CHAPTER 1

General introduction
Introduction on head and neck squamous cell carcinoma

Head and Neck Squamous Cell Carcinoma (HNSCC) is the 5th most common cancer worldwide\cite{1}. HNSCC is the general name for all cancers originating in the squamous epithelium of the upper aerodigestive tract. The tumors arise at the anatomical locations depicted in Figure 1, which include the oral cavity, pharynx and larynx\cite{2}. In the Netherlands around 2,400 new patients are diagnosed with HNSCC each year, mostly in the elderly population, with the highest incidence in the sixth decade of life. Men are mostly affected, but the incidence in females is rising, probably related to changing life style factors.

1.1 Risk factors

The exposure to carcinogenic substances, in particular alcohol and tobacco are still the main risk factors for HNSCC. However, as a result of different ethnic, socio-economic and life-style factors the incidence and preferential anatomical location of HNSCC varies over the world. An example hereof is the betel chewing in India which causes a high incidence of tumors in the oral cavity. In the Western world the most common risk factors for development of HNSCC are still tobacco smoking and excessive consumption of alcohol-containing beverages. Tobacco smoke contains besides nicotine and tar, over 4,000 different chemicals including multiple carcinogenic compounds. These compounds are for example benz-(a)-pyrene and nitrosamines, which are known to give specific guanine transversions and may cause mutations in oncogenes or tumor suppressor genes\cite{3,4,5}.

Tobacco smoking and alcohol consumption are independent risk factors, but act synergistically when combined\cite{6,7,8}. By estimate, the risk increases by 2 to 9 times in indi-
individuals who either smoke tobacco or consume alcohol-containing beverages\textsuperscript{[6,7]}, but increases to 80 times in individuals who combine these habits\textsuperscript{[8]}. It is estimated that around 75\% of all HNSCCs are associated with smoking and excessive alcohol consumption\textsuperscript{[6]}. The effects of both risk factors on the different anatomical subsites vary with smoking mostly associated with glottic cancer and alcohol consumption with oral cancer\textsuperscript{[8]}.

Besides exposure to carcinogenic chemical compounds also viral infections with Human Papillomavirus (HPV, mainly oropharyngeal tumors) or Epstein Barr Virus\textsuperscript{[9]} (EBV, mainly nasopharyngeal tumors specifically in the Asian population) are risk factors for HNSCC. HPV accounts for \textasciitilde 10\% of HNSCC in the Netherlands\textsuperscript{[10,11]}. Finally, there is a population with a strong genetic predisposition for the development of HNSCC, i.e. patients suffering from Fanconi anemia (FA). Patients with FA have a 500-1,000 times increased risk for developing HNSCC\textsuperscript{[12]}. Both the role of HPV in HNSCC and FA-related genetic predisposition will be discussed in more detail below.

1.2 Diagnosis and tumor classification
HNSCC is diagnosed by physical examination and histopathological evaluation of a tissue biopsy. HNSCC is classified according to the TNM (Tumor, Nodal and Metastasis) system of the International Union Against Cancer (IUAC). The TNM-anatomical classification is based on 3 clinical parameters: primary tumor size and extent (T), the presence or absence and size of regional lymph node metastasis (N) and the presence or absence of distant metastasis (M). The clinical staging occurs by palpation, panendoscopy, ultra-sound fine needle aspiration cytology (USgFNAC) and imaging techniques as CT, MRI or PET. When the tumor is treated by surgery, additional histological examination is carried out on the specimen resulting in a pTN staging, the most reliable prognostic factor at present. Based on the TNM system, tumors are classified in clinical disease stages. Small tumors confined to the primary site and without metastasis are stage I (<2 cm), or stage II (2-4 cm). Larger tumors and tumors that have metastasized to the regional lymph nodes or distant sites are of disease stage III or IV. The above described criteria apply to tumors located in the oral cavity and pharynx only. Staging systems evolve and molecular or biological features might be added to improve the staging and become relevant for the clinical management of HNSCC patients\textsuperscript{[13]}.

1.3 Treatment and survival.
Early stage tumors are mostly treated by single modality therapy, usually surgery or radiotherapy. However, patients with advanced disease stage are mostly treated with a combined regimen using surgery and postoperative (chemo)radiotherapy or concomitant treatment with radiotherapy and chemotherapy or biological therapy. Treatment-related morbidity for the advanced stages can be severe and may itself have major impact on the quality of life.
Locoregional control of HNSCC has improved over the last decades, but survival has not increased accordingly\[1\]. When diagnosed at early disease stage (stage I or II), the overall survival is relatively high for a disease that strikes in the latter decades of life (www.ikcnet.nl; Figure 2, survival of oral cancer patients according to disease stage). However, survival remains disappointing when HNSCC is diagnosed at an advanced disease stage (stage III to IV). Unfortunately, two thirds of HNSCC patients present with advanced disease stage and despite extensive surgery and combined treatment modalities the survival rates leave much to be desired.

A major problem in the clinical management of HNSCCs is the frequent occurrence of tumor relapses in the same or adjacent anatomical region even when the surgical margins were histologically tumor-free. These local relapses are clinically assigned as local recurrences (LR) if they develop within 3 years and at <2 cm distance of the primary tumor. Relapses that do not meet these criteria are assigned as second primary tumors (SPT)\[14\], and these may occur in the same or adjacent anatomical area but also frequently in the lung. Treatment of local relapses is difficult as they develop in previously treated tissues and not uncommonly lead to disease-related death\[15,16,17,18\].

The high occurrence of local relapses in HNSCC patients can be explained by two different mechanisms; minimal residual cancer \[19,20\], which is the presence of so few tumor cells in the margins that they escape histological detection, or field cancerization\[15,21,22,23\], which will be explained in more detail below as this is highly relevant for the studies described in this thesis.

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**Figure 2.** Overall survival according to stage of oral cancer patients (age-adjusted) in the Netherlands (1999-2007) stratified for disease stage at diagnosis. (Source: IKC The Netherlands).
2 Human Papillomavirus in HNSCC

In the last decades it has become clear that some viruses are able to cause oncogenic transformation of human cells. One of those viruses is the Human Papillomavirus (HPV). HPV is a strictly epitheliotrophic DNA virus of which nowadays over 100 subtypes have been identified\[24,25\]. Most HPV types are benign and may cause warts or other benign lesions, but a number of HPV types are oncogenic and are defined as high-risk HPV types\[26\]. Although many individuals infected with high-risk HPV induce a proper immune-response and clear the infection, others do not\[27\] and may eventually develop a tumor induced by the infection. The viral genes that play an important role in the oncogenic transformation of human cells are the E6 and E7 genes\[28\]. These genes encode the E6 and E7 onco-proteins that inactivate the p53 and Rb proteins, respectively, causing cell cycle entry and apoptosis inhibition to support viral replication\[25\].

HPV has been recognized as the primary cause for cervical cancer, with HPV16 and HPV18 causing the very large majority of these cancers. Since the late eighties and early nineties of the previous century a role of HPV in HNSCC was suggested. Later studies in 2000, 2002 and 2004 strongly supported the hypothesis that HPV, in particular HPV16, might play a role in a subgroup of HNSCC\[25,29,30,31,32\]. HPV-positive tumors are mostly found in the oropharynx and some in the oral cavity. In the Netherlands (Amsterdam area), 5% of the tumors of the oral cavity and 16% of those in the oropharynx were shown to be HPV-induced\[33\]. However, the prevalence of HPV in HNSCC seems quite variable in different geographical regions since in the Baltimore area 72% of the oropharyngeal tumors were found to be HPV-positive\[34\]. In a recent meta-analysis the large difference in HPV prevalence has been indicated, and is as yet not explained\[35\].

2.1 HPV detection

As indicated above, the prevalence of HPV in HNSCC reported in different studies from several parts of the world varies considerably, in fact ranging from 0-100%. One of the explanations is related to the site of the studied tumors as the prevalence is highest in oropharyngeal tumors, low in oral cavity and almost absent in laryngeal and hypopharyngeal tumors, however, also technical aspects of HPV detection play an important role. HPV is not easily cultured in vitro and presence or absence of the virus in tumor specimens usually relies on the detection of the viral genome using DNA PCR-based assays. The many available DNA PCR-based tests are extremely sensitive and can detect a few DNA copies in a sample. If HPV is causally involved in tumorigenesis it is to be expected that the virus is present in every malignant cell in the tumor and viral loads of 1 copy per cell or higher are to be expected, or 0.5 or higher assuming that the percentage of tumor cells in a sample consists of 50% neoplastic cells. It is therefore questionable whether low viral loads detected by these very sen-
sitive PCR method reflect a true oncogenic infection. Low viral copy numbers might not reflect HPV involvement in the initiation and maintenance of the tumor, but may be related to a coincidental productive viral infection or technical artefacts \cite{10}.

Based on the known biological role of HPV in cervical cancer and the critical association of expression of the viral E6 and E7 genes with oncogenic transformation, van Houten et al. suggested that E6/E7 expression analysis is a more reliable method to detect a biologically relevant HPV infection \cite{31}. The presence of HPV E6 and E7 mRNA in the tumor sample was also shown to coincide with characteristic genetic patterns and expression array profiles \cite{33,36,37,38}. HPV E6/E7-positive HNSCCs only show few allelic imbalances scattered over the genome while HPV-negative HNSCCs show many allelic imbalances in particular encompassing chromosome arms 3p, 9p and 17p. In addition, in 60% of HNSCCs a mutation in TP53 can be detected, whereas the presence of HPV E6/E7 mRNA coincides with the absence of mutations in the TP53 gene. The absence of TP53 mutations in tumors with HPV E6/E7 expression makes biological sense as the HPV E6 oncoprotein inactivates p53. Tumors with transcriptionally active HPV that reflects causal viral involvement, are thus typically p53 wild type \cite{33,36}.

Hence, HNSCC tumors can be classified in two molecularly distinct subsets. Tumors with transcriptionally active HPV showing few genetic aberrations and absence of TP53 mutations, and tumors lacking HPV that generally show many genetic aberrations encompassing whole chromosome arms and frequent TP53 mutations. These data strongly support the relevance of E6/E7 expression analysis to assess HPV involvement. However, detection of E6 or E7 mRNA in tumor tissue can only be performed on fresh or frozen biopsies. Although recently a method for E6/E7 transcript detection in paraffin embedded tissues was developed, it is only suited for HPV16 \cite{11}.

Moreover, the analysis of paraffin-derived RNA remains problematic. Using two independent methods suited for analysis of formalin-fixed paraffin-embedded specimen an algorithm for HR-HPV detection has been developed, which combines satisfactory performance with the option for high-throughput analysis. This procedure combines the use of p16\textsuperscript{INK4A} immunostaining (a protein upregulated by HPV infection), followed by GP5+/6+ DNA PCR (detection of HR-HPV using an EIA-based method) on just the p16\textsuperscript{INK4A} positive cases \cite{11}. A positive analysis result can be confirmed by investigation of surrogate markers such as a the specific genetic profile and the p53 mutational status, also methods that can be carried out on tumor DNA isolated from FFPE specimen.

### 2.2 HPV in HNSCC and outcome

It is generally accepted that HPV-positive HNSCCs have a better prognosis than HPV-negative tumors, but there remains some controversy \cite{39}. A recent meta-analysis on the prognosis of HPV-positive HNSCCs suggests that these virally induced tumors have a more favorable prognosis, but only for the oropharyngeal SCCs \cite{40}. In another recent prospective trial of oropharyngeal and laryngeal SCCs the better survival of
HPV-positive SCCs was confirmed\(^\text{[42]}\). Whether this depends on a better response of HPV-positive tumors to therapy or other biological properties is not known. Two small studies indicate that HPV-positive tumors respond better to induction chemotherapy\(^\text{[42]}\) (platinum combined with 5-FU) and radiation\(^\text{[43]}\). Another study did not observe an improved response of HPV-positive tumors to radiation, but overall survival of patients with HPV-positive tumors was still better\(^\text{[44]}\).

Two main factors overshadow these outcome data. The first factor is the lack of a standardized and validated HPV testing approach. Some investigators even used very sensitive nested DNA PCR approaches, which makes it likely that they scored many cases positive in which HPV is not causally involved. The second factor is that many studies are relatively small and do not allow to analyze the effects of cofactors such as smoking and treatment. The awareness of the HPV-testing problem in this research field and the recent introduction of more reliable high-throughput HPV test methods hopefully will help to solve these issues in the future. Recently a first study was published that clearly demonstrated that prognostic risk groups can be defined in oropharyngeal carcinomas on basis of TNM stage, HPV presence and smoking behavior\(^\text{[135]}\).

How important reliable testing for HPV is, will be illustrated by the observations on head and neck tumors in Fanconi anemia patients discussed in the next section.

3 Fanconi Anemia

3.1 Clinical features of Fanconi anemia

Fanconi anemia (FA) is an inherited autosomal recessive or X-linked (FANC-B) disease with a prevalence of 1-5 per million births. The life expectancy of FA patients is on average 20 years\(^\text{[45]}\), but is rising due to improved clinical management. The clinical features of the disease include congenital malformations of the skeleton, mostly the thumbs or radii, but also of the kidneys, heart and other organ systems\(^\text{[45]}\), and bone marrow failure. FA patients are also genetically predisposed for malignancies, in particular acute myeloid leukemia (AML) and solid tumors. These solid cancers are predominantly SCCs in the head-neck and anogenital region\(^\text{[12,45,46,47,49,50,51,52]}\).

FA patients frequently undergo bone marrow transplantation (BMT) or stem cell transplantation (SCT) to treat the bone marrow failure that can be life threatening, but also to treat or prevent AML when premalignant clones arise in the bone marrow. Initially, transplantation results in FA patient were disappointing, but as a result of improved protocols adapted for FA patients, bone marrow transplantation became quite successful. However, as more patients have an increased life expectancy due to the success of BMT to control haematological failures, patients develop more frequently SCCs, which is rapidly becoming a major cause of mortality in FA patients. Additional risk factors for development of SCC in FA patients are BMT and BMT-related Graft versus Host Disease (GvHD)\(^\text{[46,50,53]}\).

Treatment of SCC in FA patients is cumbersome as these patients do not sustain the
regular treatment regimens containing radiotherapy with or without cisplatin-based chemotherapy. Hence, successful clinical management is limited to surgery of early stage tumors, which emphasizes the importance of surveillance and early detection of cancer and precancer.

### 3.2 Cellular features of Fanconi anemia

FA cells display chromosomal instability, contributing to the genetic predisposition of FA patients to cancer. FA is caused by defects in the FA/BRCA pathway, which is part of a DNA damage response pathway, particularly activated for repair of DNA cross-links. Consequently, FA cells are particularly sensitive to DNA cross-linking agents such as cisplatin, mitomycin C (MMC) or diepoxybutane (DEB). When challenged with DNA cross-linkers, FA cells arrest at the S/G2 phase, probably in response to the inhibited DNA repair. Figure 3 shows a schematic representation of the FA/BRCA DNA repair pathway. The FA complex consists of several proteins; of which 13 have been identified to date. These proteins FANCA, -B, -C, -D1 (BRCA2), -D2, -F, -G, -I, -J, -K, -L, -M, and -N act together with BRCA1, HES1, P24 and P100 to form the FA/BRCA pathway. The first step in the FA pathway after DNA damage is the formation of the so-called FA core-complex, consisting of FANCA, B, -C, -E, -F, -G, -L and –M in the nucleus. In the assembled FA core-complex FANCL catalyzes the mono-ubiquitination of FAND2 and FANCI. This is the crucial step to initiate DNA repair, after which BRCA1 and other DNA repair proteins are recruited at the site of the damaged DNA.

![Figure 3](image.png)

**Figure 3 Schematic representation of the FA proteins including the FA core complex.** After cytoplasmic assembly of FANCA, -B, -L, and -G, this complex is transported to the nucleus where in response to DNA damage FANCC, -F and -E complete the complex, allowing mono-ubiquitination of FANCD2 and FANCI finally resulting in repair of DNA damage.
3.3 SCC in FA patients

In general, FA patients have a 500-1,000 times increased risk for developing SCC\cite{12}, in particular SCC in the mucosal linings of the head-neck region. FA patients develop SCC at relatively young age, the youngest even at 11 years. In the Dutch SKION guidelines for FA patients it is indicated that the oral cavity should be screened from 10 years of age every 3 months by an experienced head and neck surgeon, and the anogenital region of female patients should be inspected by a gynecologist once a year starting at the age of menarche. The risk factors for SCCs in sporadic patients are exposure to environmental carcinogens (tobacco and alcohol) or infection with HPV. The observation that only a small subgroup of FA patients (16\%\cite{57}) use tobacco or alcohol containing beverages, together with the observation that these tumors preferentially occur at the head-neck and anogenital region, led to the hypothesis that HPV infection might play a critical role in FA SCC\cite{12,57,58,59}. In a first study it was reported that 15/18 HNSCC and 6/7 anogenital SCC of FA patients were found to be HPV-positive (cut-off level 0.1 viral copies per cell) and none of these SCCs showed a TP53 mutation (exon 4-9), supporting HPV involvement, as explained above. The control group matched for age, sex and tumor site, showed only 18/50 HPV positive tumors (5/14 anogenital and 13/36 head and neck SCC). Additionally, in contrast to the FA-SCCs, in the control group 36\% of the tumors had TP53 mutations. For HNSCC only, the figures were 0/18 for HNSCCs in the FA patients and 12/31 (39\%) in the controls. Previously it was shown that a polymorphism at amino acid 72 in exon 4 of the TP53 gene might be associated with HPV involvement in sporadic SCC as the Arg72 p53 protein would be degraded more easily by the E6 protein of HPV16 or HPV18\cite{60}. In the study by Kutler et al. analysis of this polymorphism showed that a greater proportion of the FA patients carried the Arg/Arg polymorphism than the Pro/Pro or Pro/Arg when compared to the sporadic control patients. The highly present Arg/Arg polymorphism could therefore be the explanation for the high prevalence of HPV induced SCC in FA patients\cite{57}.

Notwithstanding the above described data there are clinical arguments against a role of HPV in FA-SCC. First, FA HNSCCs arise mostly in the oral cavity\cite{61}, while in the sporadic population mainly oropharyngeal tumors and in particular tonsillar carcinomas are HPV-positive\cite{33}. Second, HPV-induced HNSCCs seem to be caused by sexual behavior, but FA patients are generally young at the time of diagnosis or have severe FA symptoms and are often not sexually active. Lastly, the data on HPV in the reported study raises questions. The HPV viral loads (the number of viral genome copies per cell) detected in these SCCs were highly variable, ranging from 0.2 to 249 viral copies per cell (median viral load HNSCC 2.46 (0.16–7.86), anogenital SCC 68.3 (0.54–249.3)). In tumors with very low viral loads (<0.5 viral copy per cell) it is questionable whether HPV is the causative factor\cite{10}. As explained above the analytical sensitivity of the DNA tests are often too high, and detect productive coincidental viral infections instead of a true oncogenic infection\cite{11}.

To support the observation that specific FA SCCs are caused by HPV, additional sur-
rogate markers should be used, especially p16 immunostaining, the patterns of allelic loss or genetic profiles that all might give a good indication as explained above. We also need to consider that the HPV prevalence differs considerably between the US and EU at least in the sporadic HNSCC population, and it remains a question whether the data obtained in studies on FA patients in the US could be extrapolated to the EU FA population. It remains a highly relevant question as preventive HPV vaccination might be very beneficial in these patients to prevent HPV-induced SCC.

4 Head and neck carcinogenesis

Just as for all cancers, it is now generally accepted that development of HNSCC is a multi-step process, resulting from the accumulation of both genetic and epigenetic alterations such as chromosomal changes, mutations, promoter methylation or gene amplifications. The progression from normal mucosa to carcinoma can be observed as morphological changes in the mucosal epithelium by routine microscopic examination of biopsies or surgical margins. These changes are graded according to the standard criteria of the World Health Organization as mild, moderate or severe dysplasia. Increased proliferation, mitotic figures and loss of differentiation over the layers are the main morphological features characterizing dysplasia (Figure 4).

![Figure 4. Schematic representation of the architecture of the non-keratinizing normal oral mucosa and different stages of dysplasia. In the normal mucosa, cells divide in the basal and parabasal layers, then differentiate and move upwards. Mild dysplasia affects only the basal layer, moderate dysplasia includes 2/3 of the mucosal layer, and severe dysplasia all layers.](image)

In 1996, a first genetic progression model for HNSCC was presented, in which the accumulation of particular genetic changes was associated with increasing histological abnormalities. Allelic loss at 9p21 was thought to be the first genetic change from normal oral mucosa to a benign mucosal hyperplastic lesion. Dysplastic lesions had additional losses at 3p21 and 17p13. Subsequent losses at 11q, 13q and 14q were detected in carcinoma in situ. Invasive cancers also displayed allelic loss at 6p, 4q and 8p. Many potential cancer genes are located at these frequently altered chromosomal regions including several genes involved in cell cycle control. The locus 9p21 contains the p16/p14 gene, a gene encoding both the p16 and p14 proteins. The TP53 gene is located at 17p13 and the cyclinD1 gene at 11q13. Up to now, 3p21 is not associated with a particular tumor suppressor gene.
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However, the initial progression model heavily relied on histological grading; the microscopical evaluation of the abnormal appearance of the tissue architecture of the mucosal layers. It is well known that histological grading is somewhat subjective, with high intra- and inter-observer variability\textsuperscript{[68]}. In addition, this model did not describe the earliest mucosal changes before morphological changes are detected. A refined genetic model was proposed by Braakhuis et al.\textsuperscript{[22,69]}. This model focuses more on the genetic changes, and also incorporates the earliest changes detectable by molecular methods. It describes a stem cell which feeds a clonal unit (~200 cells) of the normal mucosa consisting of transit amplifying daughter cells and differentiated cells. Genetic damage of the stem cell causes a change in the clonal unit. These genetically damaged clonal units can be detected by immunostaining of $TP\textsubscript{53}$, and mutation in the $TP\textsubscript{53}$ gene is thought to be one of the first steps. Additional genetic hits cause more damage to that particular clonal unit, and result in lateral displacement of the mutated clonal unit over the normal mucosal epithelium, thereby creating a field of genetically altered cells that might reach dimensions of up to 10 centimeter in diameter. These fields are genetically characterized by $TP\textsubscript{53}$ mutations and loss of chromosome arms 3p, 9p and 17p, which are found in 60-80\% of the preneoplastic mucosal changes and invasive HNSCCs \textsuperscript{[20,22,23,70,71]}. Accumulating genetic damage in these fields finally cause the invasive carcinoma\textsuperscript{[69]} (Figure 5).

Hence, HNSCC tumors seem to develop in preneoplastic fields. When the tumor is diagnosed, it might be surgically removed. However, preneoplastic fields can reach dimensions of several centimeters in diameter and are not visible in most cases (~80\%). Consequently, they are often not completely excised when the tumor is removed. These fields that stay behind cause local relapses, clinically assigned as local recurrences and second primary tumors. These local relapses are clonally related to both the surrounding preneoplastic field and the primary tumor\textsuperscript{[23,70,72]}.

4.1 Visible lesions; leuko- and erythroplakia

The large majority of HNSCCs arise \textit{de novo} without preceding visible changes in the mucosa. However, some tumors are preceded by visible precursor lesions, mostly diagnosed in the oral cavity. This subgroup of fields (estimated 20\%) is visible to the naked eye as white (leukoplakia) or red (erythroplakia) changes in the mucosal epithelium. Leukoplakia is formally defined as “a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion”\textsuperscript{[73,74]}. The reported
prevalence of leukoplakia ranges from one to eight percent depending on gender and age\textsuperscript{[75]}, but these numbers might be overestimated due to the fact that lesions are incorrectly defined as leukoplakia, and the most recent estimation of the prevalence of leukoplakia is 0.5\%\textsuperscript{[76]}. Malignant transformation can occur in leukoplakias, but the frequency and time to progression is different between study populations\textsuperscript{[75]}. In a cohort study in Amsterdam the malignant transformation rate was 2-3\% per year, accumulating to 50\% after 200 months\textsuperscript{[77]}. The prevalence of erythroplakia lesions is low and reliable data is scarce. The rate of malignant transformation is high and most, if not all, erythroplakias will finally give rise to a carcinoma, and should be treated. Moreover, erythroplakias generally give more clinical symptoms, whereas leukoplakias in general do not give additional symptoms\textsuperscript{[76]}.

The current clinical management of leukoplakias consists of clinical inspection, biopsy and microscopic examination for dysplastic changes. Interventions are excision, laser (CO\textsubscript{2}) evaporation or watchful waiting depending on the extension of the lesion, the clinical symptoms and histology. All patients will undergo frequent surveillance, every 3 months when dysplasia is confirmed, or every 6 months, when dysplasia was not detected in the biopsy\textsuperscript{[76]}. Many patients finally will develop a SCC in the lesion, despite intervention. Unfortunately, the current intervention with excision or laser evaporation seem not to result in a significant longer time to progression\textsuperscript{[77]}. The lesions frequently recur after treatment or tumors develop outside the visible lesion. New and other modalities also for these visible lesions are urgently needed to improve treatment results.

In conclusion, treatment options for preneoplastic fields are very limited. If the lesion is visible (in only 20\% of the cases) it can be removed by laser evaporation or surgical excision. However, when the lesions are large they cannot be excised and other options for treatment are not available until now. Some chemopreventive agents are currently being tested such as freeze dried black raspberry gel. These preparations contain several chemopreventive substances, and by \textit{in vitro} studies it was shown that these could inhibit survival pathways, stimulate apoptotic pathways and reduce production of proangiogenic cytokines. This led to a clinical trial in patients with dysplastic lesions resulting in an improvement by a decrease in number of allelic losses and dysplasia scores in a subgroup of the patients studied\textsuperscript{[78]}. Although some effect of this gel was observed, new treatment modalities are urgently needed. Besides this study there are still many preventive trials running (www.clinicaltrials.gov). A relatively successful approach was the use of oncolytic adenoviruses that will be described in more detail below.

\section*{4.2 Identification of preneoplastic lesions}
As has been discussed, the majority of preneoplastic lesions is not visible by the naked eye, which hampers the detection and analysis of these lesions. As described,
biopsies and surgical margins of excised invasive carcinomas can be analyzed by histological examination, immunostaining\textsuperscript{[79]} and genetic analysis to identify the fields. However, these are focused approaches and screening other sites in the oral cavity or oropharynx is less straightforward. Taking random biopsies of the oral mucosal tissue for routine investigation is invasive and causes discomfort, and visualization of these preneoplastic fields might therefore be a great aid to the clinician. Recently it was shown that auto-fluorescence of the preneoplastic cells might be exploited for that purpose. Although this research field is still in its infancy the visualization approaches confirmed the existence and the dimensions of these preneoplastic fields\textsuperscript{[80,81]}.

An alternative to detect preneoplastic fields in these patients might me to use non-invasive genetic screening\textsuperscript{[82]}. Cells can be brushed from the mucosa and investigated by allelic loss analysis. Such an assay can predict the presence of preneoplastic fields with a sensitivity of approximately 50% and a specificity of 100%. Sampling by brush is non-invasive and does not give discomfort allowing analysis of the oral cavity\textsuperscript{[83]}.

In conclusion, HNSCCs arise within preneoplastic fields that are non-visible in most cases. We can detect these fields as visible lesions in some cases, but also by auto-fluorescence imaging methods, in brushed samples by genetic analysis, and in tissue samples by routine histology, immunostaining and genetic analysis. Patients with an increased risk for having these preneoplastic fields include patients treated for oral cancer, who are at risk for development of a local relapse adjacent to the index tumor but also elsewhere in the oral cavity; patients with oral leukoplakias who may develop primary SCCs also outside the visible lesion; and FA patients who have an extremely high risk for developing SCCs mainly in the oral cavity. These patients might benefit from early detection of oral precancer by brushing at regular time intervals. However, there are no treatment methods for the majority of preneoplastic fields. The only intervention at present is increased surveillance and watchful waiting till visible lesions develop which can be excised. New effective treatment modalities are urgently needed. An interesting new treatment option for preneoplastic fields in the oral mucosa is rinsing with an oncolytic adenovirus, and an overview of these agents will be provided below.

5 Adenoviruses

Adenoviruses are epitheliotrophic viruses that mainly infect the upper airways. The most common symptoms of adenoviral infection include nasal congestion rhinorhea, cough and conjunctivitis. Also a latent infection in lymphoid tissues may occur. Although most adenoviruses do not cause any serious complications some adenoviruses of subgroup A (Ad12, Ad18 and Ad31) seem to have oncogenic potential in hamsters, but this has not been described for humans\textsuperscript{[84,85]}.

Adenoviruses are non-enveloped, icosahedral viruses with linear double-stranded DNA genomes of approximately 36 kb. The adenovirus uses the host cell machinery to
replicate its viral genome in the nucleus and lysis of the host cell occurs after progeny has been produced. The capsid of the adenovirus consists of 252 hexon capsamers with 12 penton bases to which the fiber of the virus is attached (Figure 6).

Today, 51 human adenoviral serotypes have been identified. The human adenoviruses can be subtyped to subgroups A to F according to their hemagglutination properties, oncogenicity in rodents, DNA homology and genomic organization. The subgroups each contain one or more serotypes. For example subgroup C contains serotypes 1, 2, 5 and 6. There is some correlation between subgroups and the tissues they infect, for example B1, C and E cause respiratory infections, F causes gastroenteritis and D subtypes are associated with keratoconjunctivitis.

The entry of the adenovirus in the host cell generally involves the attachment of the virus to a primary receptor. The primary receptor for adenovirus type 5 (Ad5) is the Coxsackie Adenoviral Receptor (CAR), which is bound by a discontinuous domain in the C-terminal knob domain of the fiber protein. Integrins, such as αvβ3 and αvβ5, serve as secondary receptor facilitating adenoviral entry in the cell. They are bound by RGD motifs present in the penton proteins located at the base of the protruding fibers. Other receptors known to be used by Ad5 and other serotypes include CD46, CD80/CD86 and sialic acid. In addition, indirect binding to cells occurs via soluble factors in the blood, such as complement and clotting factors.

5.1 Adenovirus life cycle

The adenovirus life cycle can shortly be described in 7 steps: 1) binding to and entry of the adenovirus in the cell, 2) delivery of the viral genome to the cell nucleus, 3) early adenovirus gene expression, causing initiation of S-phase in the host cell and inhibition of apoptosis, 4) viral DNA replication, 5) shutdown of host cell protein synthesis and selective synthesis of structural adenovirus proteins, 6) viral particle assembly and genome incorporation in the nucleus, and 7) induction of cell death and viral progeny release. The adenoviral genome can be grossly divided into early and late genes based on time of transcription after infection. The early genes modulate the...
biology of the host cell to facilitate the replication of virus DNA and the transcription and translation of the late genes. These late genes encode proteins for the assembly of the viral particle. The virus largely relies on the DNA replication-machinery of the host cell. To this end, various viral genes interfere with the regulation of cell cycle and DNA replication.

The first adenoviral region transcribed immediately after infection is E1A. E1A encodes proteins that transactivate other early adenovirus genes and that induce S-phase of the host cell\[88,89\]. In normal resting cells, Rb is unphosphorylated and binds E2F. Upon phosphorylation by cyclin-CDK complexes Rb releases its binding partner E2F resulting in cell cycle entry. E1A proteins directly bind Rb, thereby releasing E2F resulting in transcription of adenoviral E2A and cell cycle genes causing S-phase entry\[89,90,91,92\]. E1A also upregulates p53 by releasing E2F, which stimulates p14, which releases the inhibitory effect of MDM2 on p53, finally resulting in stabilized p53 protein. Another main interaction of E1A is with p300/CBP. This protein is an important activator of transcription and interacts with many transcription factors. E1A thus regulates by use of many host cellular proteins the first steps in the adenoviral life cycle.

The two different proteins encoded by the E1B region are E1B-19kD and E1B-55kD. These E1B proteins inhibit apoptosis and induce host cell protein shut-off. One function of E1B-55kD is the binding to p53, thereby blocking p53-dependent apoptosis and by co-operation of E4-orf6 p53 is targeted for degradation\[89,92,93\]. The induced p53-independent apoptosis pathway mediated by TNF-α is inhibited by E1B-19kD\[86,92\]. The E2 gene region encodes the DNA binding protein (DBP)\[94\], precursor terminal protein (pTP) and DNA polymerase (pol) involved in DNA replication and protecting the integrity of the linear dsDNA genome\[86\]. The E3 proteins take care of immune response regulation and cell lysis after completion of replication\[95\]. The E3-gp19K protein allows the adenovirus to replicate in the cell without alarming the immune system by downregulating MHC class I molecule expression on the cell surface that might otherwise activate specific CD8 T-cells by presenting adenovirus peptides. The cell is thereby not recognized by the immune system as being infected and the replication cycle of the virus can continue. Other E3 proteins (RID α and β) downregulate the receptors for the so-called death-ligands TRAIL, TNF-α and Fas-ligand to prevent apoptosis of the cell. Additionally, the E3 region encodes ADP, the adenoviral death protein, which induces cell lysis. Despite it being part of the E3 region, the ADP gene is expressed primarily in the late phase of infection. Overexpression of ADP from otherwise complete E3-deleted adenovirus resulted in increased viral spread and increased anti-tumor activity\[96,97,98\].

The E4 proteins mostly encode proteins that are involved in controlling viral replication and host cell protein synthesis shut off, to allow efficient adenoviral protein production\[88\]. The late genes are predominantly involved in the synthesis of structural proteins of the adenoviral capsid, as hexon, penton and fiber proteins\[86\].
5.2 Adenoviruses for use in virotherapy
The use of adenoidal-pharyngeal-conjunctival (apc viruses), nowadays called adenoviruses, for treatment of cervical carcinoma was first described by Huebner et al.\cite{99} based on previous observations that these viruses were oncolytic in HeLa cells, a cell line originating from a cervical cancer. The viruses were used in 30 patients to investigate their effect in vivo. In 16 cases increased necrosis in the tumor tissue was observed, which was not observed if inactive virus was used. So although Huebner\cite{99} used wild type adenoviruses (several subtypes) without any specificity or selectivity towards cancer cells, there was effectivity of adenoviral infection on tumor cells.

Virotherapy has long been forgotten as an option for cancer treatment. Renewed interest in the use of adenoviruses started with the rise of molecular biology. During that time the knowledge on the genes involved in cancer increased tremendously as well as the knowledge on the interaction of the adenovirus with the host cell. Use of both started the development of new adenoviral backbones suited to be used in cancers with specific genetic aberrations.

5.3 Conditionally replicating adenoviruses
Conditionally replicating adenoviruses (CRAds) are developed as new anti-cancer agents. CRAds are genetically engineered adenoviruses that only replicate in cancer cells and destroy these cells by normal adenoviral replication processes. Progeny virus released from destroyed tumor cells can infect neighbouring tumor cells. Via several rounds of replication and cell lysis all tumor cells might eventually be eradicated, and thus the tumor be destroyed. The selective replication in cancer cells and the prevention of replication in normal cells is discussed in the next section.

5.4 Selectivity
Conditionally replicating adenoviruses are designed to replicate only in tumor cells. The specific properties of cancer cells can be exploited to achieve selective replication. One way to design CRAds is to modify the Ad genome in such a way that viral functions that are essential for replication in normal cells, but are redundant in tumor cells, are deleted. For example, genes responsible for activating the cell cycle or preventing early apoptosis through p53 or pRb binding can be mutated. In 1996 the ONYX-015 was introduced as an adenovirus only replicating in cells in which the p53 pathway is inactivated, for example by mutation\cite{100}. However, the ONYX-015 was shown to replicate also in tumors also with wild-type p53. An explanation for this phenomenon is given in the fact that the E1B55K is also involved in the functioning of late viral proteins. Due to the loss of E1B55K the export of these late viral RNAs to the cytoplasm is affected in normal cells. Tumor cells which allow ONYX-015 replication (irrespective of their p53-status) have an increased export of these late proteins, resulting in virus assembly\cite{101}. Another option to achieve selective replication is abolishment of binding of E1A to Rb\cite{102,103}. CRAds with Rb-binding deficient E1A are unable to
sequester Rb from E2F. Release of E2F to allow virus replication thus has to be done by other means. In tumor cells, Rb is frequently deficient, hypophosphorylated, or sequestered by other cellular or viral proteins associated by the malignancy. Hence, in cancer cells the replication of this type of CRAd should thus not be hampered, in contrast to normal cells with an intact Rb pathway\[102,103\].

Another approach is the production of early adenovirus proteins essential for viral replication being suppressed in non-malignant cells, by driving their expression from a tumor-selective promoter. Examples of selective promoters used to construct such CRAds are PSA (CV706)\[104\], Survivin\[105,106,107,108\], Cox2\[109\] or CXCR4\[110\]. An example of the clinical effect of these promoter driven viruses is the use of CV706, in which the promoter of PSA regulates E1A, to treat prostate cancer. The virus was safe to use by intra-prostatic injections, without serious adverse events, and even showed partial responses in some patients, with decrease in PSA levels.

While each of these manipulations of the adenovirus genome provide a level of cancer-specificity, none have been reported to completely abort replication in non-malignant cells. Therefore, oncolytic adenoviruses were made that incorporate multiple modifications. In general, such viruses exhibited a more strict tumor-selective replication\[111,112,113\].

Finally, to increase infection of a specific cell type, adenovirus receptor recognition has been modified. To this end, changes have been made in the adenoviral capsid, using for example fibers of other serotypes\[114,115,116,117,118,119\]. More elegant is the use of specific targeting moieties. This can be achieved by adding bispecific antibodies or addition of binding motifs in structural proteins of the capsid\[120,121,122,123,124,125,126\]. Bispecific antibodies have already been developed for retargeting adenovirus type 5 towards other cell surface expressed antigens. The antibodies bound effectively on one hand to the adenoviral knob and on the other to either EGFR or CD40. Moreover, the bispecific moiety could be expressed under control of a CMV promoter from the genome of a CRAd, resulting in an effective retargeting of the CRAd over several rounds of propagation\[120,125,127\].

5.5 Replicating adenoviruses in clinical trials\[128\]
ONYX-015 was one of the first CRAds used to treat patients, especially patients with HNSCC. One of the first phase I studies with ONYX-015 for HNSCC showed that intratumoral injection of the virus at high particle titer (10\[^{11}\] PFU) was safe, with limited toxicity. Although not the purpose in this phase I study, effects of ONYX-015 could be observed as stable disease and partial response\[129\]. Phase II studies in HNSCC patients performed by Nemunaitis and co-workers used ONYX-015 in HNSCC patients, either or not in combination with chemotherapeutic drugs (cisplatin and 5FU)\[130,131,132,133\]. As a single treatment using 10\[^{10}\] PFU per injection (5 or 10 injections per patient) intratumorally, several complete responders were observed, as well as partial responses and stable disease\[133\]. Moreover, ONYX-015 was shown to replicate only in the tumor,
which indicated selectivity of the backbone for tumor cells\textsuperscript{[134]}. Use of ONYX-015 in combination with 5-FU and cisplatin for recurrent HNSCC showed an improved loco-regional control compared with historical controls only treated with chemotherapy\textsuperscript{[131]}. Additionally, the observed side effects were not greater than grade 3, establishing that ONYX-15 is quite safe. In conclusion, the results from these clinical trials using ONYX-015 were encouraging, but could still be improved.

ONYX-015 was not only used as tumor therapy, but was also applied as a mouthwash therapy in patients diagnosed with histologically defined dysplastic leukoplakia lesions of the oral cavity\textsuperscript{[134]}. Patients were asked to rinse their mouth for 30 minutes with a solution containing ONYX-015. In total 19 patients were treated and 7 showed a complete response: a histologically confirmed regression of the dysplastic lesion. Unfortunately, some of the patients had a relapse of the dysplastic lesion after cessation of therapy. This mouthwash trial did provide a lot of information on the possibility to use adenovirus for non-invasive treatment, side effects and efficacy of mouthwash. However, improvements can still be made, because anti-tumor effects only lasted during treatment and much more powerful CRAds than ONYX-015 have been developed more recently.

In conclusion, ONYX-015 has shown selective replication in HNSCC and preneoplastic cells, but testing of next generation viruses is worthwhile. Although the results of the clinical trial using mouthwash were somewhat disappointing, they are encouraging enough to provide incentive for further development of oncolytic adenoviruses for treatment of HNSCC. Combination strategies with chemotherapy and radiation also deserve further consideration, as the clinical trials performed did not show increased toxicity caused by the adenovirus and tumor regression was mostly increased in these combined therapies.
6 Outline of this thesis

HNSCC is caused by carcinogen exposure (smoking and alcohol consumption), infection with the human papillomavirus (HPV) and genetic predisposition such as in Fanconi anemia (FA). It has been well established that HPV-infected tumors are a separate entity within HNSCC both at the molecular and clinical level. It is at present unclear whether HNSCCs in FA patients also form a separate entity. Both the etiology (a DNA repair defect) and the age of onset are unusual when compared to HNSCCs in sporadic patients. Preliminary data further suggested a much higher HPV-prevalence in tumors of FA patients. Specific molecular characteristics of SCCs in FA-patients might have important consequences for the clinical management of these tumors in this distinct patient group.

In Chapter 2 the generation of 4 FA HNSCC cell lines is described, and these were compared at the molecular level to HNSCC cell lines of sporadic patients. The results suggested that SCCs in FA patients are not very different at the molecular level from sporadic SCCs and do not show a higher frequency of HPV involvement. However, this study was performed using cell lines, and we therefore investigated in addition primary tumor specimens of FA-SCC patients and sporadic SCC patients. The results are described in Chapter 3 and confirmed our findings in the cell lines. The data suggested that SCCs in the sporadic population and in FA patients seem to follow the same carcinogenesis route, targeting the same cancer genes with a role of HPV in particularly anogenital cases. This suggests that SCC in FA patients can be treated with the same approaches applied in sporadic cases, except for DNA cross-linking agents or other drugs that cause synthetic lethality with FA defects, as these will cause severe toxicity.

HNSCC develops in genetically defined preneoplastic fields in the mucosal linings. A minority of these fields (20%) is visible as a leukoplakia or erythroplakia lesion, but the large majority is not recognized by the naked eye (~80%) and does not give any other clinical symptom. When a tumor arises it is often treated by surgical excision, combined with postoperative radiotherapy for more advanced disease, but the preneoplastic field stays behind in approximately 25% of the cases, causing a high risk for local relapse. Hence, these fields cause primary tumors and relapses and an intervention to prevent malignant transformation is urgently needed. A problem at present is that we have no access to cell lines of preneoplastic fields to evaluate therapeutic strategies, and we also miss information on the cancer-associated phenotype of these preneoplastic cells. In Chapter 4, the generation of a preneoplastic cell line from a field is described. Mucosal biopsies surrounding excised tumors were cultured and studied genetically and phenotypically. Of 18 cultures analyzed, four showed genetic changes, and one developed into a preneoplastic line. This sample showed allelic losses at multiple chromosomes, a mutation in TP53, and appeared immortal. It was used for subsequent experiments next to HNSCC cell lines of sporadic and FA patients. Mouthwash therapy with conditionally replicating adenoviruses (CRAds) is consid-
ered a promising treatment approach to eradicate fields, but initial clinical trials leave much to be desired. We hypothesized that the infection of the adenovirus could be hampered and that enhancement of infection might improve the efficacy of oncolytic adenoviruses, particularly when applied for treatment of preneoplastic fields by an oral rinse. In Chapter 5 we studied this in an organotypic model and found that infection is indeed decreased which might relate to the observation that the expression of the adenoviral receptor CAR seemed to be lost on the most superficial layers of the oral mucosa. To improve infection we evaluated the value of immunological retargeting using squamous-cell specific antibodies.

In Chapter 6 we studied which genetically manipulated adenoviral backbone is most suited for treatment of preneoplastic mucosal fields. Eleven CRAds with different modifications conferring cancer-selective replication and effective cancer cell kill were compared for their cytotoxicity against normal cells (fibroblasts and keratinocytes), HNSCC cell lines (3 sporadic cell lines and 2 FA cell lines) and the preneoplastic cell line generated in Chapter 4. In Chapter 7 the results of the research reported in this thesis are discussed.
General introduction

References

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