Targeted chemoprevention of head and neck cancer

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Chapter 7

General discussion and future perspectives

Despite application of advanced invasive multimodality treatment protocols, HNSCC patients often develop local relapses, recurrent disease in the neck and distant metastasis. The 5-years survival rate only moderately increased during the past decades[1], especially when the increased prevalence of HPV-induced tumors that have a more favorable prognosis is taken into account. Hence, there seem to be pathobiological barriers to advance the clinical management of HNSCC patients, specifically those with an HPV-negative tumor, and one of these barriers relates to the carcinogenic process.

Since 1953 it is known that primary HPV-negative tumors find their origin in precancerous mucosal changes also coined as fields[2]. When postulating this model, the authors stated: "*From the foregoing observations it would appear that epidermoid carcinoma of the oral stratified squamous epithelium originates by a process of 'field cancerization', in which an area of epithelium has been preconditioned by an as-yet-unknown carcinogenic agent*"[2]. Nowadays, the key carcinogenic agents have been identified, as well as the genetic changes involved in the process of field cancerization and cancer development. Studies by Califano *et al*. and many others resulted in a carcinogenesis model for HNSCC including losses at chromosome sides 3p, 9p and 17p as early events[3–6], and a variety of other changes as late events. In fact, these specific early alterations are considered as most accurate predictors of malignant transformation in precancerous fields visible to the naked eye as lesions[7]. However, the exact process of normal squamous cell replacement by genetically altered cells in the mucosal lining is still an unsolved puzzle. Since specific squamous stem cell markers are not available at present, the process of cell renewal in the oral mucosa is described in less detail as for instance for the intestinal tissue[8], but we may assume and recent data indicate that it is most likely a similar dynamic process in the oral mucosa.

For a long period of time it was assumed that the stem cells in the oral mucosa were organised according to an invariant asymmetry model[9], with hierarchical organization of stem cells, transit amplifying cells and differentiating cells. In this model stem cells in the basal layer divide with a slow rate into a stem cell and a rapidly proliferating transit amplifying cell, and are together with the differentiated cells organized in epithelial progenitor units[10,11]. However, a recent study showed that the oral buccal mucosa is more likely maintained by a population of oral epithelial progenitor cells (OEPC) actively dividing in the basal layer, most likely according to the population asymmetry model with neutral drift dynamics[9]. While another study showed a niche in the hard palate where symmetric divisions appear to maintain the population[12], indicating that there are differences in the various regions of the mucosal lining. How these models relate to carcinogenesis of HNSCC is unclear. For colorectal cancer, a model has been proposed for the effects of mutations on a population of cells by adapting the neutral drift model[13,14]. Effects that were non-neutral, like mutations,

where incorporated in this framework and gave rise to an advantage for mutated cells to replace their neighbouring wild-type cells[8]. This could be due to increased cell cycle rates of these mutated stem cells. Expansion of the mutations outside a certain crypt is in colorectal cancer most likely caused by crypt fission, resulting in some form of field cancerization and the formation of polyps. Interestingly, these proliferation advantages causing crypt replacement are not absolute, explaining why many mutated stem cells might disappear during turnover[14], thereby reducing the risk for colorectal cancer[13–15]. Of note, several other hallmarks of cancer such as overcoming apoptosis were not included in this model yet[8]. Since Jones *et al*. suggested that neutral drift dynamics appear at least in some regions of the oral mucosa as well[9], similar effects of mutations in the oral mucosal lining might be expected. Van Houten *et al*. demonstrated, already in 2002, the presence of patches of mutated p53 in the surgical margins of HNSCC patients, indicating a complete replacement of the clonal units by mutated p53 cells[16]. It was assumed that the stem cell forming this unit was mutated in line with the theories at that time, but in line with the more recent studies a progenitor cell might have gained a mutation and outcompeted the other progenitor cells in that unit. Factors that determine the advantage of mutated cells relative to the wild-type cells are not known for oral mucosal cells, but increased proliferation seems less likely as we showed that proliferation rates of normal oral keratinocytes, precancer and cancer cells are comparable at least *in vitro*[17]. For cells of the skin it was shown that UV exposure stimulated expansion of mutated cells and in esophageal epithelium similar effects were seen after low-dose ionizing radiation or hydroxyl peroxide (H_2O_2) exposure; both induced clonal expansion of p53-mutated cells over p53 wild-type cells[18,19]. Interestingly, it was shown by the latter study that treatment with the antioxidant N-acetyl cysteine (NAC) abolished the competitive advantage of mutated cells after radiation or H_2O_2 exposure. These observations may suggest an alternative way of cancer prevention, by stimulating wild-type cells in favor of mutated cells[19]. However, a large clinical chemoprevention trial in both lung and head and neck cancer patients showed no effect in reducing recurrences or second primary tumors upon treatment with NAC[20]. In theory an attractive strategy, but extrapolation to the clinic is always a challenge, nonetheless it would be important to further unravel the underlying replacement mechanism that drives the field cancerization process in the oral mucosa following genetic changes such as mutations. This might shed more light on alternative prevention strategies.

From the genetic analyses we could deduce that field cancerization can lead to large fields of mutated cells in the head and neck region, up to 10 cm in diameter[6]. Some of these precancerous fields are visible as a leukoplakia or erythroplakia lesion. Other precancerous changes are not visible to the naked eye, although most are microscopically detectable as either classical or differentiated dysplasia[21]. More fields

can be visualized when using autofluorescence or other imaging techniques and all fields are detectable per definition by the presence of cancer-associated genetic changes by analyzing biopsies or brushed cells[4,22–24]. Nowadays the genetic landscape of HNSCC has been unraveled in large detail[25,26], and it becomes increasingly important to study these precancerous changes in parallel, to investigate the natural history of the disease as well as to analyze the impact of these precancerous changes on the clinical management of these patients. As noted, these fields may have dimensions of up to 10 cm in diameter, and when not visible escape detection and may stay behind when patients are treated for the index tumor, causing local relapses and second primary tumors during follow-up. Thus characterization, diagnosis and risk assessment of precancerous mucosal changes remains an important topic of investigation.

However a major issue remains: how to treat these abnormalities? Small visible lesions can be excised, but efficacy is questionable as lesions recur or tumors arise elsewhere. Larger lesions and precancerous mucosal changes not visible to the naked eye, cannot be treated at present, although fluorescence-guided excision showed interesting preliminary results[23]. Hence a logical approach seems systemic drugs, and since many years small molecule inhibitors have been tested in trials, such as retinoids[27,28]. Systemic drugs need to be tolerated well to facilitate long term treatment. However, all previous studies did not lead to implementation: the drugs were not effective and/or too toxic[27,28], and the latter is not acceptable for a chemopreventive strategy. Targeted agents may be much more interesting as they may combine high efficacy with low toxicity.

A prerequisite to develop and test targeted treatments is the availability of cellular models, from visible[29–31] as well as invisible precancerous fields, as now presented in **Chapter 2**. It was already known that *TP53* and *CDKN2A* encoding $p16^{INK4A}$ are frequently affected in precancerous lesions and indeed more extensive genetic analysis of the precancer cell cultures confirmed a frequent loss of 9p (*CDKN2A*) and mutations in *TP53*. In addition, mutations in *NOTCH1* were detected in these early stage cell cultures, a novel finding. In another recent study several cell cultures of solely visible precancer samples have been genetically analyzed[32], with some samples overlapping with our study (all the (erythro)leukoplakia samples included in **Chapter 2**, $n = 5$). The early appearance of *NOTCH1* alterations was observed as well in this study, although *NOTCH1* seemed more often affected by copy number alterations (CNAs) than loss-of-function mutations in their panel[32]. In general, *NOTCH1* inactivation by a mutation is found in about 20% of the HNSCC tumors[25]. The exact role of *NOTCH1* in HNSCC development remains unclear. Knockout of *Notch1* in mice resulted in changes in differentiation of keratinocytes and an increase in proliferation of the cells, probably related to β-catenin activation, which supports

its role as tumor suppressor gene[33]. However, Veeramachaneni *et al*. noticed that many of the CNAs were gain of function changes, confirming a dual oncogenic and tumor suppressive role for *NOTCH1*, as previously already suggested[32,34]. More functional studies to determine the exact role of *NOTCH1* in cancer progression of HNSCC will be required to elucidate this apparent dual role. The remarkable role of *NOTCH1* is further supported by the recent observation in esophageal tissue, where it was shown that aged mucosa demonstrated more mutations in *NOTCH1* than tumors originating from the same area[35].

Besides mutations and CNAs, also epigenetic modifications such as chromatin remodeling and DNA methylation likely play a role in carcinogenesis and tumor progression. Overexpression of methyltransferase 3B (*DNMT3B*) in leukoplakia and erythroplakia lesions, for example, predicts the risk for oral cancer development[36]. *DNMT3B* is a *de novo* DNA methyltransferase and its overexpression is correlated to a CpG island methylator phenotype (CIMP), a phenotype with widespread methylation of CpG islands, in colorectal cancer[37]. The same phenotype is also seen in some HNSCCs[38] and might be an early event in HNSCC development. Indeed, promotor methylation of several genes was increased when comparing normal mucosa samples to samples with severe dysplasia in the oral cavity[39]. In addition, in a study of 24 leukoplakia lesions it was demonstrated that the promotor region of at least three genes was hypermethylated in relation to malignant progression[40]. Moreover, in a meta-analysis of existing literature it was shown that hypermethylation of the promotor region of *CDKN2A* is associated with carcinogenesis as well as metastasis of HNSCC[41]. The same accounts for the opposite state of hypomethylation; *LINE1*, a group of long interspersed nuclear elements, displayed lower levels of methylation in precancerous changes and this hypomethylation state was positively correlated to the risk of tumor development[40]. All these studies indicate a role for epigenetic modifications in oral field cancerization and suggest that epigenetic analysis might be valuable for the risk assessment of precancerous lesions. In addition, targeting these modifications might serve as possible treatment strategy as well[42].

Gradually the molecular background of precancerous changes is being elucidated, although there are still many open questions. As described above, convincing events thus far are the involvement of p53 and $p16^{INK4A}$ alterations in the early stages of progression. Inactivation of both proteins allows major changes in the regulation of the G1/S cell cycle checkpoint resulting in unscheduled replication by the release of cells into the S-phase. This will lead to replication stress usually causing DNA damage, which normally elicits a response by activation of p53, which is now abrogated. Consequently, cells become dependent on S-phase regulation, the G2-M checkpoint and M-phase regulation for solving their replication problems and ensuring proper cell division[26,43]. We demonstrated that multiple regulators of cell cycle progression or related processes

form suitable druggable targets for treatment of precancer cells as well as HNSCC cells (**Chapters 3-5**), as was previous also shown for other cell cycle related targets[44–47].

A major limitation of targeted approaches might be drug resistance, a phenomenon well -studied in cancer cells while information on precancer cells is lacking. Replication stress and genomic instability are already noted in precancer cells[48,49], factors that likely facilitate drug resistance. A solution to circumvent resistance might be combining inhibitors for suitable targets, which perhaps increase efficacy and prevent resistance as has been shown previously in cancer cells[50]. By for instance combining the induced replication stress after gemcitabine treatment as a consequence of decreased dNTPs levels, with the inhibition of the compensatory mechanisms of fork stabilization by targeting ATR, CHK1 and WEE1 with specific inhibitors[51–54], synergistic effects might be obtained[50,54]. A currently almost completed phase I trial showed already effects of low levels of gemcitabine combined with the CHK1 inhibitor SRA737, lowering the toxic effects of gemcitabine treatment by the use of low dosages (study number: NCT02797977)[55]. Combining different inhibitors is upcoming, although caution should be taken towards toxicity. There should be a rationale behind combinations and companion diagnostic biomarkers to predict which patient group would benefit are desirable, especially for chemopreventive strategies.

With an efficient delivery system to specifically target cancer or precancer cells, the RNAi tools that we exploited could also be used as therapeutic agents. siRNAs are very specific small molecule inhibitors and miRNAs with a broader spectrum of activity could also qualify to target precancer cells. Candidate miRNAs mimics that are lethal to precancer cells are on the shelf (**Chapter 6**), but await additional validation. However, despite many years of research, treatment strategies using RNAi molecules are still in its infancy. Many variants of nanoparticles have been investigated for *in vivo* delivery of siRNA and miRNA molecules[56,57]. Ideally, these particles need to be able to protect the molecules from degradation and deliver the RNA molecules to the tumor or precancerous lesion[58]. Results with the first FDA-approved siRNA-based inhibitor, Patisiran, targeting transthyretin (TTR) in patients with hereditary TTR-mediated amyloidosis demonstrated only mild to moderate adverse effects, providing confidence for more successful clinical applications of RNAi[59]. Patisiran was followed by FDA and EMA approval for Givosiran that targets aminolevulinate synthase 1 (ALAS1) in patients with acute hepatic porphyria[60]. Others, such as Inclisiran that results in sustained reduction of low-density lipoprotein cholesterol levels by targeting proprotein convertase subtilisin–kexin type 9 (PCSK9)[61], are in an advanced stage of clinical development. However, in general siRNA and miRNA applications in the clinical setting have been shown to be extremely complex and ineffective. If this could be solved, there are many siRNAs and miRNAs on the shelf that could be employed to treat cancer and precancer, alone or in combination.

As described above *in vitro* precancer cell models, established as reported in this thesis (**Chapter 2**) and previous publications[30,31,62], have been instrumental to identify druggable gene targets. To translate the results to clinical studies, an *in vivo* model would be important. Our precancer cultures did not show growth in immuno-deficient mice, in fact an important indicator for the precancerous state of these cultures. However, the lack of an *in vivo* model obviously hampers preclinical drug testing as chemopreventive strategy. There are mouse models used for studying oral tumor development by using 4-NQO on the tongue, but these models are somewhat unpredictable and have been poorly genetically characterized. In addition, the tumors that develop inhibit food intake of the mice, causing animal suffering and hampering exploitation of such models. As an alternative, we focused on the chick embryo chorioallantoic membrane model (CAM-model), but for precancer cells success was limited while we and others demonstrated that it is successful for HNSCC cell lines[63,64].

Besides application of experimental drugs, drug repurposing might form an alternative and more efficient strategy, and fortunately, for some of the promising candidate genes identified in **Chapters 3-5**, inhibitors are available and already tested in clinical trials for HNSCC or other cancer types[65–68]. Currently, there are active trials ongoing for all three targets described in the subsequent chapters[69]. However, all drugs identified as promising agents are still experimental, and we depend on the registration of these agents for specific indications to allow repurposing. Currently, we depend on the pharmaceutical industry to initiate clinical chemoprevention studies to study efficacy and safety.

Which patients would form the ideal target population for a chemopreventive strategy? In fact, all patients with a precancerous mucosal change would qualify. For chemopreventive purposes, inhibitors have to be available for oral or topical administration[70]. In addition, treatment should have very limited toxic effects. On the other hand, we can define a high-risk group for cancer development using a simple 3p, 9p and 17p loss of heterozygosity analysis by microsatellite PCR, allowing some toxicity of the treatment regimen. A first HNSCC-preventive clinical trial should therefore focus on leukoplakia patients with genetic changes present in their lesion most particularly chromosomal changes of 3p, 9p and 17p[7], and on treated oral cancer patients with confirmed precancerous changes in the surgical margins with similar genetic changes. The risk for such patients to develop cancer or recurrent disease within five years may be 60%[7], allowing an efficient trial design and allowing some toxicity considering the high risk for tumor development. Phase I/II trials should focus on dose finding and efficacy assessment by intermediate endpoints such as a decrease in the dimensions of the visible lesions, decreasing histological grades and disappearance of genetic changes in the surgical scar. The latter could be assessed

using brushing strategies[24]. In a randomized phase III trial, cancer or recurrence development should be taken as clinical endpoint. These trials are well feasible as there are no effective approved competing treatments at present. A brush test to identify invisible changes could be an interesting companion diagnostic tool to select patients from other high-risk groups such as Fanconi anemia patients, but also heavy smokers at increased age apply. As mentioned in the introduction of this thesis (**Chapter 1**) patients with HPV-positive tumors were not part of the main focus, mainly because of the relative favorable prognosis for this group of patients. In addition, thus far there are no indications that field cancerization forms an important process prior to HPV-induced tumor development, at least not in non-dysplastic tissue and dysplasia is rarely seen[26,71,72].

In our opinion, clinical trials with one of the proposed inhibitors that specifically inhibit precancer cells *in vitro* seems the next logical step. Obviously clinical trials are expensive, time consuming and cost a lot of effort. Also the toxicities of the drugs, that might have to be taken for a long period, is a serious issue for consideration. The respective companies have been approached, but this has not resulted in the initiation of trial designs so far. The fate of many promising inhibitors is unclear at present, since the large number of immunotherapy trials restrains available resources. Although further research is necessary, the clinical need is obvious, the promising inhibitors are on the shelf and the biomarkers to select the patients are ready.

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