CHAPTER 7

General discussion
After many years of research we can safely conclude that head and neck cancers develop in preneoplastic fields, although this is still unclear for tumors arising by HPV infection. These fields are most likely preceding the invasive carcinomas and due to the dimensions of these fields and the fact that the large majority is not macroscopically visible, they frequently stay behind when the tumor is excised, causing a high risk for local relapse. Populations at risk of having these fields include leuko- and erythroplakia patients, previously treated head and neck squamous cell carcinoma (HNSCC) patients and Fanconi anemia (FA) patients. The fields can at present be diagnosed by analyzing tissue samples with genetic markers and by immunostaining. However, it would be helpful to develop tools to visualize these fields, and initial data using auto-fluorescence are promising[1]. The major question still remains how to treat these fields. Obviously, increased surveillance will be an option, but not very attractive, and treatment approaches are urgently awaited.

Previous research, but also the data in this thesis and in keratinocyte transformation models show that the first genetic hits in head and neck cancer progression seem p53 mutation, inactivation of p16 (9p loss), activation of cyclinD1 (11q amplification), and likely 3p loss (gene unknown)[2,3,4], changes that abrogate the p53 and pRb pathways. HPV, which is known to cause a molecular and clinical separate class of tumors particularly in the oropharynx, also hits the p53 and Rb pathways by its E6 and E7 genes thereby causing oncogenic transformation[5]. The viral E6 and E7 onco-proteins directly inactivate the p53 and pRb pocket proteins, respectively, causing an S-phase environment and apoptosis inhibition to support viral replication[6].

In this thesis the molecular carcinogenesis and the role of HPV in squamous cell carcinoma (SCC) in FA patients has been investigated. FA patients have an increased risk for developing squamous cell carcinomas, particularly of the head and neck. Few years ago the question arose whether FA-SCC were induced by high-risk human papillomavirus (HPV) infection. This hypothesis was mainly based on the localization of the SCC[7] in FA patients as they present with tumors of the head and neck or the anogenital region, sites where HPV is known to play a role in carcinogenesis. Our data (we reported 0/16 FA-HNSCC HPV-positive while 15/18 were reported positive by Kutler et al.[8]) have created a lot of confusion. Most likely explanation is that there is a considerable regional difference. Tumors tested by Kutler et al. were from patients in the USA while our samples were mainly from patients in the EU. It has been reported that the HPV prevalence in the sporadic HNSCC population differs markedly in various regions. In the Baltimore area 72% of the sporadic oropharyngeal SCCs are HPV-positive, while in the Amsterdam area only 16% was HPV-positive, and this regional difference seems also apparent in FA-SCC[9]. Based on the research described in this thesis we have to conclude that despite the fact that both the etiology (genetic predisposition vs carcinogen exposure) and the age of onset differ tremendously between FA-SCC and sporadic SCC, the tumors seem to follow more or less the same
cancerogenesis routes at the molecular level. This is an important observation as it implies that established and experimental treatments developed for sporadic SCC, likely will be effective for FA-SCC as well. Obviously FA patients remain extremely sensitive to DNA cross-linking agents.

The p53 and pRb pathways are very attractive targets for therapy of preneoplastic fields as these pathways are involved both in the HPV and non-HPV induced HNSCC. A potential treatment modality specifically targeting p53 and pRb pathways are oncolytic adenoviruses. Adenoviruses exploit their E1A and E1B genes to block these same pathways as HPV does to create an S-phase environment for viral DNA replication and inhibit the p53-induced stress response. The resulting deregulation of the cell cycle is also a typical characteristic of many if not all tumors, and the idea emerged that viruses with modified E1A or E1B genes might therefore specifically replicate in these cells, and not in normal cells.

In this thesis, the possible exploitation of oncolytic adenovirus to treat preneoplastic fields has been evaluated. One of the viruses initiating this research field was ONYX-015 in which the E1B-55K gene was deleted, thereby abrogating adenovirus-mediated p53 degradation. The virus has been successfully tested in the clinical setting for treatment of HNSCC by injection and of preneoplastic lesions by an oral rinse\cite{10,11,12}. Treatment of preneoplastic lesions by an oral rinse was only moderately effective. In 7 of 19 treated patients an effect was seen determined by regression of the lesion or a decreased grade of dysplasia. Remarkably there was hardly any toxicity reported, while we clearly showed in this thesis that keratinocytes are very sensitive to adenoviral vectors, at least in vitro. The data presented in this thesis suggest, however, that the therapeutic window in the latter study might not relate to specific replication but to specific infection. In the setting that adenoviruses are applied as an oral rinse, it seems that the tissue architecture of the oral mucosa is the most critical factor determining selectivity. Due to differentiation of the mucosal linings the expression of the receptors relevant to adenovirus infection, CAR and integrins, is lost in the differentiated layers, thereby inhibiting native infection in healthy mucosa. Also keratinization of most superficial cells might add to resistance for adenoviral infection. Preneoplastic tissues loose this differentiation capacity, the cells become more ‘basal’ cell like and keep expression of CAR and integrins, particularly when they reach a progression state histologically recognized as severe dysplasia or carcinoma in situ. This might allow increased infection of preneoplastic cells compared to normal cells that are not infected.

This de-differentiation dependent selective infection might be the reason why ONYX-015 was effective in some patients with preneoplastic fields without toxicity. This suggests that only moderate/severe dysplastic fields can be targeted specifically, a hypothesis that should be studied in future clinical trials. Of note, many preneoplastic fields have not yet lost the normal tissue architecture despite a mutation in TP53.
and allelic losses of 9p, 3p and other chromosome arms. In fact 4 out of 5 fields that caused a local relapse were graded as mild dysplasia\[^{13}\]. This creates a difficult problem and likely necessitates to enhance adenoviral infection using a retargeting approach as described in this thesis. The drawback of retargeting is that this may also increase toxicity to the normal oral mucosa, which is apparently highly sensitive to adenoviral infection, at least \textit{in vitro}. Strategies that might reduce this toxicity are 1) identification and exploitation of surface proteins that are specific for preneoplastic fields, and 2) modification of the viral backbone to enhance selective replication. Preneoplastic-specific retargeting of the adenovirus is very attractive but might be difficult as in a recent proteomics study aimed to identify differentially expressed proteins in normal and preneoplastic lesions, only small differences in protein profiles were detected\[^{14}\]. Moreover, to retarget the adenovirus a protein with high expression in the preneoplastic field and low or absent expression in normal tissue and not vice-versa is required, which limits differentially expressed candidates.

Summarized, retargeting the virus seems at present the most promising approach for oral rinse application. Enhanced infection might remain critical for the fields that show more or less normal differentiation with low CAR and integrin expression in the superficial cells. The constructed bispecific antibody E48-S11 enhanced infection markedly, and retargeting may enhance infection of both HNSCC and preneoplastic lesions. By enhancing the infection of the mucosal layers the use of selective backbones will become more important. Keratinocytes were shown to be very vulnerable to adenoviral infection, at least in the models we have used. The backbone should be chosen in relation to the selectivity of the virus towards normal keratinocytes and not, as in many studies, in relation to normal fibroblasts that are relatively resistant against adenoviral infection. Notwithstanding, we should also not over-interpret our findings as our data were collected in flat bottom culture models of highly stimulated and proliferating keratinocytes. Clinical toxicity might be much less severe in vivo than predicted from these \textit{in vitro} models.

Apart from virotherapy, chemoprevention has been explored to treat preneoplastic lesions. Chemoprevention is defined as using natural, synthetic or biological agents to suppress, reverse or prevent progression to cancer\[^{15}\]. Chemo-preventive strategies were initially focused on retinoids (vitamin A related compounds)\[^{16}\]. Although the first results were encouraging\[^{16}\], these drugs have never been implemented in clinical practice, mainly due to their severe side-effects. In addition, trials with beta-carotene, a potent antioxidant were negative\[^{17}\]. Today several chemoprevention trials are running in various phases of completion. Freeze-dried black raspberries are used for treatment of dysplastic lesions of the oral cavity by topical application. In this phase II trial a decrease in the dysplasia grade was observed in various patients, and was claimed to be treatment related\[^{18}\]. In addition there are many other trials ongoing for prevention of oral cancer, mainly in patients with leukoplakia with dyspla-
sia or genetic changes (www.clinicaltrial.gov). The Bowman-Birk concentrate[19,20,21], a soybean derived protease inhibitor, was shown to have some effect on leukoplakia. In another trial the systemic use of erlotinib is studied to prevent malignant transformation. This tyrosine kinase inhibitor targets the EGF receptor, which is considered to be upregulated in HNSCC. The positive effect of erlotinib in clinical trials in combination treatment with standard regimens for HNSCC has been proven[22,23,24]. However, EGFR targeting in preneoplastic fields is difficult as also the normal oral mucosa and skin express EGFR, causing a toxic effect to the normal tissues.

Another class of drugs tested is the non-steroidal inflammatory drugs (NSAIDs). Agents like celecoxib, ketorolac and sulindac inhibit the COX-2 enzyme, which might be the main target for the cancer preventing effect observed in some previous studies[25,26,27]. Only the trial using ketolorac is based on an oral rinse, while the other drugs are given systemically. The third class of drugs (including piaglitazone[28] and rosiglita-zone, both given systemically) are binding PPAR-γ, which leads to a decrease in NF-κB, an important survival factor in cells. These systemic treatments might cause relatively high toxicities in comparison to topically applied drugs.

The normal function of the mucosal tissues is to serve as a barrier for environmental chemical agents and cellular entrance of agents might therefore be hindered when topically applied by mouth rinse or gels, whereas systemic treatment can finally reach all cells. Depending on expected toxicity the choice for either systemic or local treatment should be made for each drug. As many fields are mild dysplastic and might have retained their barrier function, systemic treatment seems preferable. Likely p53 and genes in the pRb pathway or genes that are synthetic lethal with the cellular defects caused by the abrogation of these pathways, are at present preferable as therapeutic targets.

Based on our data most chemopreventive strategies for sporadic HNSCC might also be useful for FA patients. In this thesis we show that the carcinogenic route of FA-HNSCC seems comparable to that of sporadic HNSCC with activation and inactivation of the same cancer genes and cancer-associated loci. Whether this conclusion holds when more refined genetic analysis platforms are employed remains to be determined. A special concern when treating FA patients is the inherited susceptibility for DNA-damaging agents particularly cross-linkers such as cisplatin. Although the tumors retain their FA phenotype as we showed in the cell lines, the normal cells of FA patients are also very sensitive to these agents. Preventive strategies using adenoviruses to eradicate preneoplastic fields in these patients might be very worthwhile. Our data show that the selected adenoviral backbones seem comparable in effectiveness in both sporadic and FA-HNSCC. Early detection of the fields and prevention of SCC is the best chance for these patients.
References


