity and the paranasal sinuses are rare and the incidence has been estimated to less than 1/100000 [1, 2]. They account for approximately 0,1% of all malignancies and 5% of head and neck malignancies in Sweden [3]. The mean age at diagnosis is over sixty years, but varies among the different histological types [1, 4].
Some studies have shown that apart from sinonasal malignant melanomas (SNMM), sinonasal malignancies (SNM) are more common among men but that the incidence among men is decreasing and closing in on the female incidence [5-7].
Whether incidence or frequency of the different tumour types has changed over time in the Swedish population has not previously been investigated.

Aetiology

SNMs consist of different histological types and the most common is squamous cell carcinoma (SCC). Smoking, alcohol, and formaldehyde seem to be the main aetiological factors associated with sinonasal SCC while adenocarcinoma foremost has been associated with exposure to dust from hard wood and leather [8-14]. It has also been suggested that sinonasal malignancies are associated to heavy air pollution[15].
Ebstein Barr virus (EBV) and human papillomavirus (HPV) have been studied as aetiological factors for different histological types of SNM but results have been diverging and inconclusive [16-20].

Diagnostic features

Patients with malignancies in the nasal cavity and paranasal sinuses tend to present at a rather advanced stage. The symptoms are heterogeneous and non-specific ranging from unilateral nasal blockage and epistaxis to septal perforations, ulcers, pain, facial swelling or epiphora [21].Not seldom has there been a “doctors delay” and it is important to keep in mind that unilateral symptoms and unilateral polyps must be considered malignant until investigation proves otherwise.

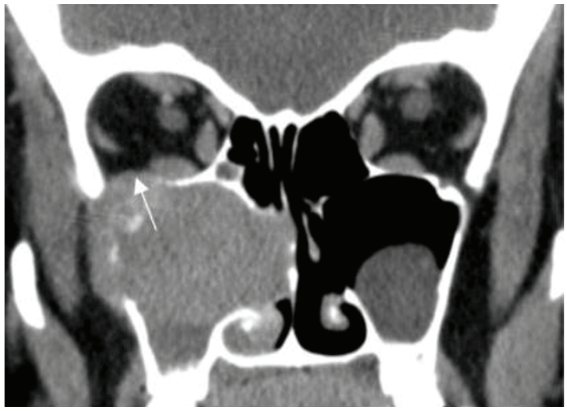

Figure 1a: *DT scan of an advanced SCC with orbital engagement.*

Figure 1b: *MR picture of the same tumour as figure 1a.*

SNMs are diagnosed by histopathological analysis of tumour biopsies. However, the diagnosis is difficult and there is a risk of misclassification [7]. Local spread and metastasis is assessed by imaging, both by computed tomography (CT) and magnetic resonance imaging (MRI).

Histological classification

The majority of tumours found in the nose and adjacent sinuses are derived from the mucosa. Some tumours such as SCC, adenocarcinoma, undifferentiated carcinoma and transitional cell carcinoma derive from the epithelial cells while other tumours derive from cells and glands found within the mucosa, for example adenoid cystic carcinoma, mucoepidermoid carcinoma and SNMM. SCC is the most common SNM, followed by adenocarcinoma and SNMM [1, 22, 23]. The proportion of the different tumour types and how their distribution has changed over time in the Swedish population is unknown.

Treatment

In general, the sinonasal malignancies require a combined treatment with both radical surgery (when possible) and radiotherapy (RT) or, more seldom, one of the treatment modalities. There is a consensus that the treatment of choice for most sinonasal cancers is, whenever required and possible, complete surgical resection followed by postoperative radiotherapy with or without chemotherapy [24]. Treatment with both surgery and RT is challenging due to the short distance to the brain and brainstem, the eye, vital vessels and cranial nerves and both are associated with serious complications [25]. Because of the rarity of the disease, the many histological types and different tumour extension it has been difficult to develop treatment guidelines. However, an international position paper has made recommendations of how to manage the different sinonasal malignancies [26]. Individual treatment is tailored at multi-disciplinary conferences based on these recommendations.

Prognosis

According to an international comparison study, mortality rates between 2004 and 2008 varied from 1.3 per million in Sweden to 3.3 per million in Denmark [2].
There is a tendency towards improved survival for SNM over time [1, 2]. Patients with SCC often present with a locally advanced disease and 2-5% have nodal involvement, both contributing to a poor prognosis [24, 27]. The improvement of prognosis may possibly be related to the shift of location where over time more tumours are diagnosed in the nasal cavity, as compared to the paranasal sinuses [2, 4].
Prognosis for different histological types of SNM other than the most common, SCC, adenocarcinoma and SNMM have not been reported on a population-basis [2, 4, 7]. Women tend to have a higher overall survival than men, with a male to female age-standardised mortality rate ratio of approximately 2.0 [2].
Among the tumour infiltrating lymphocytes (TILs), T regulatory cells (T-regs) have been studied as prognostic factors but the reports are few and no conclusions can be drawn [28, 29]. An association between prognosis of SNM and epidermal growth factor (EGFR), among other markers, has been reported [30, 31].

SINONASAL PAPILLOMA

Sinonasal papilloma arise from the ectodermal derived mucosa of the nasal cavity and the paranasal sinuses, also sometimes referred to as the" Schneiderian Membrane" [32].
Histologically these are divided into three different groups: exophytic papilloma, inverted papilloma and oncocytic papilloma.[33]

Exophytic papilloma

Exophytic papilloma, also called everted or fungiform papilloma, are the most common sinonasal papilloma and account for approximately 50% of sinonasal papilloma. They often appear in the third to fifth decade of life and there is a male predominance. An association with HPV in over 80% of the papilloma has been reported in many studies [34-36]. They usually originate from the septal mucosa [37]. The exophytic papilloma have a propensity for recurrence but have not been reported to undergo any malignant transformation. Histologically they typically consist of broad, exophytic branches of thickened well-differentiated stratified squamous epithelium surrounding a fibro-vascular core. Mucus cells or cysts and microabscesses can be seen throughout the epithelium [37].

Inverted Papilloma

Inverted papilloma (IP) is also called transitional cell papilloma, inverting papilloma and Schneiderian papilloma although all papilloma can be referred to as Schneiderian. IP represent less than 50% of the sinonasal papilloma. IP is a benign neoplasm originating from the sinonasal mucosa with an extensive growth-pattern, a risk of recurrence and an association with malignant transformation as will be described.

Oncocytic papilloma

Oncocytic papilloma, also called cylindrical cell papilloma, have enlarged epithelial cells that possess abundant mitochondria resulting in a granular eosinophilic cytoplasm[33]. They are rare and account for approximately 5% of sinonasal papilloma. They usually originate from the lateral nasal wall. There is no difference in incidence due to gender and they often appear in the 5th-6th decade of life. Oncocytic papilloma can be associated with SCC or mucoepidermoid cancer [38]. They exhibit both exophytic and endophytic patterns with several layers of pseudostratified columnar cells containing uniform small dark round nuclei and eosinophilic cytoplasm. Intraepithelial mucinous cysts and microabscesses can be detected [37].

INVERTED PAPILLOMA

Incidence and epidemiology

The incidence of IP varies in different studies, most of which are not population-based, but has been estimated to approximately 0,5-1.5/100000 person-years (py) [39, 40]. According to the number of referrals of patients with IP, the incidence seems to have increased [41]. The age of onset varies widely, from adolescence to over 90 years, but the highest incidence is during the fifth-sixth decades of life with a reported male to female ratio of 2:1 to 5:1 [42, 43].

Aetiology

Different aetiological factors for inverted papilloma have been proposed, such as smoking, outdoor and industrial occupations and chronic rhinosinusitis [33, 44, 45]. A viral association of EBV virus has also been presented[46]. HPV has been proposed as an aetiological factor although the results and interpretations of its involvement in the development of IP are very diverse ranging from no association to a high association [47-49]. Thus, there is presently no consensus regarding the association of IP with HPV or whether HPV is an aetiological factor for IP or not.

Diagnosis

Diagnosis is made by clinical examination with endoscopy, biopsy for histological analysis and imaging for evaluation of tumour extension [26].

Symptoms and clinical findings

Patients with IP have unspecific symptoms, most often unilateral nasal obstruction, less commonly a unilateral nasal mass, epistaxis, sinusitis, anosmia, rhinorrhoea or pain [37, 43]. If the tumour engages the sphenoid sinus patients can suffer from neurological or visual symptoms [50]. In rare cases IP has grown into the orbit or skull base where patients have presented with related symptoms such as diplopia and headache [51]. Some patients are asymptomatic and their tumours are found en passant. IP is best visualised endoscopically. The IP is often cerebriform, pink-greyish, firm, bulky and lacks translucency (Figure 2) [52]. This differentiates them from the sinonasal polyps. However, they may have a polypoid appearance and IP or malignancy should be suspected in all patients with uni-lateral polyposis. Polyps should therefore be sent for histology to avoid misdiagnosing of an IP or a malignancy [53, 54]. Because IP often is slow-growing, there is often a patient delay with an estimated mean duration of 3,9 years [55].

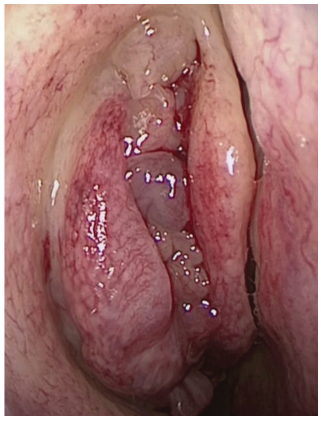

Figure 2: *Endoscopic view of IP.*

Imaging

Radiological imaging serves the purpose of determining the extension of the tumour and to identify the original site for the upcoming surgery. CT is the primary imaging modality and typically shows a unilateral homogenous mass extending from the middle meatus through a widening of the maxillary ostium to the adjacent maxillary sinus. Bone erosion can be found in IP but malignancy must be suspected, especially when there is considerable bone destruction. Hyperostosis is highly correlated to the site of origin of the tumour and calcifications are common and can be seen in approximately 20% of cases [56-58].

It can be difficult to determine tumour from inflamed mucosa or retained mucus why MRI is a helpful complement. On MRI, IP can appear as cerebriform circumvolutions, often with a columnar pattern, and remodelling of the bone can sometimes occur [59, 60].

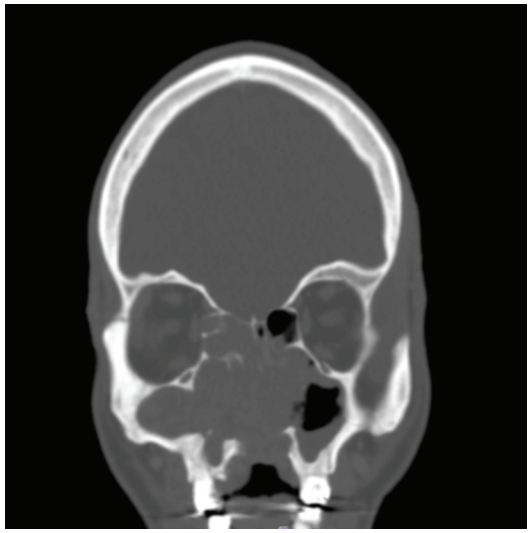

Figure 3: *Advanced recurrence of IP with destruction of the hard palate.*

Histology

A biopsy is always warranted for histological evaluation. In inverted papilloma, the epithelium inverts into the underlying stroma but with a preserved basement membrane. Histologically the invaginating sinonasal columnar epithelium undergoes squamous metaplasia and therefore it can be variably squamous, squamoid, transitional or ciliated columnar. The thickened epithelium has a prominent down-growth of elongated, rounded epithelial masses sometimes exhibiting an elephant foot appearance (figure 4) [61].The desmosomes (intercellular bridges) are preserved. Some IPs have mitotic figures but never atypical or numerous. Microscopic cysts are seen throughout the neoplastic epithelium containing cell debris, macrophages and mucin [32]. Neutrophil infiltration is seen in a majority of cases along with microabscesses. Areas of mild to severe dysplasia can occur, with cells characterised by anisocytosis, poikilocytosis, hyperchromatism and presence of mitotic figures [37, 62].

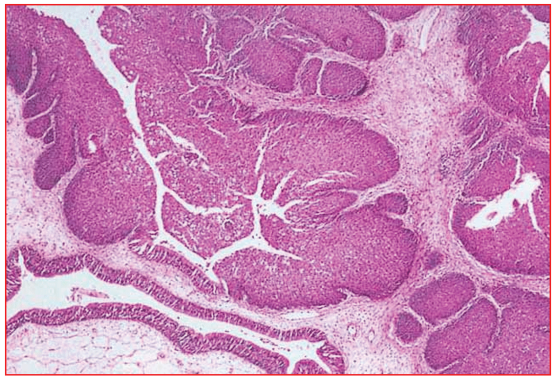

Figure 4: *Histological appearance of IP.*

Site of origin

IPs most often have their site of origin on the lateral wall of the nasal fossa, the maxillary sinus and the ethmoid region. The frontal and sphenoid sinuses are less commonly seen as site of origin [63]. Not rarely does IP extend into adjacent sinuses. It has also been reported that IPs extend into the orbit. However, this should give rise to suspicion of malignancy as should a bilateral manifestation of the tumour whereas IP most seldom is bilateral [64, 65].

Staging

In the three major staging systems, Krouse, Han and Cannady, the stage is defined according to the location and the extent of the tumour and Krouse also includes associated malignancy (Figure 5 a- c) [66-68]. Krouse is the most commonly used staging system and tumour stage is believed to correlate with outcome [69]. However, a new study suggests that Han and Cannady better predict recurrences [70].

Krouse Staging system	
T1	IP is confined to the nasal cavity.
T2	IP involvement of the ostiomeatal complex, ethmoid sinus, and/or medial maxillary sinus; with or without involvement of the nasal cavity.
T3	IP involvement of lateral, inferior, superior, anterior, or posterior walls of the maxillary sinus, the sphenoid sinus, and/or the frontal sinus.
T4	IP involvement beyond the sinonasal cavities or associated with malignancy.

Figure 5a: *Krouse staging system*[66]

Cannady Staging system	
Group A	IP is confined to the nasal cavity, ethmoid sinuses and/or medial maxillary wall.
Group B	IP involvement of any maxillary wall (besides the medial wall), frontal sinus or sphenoid sinus.
Group C	IP involvement beyond the sinonasal cavities.

Figure 5b: *Cannady staging system*[67]

Han Staging system	
Group I	IP is confined to the nasal cavity, lateral nasal wall, medial maxillary sinus, ethmoid sinus and/or sphenoid sinus.
Group II	IP involvement of lateral to medial maxillary wall.
Group III	IP involvement of the frontal sinus.
Group IV	IP involvement beyond the sinonasal cavities.

Figure 5c: *Han staging system*[68]

Treatment

IP is treated by surgery. During surgery, it is important to resect the entire tumour with adjacent normal mucosa, to drill underlying periosteum and hyperostotic bone at the attachment site of the tumour and to remove all affected bone to minimise the risk of recurrence [71].

Previously, IP was considered best treated with an open en bloc resection. Since the 1980s the endonasal endoscopical approach has evolved and is now advocated for the majority of cases because of better visibility, good access and less morbidity than with an open approach without affecting the recurrence rate negatively. Sometimes it is combined with an external approach, depending on the location and extent of the tumour. Most authors agree that tumours limited to the middle meatus, ethmoids, middle maxillary wall, sphenoid, frontonasal recess, medial frontal sinuses and skull base can be managed endoscopically [69, 72]. The resection should also permit a good endoscopic view of the tumour area for future follow-up. Radiotherapy has no place in the treatment of IP except for rare, difficult or recurrent cases [73].

Recurrence

IP has a high propensity for recurrence, often estimated to approximately 15-30%, but the recurrence rate varies largely in different studies [43, 74, 75]. However, even though factors such as location, multicentricity and extent of tumour growth affect the results, recurrences are much related to the surgical treatment and to what extent the IP is radically resected [76]. Most recurrences occur within the first two to three years at the original site [77]. In a meta-analysis from 2006, contemporary recurrences after endoscopic resection was 12% vs 20% for non-endoscopically treated IPs [78].

Because of the relatively high recurrence rates in IP, patients should be followed for at least three years [26, 65]. Previous recurrences, risk of inadequate resection or signs of aggressive behaviour of the tumour should impel physicians to prolong the follow-up time.

Malignant Transformation

An association between IP and SCC, mainly well-differentiated SCC, has been identified[79]. It can sometimes be difficult to separate IP from cancer. Histological signs of malignant transformation are increased hyperkeratosis, squamous hyperplasia, increased nuclear to cytoplasmic ratio, atypical mitosis, high mitotic index, and especially loss of polarity and invasion through the basement membrane [80, 81]. Radiologically, significant bone erosion or bone destruction and focal loss of cerebriform pattern are malignant signs [82].
Different studies show diverging results regarding the proportion of IP that malignifies but most articles report a malignant transformation of approximately 5-10 % [83, 84]. SCC is found both synchronously and metachronously (diagnosed in the same area as the IP but later). In a review from 2010, 6,8% of patients with IP had synchronous carcinomas while 3% developed metachronous carcinomas [26].
Many factors have been studied for their role in the pathogenesis of the malignant transformation of IP [85]. HPV is among the most studied but the results are diverging and whether HPV has an active role in the malignant transformation of IP or not is still unclear [86]. Other markers such as over-expression of oncoproteins, p16, p53, EGFR have been proposed as causative factors although their roles as such remain uncertain [86-88].

HPV

Background

There are over 200 different types of HPV which all belong to the papillomaviridae family. The papillomaviridae family includes 16 different genera where the human papillomaviruses belong to five of the genera (alpha, beta, gamma, mu and nu). Among these the beta genus contains most HPV types associated with cutaneous tumours while the alpha genus contains the viruses mainly associated with the development of mucosal tumours. The alpha HPV can be divided into low risk (LR) and high risk (HR) depending on their ability to induce malignancies. LR HPV can give rise to benign conditions such as anogenital warts and respiratory papillomatosis (HPV6 and HPV11) and common skin warts (HPV1 and HPV2) [89].
Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are listed as HR by the International Agency for research on Cancer (IARC)/ World Health Organization (WHO) for their causative role in the development of especially cervical cancer, anogenital cancer and oropharyngeal cancer [90, 91].

Viral structure

HPV is a small (55nm in diameter) non-enveloped, capsid-encased, double-stranded DNA virus with a genome of approximately 8000 base pairs. The genome consists of an early region (E), that codes for E1-2 and E4-7 proteins, required for viral gene expression, replication and survival, a late region (L) coding for the capsid proteins L1 and L2, and a long control region (LCR), a non-coding region which regulates the viral gene expression and replication.

HPV and carcinogenesis

HPV is transmitted from skin-to-skin or mucosa-to-mucosa. In cervical infections, it is transmitted sexually but in oropharyngeal infections the route of transmission is not as clear although sexual contact has been suggested [92]. The transmission route to the sinonasal mucosa is sparsely studied. HPV infects the mucosal epithelial cells in the basal layer. This mechanism is not fully understood but possibly by entry through micro traumatised epithelium [93]. Often, HPV-infection heals by interference of the human immune response but in a minority of cases the infection results in an asymptomatic carrier state which later may lead to dysplasia and malignancy. The replication of the HPV virus takes place in the host nucleus, dependant on S-phase entry [94]. The integration of the viral genome leads to expression of its three oncoproteins; E5, E6 and E7. These oncoproteins play a key role in the malignant transformation.

- The mode of action for E5 is still unclear, but in vitro studies have showed that E5 may increase EGFR levels as well as modulate the EGFR signalling pathway. E5 can thereby exhibit a weak transforming activity [95]. The E5 coding sequence is often deleted from the viral DNA during integration into the host genome. Therefore, E5 may not be required for the late stage of carcinogenesis in the significant way that are E6 and E7 [96].

- E6 cooperates with E7 and affects the cell apoptosis process thereby promoting viral DNA replication. The most well characterized target of E6 from high-risk HPV types is the tumour-suppressor protein p53. P53 usually acts by arresting cells in G1 to allow host DNA to be repaired or else to undergo apoptosis. E6 protein forms a complex with an E3 ubiquitin ligase and ubiquinates (degrades) p53. Cells expressing E6 thereby lose their p53 checkpoint function and are therefore susceptible to genomic instability [96].

- E7 binds to the cullin 2 ubiquitin ligase complex and ubiquitinates the retinoblastoma tumour suppressor protein (pRb). This induces degradation of pRb resulting in uncontrolled G1/S phase of the cell cycle. The absence of the pRb function induces a release of transcription factors and transcription of S-phase genes occurs, leading to cell proliferation. In summary, in a HPV infected cell, pRb is inactivated by E7 and consequently S-phase is induced [96]. The inactivation of pRb results in an overexpression of p16INK4a (p16) since pRb normally inhibits transcription of p16. Upregulation of p16 can also be mediated by E7 independently from pRb inactivation.

HPV and p16

HR HPV positive malignant tumours, especially cervical, oropharyngeal and anogenital cancers, are often p16 positive [97, 98]. Hence p16 is frequently used as a surrogate marker for HPV in these tumours [99]. However, its function as a surrogate marker in OPSCC is under debate [100, 101]. Furthermore, its role as surrogate marker in IP has not been assessed.

HPV and immune response

When infected by a virus, keratinocytes start secreting pro-inflammatory cytokines. This attracts antigen-presenting cells such as dendritic cells (DC) and macrophages. DCs also engulf viral products. This promotes DC activation. Mature DCs migrate to the lymph nodes where they present the foreign HPV proteins, degraded into peptides, on the major histocompability complex (MHC, in humans also referred to as human leukocyte antigen-HLA). Upon presentation, DCs also bind to cytotoxic T cells or helper T cells and interact with and activate their respective CD8+ or CD4+ receptors. CD4+ cells mature into Th1 or Th2 cells where the Th1 cells help promote maturation of CD8+ cells and activate macrophages and NK cells [102].

It has been suggested that the T cell response, comprising both CD4+ and CD8+ cells, are required to kill HPV infected cells. An immune response with infiltration of TILs in HPV-positive OPSCC has been correlated with clinical outcome and it would be beneficial to determine if the same relation is valid for IP [103, 104].

HPV detection methods

There are three methods mainly used for detection of HPV; polymerase chain reaction (PCR), in situ hybridisation (ISH) and detection of p16 by immunohistochemistry (an indirect method). Detection of viral proteins and serum anti bodies against viral proteins can also be used.

- *Polymerase chain reaction (PCR):* PCR is often done with a consensus primer, also called, general primer, that's to say a primer that can detect different HPV types by binding to highly conserved regions within the viral genome. Among these, broad spectrum Gp5+/6+ is often used because of its high sensitivity [105]. After DNA amplification, commonly a probe-based technique is used to type the PCR product (eg Luminex) [106].

- *In situ hybridisation (ISH):* In ISH, a biotinylated probe is hybridised to the viral DNA, after which the signal is amplified. The presence and localisation of the virus is thereafter visualised under a light microscope [107].

- *detection of p16 by immunohistochemistry (IHC):* p16 is most often upregulated in HPV infected cells and often lost in HPV negative tumours [107]. It is therefore used as surrogate marker. IHC is an antibody-based method to identify proteins in tissues and cells. The indirect method where antibody-protein conjunction is detected with an enzyme-labelled polymer conjugated secondary antibody, has a better sensitivity than the direct method where a labelled antibody reacts directly with the antigen. After incubation with a substrate, a positive immunoreactivity occurs. If 3,3-diamino-benzidine (DAB) is used as substrate, a brown colour can be seen. To provide contrast to the sections and to visualize the nuclei, the slides are usually counter stained with haematoxylin. To rule out unspecific background staining, negative controls for mouse and/or rabbit primary antibodies are used. To summarise, a solution with fluorescence marked antibodies are added to tumour tissue and thereafter visualised by microscopy. The value of p16 as a surrogate marker in HPV associated malignancies is as mentioned under debate. Since approximately 14% of HPV negative oropharyngeal tumours have upregulated p16, the use of p16 as surrogate marker for HPV in OPSCC should not be advocated [100].

HPV in sinonasal SCC and IP

Some reports have found an association between HPV and SCC and suggest that HPV is a positive prognostic factor [91, 108]. It has also been suggested that HPV is associated with the prognosis of IP and that HPV positive IP has an increased risk of recurrence and malignant transformation [109, 110]. Although, for IP, as opposed to malignant tumours, LR HPV positivity has mainly been reported [111].

TUMOUR INFILTRATING LYMPHOCYTES (TILs)

Tumour infiltrating lymphocytes are B cells and T cells who have left the bloodstream and infiltrate the tumour or its stroma. They have, for different malignancies such as breast cancer, colon cancer, malignant melanomas and head and neck cancers, been associated with tumour prognosis and clinical outcome [112, 113].

CD8 positive T cells

CD8 is a protein expressed by, and thus a marker for, cytotoxic T cells. These are lymphocytes which can kill endogenous cells, with help from the CD4+ T regulatory cells, by induction of apoptosis or by cell lysis. Cytotoxic T cells have the ability to destroy virus-infected cells and tumour cells. Cells infected by viruses synthesize viral proteins presented on its MHC-1 molecule, to which the T cell can bind.

It seems that a high level of CD4+ and CD8+ TILs improves overall survival in head and neck squamous cell carcinoma (HNSCC) [114]. This phenomenon is seen in many solid tumours. It has further been shown that the morphological pattern of TILs is important and that it differs between HPV positive and HPV negative cancers [115, 116].

Forkhead box protein 3 positive T cells

Forkhead box protein 3 (Foxp3) is expressed by a subgroup of the CD4+ T helper cells called T regulatory cells (T-regs). They control and regulate the specific immune response, inhibit the activation of T helper cells and suppress the antigen presenting cells so that the T helper cells are deprived the signals promoting mitosis and maturation. Instead tolerance towards the antigen appears. Studies indicate that in viral infections, the protective function of CD8+ T cells is compromised by the presence of Foxp3+ cells [117].

According to some reports, tumour infiltration of Foxp3+ TILs improves survival while others have found that a high CD8+/Foxp3+ ratio is beneficial [103, 114, 118].

CD8 and Foxp3 in IP

CD8+ and Foxp3+ TILs might be prognostic factors in head and neck malignancies. Although seemingly frequent in IP and in associated SCC they are sparsely studied in those tumours.

BIOMARKERS

Biomarkers are defined by the National Cancer Institute (NCI) as a biological molecule found in tissue, blood or body fluids that is a sign of a normal process, a condition or a disease [119]. They can be used to measure level of disease or effect of treatment and some markers, like EGFR and stathmin, are proteins related to the cell-cycle.

Epidermal growth factor receptor

The EGFR is a trans-membranous tyrosine kinase cell surface receptor belonging to the erbB family. EGFR is activated when its ligands, among them epidermal growth factor (EGF) and tumour growth factor-α (TGFα), bind to its extracellular parts leading to its dimerization. This stimulates phosphorylation of tyrosine kinase which in turn activates numerous signalling pathways leading to a modulation of functions such as DNA synthesis, cell migration, cell proliferation, angiogenesis and apoptosis [120].

EGFR and tumour prognosis
Overexpression and mutation of EGFR is associated with a wide variety of tumours and a poorer prognosis [121]. Interruption of EGFR signalling, either by blocking EGFR binding sites on the extracellular domain of the receptor or by inhibiting intracellular tyrosine kinase activity, can prevent the growth of EGFR-expressing tumours and improve the condition in head and neck cancer patients [122].

Stathmin

Stathmin is an oncoprotein (oncoprotein 18) important in the regulation of the cell cycle. During the M-phase of the cell cycle, the mitotic spindle, composed of microtubules built up by alfa- and beta- tubulin, is responsible for aligning the cell chromosomes, segregating the cell chromatids to form two identical sister cells. Polymerisation and depolymerisation of microtubules respectively causes it to grow and disintegrate. Stathmin, when binding to tubulin, inhibits polymerisation and microtubule assembly. When stathmin is phosphorylated the level of tubulin in the cytoplasm increases and this promotes microtubule assembly [123].

Stathmin and tumour prognosis
When mutated or abnormally activated, stathmin can cause transformation of normal cells to cancerous cells by uncontrolled proliferation. If stathmin is unable to bind to tubulin, it allows constant microtubule assembly and therefore constant mitotic spindle assembly. With a deficient regulation of the mitotic spindle, the cell cycle is capable of cycling uncontrollably leading to unregulated cell growth characteristic of cancer cells.
Mutation and overexpression of stathmin has shown to be associated with numerous human cancers and their prognosis [123, 124].

EGFR and stathmin in IP

In the few studies investigating the association between EGFR and IP, it seems that overexpression or mutation of EGFR is associated with IP and of SCCs derived from IPs, as opposed to SCCs without IP [125-127]. The one study assessing the association between EGFR and HPV in IP found EGFR mutations and HPV to be mutually exclusive prognostic factors in IP [128]. Since stathmin is an oncoprotein and associated with prognosis of malignancies it is an interesting marker to study in IP. However, reports on the association between stathmin and IP are sparse. One study found stathmin to be a valuable prognostic marker [129]. Therefore, further investigations on stathmin and IP are warranted.

AIMS OF THE THESIS

The overall aim was to study the incidence trends of SNM and IP and also the malignant transformation and prognostic factors of IP on a population basis.

Specific aims:

Paper I: The aim of this study was to determine the incidence trends and survival trends for different histological subgroups of SNM in Sweden 1960-2010.

Paper II: In this study, the intent was to investigate the changes in incidence and the malignant transformation of IP on a population-basis, in Sweden 1960-2010.

Paper III: The purpose was to analyze the presence of HPV and the infiltration of CD8+ T cells and Foxp3+ T-regs in specimens of IP and to analyze their correlation to prognostic outcomes. The possibility of using p16 as a marker for high risk HPV in IP was also investigated.

Paper IV: This study aimed to assess if stathmin or EGFR correlate with HPV, dysplasia or recurrence of IP.

MATERIALS AND METHODS

SUBJECTS AND MATERIALS

All studies were conducted according to the ethical permission 2012/49-31/2 from the Regional Ethical Review Board at the Karolinska Institute, Stockholm, Sweden.

Paper I. Patients were identified from the Swedish Cancer Registry (SCR). All patients diagnosed with SNM between 1960-2010 with the codes 160.0, 160.2, 160.7, 160.8 and 160.9 according to the ICD-7 were included. The SCR has a coverage of approximately 96% of all cancers diagnosed in Sweden but the coverage was more uncertain during the first two years of its existence (1958-1959). Therefore, patients diagnosed from 1960 onwards were included [130, 131]. From the SCR, we also retrieved information on the patients gender, age at diagnosis and the histological type and localisation of the tumours.

Information on the patients identified in the SCR from the emigration, immigration and causes of death registers, from Statistics Sweden enabled identification of patients lost to follow-up and to perform survival analysis. For the survival analysis, patients were followed until the end of 2012. From Statistics Sweden (Statistical Yearbook of Sweden) information on annual population and gender distribution to calculate incidence rates was retrieved.

Paper II. From the SCR, patients with tumours diagnosed from 1960-2010 with the histology code for true papilloma, in the sinonasal area, numbered 160.0, 160.2, and 160.7–160.9 according to the ICD-7 were included and information on their gender distribution and age at diagnosis was collected. Data was also retrieved on patients with IP who were diagnosed with SCC in the sinonasal mucosa synchronously or later than the IP diagnosis. Information for analysis of loss to follow-up and information for calculations of incidence rates were obtained from the same registers as in paper I.

Paper III. Subjects were patients diagnosed with IP in Stockholm 2000-2010 identified from the SCR. The study base consisted of 126 patients. Formalin fixed, paraffin embedded (FFPE) blocks with specimens of IP from these patients were obtained from the Stockholm Medical Biobank (SMB). After histological re-evaluation of the original diagnosis by a qualified pathologist for the cases where blocks were not missing or slides not unusable, 98 cases out of 126 were obtained for further analysis.

Patient data including age at diagnosis, gender, recurrence data, follow-up time, malignant transformation and information on reported surgical margins was obtained from the medical records. Tumours where the surgeon reported uncertain radicality of the tumour resection or where the question of radicality was not mentioned were considered as having positive surgical margins. Data on dysplasia were obtained from the histopathological reports.

Paper IV: Subjects and their clinical data were the same as for paper III.

METHODOLOGY

HPV DNA detection by PCR

In paper III-IV, HPV DNA was extracted from 30µm sections (2x15µm) from FFPE IP tumour blocks. DNA was purified using the Roche High Pure FFPET DNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers instructions. Blank controls were included in parallel to detect cross-contamination.
The DNA detection method consists of a multiplex PCR followed by a Luminex-based assay for 27 HPV types, described below [105, 106].

For the PCR, broad-spectrum general primers, GP5+/6+ primers (BGP5+/BGP6+) were used. These bind to the well-conserved L1 region of the HPV genome. 10µl of extracted DNA from the FFPE material (tumour or blank control) was added to a 50µl reaction mixture containing biotinylated primers in the HPV genotyping kit and Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany). A denaturation step during 15 minutes at 94°C was followed by 40 cycles of amplification. These cycles each consisted of a denaturation step of 20 seconds at 94°C, an annealing step at 38°C during 90 seconds and an elongation step at 71°C during 80 seconds. The final elongation step of the last cycle lasted for 4 minutes [115]. DNA from SiHa cells were used as HPV-positive control and primers of the housekeeping gene ß-globin were used to confirm presence of amplifiable cellular DNA. RNAse free water was used as a negative control.

The amplified DNA was then analysed using a bead-based assay in a Magpix instrument (Luminex Corp). The assay covers 27 HPV types (HPV 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73 and 82) and ß-globin. To summarize, magnetic beads with 27 different colours were attached to probes specific for, in this case, DNA of the 27 HPV types. The PCR products were denaturised and hybridized to the bead-probe complex. Unhybridized DNA was washed away and the PCR products were stained with a fluorescent dye. After another washing, the complexes were analysed in the Luminex 100 analyzer (BioRad Laboratories, Hercules, USA) by two lasers. One laser evaluated the colour of the beads, hereby determining which HPV types were present. The second laser detected the fluorescent dye and semi-quantified the amount of DNA so that the result was expressed as median fluorescence intensity [106].

Immunohistochemistry

The IHC procedure consisted of 4µm FFPE sections which initially were de-paraffinised in xylene and rehydrated in ethanol followed by antigen-retrieval in citrate buffer in a microwave oven. Thereafter, endogenous peroxidase activity was extinguished with hydrogen peroxide and the slides were treated with horse serum to block unspecific binding sites. Then the slides were incubated with the primary antibody (see below) after which a biotinylated secondary anti-mouse antibody (anti-rabbit for EGFR) was applied followed by incubation with an avidin-biotin complex. The slides were then developed in DAB 3.3' diaminobenzidine (DAB) and counterstained using haematoxylin. Evaluations were done using light-microscopy with researchers blinded to clinical data and prognosis related to the tumours assessed. Discrepant opinions were solved by consensus.

p16

p16INK4a overexpression was analysed using the p16 antibody (clone E6H4, undiluted, CINtec®, Ventana CINtec® p16 Histology, Roche AB, Stockholm, Sweden). The fraction of positive cells and staining intensity were evaluated and a consensus was formed for each sample. p16 positivity was defined as a strong and diffuse cytoplasmic and nuclear staining in >70% of all cells of the lesion [132].

CD8+ and Foxp3+ infiltrating lymphocytes

Analyses of CD8 and Foxp3 positive immune cells were performed in a similar way using the mouse monoclonal antibodies anti-CD8 (dilution, 1:40; clone 4B11; Novocastra Laboratories) and anti-Foxp3 (dilution 1:100, clone 236A/E7; eBioscience) respectively [103]. CD8 and Foxp3 positive immune cells infiltrating the lesion were evaluated by counting positive cells in 10 randomly selected high-power fields (40×) per sample. The ratio of tumour infiltrating CD8+ and Foxp3+ cells was also calculated for every lesion.

EGFR

EGFR expression was analysed using the rabbit monoclonal antibody, anti- EGF Receptor XP® (clone: D38B1, dilution: 1:50, Cell Signaling, Massachusetts, USA) [101]. Positive EGFR staining was defined as membranous staining with strong intensity. Fraction of EGFR positive tumour cells in the slide (closest 10%) was evaluated.

Stathmin

Stathmin expression was analysed using anti-Stathmin 1 RabMab® (clone: EP1573Y, dilution: 1:250, Abcam, United Kingdom). Positive stathmin staining was defined as a strong cytoplasmic staining. Fraction of stathmin positive tumour cells in the slide (closest 10%) was evaluated.

STATISTICAL ANALYSIS

Paper I. The subjects included were divided into three different age groups; <55, 55-69, ≥70. For the incidence-rates, the calendar period was divided into five-year-periods. Incidence rates were calculated by dividing the number of cases by the total population in each age group for every five-year-period. The rates were adjusted both to the World Standard Population of 1966 and the Swedish Standard Population of year 2000.

Relative survival was further analysed, defined as the observed survival in the study- population divided by the expected survival of a group in the general population comparable regarding age. The relative survival thereby measures excess mortality as a result of cancer without relying on information on cause of death[133]. To estimate the expected survival, the Ederer II method was applied on data from the Swedish population life tables stratified by age, gender and calendar period [134]. Relative survival was analysed by decade and the three defined age-groups were used to estimate the age-standardized relative survival [135].To adjust for effects of age at diagnosis, year of diagnosis and gender on the excess mortality rate ratio a multivariable Poisson regression analysis was conducted.

Paper II. Because of the low incidence of IP, the incidence was reported per decade. The study period was divided into decades beginning in 1960. Incidence rates were calculated by dividing the number of cases in each calendar period by the total average population in each age group for each decade. The incidence rates were calculated for the population as well as sex specifically. For comparison, the rates were also age-adjusted to the Swedish Standard Population of year 2000.
The incidence of SCC among patients with IP was calculated as a proportion and a standardised incidence ratio (SIR). SIRs were calculated for SCC in patients diagnosed with IP by dividing the observed numbers of SCC in this group by the expected numbers of SCC based on person-years at risk and population incidence.

Paper III. For differences in categorical date we used the Pearson Chi-square and the Fisher exact test and for continuous data, the Mann Whitney U-test. Time to recurrence was measured from the date of diagnosis until a documented recurrence of IP, when the patients were considered a case. Patients lost to follow up or dead were censured. All patients were considered as tumour free after surgery, independent of reported surgical radicality. Patients without any follow-up were censored day 0. The Kaplan-Meier estimator was used to estimate survival and differences in survival was assessed using the log-rank test. Hazard ratios (HR) for recurrence were calculated by univariable and multivariable Cox regression. The multivariable model included age, gender, HPV and radicality as covariables.

Paper IV. Pearson Chi-square and the Fisher exact test was used for categorical data and the Mann Whitney U-test was used for data which did not have a normal distribution. Recurrence was assessed in the same way, by the Kapplan-Meier estimator and Cox regression analysis.

P-values of <0.05 were considered statistically significant in all studies.
For paper I and II, statistical analysis was performed in SAS 9.2, STATA (StataCorp, 4905 Lakeway Dr, College Station, TX 77845, USA), and Excel. In paper III and IV, SPSS (SPSS Statistics for Mac, Version 21.0. Armonk, NY: IBM Corp. USA) and STATA (StataCorp, 4905 Lakeway Dr, College Station, TX 77845, USA) were used.

RESULTS

PAPER I

Frequency of sinonasal malignancies

3 221 patients with non-lymphoid SNMs were included. The mean age at diagnosis occurred during the seventh decade of life for all histological subtypes except for SNMM which had the highest mean age at diagnosis (71.7 years).

The predominant histopathological type was SCC followed by adenocarcinoma and SNMM. There was a male predominance except for SNMM and adenoid cystic carcinoma, where the female gender was predominant (55% and 53%, respectively).

The two most common anatomical localisations for the tumours were the nasal cavity (49.9%) and the maxillary sinuses (31.4%). During the study period, the proportion of tumours originating from the nasal cavity compared to tumours from the maxillary sinuses increased. SNMM had a higher frequency of tumours located in the nasal cavity compared to SCC.

Incidence of sinonasal malignancies

The incidence rate of SNM decreased from 1.19 in the first decade of the study period to 0.86/100000 py in the last decade studied and the trend observed was similar for men and women. The incidence of SCC as well as undifferentiated carcinoma decreased whereas SNMM was the only sinonasal malignancy that increased in incidence (from 0.04 to 0.15/100000 py) during the study period. This resulted in a shift in distribution of histological types of SNM (Figure 6a and 6b).

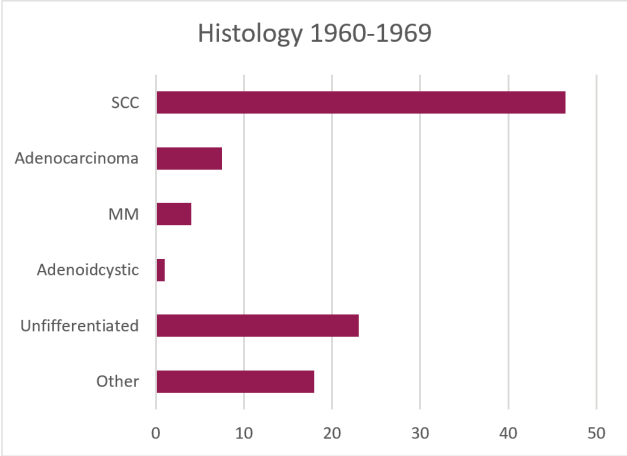

Figure 6a: *Proportion (%) of different histological types of sinonasal malignancies 1960-1969.*

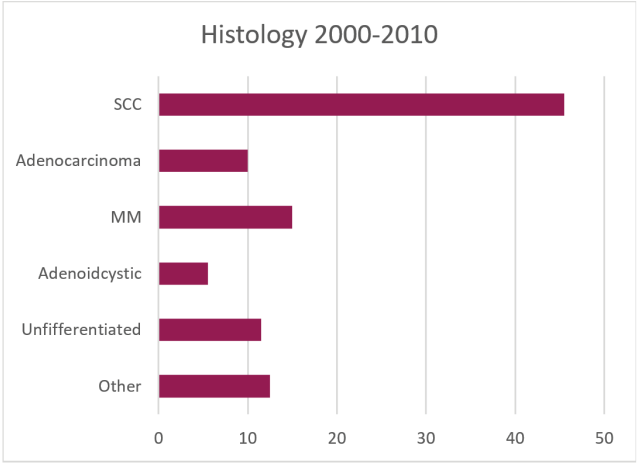

Figure 6b: *Proportion (%) of different histological types of sinonasal malignancies 2000-2010.*

Survival rates for sinonasal malignancies

The 5-year relative survival data was evaluated for all patients diagnosed from 1960 to 2010. 36 patients were lost to follow-up. The overall relative survival tended to improve during the study period. This trend was seen among all histological types except for sinonasal undifferentiated carcinomas.

The patients with adenocarcinoma and SCC had a 5-year relative survival of 56% and 46%, respectively. SNMM and undifferentiated carcinoma were associated with a poorer prognosis with 5-year relative survival of 27% and 37%, respectively. Patients with adenoid cystic cancer had the best 5-year relative survival (58%). Regarding survival in relation to localisation of the tumour, patients with tumours originating from the nasal cavity had a better prognosis than patients with tumours originating from the maxillary sinuses (mean overall survival time 84.2 versus 55.2 months; $p < 0.0001$).

PAPER II

The mean age at diagnosis for IP was 55.1 for the whole study population, 54.7 for males and 56.1 for females, and was stable over time. The male-to-female ratio was also rather constant over time from the seventies onwards (2.4–3.3:1). The incidence of IP increased over time from 0.01/100000 py in the sixties to 0.33/100000 py in the first decade of this century (figure 7). The same trend was observed for males and females.

In all, 814 persons were diagnosed with IP during the study period. Among them, 11 (1.35%) were diagnosed with SCC or SCC in situ. This corresponds to an incidence of SCC over the time period of 111/100000 py among patients with IP as compared to 0.37/100000 py in the general population. Only in males did IPs undergo a malignant transformation, which was statistically significant. For men with IP, this represented a proportion of 1.88% that developed SCC or an incidence of 157.8/100000 py as compared with 0.46/ 100000 py in the general male population.

For the whole IP cohort, the SIR was 142.76 ($p < 0.05$), which also demonstrates an over-representation of SCC among patients with IP. When analysing the SIR stratified by duration, we found no evidence of reverse causality.

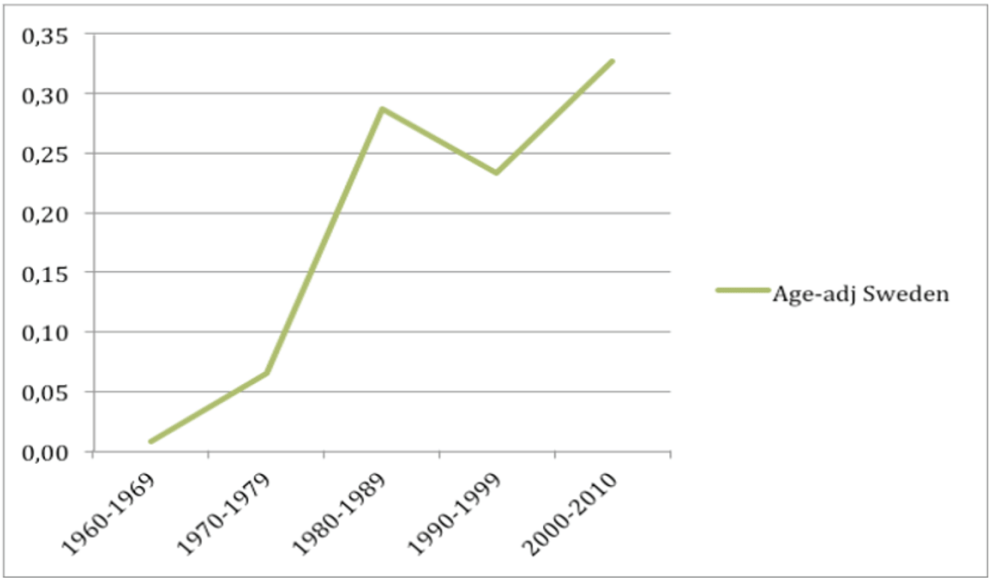

Figure 7: *incidence of IP /100000 py 1960-2010*

PAPER III

Of 126 patients reported with IP during the period, 28 cases were excluded from the study because they were re-evaluated as non-IP, diagnosis was unsure at re-evaluation, cases were missing or too little material was left to be representative of the tumour or the patients registered with IP tumours were not found in the medical records. Excluded cases did not differ significantly regarding sex and age at diagnosis.

HPV prevalence and p16 overexpression
12 of the 98 tumours (12.2%) were HPV DNA positive. 11 had low-risk HPV (HPV 6 and 11) and one had high-risk HPV (HPV 45). Of all 98 specimens, only the HR HPV specimen had a p16-overexpression (>70% positive cells).

Patients with HPV positive tumours were significantly younger (mean age 45.6 years vs. 59.6 years, $p = 0.003$). In the histopathological reports the HPV positive tumours were described more often with areas of mild to moderate dysplasia compared to the HPV negative tumours (25.0% vs. 8.1%) although this difference was not statistically significant. Two patients with HPV negative IPs were diagnosed with SCC.

CD8 and Foxp3 immune cell infiltration
Patients with HPV positive lesions showed a tendency of higher infiltration of Foxp3 positive lymphocytes as compared to HPV negative lesions. However, this difference was not statistically significant. No differences in Foxp3 infiltration or CD8 positive immune cell infiltration or CD8/Foxp3 ratio were observed with regard to dysplasia. Nor were there any differences regarding CD8 positivity or CD8/Foxp3 ratio and HPV status.

Recurrence, HPV status and immune cell infiltration
Among the patients included in the study, the follow-up time ranged from 0-12 years with a median of 5 years. 40/98 (40.8%) patients had a recurrence of their IP. 10/98 patients (11.2%) had their first recurrences after 5 years or more even though 40 patients were lost to follow up.

Patients with reported positive or uncertain surgical margins had a significantly higher recurrence rate as compared to patients with reported radical surgery (log-rank test $p=0.001$). The hazard ratio (HR) for recurrence for tumours with negative compared to positive surgical margins was 0.36 (95% CI 0.18-0.71). Adjustment for age, gender or HPV did not affect the HR in the multivariable analysis. Among patients with reported radical surgery, 17.8% of patients had recurrences within 5 years and 6.7% after 5 years or more.

Patients with HPV positive lesions tended to have a lower 5-year recurrence rate, but this was not a significant difference (log-rank test: $p = 0.17$).

PAPER IV

EGFR

57.1% of the specimens expressed EGFR and the range of EGFR expression was 0-90%. 91.7% of HPV positive IPs expressed EGFR, as compared to 52.3% of HPV negative IPs ($p=0.01$) and the median proportion of EGFR-positive cells in HPV positive tumours was also higher than in HPV-negative tumours (35% vs. 10%, $p<0.010$). Age or gender did not influence the results. No statistically significant association was seen between EGFR-positivity and recurrence. There was no difference in EGFR expression in relation to dysplasia.

Stathmin

Staining for stathmin failed for two tumour specimens. Therefore, 96 tumours were included for analyses. All IPs expressed stathmin and the range of stathmin positive tumour cells was 10-100%, with a median expression of 20%. Stathmin expression was further categorized into low and high expression with 50% as a cut-off level. In total, 15.6% of the tumours had high expression of stathmin.

A higher proportion of IPs with dysplasia had a high expression of stathmin as compared to those without dysplasia (40.0% vs. 12.6%, $p=0.045$). When analysing the relation between stathmin expression and recurrence, patients with IPs with high expression of stathmin tended to have earlier recurrences, than those with a low stathmin expression although this difference was not statistically significant (log rank test: $p=0.053$).

No difference in level of stathmin expression in HPV positive as compared to HPV negative IPs (27.3% vs 30.2%, $p=0.25$) was observed. Nor did age or sex influence the level of expression of stathmin.

Besides tumour tissue, 73 of 96 slides also contained normal mucosa. The mucosa in 28 of 73 slides (38.4%) did not express stathmin and in the remaining slides (61.6%), the mucosa expressed stathmin weakly, far from strong cytoplasmic staining which was the definition of positive stathmin staining in the study.

DISCUSSION

The aim of this thesis was to investigate the epidemiology and prognosis of IP and prognostic factors for IP on a population basis. Moreover, the aim was to investigate epidemiological aspects of SNM in the Swedish population as a background information to the epidemiology of IP. The development and behaviour of IP is still relatively unknown and under debate. With this thesis, the knowledge of this rare tumour has hopefully increased somewhat.

Epidemiology and prognosis of SNM (Paper I)

This study is one of few, large population based studies of SNM in recent years. According to the results, the incidence of SNM has decreased in Sweden during the study period (1960- 2010). However, the incidence of SNMMs increased significantly. A slight tendency towards an improved 5-years relative survival of patients with SNM over the last 50 years was also found.

The most frequent sinonasal malignancy during the study period was SCC followed by adenocarcinoma. The same relation was shown in other population-based studies [1, 2, 6] . However, when comparing incidences over time, SCC was still the most common malignancy but SNMM, became the second most common tumour at the end of the study period. This increase in incidence of SNMM has also been observed in other investigations [7, 136]. The difference in distribution of the different histological types might reflect an actual change of incidences but can also be influenced by improved histopathological methods. New diagnostic tools such as immunohistochemistry, introduced in the 1980s, and analyses of oncoproteins and other tumour markers, mostly introduced in the 1990s onwards, have increased the accuracy of identification of certain tumours. Therefore, tumours that were classified as undifferentiated in 1960 are now likely to have acquired a specific histological diagnosis. For SNMMs there still seems to be a real increase in incidence [7].

The decrease in other SNMs in this study, similar to other studies, could be related to a decrease in etiological factors. For SCC, reduced smoking and a reduction in occupational exposures such as formaldehyde and for adenocarcinomas reduced levels of hard wood and leather dust (related to better working conditions) may be reflected in the decrease in incidence [2, 6, 8, 9, 137].

The increasing proportion of SNMs originating from the nasal cavity corresponds to observations in other studies [1, 2, 6, 138]. The reason for this is unknown. Possibly better diagnostic tools (such as endoscopy, CT and MRI) and earlier diagnosis (due to diminished doctors' and patients' delay), at the end of the study period as compared to the beginning of the study period, could give a more reliable origin of the tumour with reduced misclassification. Some of the tumours in the nasal cavity (SCC and SNMM) may have been misclassified as they might have originated from the skin in the vestibulum nasi. The increased proportion of tumours originating from the nasal cavity may also be related to the increase of SNMMs which were more common in the nose than in the paranasal sinuses.

A significantly better survival for patients with tumours originating from the nasal cavity compared to other location, consistent with other studies, was found [1, 139]. Presumably this relates to the fact that tumours in the nasal cavity generate symptoms, such as nasal blockage earlier and therefore are diagnosed earlier. There is a slight increase in relative 5-year survival for SNMs, possibly related to the shift with more tumours originating from the nasal cavity. However, new treatment strategies and

modern diagnostic tools, seem not to have significantly ameliorated treatment outcome but possibly, they may have reduced therapy-related side effects. Unfortunately, there was no data on how the tumour stage of the different SNMs changed over the study period and how this may have influenced the results.

The strengths of this study are the size, and that it is based on data from a nationwide population based cancer registry, the SCR, with a uniquely high coverage rate [131, 140]. The weakness is that due to the rarity of SNM, and especially some of the histological types, conclusions may be difficult to draw.

Epidemiology and prognosis of Inverted Papilloma (Paper II)

This population-based study covering the Swedish population from 1960-2010, found that the age standardised incidence of IP increased by approximately 400%, from 0.01 to 0.33/100000 py.
An over-representation of SCC among patients with IP (SIR 142.76, $p < 0.005$) was also found. Interestingly, for patients with IP, the malignant transformation only occurred in men, in a proportion of 1.88 % which corresponds to a 300-fold increased incidence compared to the general male population.
In our study, two individuals (18%) with IP had synchronous SCC and nine (82%) had metachronous SCC. The results regarding the incidence of IP are roughly similar to hospital based data presented from Denmark and in a European position paper on the endoscopic management of sinonasal tumours [26, 39]. There are, however, some differences. These could be due to chance but may also show the advantage of a higher coverage in a population- based study otherwise to less accurate histo-pathological diagnosis or under-reporting to the SCR.

IP increased over time as opposed to SNMs (SNMMs excluded) which in the first paper were found to decrease during the same time-period [141]. However, the results could be influenced by an under-reporting to the SCR during the earlier part of the study period. This could also be explained by a higher detection rate of IP because of a better awareness of IP among ENT physicians in the recent decades and also by the increased use of endoscopic sinus surgery for diagnosis and treatment. Another possible explanation is that over time more sinonasal biopsies were sent for histo-pathological assessment which signifies that an increased number of tumours could be reported to the SCR by the pathologists. Histopathological evaluations have generally improved over time and this may also have led to an increased reporting to the SCR. The accuracy of the SCR has been studied for malignant tumours [130, 131]. IP is considered a precancerous lesion and the accuracy of the reporting of IP has not been studied per se. The reporting rate for IP may, therefore, be lower than for true malignant lesions, especially in the beginning of the study period. There may however also be some unidentified aetiological factor that partly explains the increase in incidence of IP.

As for the risk of malignant transformation of IP, diverging results have previously been reported to range from 1–53 %, where most studies have reported a considerably higher proportion of malignant transformation than the 1.35 % that was found in this population-based study [142-146]. This discrepancy may be explained by the fact that earlier hospital-based studies from referral centres could have studied patients with larger, more aggressive or recurrent IP tumours, where smaller or less aggressive IPs might not have been included. It may also be due to differences in populations on which the studies have been conducted. There is also a risk that our lower reported proportion of malignant transformation among IP is caused by an underreporting of IP in specimens of SCC. This

would both give an incorrectly low incidence rate of IP and an incorrectly low rate of synchronous SCC among IPs. However, a true comparison between studies on different populations would require new studies presenting SIR, since difference in age or gender distribution may differ.

Conducting a register study with a register covering a whole population is a strength. However, we cannot be asserted that the coverage of IP is as high as for malignant tumours especially regarding the reporting of IPs with synchronous SCCs and this could affect the analyses. Furthermore, when IP specimens were re-evaluated in paper III and IV, six cases of exophytic papilloma reported to the SCR as IP were found. Among the 814 diagnosed IPs found in the register, it must be suspected that there are other such misclassified cases.

IP, HPV and infiltration of CD8 positive and Foxp3 positive TILs (Paper III)

In paper III, the proportion of HPV positive IPs was fairly low compared to proportions reported in other studies[111]. Patients with HPV positive IPs seemed to differ in that they were younger at diagnosis and tended to have higher infiltration of Foxp3+ TILs. Moreover, only the HR HPV positive specimen was p16 positive. Also, in this study, the recurrence-rate was higher in patients who had not undergone radical surgery, which was expected, and a considerable proportion of all recurrences occurred after more than five years after surgery.

Among patients diagnosed with IP in Stockholm 12.2% had HPV positive tumours. This proportion is lower than in many other studies. In a meta-analysis from 2013, Syrjanen and Syrjanen found the point-estimate for HPV prevalence to be 37.8% with prevalence ranging from 0-100% [111]. There are different possible explanations regarding which factors that might influence these differences in HPV prevalence, such as HPV detection method (in situ hybridization, p16-analysis, PCR) or the size of the study [111]. The prevalence of HPV and HPV positive tumours can also differ in different study populations from different geographical regions as has been reported for HPV prevalence in tumours in other anatomical sites [147]. The increase in incidence of IP and in parallel, the increase of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) brings up the question of whether the increased incidence of IP is related to HPV as it is for OPSCC [148]. However, because IP is such a rare tumour and the proportion of HPV-positive tumours is low, it is difficult to assess if the incidence of HPV-positive tumours is increasing or not. An international collaboration with a larger study-population would be required for such research.

Furthermore, misclassification is a plausible cause for differences in proportion of HPV- positive IP since there are histological resemblances between IP and other types of sinonasal papilloma where exophytic papilloma have a stronger association to HPV [111]. Occasionally, there are uncertainties in regard to what kind of papilloma a study is really referring to. Like in previous studies, we found that LR HPV-6 and HPV-11 were the most frequent types [149-151]

Moreover, p16-positivity was only found in the one IP specimen that was HR HPV positive. This might indicate a possible correlation between HR HPV and p16 positivity in IP as has been demonstrated in OPSCC. Rooper et al. found such a correlation for inverted papilloma as opposed to Kim et al [88, 152]. However, in the latter study, the cut-off for p16 positivity was set at a lower level (10%). P16 positivity is of course dependant of the cut-off of level. A level of 70% has been validated for OPSCC and should most likely be the same for IP [132, 153]. Since only one specimen was HR HPV positive in our study conclusions cannot be drawn and more studies on p16 and HPV are needed to analyse if p16 can serve as a surrogate marker for HR HPV in IP.

Patients with HPV positive IPs differed from patients with HPV negative tumours in that they were diagnosed with IP at a significantly lower age. Patients with HPV positive IP more often had tumours with reported light to moderate dysplasia and a higher infiltration of Foxp3+ TILs than patients with HPV negative IPs. However, these differences were not statistically significant. This might be related to the small number of HPV positive IPs in the study. Patients with HPV positive IPs had a higher proportion of radical surgery according to the medical reports. This might indicate that their tumours were less extensive or less aggressive as has previously has been suggested for sinonasal SCCs [91]. Moreover, conclusions regarding the correlation between HR HPV and prognosis in IP could not be drawn since only one HR HPV tumour was found.

A majority of studies conducted to compare HPV prevalence in normal mucosa and in nasal polyps could not detect HPV in normal mucosa. [154-157].

Presence of HPV in IP and differences in patient and tumour characteristics in HPV positive compared to HPV negative tumours in this study might suggest that HPV has some causative effect in the HPV positive IPs.

Furthermore, this study aimed to assess if, as has been hypothesised for OPSCC, HPV stimulates an effective anti-tumoural response comprising activation of CD8+ cells and Foxp3+ cells or a high CD8+/Foxp3+ ratio affecting the prognosis of IP tumours [103, 115, 158]. It has previously been shown that IP induces a marked immune response. [29, 159, 160]. This study found an overexpression of Foxp3 in HPV positive IPs as compared to HPV negative tumours but the difference was not statistically significant. The difference might however have reached statistical significance if the HPV positive tumours were more numerous. A potential statistically significant difference could have indicated an activation of the immune system by the HPV virus. However, regarding prognosis, no statistically significant differences in expression of Foxp3, CD8+ or the CD8+/Foxp3+ ratio were found in patients with dysplasia or recurrence compared to those without.

The recurrence rate, 40.8%, was high compared to previous studies [78, 161]. This is partly caused by the inclusion criteria where included patients could have unknown or unexpected IP histology and where surgery sometimes was undertaken only for diagnostic purpose thus leading to a partial resection of the tumour. The recurrence rate was significantly lower in patients who had negative surgical margins (23%). Notwithstanding, the still relatively high recurrence rate may partly be due to the surgical technique [162]. The high recurrence rate was also influenced by the, for some cases, long follow-up time where 10% of the recurrences occurred after five years. 17.8% of the patients who were reported to have undergone radical surgery had recurrences within 5 years from diagnosis. This is assumedly the proportion that should be compared to results from other studies reporting recurrences in IP where the diagnosis was known before surgery and the follow-up time did not exceed 5 years.

Limitations in this study were that the study population and the different subgroups were relatively small. Secondly, the recurrence rate of the whole study population is probably overestimated, due to the study design where patients having surgical diagnostic resections were considered having undergone surgery. However, there is also a considerable strength in that there was no referral bias, since the study was population-based.

IP and EGFR and Stathmin Positivity (Paper IV)

A significantly higher expression of EGFR was found in HPV positive compared to HPV negative tumours. Furthermore, a significant association between high expression of stathmin and dysplasia and also a tendency towards an association between stathmin and recurrence in IP were observed.

Like in previous studies with a similar EGFR-positivity definition, EGFR expression was higher in HPV positive than in HPV negative IPs [163-165].This might strengthen the hypothesis, that there are IPs where HPV infection is an aetiological factor, possibly among others, that generates a different entity of IPs than the HPV negative tumours. Possibly by increasing expression or activation of EGFR protein since it has been proposed elsewhere that in tumours harbouring LR HPV types, the E5 oncoprotein plays an important role in the oncogenic transformation of the tumour, mainly by stimulating the EGFR pathway signalling [87, 95, 128].

Moreover, it has been reported that HPV negative specimens had EGFR mutations and that IP associated SCCs possibly require either overexpression or mutation of EGFR [128]. Also, a tendency of higher expression of EGFR has been reported in IPs with carcinomas than in IPs without [164]. It has been suggested that EGFR-inhibitors could be tested in LR HPV positive IP's but considering the costs and side effects of such treatment, administration of EGFR- inhibitors should probably be reserved for patients with EGFR-positive IPs with either malignant transformation or with extra-sinonasal growth where radical surgery would be too mutilating [163]. Irreversible EGFR-inhibitors have been tested in vitro on SCC derived from IP with promising results [127].

Association between EGFR expression and light to moderate dysplasia was not found. A recent study reported increased EGFR expression in IP with severe dysplasia and in IP with associated SCC as compared to normal mucosa and also compared to IP with mild to moderate dysplasia. However, there was no comparison with IPs without dysplasia [87]. In this study, there were unfortunately no specimens with reported severe dysplasia which would have been beneficial to the study. No association between expression of EGFR and recurrence was found.

27% of the HPV positive IPs were stathmin positive which of course was related to the cut-off level for stathmin positivity (50%) in the study. For stathmin, there does not seem to be any consensus regarding this cut-off level as there is for p16 positivity. Levels for stathmin positivity vary in different studies [166, 167]. A cut-off level of 50 % was chosen in this investigation in respect to the distribution in the study population and because it has previously been used as a cut-off level, among others [168, 169]. We did not include grading of intensity of staining as a parameter in the assessment of stathmin positivity which might have influenced the results.

Stathmin was more intensely expressed in the tumours as compared to the normal mucosa, which showed no, or very weak stathmin staining. This is in line with results from a previous study [129]. We also found a higher expression of stathmin among specimens with dysplasia than in specimens without and there was the same tendency for stathmin in recurring and non-recurring tumours. These results are also similar to what has been found in reports on malignant tumours such as oesophageal and hypopharyngeal cancer, where a high stathmin expression seems to be correlated to a poorer prognosis [170, 171]. If these results could be reproduced, stathmin could be a be a possible marker of prognosis or even a treatment target for recurring cases or tumours which, because of their extent or closeness to vital structures, are surgically challenging.

CONCLUSIONS

The main findings of the thesis were:

- The overall incidence of sinonasal malignancies has decreased in Sweden from 1960 to 2010. However, the incidence of sinonasal malignant melanomas has increased during the same period. SNMM is now the second most common SNM in Sweden.

- There was a trend towards better survival for all SNMs except undifferentiated carcinoma during the time-period.

- There was a more than tenfold increase of incidence of IP in Sweden 1960-2010.

- In this population-based study, the risk of malignant transformation of IP was lower than previously has been presented but significantly higher than the risk of being diagnosed with SCC in the general population.

- Infiltration of Foxp3+ and CD8+ TILS alone or in combination with HPV did not affect the prognosis of IP significantly.

- Patients with HPV positive IP were diagnosed at an earlier age than HPV negative tumours and HPV positive IP showed a non-statistically significant tendency of a higher proportion of dysplasia and later recurrences. HPV positive IP also had a significantly higher expression of EGFR.

- Normal mucosa adjacent to the tumours expressed stathmin weakly or not at all. IPs with high expression of stathmin had significantly higher proportion of dysplasia and a trend towards earlier recurrence was observed. HPV-status did not affect the stathmin expression.

FUTURE PERSPECTIVES

Inverted papilloma is a rare tumour and one of the aims in this thesis was to assess its incidence on a population basis using the SCR. However, more population-based studies are needed to confirm the results. Preferably they should present SIRs for an optimal between-populations comparison.

A relatively low proportion of IPs associated with SCC and especially few synchronous malignancies were found. This could be an effect of pathologists not registering IP when there is synchronous SCC in a same specimen. Therefore, analyses of SCCs in the Stockholm Biobank to investigate if they also harbour non-reported IP would be valuable.

Since IP is a rare tumour, larger interregional and international studies are needed to analyse the interaction between the immune system and the tumour. It is possible that, like in other studies, statistical significance was not reached because of the relatively low number of cases analysed in the different subgroups in the study-population. Likewise, larger studies are required to assess the correlation of high risk HPV and p16.

Furthermore, the proportion of HPV-positive tumours is expected to decline over time with the introduction of vaccines. These cover the most frequently found HPV types in IP (both high risk and low risk types) why a shift towards more HPV negative IPs in the future should be expected. If HPV positive tumours have a different behaviour and prognosis than the HPV-negative tumours, the eventual disappearance of HPV positive tumours will affect the overall prognosis of IP. For rare recurring or inoperable cases among the non-HPV driven tumours, it would be beneficial to study treatment targets, such as stathmin or other markers in order to possibly introduce medical treatment for these selected cases.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Inverterat papillom (IP) är en godartad tumör som utgår från slemhinnan i näsa och bihålor. Tumören har fått sitt namn efter den histologiska bilden som man ser vid mikroskopisk undersökning av vävnaden där en förtjockad slemhinna växer ner med tjocka kolvar i den underliggande vävnaden. Inverterat papillom är en ovanlig tumör men riktigt hur ovanlig den är har man inte vetat då de studier som gjorts inte baserat sig på hela befolkningar utan endast på de patienter som remitterats till vissa sjukhus. Män drabbas ca tre gånger så ofta som kvinnor medan barn och ungdomar sällan drabbas. Inverterat papillom är oftast ensidigt och vid klinisk undersökning ter det sig som en rödgråaktig oregelbunden, lättblödande massa. Tumören kan växa igenom ben och brosk och därmed växa utanför näsa och bihålor in i till exempel ögonhåla och skallbas. Behandlingen utgörs idag nästan uteslutande av kirurgi.

Inverterat papillom har en tendens att återkomma och återfall beror till stor del på den kirurgiska radikaliteten, dvs hur väl man har fått bort hela tumören. Vilka övriga faktorer som påverkar återfallsrisken är inte känt. Tumören klassificeras som ett förstadium till cancer. Tidigare studier har visat att skivepitelcancer har funnits samtidigt eller uppkommit senare hos i snitt 10 % av patienterna.

Bakomliggande orsaker till inverterat papillom är okända men man har spekulerat i att allergi, kronisk bihåleinflammation, luftföroreningar och virusinfektioner bidrar till uppkomsten av tumören. HPV (humant papillomvirus) har föreslagits som riskfaktor på grund av orsakssambandet mellan HPV och papillom (vårtor) huden och i slemhinnan i strupen och svalget samt sambandet mellan HPV och cancer och förstadium till cancer i svalget, i genitalierna och kring anus. De relativt få studier som är gjorda på inverterat papillom och HPV har dock inte givit någon entydig bild.

Cancer i näsa och bihålor är en sällsynt cancerform i Sverige och orsakar idag cirka 0–1% av alla cancerfall och utgör cirka 5% av all huvudhalscancer. Hur förekomsten av cancer i näsa och bihålor har utvecklats över tid i Sverige är inte känt sedan tidigare.

I avhandlingens första delarbete undersökte vi hur insjuknandet i cancer i näsa och bihålor har förändrats från 1960–2010 i hela den svenska befolkningen med hjälp av det svenska cancerregistret. Vi fann att insjuknandet i näs- och bihålecancer minskat något generellt men att maligna melanom i näsa och bihålor ökat under denna period. Fem-årsöverlevnaden har endast marginellt förbättrats under undersökningsperioden.

I andra delarbetet analyserade vi, också med hjälp av cancerregistret, hur insjuknandet i IP har förändrats över tid och hur många av dem som egentligen blir elakartade. Vi fann att insjuknandet ökat markant över tid men att övergången till cancer var lägre än vad som tidigare rapporterats.

I det tredje delarbetet ville vi utröna om vi kunde hitta något samband mellan IP och HPV samt om infiltration av vissa immunförsvarsceller (så kallade CD8-positiva och Foxp3-positiva celler) i tumören hade någon påverkan på prognosen. I vår studie skiljde sig patienter med HPV förekomst från de som inte hade HPV i sina tumörer på flera sätt vilket kan tyda på att HPV påverkar tumörens uppkomst och beteende. Däremot verkade inte immunförsvarscellerna närvaro påverka tumörens prognos nämnvärt.

I det sista delarbetet analyserade vi uttryck av två proteiner, EGFR och stathmin, är relaterade till cellcykeln. När de är överuttryckta (producerade i överskott) eller är muterade (förändrade) kan de bidra till utvecklingen av en vanlig cell till en cancercell. Resultaten visade att det fanns en relation mellan HPV och EGFR i IP medan stathmin verkade vara associerat med tumörernas prognos för hela gruppen IP.

Sammanfattningsvis fann vi att inverterat papillom är en ovanlig tumör som ökar i förekomst. Det finns en liten risk att den omvandlas till en elakartad tumör. HPV kan kanske bidra till dess utveckling och då möjligen genom överuttryck av ett visst protein, EGFR. Ett annat protein, stathmin, kan till synes också påverka tumörens beteende. Förhoppningsvis kan man i framtiden behandla IP med mediciner som riktar sig mot dessa eller andra proteiner men mer forskning inom detta område behövs. Vi fann även att insjuknandet i de flesta cancerformer i näsa och bihålor minskat i Sverige sedan 1960 talet men insjuknandet i malignt melanom i näsa och bihålor har ökat. Tyvärr har inte prognosen för näs- och bihålevcancer förbättrats nämnvärt.

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