CHAPTER 8

Summary
The lack of substantial improvement in prognosis of head and neck cancer patients despite advances in treatment is of notable concern. This can be attributed to late presentation, failure of advanced lesions to respond to treatment, development of local relapses and lack of suitable markers for screening or personalised therapy. Risk factors of HNSCC are carcinogen exposure (tobacco and alcohol), infection with the human papillomavirus (HPV), and genetic predisposition, particularly in Fanconi anemia (FA) patients. Previous research has revealed that head and neck squamous cell carcinomas (HNSCC) frequently, if not always, develop in preneoplastic fields in the mucosal linings of the upper aerodigestive tract. In a minority of cases these fields are macroscopically visible as white (leukoplakia) or red (erythroplakia) lesions in the mucosa. The preneoplastic fields often stay behind when the tumors are excised and cause a high risk for local relapse\(^1,2,3,4\). A major challenge is to eradicate these fields or to prevent their malignant transformation especially in high risk populations such as patients with leuko- and erythroplakia, FA patients or patients who have been treated previously for head and neck cancer. These fields cause both primary tumors and local relapses.

In Chapter 2 four FA-SCC cell lines have been generated from primary head and neck tumors of FA patients and molecularly characterized, and the results compared to seven cell lines of sporadic HNSCCs. None of the cell lines in this panel of FA and sporadic HNSCCs was positive for any of the high-risk HPV types. When the genetic changes were compared, these seemed not to differ between FA-SCC and sporadic SCC. Based on these analyses, we concluded that FA HNSCC seems not to be induced specifically by HPV infection and appears to follow more or less the same carcinogenic route as sporadic HNSCC.

Our results based on these cell lines are in direct contrast to data published previously\(^5\). However, genetic differences may have been introduced by culturing, and we therefore set out in Chapter 3 to investigate primary tumor tissue specimens of FA patients. In total 21 FA-SCC of 19 patients (16 HNSCC and 5 anogenital) were analyzed for high-risk HPV\(^6,7\), surrogate markers for HPV infection, and genetic characteristics. In total 2 of 21 SCC were shown to contain HPV, one with HPV16 and one with HPV33, and were likely induced by HPV infection based on the surrogate markers and genetic profile. Both these FA-SCCs originated in the anogenital region. The 16 SCCs of the head and neck region were all HPV-negative, which was supported by the surrogate markers. Importantly we could also show that, based on the genetic data, FA-SCC does not seem to be a different class of tumors at the molecular level when compared to sporadic HNSCC, suggesting that diagnostic and treatment strategies suitable for preneoplastic fields in the sporadic population might be adopted for the FA population as well.

The second major aim of this thesis was to develop a treatment for preneoplastic fields by generating an oncolytic adenovirus that specifically replicates in cancer or
precancerous cells, and eradicates them. However, the efficacy of oral rinses with oncolytic adenovirus ONYX-015 left much to be desired, and we hypothesized that it could be improved by increasing both the infection of oral keratinocytes using an immunological retargeting approach, and by selecting an adenoviral backbone that is more powerful to eradicate these fields specifically. A problem that we faced was that although we had access to multiple HNSCC cell lines, we did not have access to precancerous cell lines, the real target cells. Therefore we decided to generate preneoplastic cell lines that might be exploited for selecting a suitable adenovirus or other treatments, and the results of these attempts are described in Chapter 4. Mucosal biopsies of excised specimen were cultured to obtain preneoplastic cells. Three cultures of 18 tested showed an extended lifespan or clonal expansion as well as genetic changes. Of these three short term cultures one immortal preneoplastic cell line was obtained that showed loss of at least two chromosomal arms (3p and 9p) and a TP53 mutation. Our explanation that only one preneoplastic cell line was obtained is that loss of only one or a few selected chromosomal regions is not sufficient for a keratinocyte to progress into an immortalized cell line. The cell line obtained (VU-preSCC-M3), which was used as a preneoplastic model in the next Chapters, had already many changes, but nonetheless displayed a growth pattern in organotypic models that closely resembled normal mucosa.

Optimization of virotherapy for the treatment of preneoplastic fields in the oral and oropharyngeal mucosa by increasing the infection of the oral mucosa was the main focus of Chapter 5. As a few previous reports already suggested, we confirmed that adenoviral infection of the oral mucosa is hampered by the tissue architecture. During differentiation of the keratinocytes the expression of the adenoviral receptor CAR is decreased and infection is markedly reduced. To improve the infection of the oral mucosa we searched for another surface protein abundantly expressed on the superficial cells of the normal and mild/moderate dysplastic oral mucosa that might be suited for retargeting the adenovirus. The antigen Ly6-D, which is recognized by monoclonal antibody E48, seemed the best candidate for retargeting the adenovirus, and we constructed a bispecific antibody E48-S11 that recognized Ly-6D and the adenoviral knob. It was tested both in flat-bottom and organotypic cell cultures, and was shown to enhance adenoviral infection.

Finally, the oncolytic adenovirus should preferably only replicate in (pre)neoplastic mucosal cells and not in normal keratinocytes or fibroblasts. The development of ONYX-015 and other adenoviruses showed that conditionally replicating adenoviruses (CRAds) could be engineered that make use of specific tumor cell properties for selective replication. Various strategies have been followed to design CRAds. These include deletions or mutations of adenoviral genes, insertion of transgenes, or use of tumor selective promoters driving viral genes. As the effect of all these different changes in the viral genome on the various cell types and tissues is not well cha-
racterized, we performed the study described in Chapter 6. We analyzed a panel of CRAds in HNSCC cell lines of sporadic and FA patients and normal oral keratinocytes and fibroblasts. Most striking observation in this study was the high vulnerability of normal oral keratinocytes to adenoviral infection in contrast to normal fibroblasts, which are relatively resistant. The ONYX-015 virus, which has been tested in HNSCC patients, was included in this comparative study, and was also relatively toxic to the normal keratinocytes in vitro although to a lesser extent than some other CRAds. In our study the most selective adenovirus seemed a CRAd with the survivin promoter driving E1A protein expression, an intact E3 region, and with a fiber of which the tropism was modified using an RGD motif. In Chapter 7 the data presented in this thesis are discussed in a larger perspective.
References


