Targeted chemoprevention of head and neck cancer

de Boer, D.V.

2021

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Chapter 1

General introduction

Head and neck squamous cell carcinomas

Annually around 700,000 patients are diagnosed with head and neck squamous cell carcinoma (HNSCC) worldwide[1]. In the Netherlands approximately 3,000 HNSCC patients are diagnosed every year[2]. These tumors arise in the mucosal lining of the upper aerodigestive tract: i.e. the oral cavity, oropharynx, larynx and hypopharynx. Most important risk factors are smoking and excessive alcohol consumption. Risk is related to pack years of smoking and unit years of alcohol consumption, with a synergistical effect upon combination of both[3]. Besides chemical carcinogens, infection with a high-risk type of the human papillomavirus (hrHPV) can cause HNSCC as well, most particularly in the oropharynx[4]. Furthermore, some genetic disorders, such as the genome instability syndrome Fanconi anemia (FA), predispose the affected individuals for tumors in the head and neck region[5,6].

After initial presentation, the diagnostic workup for HNSCC consists of computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) on indication, supplemented with examination under general anesthesia. Regional metastases in the lymph nodes of the neck are diagnosed by palpation, imaging, ultrasound-guided fine needle aspiration cytology and sentinel node biopsy. When surgery is applied as upfront treatment, microscopic examination of the surgical specimen is added to the diagnostic procedures. Depending on the diagnostic findings, tumors are staged according to the eighth edition of the Tumor-Node-Metastasis (TNM) staging system and classified in disease stages I to IV[7].

The first line treatment strategy depends on the stage of the tumor at time of diagnosis, the site of the tumor, and other relevant diagnostic findings. In addition, patient-related factors such as age and comorbidities are considered in treatment planning. When surgery is applied the histological findings of the specimen are highly relevant as well. Early stage tumors, diagnosed in 30% of the patients, are primarily treated with radiotherapy (RT) or surgical resection alone, while for more advanced stage tumors surgery is combined with post-operative radiotherapy or chemo-radiotherapy, or upfront chemo-radiotherapy is applied with surgical salvage for residual disease. Oral cancers are typically treated by upfront surgery. In general, cisplatin is the primary choice for concomitant chemotherapy[7–9].

For HPV-negative tumors the five-years survival rate remained around 60% over the last decades[1]. Despite innovation and improvements in diagnostic and treatment strategies, such as sentinel node biopsies and transoral robotic resections[10,11], the rate of locoregional recurrences, distant metastases and second primary tumors, remains high. A local recurrence presents within a distance of two cm from the original tumor and within a timespan of less than three years[7]. Second primary tumors develop further than two cm from the original tumor or later than three years after the first tumor, and occur in approximately 2-3% of the patients per year[12]. In patients presenting with

relapsed disease, treatment for cure is difficult and often these patients only qualify for palliative care to extend their life expectancy and/or improve quality of life as much as possible[13].

New therapeutic options are urgently awaited to eradicate the tumor cells and prevent relapses. However, identification of therapeutic targets in HNSCC is hampered by the fact that mutations in oncogenes rarely occur, and on the other hand because HNSCC tumors are very heterogeneous at the molecular level[7]. The Cancer Genome Atlas (TCGA) consortium published a comprehensive genomic overview of the mutations and numerous chromosomal gains and losses in a large HNSCC cohort, which demonstrated the large variety of molecular changes detected in HNSCC. At least 50-100 candidate cancer genes have been reported that might play a role in HNSCC. Most HPV-negative tumors share a loss of function of *TP53* and inactivation of *CDKN2A*. In HPV-positive tumors *PIK3CA* mutations are most frequent[14]. Besides these somatic mutations several copy number alterations (CNAs) are acquired in the majority of HNSCCs (HPV-positive and HPV-negative), most often loss of 3p, 4p and 18q and gain of 3q, 5p and 7p is seen. Some CNAs are associated with HPV-status, like loss of 9p in HPV-negative tumors and gain of 9q in HPV-positive tumors[14,15]. In addition, a small subset of the HPV-negative tumors display a copy number silent profile, these tumors are also often *TP53* wild-type, and typically contain *HRAS* and *CASP8* mutations[7,14].

The comprehensive overview of the TCGA can be exploited for the identification of druggable targets for therapy, but currently the available options for HNSCC are very limited. One FDA-approved strategy is blocking the binding of epithelial growth factor (EGF) to its receptor (EGFR) by cetuximab, typically applied for patients who are unfit to receive cisplatin. However, recorded responses to cetuximab are generally modest, and recently it was shown that cetuximab is inferior to cisplatin when combined with radiotherapy in HPV-positive tumors[16–19]. Immune checkpoint inhibition seems at present a more promising strategy for HNSCC treatment. A variety of immune checkpoint inhibiting antibodies have been approved for various tumor types including HNSCC, although efficacy in HNSCC is still limited with less than 20% of the patients responding[20,21].

In general, HPV-positive tumors have a more favorable prognosis compared to the HPV-negative tumors. In a recent Dutch cohort study it was shown that the five-years overall survival of patients with an HPV-positive tumor is 79% versus 43% in patients with an HPV-negative tumor[22]. Therefore the direction of clinical research for these tumors is currently more focused on treatment de-escalation[23–25]. Although some HPV-positive tumors still recur and some patients still die of the disease, the risk is relatively small when applying current treatment protocols. The major challenges to

improve treatment relate to patients with HPV-negative tumors, in this thesis we will focus on this particular subgroup.

Carcinogenesis

Cancer arises by the accumulation of (somatic) genetic and epigenetic changes causing the malignant transformation of a normal cell into a tumor cell, a process defined as multistep carcinogenesis (BOX 1)[26]. Fortunately, there are several barriers that need to be taken before a normal cell becomes a cancer cell. These phenotypic capabilities that must be overcome, known as the 'hallmarks of cancer', have been postulated by Hanahan and Weinberg[27]. Multiple mutations in relevant signaling pathways need to be accumulated to allow the transformation to a malignant cell. The molecular changes to overcome these barriers in HNSCC show a large variety. The number of driver genes linked to HNSCC development, is consequently estimated between 50 and 100 candidates. The large majority of these genes are mutated in only a low percentage of tumors and the functional consequences are mostly unknown. Therefore, many of these genes remain candidate driver genes. The most frequently changed and consequently well-established drivers in HNSCC are *TP53, CDKN2A, FAT1* and *NOTCH1*[4,7,14].

General multistep models of a tumor type are deduced from descriptive genetic analysis of tumor and precursor changes, followed by functional experiments. Also HNSCC is preceded by precancerous changes. The precursor stages of HNSCC were first described by Slaughter *et al.* already in 1953, and explained as a process of 'field cancerization' in the upper aerodigestive tract[28]. This field cancerization concept was based on the frequent occurrence of morphologically abnormal tissue surrounding HNSCCs, the existence of multiple tumors in patients, and the persistence of the affected tissue after treatment[29].

The first genetic progression model for HNSCC development was proposed by Califano *et al.* and based on PCR-based microsatellite marker analysis of a subset of chromosomal loci[30]. They studied microscopic abnormalities detected in the mucosal epithelium indicated as dysplasia, and graded as mild, moderate, severe dysplasia or carcinoma *in situ* (CIS). By studying these genetic markers a start of unraveling the molecular basis of mucosal carcinogenesis was made and early genetic events were identified, such as loss of heterozygosity (LOH) at chromosome arms 3p, 9p and 17p. These early changes were detected in benign squamous hyperplastic and dysplastic lesions, while later events such as LOH at chromosomes 11q, 4q, 8p/q, 14q, 13q and 6p were detected in tissue sections defined as CIS and invasive lesions[30]. Generally, the severity of the morphological changes correlates with an increasing number of chromosomal alterations[30,31].

The driver gene located at chromosome 17p, of which the loss of function is identified as an early event in tumorigenesis, is *TP53*. Mutations in *TP53* might be considered as a gatekeeping mutation in HNSCC[7]. Previously, Van Houten *et al.* detected small focal patches of *TP53* mutated cells in surgical margins of HNSCC patients as the apparent very first sign of malignant transformation[32]. These focal patches may progress into larger fields of mutated cells, and finally give rise to cancer[4]. Loss of functional *TP53* together with loss of *CDKN2A* (located at chromosome arm 9p) is most predominantly present, in about 84% and 58% of HPV-negative tumors, respectively[14]. In addition to these two early events, HPV-negative tumors contain many other mutations and chromosomal losses and gains.

BOX 1: Cancer progression. Cancer development starts when a normal primary mucosal cell acquires the first, gatekeeping mutation. This mutation allows the cell to obtain a growth advantage relative to its neighboring cells, the so-called breakthrough phase[26,33,34]. Over time these cells accumulate and form a precancerous lesion. Mutations providing a growth advantage are known as driver mutations. Nowadays, there are about 200 identified driver genes for the common cancer types[33,34]. After acquiring the initial driver mutation, additional hits can give rise to the invasive properties of the affected cell. Once gained, the cell is able to invade into the normal surrounding tissue[34].
Besides mutations in the driver genes, every cell acquires extra mutations as a result of cell division, exogenous factors or other cellular processes. Most of these mutations do not contribute to the development of cancer, and are called passenger mutations[35]. Although these passenger mutations may impact the fitness of the cells and provide neoantigens that can be recognized by immune cells[36]. Next to the point mutations appearing in every cell, several chromosomal changes are present as well. Most solid tumors contain several chromosomal aberrations, losses and gains of parts or whole chromosomes, causing aneuploidy. A third type of alterations accountable for or at least involved in tumor progression are the epigenetic changes. Alterations in methylation status of coding genes can cause an advantage and an increased growth rate as well[33].

Precancerous lesions in the mucosal lining

As described above precancerous fields consist of a group of cells in the mucosal epithelium containing cancer-associated alterations. Typically, these cells still lack invasive growth abilities and consequently do not show metastatic behavior[4]. Some precancerous fields, manifest as visible lesions in the mucosal lining, either as white patches (leukoplakia) or red patches (erythroplakia).
Leukoplakia is defined as a predominantly white plaque of questionable risk, having excluded (other) known diseases or disorders that carry no increased risk for cancer[37]. There are two main subtypes of leukoplakia, designated as homogeneous or non-homogeneous. Especially the non-homogeneous lesions tend to have a higher risk of malignant transformation[38–40]. A third subtype, the proliferative verrucous leukoplakia (PVL), is defined as multifocal leukoplakia. This type of lesion presents as a non-homogeneous plaque with a verrucous keratotic surface. It is most common in women and quite aggressive, since 60-100% of these lesions

progress into oral carcinoma[41,42]. All subtypes together have a prevalence of 1-5% in the general population[43]. Transformation of a leukoplakia lesion into a squamous cell carcinoma occurs in approximately 1-2% of the patients in case of a homogeneous lesion, this percentage is higher for patients with a non-homogeneous lesion[44,45]. In a recent retrospective study at the VU Medical Center, the annual transformation rate was as high as 5.2% (Evren *et al.*, submitted). In general, the most important risk factor for progression of a leukoplakia lesion into a carcinoma is the presence of genetic alterations. Especially, alterations on the chromosomal arms 3p, 9p, 17p and 4q contribute to a high-risk profile. Losses of these regions are indicators for a high risk of tumor progression according to an established risk model[46]. Other factors contributing to a higher transformation risk of a leukoplakia lesion include; female gender, persistence of the lesion, the presence of leukoplakia in a non-smoker, location of the lesion, the size, the presence of C. albicans and the presence of dysplasia[44]. However, these latter risk factors do not emerge in all studies, and variation between studies is large. Assessment and determination of the severity of dysplasia is estimated according to the guidelines of the WHO[47], and changes are graded as mild, moderate or severe dysplasia. Using this classification, 19-46% of the leukoplakia lesions are designated as dysplastic[39,48–51]. Recently, it was shown that a novel form of dysplasia, named differentiated dysplasia, adds to a more accurate prediction of malignant transformation by microscopic examination. This new form was present in 39% of the reviewed samples and together with classical dysplasia adds up to a score of 64% positive for any form of dysplasia[52].
Erythroplakia lesions, are less prevalent than leukoplakia lesions but have a very high transformation rate of around 50%. These lesions mostly contain severe dysplasia, CIS or even show invasive carcinoma already upon diagnosis. Erythroplakia lesions range in size from 1-4 cm and are visible as erythematous areas of the mucosa[53]. In case of a mix of white and red patches, it should be diagnosed as erythroleukoplakia[38].
Next to these visible lesions, it has been deduced that most precancerous changes are macroscopically not visible. This perception is based on genetic studies of surgical margins and mucosal biopsies surrounding tumors[54–57]. Presence of these mucosal changes can be established by use of a microscope in case of the presence of dysplasia. However, even beyond microscopic changes, the mucosal epithelial cells may already contain cancer-associated changes without any morphological changes, albeit this may change with the recognition of the newly defined differentiated dysplasia. In these cases, mucosal abnormalities are only detectable by taking a biopsy from the suspicious area and performing genetic analysis, for instance on tissue from the surgical margins surrounding a tumor, or by brushing suspicious tissue areas in high-risk patients[54,58]. A previous study, with 28 enrolled patients, showed cancer-associated genetic changes in biopsies of ten patients (36%). In seven patients (25%)

the genetically detected fields extended into the surgical margins, indicating that these fields (partially) stayed behind in the patients unnoticed. Interestingly, the identified fields were in part genetically related to the tumors and in part genetically unrelated[54]. Subsequent retrospective studies revealed that these fields characterized by cancer-associated genetic changes explain approximately half of the local relapses in HNSCC patients, and approximately half of the second primary tumors, indicating the major clinical impact of these precancerous changes on the prognosis of the patients[59].

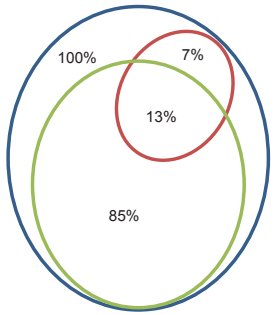

Figure 1: Schematic overview of the various precancerous mucosal changes. All precancerous changes contain HNSCC-associated genetic alterations per definition (100%, blue circle). Most of these precancerous changes are dysplastic (estimated as 85%, green circle) and can be detected using the microscope. About 20% of the precancerous changes are visible as lesions (20%, red circle), of which about two-thirds are dysplastic (13%).

Hence, as indicated in Figure 1, all mucosal precancerous changes contain cancer-associated genetic changes per definition, a subgroup is visible as dysplasia under the microscope, and a smaller subgroup is macroscopically visible. One of the current challenges is to detect all mucosal precancers in patients. Several imaging approaches are being used, since cells undergoing premalignant changes are known to contain changes in their structure and metabolic activity. These may lead to a difference in absorption of fluorescent light, which can be exploited to detect these precancerous changes because of the reduction of autofluorescence upon fluorescent spectroscopy[60]. Further analysis demonstrated the association of loss of autofluorescence with genetic changes at known involved markers, such as chromosome arms 3p and 9p[61].

Treatment of precancerous lesions

Visible lesions with limited dimensions can be removed by surgical excision, laser evaporation or cryo-based surgery[44]. Efficacy of these modalities to prevent cancer in patients is questionable as lesions tend to recur or tumors develop elsewhere[43].

For larger lesions and precancerous changes that are not visible to the naked eye, treatment is currently not an option and clinical management consists of surveillance and watchful waiting. Hence, a treatment intervention is urgently awaited. However, these mucosal precancerous changes are not invasive tumors and although treatment should be effective, toxicity has to be very limited for this preventive strategy. Radiotherapy and cytotoxic chemotherapy are therefore not indicated, alternatively several chemoprevention strategies (BOX 2) have been applied.

BOX 2: Chemoprevention. The use of synthetic, biological or natural agents could be deployed to prevent cancer development. In general, chemoprevention could be divided into three categories. Primary prevention to avoid the development of any precancer or cancer cells in a healthy individual or those with particular risk factors. Secondary prevention to avoid the transformation of precancer cells into cancer cells and tertiary chemoprevention to prevent a recurrence or a second primary tumor after diagnosis and treatment of the initial tumor[62,63]. Chemoprevention acts via several mechanisms, for instance by inducing DNA repair, modulation of signal transduction or induction of apoptosis in premalignant cells[62]. Since prevention strategies are intended to prevent and not to cure there are several eligible characteristics for the agent of choice. Toxicity needs to be manageable, it should be effective at a low dose and easy to administer. In case of HNSCC, several strategies have been explored as cancer prevention strategy in the past.

One of the most studied chemopreventive agents are the retinoids, natural and synthetic vitamin A analogs. Thanks to their role in cell proliferation and differentiation they gained interest as agents for cancer prevention. In several cancer types it was demonstrated that these retinoids are able to suppress cancer progression (reviewed in[64]). Several clinical trials were conducted in patients with oral leukoplakia lesions to reduce and remove the lesions and in some cases as prevention for second primary tumors[65–67]. Interestingly, Hong *et al.* showed positive effects of the retinoids on the prevention of second primary tumors, but they also showed that in the same patients these vitamin A derivatives were not able to prevent local, regional and distant relapses[65]. Although initial results were promising, later trials did not confirm any of the beneficial effects[68,69]. Another tested chemopreventive agent is bleomycin, an antibiotic more often used for the treatment of cancer, with initial promising results of chemopreventive activities in oral cancer[70]. However, the number of studied patients was limited and because of toxicity bleomycin is no longer studied. Another promising study treated 17 patients with high-risk precancerous lesions with cetuximab. This study showed promising results with clinical and histological responses in the treated group. However, a low number of patients was enrolled in the study[71]. A serious limitation of cetuximab is that is has to be administered intravenously at an outpatient center, which is undesirable for a chemopreventive strategy. Other agents tested were inhibitors of cyclooxygenase (COX2), administration of N-acetyl-l-cysteine, an antioxidant, and lycopene[72]. In addition, several other natural compounds

were also described to have chemopreventive properties. For instance, curcumin was shown to have an effect on several molecular pathways known to be involved in cancer development[73]. Phase I and II clinical trials with curcumin demonstrated some effects in cancer prevention, however also showed low absorption rates after administration[74,75].

Previous studies also intended to use oncolytic adenoviruses as strategy by administrating these viruses topically as mouthwash in 19 patients with visible lesions. These viruses are genetically modified and replicate specifically in cells with a p53 mutation[76]. Results of the application of ONYX-015 showed some complete responders, although after termination of the therapy some patients suffered from relapses of the lesions[77]. Meanwhile, the virus has been sold to a Chinese company and convincing clinical results have no longer been reported. The legislation of these genetically modified organisms is obviously complicated and hampers rapid introduction in the clinic.

In conclusion, despite all that has been tried, most particularly for visible lesions, currently there is no standard treatment protocol. Considering the clinical impact on development of cancer and local relapses in patients, treatment options for precancerous changes are urgently awaited. Given the low toxicity profile of targeted agents as a result of their specific mechanism of action, these agents seem most interesting at present.

Development of targeted therapy – precancer cell models

An important prerequisite for therapy development is the availability of cellular models that represent the precursor stage of HNSCC *in vitro* and *in vivo*. *In vitro,* only a few examples of successful cultured models are known today. Most of these cultures were generated from macroscopically visible lesions. Collection of biopsy material of such visible lesions with different stages of dysplasia resulted for instance in a panel of at least 19 cellular models[78–80]. Processing and prolonged culturing of these cells resulted in immortality of 7 out of 19 cultures, when kept on 3T3-feeder layers. These seven cultures all contained a mutation in *TP53*. Moreover, differences in expression of p53 and p16 were observed between the seven immortal and the twelve mortal cultures. This corresponded to the original biopsy material and confirms the importance of alterations in the expression of these two proteins. Furthermore, upregulation of cell cycle regulators and downregulation of differentiation markers was reported. In addition, loss of RAR-β was observed together with increased expression levels of telomerase[79,80].

Culturing cells from invisible precancerous changes is expected to be more complex, since 'random' biopsies should be taken from the oral tissue and cultured. The best source for these precancerous changes is the mucosa surrounding oral cancers. In a

first attempt in patients with HNSCC, biopsies of normal appearing mucosa tissue surrounding primary HNSCCs were collected and cultured[56]. In total, 57% of the collected biopsies were successfully cultured, however, only one culture contained HNSCC-associated genetic alterations when analyzed for the presence of allelic losses using microsatellite markers. The allelic losses of this precancer culture, VU-preSCC-M3, were detected at chromosome arms 3p and 9p, of which the 3p LOH was shared with the corresponding tumor material. In contrast, the LOH at chromosome arm 9p was not detected in the tumor cells. In addition, the precancer cells contained a mutation in *TP53* which was not present in the tumor. These results indicated that VU-preSCC-M3 originated from an independent field in the margin, which did not give rise to the primary tumor outgrowth[56].

Nowadays, there are no well-established precancer mouse or other *in vivo* models for the precursor stages of HNSCC. In some reports 4-nitroquinoline-1-oxide (4-NQO) was used as a carcinogen for inducing oral carcinomas in rodents and thereby mimicking human cancer progression *in vivo*[81,82]. However, the genetic validation of this model is very limited thus far, with just some analyses on the inactivation of p16, using immunohistochemistry[83].

Development of targeted therapy - target identification

In addition to the above described cell models, a larger panel of precancer cell lines from leukoplakias and especially from non-visible precancerous changes has to be established and studied to reflect the heterogeneous nature of the precancerous changes leading to HNSCC. These models can subsequently be used to develop and evaluate new therapeutic strategies. As described, for chemopreventive strategies only minimal toxicity is allowed and the use of targeted approaches seems therefore a promising strategy.

A first step in the development of such targeted treatment is the identification of druggable gene targets in precancer cells. Genomic data for precancerous lesions is hardly available, but as discussed above the invasive tumors contain mainly gene mutations in tumor suppressors and the precancers preceding them will expectantly show the same trend, although with less genetic changes per lesion. Hence, we should exploit functional genomic approaches to identify vulnerabilities of precancer cells. There are several techniques available that allow the identification of genes or pathways crucial for cancer and/or precancer cell survival. Nowadays, CRISPR-Cas systems are a widely used tool for genome editing by introducing double strand breaks (DSB) at specific DNA sites[84]. However, when the projects described in this thesis were initiated, RNA interference (RNAi) was most commonly used and CRISPR was still at its infancy[85].

The RNAi technique is based on small double stranded RNA molecules which are complementary to the transcript of a gene. These molecules recognize and inhibit gene expression by targeting the mRNA and inducing degradation of these transcripts. This appears via the RNA induced silencing complex (RISC) in the cell, the machinery used in microRNA-mediated transcript regulation.

MicroRNAs or miRNAs are small single stranded RNA molecules. These molecules are transcribed as hairpin structures, pri-miRNA, with the mature miRNA sequence in one of the stems. These molecules are then cleaved by Drosha resulting in a stem-loop precursor, pre-miRNA, with a 3′ overhang. Once exported into the cytoplasm the pre-miRNA is then processed by Dicer, resulting in an RNA molecule with double stranded RNA of about 22 nucleotides, the mature miRNA. One of the strands, the guide strand, binds to RISC which is then able to bind to the mRNA and thereby regulates gene expression of the genes containing the miRNA binding site in the 3′ untranslated region (UTR)[86].

In addition to the naturally occurring miRNAs, synthetically developed small interfering RNAs (siRNA) as well as miRNA-mimics make use of the same machinery to modulate gene expression[87]. The DNA equivalent of siRNAs are short hairpin RNAs (shRNAs), synthetic genes which are introduced into the cells by plasmids or viral vectors. Once expressed these molecules are also further processed by the internal miRNA machinery[88]. As they lend more from the miRNA processing machinery, they consequently often behave as miRNAs with many off-target effects. This could occur with synthetic siRNA molecules as well, but in practice is less frequently seen.

The specificity of synthetic siRNAs suggest that they are most interesting for targeting genes also as clinical application. However, the use of RNAi *in vivo* has been proven to be extremely difficult, because of the major obstacle of delivering the RNA molecules into the cells[87]. *In vitro,* it is a relatively simple technique to use for instance in array-based genome-wide screens to identify potential target genes. These loss of function screens are widely used to identify genes that modulate drug responses or to find new targets that could be exploited for drug development[89,90]. To do their work inside the cells, siRNAs need to be transfected usually by encapsulating them in lipophylic agents. Most cell lines allow these transfections, but some are relatively resistant or either sensitive demanding considerable optimizations. These transfections require some manipulation steps, which hamper the upscaling to array-based genome-wide siRNA screens that would encompass all ±20,000 human genes. For this reason, robotic platforms have been developed to allow and standardize these high-throughput screens that are almost impossible to be carried out manually.

Aim and outline of this thesis

Lack of effective therapeutic options for the eradication of precancerous changes results in malignant transformation to primary tumors or local relapses in patients treated for HNSCC. Small visible lesions can be excised, but frequently recur. For larger lesions and precancerous changes that are not visible to the naked eye, there are no treatment options at present. In this thesis we describe the identification of druggable targets that could be exploited to develop treatment modalities, applicable for precancerous changes as well as HNSCC cells. As a first step we generated cellular models of precancerous fields. In **Chapter 2**, we describe the collection and culturing of multiple biopsies from the mucosa surrounding HNSCCs. Proliferating cultures were genetically analyzed. Next, we used the obtained precancer cell models for target identification by performing an RNAi screen with previously identified tumor-lethal siRNAs. *In vitro* and *in vivo* validation with both the siRNAs and subsequent small molecule inhibitors, showed promising results for one of the identified hits (**Chapter 3**). In **Chapters 4** and **5** we investigated other potential hits using both RNAi and available small molecule inhibitors. In addition, we performed an array-based genome-wide screen with miRNA-mimics to identify miRNAs that could be employed for therapeutic strategies of either precancer or HNSCC cells (**Chapter 6**). Finally, the obtained results are discussed in **Chapter 7** and a future perspective is provided.

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