Addendum

Discussion and future perspective
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Nederlandse samenvatting
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Dankwoord
DISCUSSION AND FUTURE PERSPECTIVE

While head and neck squamous cell carcinoma (HNSCC) incidence is generally increasing over the past decades, overall survival has only increased modestly². New therapeutic approaches are emerging, most particularly the immune checkpoint inhibitors, but efficacy of these are still limited at present³.⁵⁰⁹.¹³⁷. Hence, effective therapeutic approaches to improve 5-years survival of HNSCC cancer patients with less toxicity than currently applied chemotherapeutic agents are still urgently awaited. Tumor-specific targeted treatment approaches seem a promising option. However, discovery of druggable cancer vulnerabilities is complicated by the lack of driving oncogene mutations in HNSCC⁵. This implies that targeted approaches need to be based on synthetic and collateral lethality to indirectly exploit tumor suppressor gene inactivation⁵,¹⁴¹,¹⁴².

Genomic profiling of a large cohort of HNSCC tumors as well as unbiased genome-wide functional genetic screening techniques⁵,³⁶⁷ shed new light on the genetic vulnerabilities of HNSCC-derived cells and emphasized the rewired cell cycle regulation, particularly in HPV-negative HNSCC. Commonly, cell cycle deregulation is linked to loss of chromosomal arm 9p21.1 containing the CDKN2A gene, amplification of 11q13 and overexpression of cyclin D1, as well as frequent mutations in TP53. The combination of alterations leads to a deregulation of the G1/S-checkpoint, which renders cells dependent on adaptive mechanisms to tolerate DNA replication stress resulting from unscheduled S-phase entry, and a tightly controlled transition from G2-phase into mitosis. Inhibition of proteins that obtained partly new functional roles in these aspects of cell cycle control such as Chk1 (Chapter 3 and ¹³⁹,³⁰⁴,³¹⁶–³²⁰), Wee1 (Chapter 4 and ¹¹⁰,³²⁷,¹²³,³³⁴–³³⁷), RNR complex components RRM1 and RRM2 (Chapter 5), or mitotic regulators as PLK1, CKAP5, SGO1, CDCAS and KIF11 (Chapter 7 and ¹²⁹,³³⁸,³³³,³⁵³,³⁵⁷) result in activation of remaining cell cycle checkpoints, accumulation of DNA damage and subsequent cell death. Despite proper proliferation in short term cultures³³³, primary non-transformed oral keratinocytes and fibroblasts are less susceptible to inhibition of these proteins and their associated pathways in vitro, most likely due to the stringent cell cycle regulation and genomic integrity (this thesis and ¹³¹,³⁵³). The uncontrolled transition to S-phase causes replication stress and DNA damage in cancer cells, which is not observed in normal cells (Chapters 3 and 4). These data indicate that interference with cell cycle progression has the potential to specifically target malignant cells and perhaps also premalignant cells, without considerably affecting normal somatic cells. Clinical studies revealed that targeting Wee1 or Chk1 in patients is indeed well tolerated in clinical trials (Chapter 1 and ¹³⁷,³³⁸,³⁴⁰,⁴¹¹–⁴¹³,³⁴²,³⁴⁵,³⁴⁶). Grade 3 or grade 3-4 toxicities are reported in the first 7 days of treatment, but this observation is an overestimate of experimental drug toxicity, since in the large majority of these clinical trials combination treatments with other (chemo-)therapeutic agents such as gemcitabine, cisplatin, or the EGFR targeting antibody cetuximab, are investigated. This makes it difficult to deduce what the real contribution to toxicity was of the targeted drugs (Chapter 1 and ³⁴⁶). Continuing this line of clinical research and novel clinical trials will be required to reveal these matters, and also to investigate for instance whether combining targeted inhibitors with radiotherapy could reveal clinical utility.
In previous research projects, we and others showed that HNSCC and premalignant cells are vulnerable to inhibition of PLK1, another regulator of the cell cycle particularly during mitosis [191,195,331–333]. The commonly used Wee1 inhibitor AZD1775, or Adavosertib (Chapter 4), might also target PLK1 with significant affinity, although reports are somewhat contradicting [207,216,419]. Nonetheless, combined inhibition of Wee1 and PLK1 might be even more potent considering their lethal effect on HNSCC (Chapter 4 and 333).

Besides these cell cycle controlling kinases and their inhibitors, we identified the RNR complex as a tumor-specific target in HNSCC. Gemcitabine is a well-known inhibitor of the RNR complex as well as a cytidine analogue, and has been clinically applied in the first-line treatment of ovarian, breast and pancreatic cancer [126–128]. Although resistance in cell lines has been reported (Chapter 5) as well as disparate responses in clinical trials have been observed in HNSCC patients with gemcitabine monotherapy [463,520,521], the identification of a biomarker for response might reinitiate clinical trials in HNSCC patients not responding to cisplatin, such as elderly patients and Fanconi anemia patients for whom cisplatin is contra-indicated [56,526,527]. It is striking that in an initial phase I clinical trial, the responses in patients with HNSCC were similar to ovarian cancer but gemcitabine was never investigated in subsequent phase II and III trials for the treatment of HNSCC [60].

Furthermore, we described the identification of other target molecules relating to cell cycle control, particularly to mitosis. Unfortunately, the lack of relevant targeted inhibitors of important proteins involved in the anaphase promoting complex (APC/C) that often show increased expression, hampers clinical applications at present (Chapter 7). Taxanes such as paclitaxel, an approved taxane-based chemotherapy used in breast cancer, binds to microtubules and thereby prevent proper chromosomal attachment to the mitotic spindle [191,516]. Since taxanes may perturb microtubule-kinetochore attachments in all dividing cells and impacts the microtubule network in neural cells, acute grade 3-4 toxicities are common. Nonetheless, a subgroup of HNSCC seem to benefit from taxane treatment, and this might be worth pursuing in HNSCC, awaiting more specific targeted agents for mitotic proteins (an overview of HNSCC taxane studies are given in 199).

Somewhat unexpectedly, we found that HPV-positive HNSCC cell lines were less vulnerable to disruption of Chk1, Wee1, RNR complex and mitotic regulators (Chapter 2, 3 and 5), while others studied these proteins in a panel of HPV-positive HNSCC cell lines and concluded that those were susceptible to Chk1 and Wee1 inhibition compared to responses of normal fibroblasts [116,321]. High risk HPV genes E6 and E7 disturb the proper function of p53 and pRb proteins, which theoretically leads to a comparable deregulation of the G1-checkpoint as a TP53 mutation and CDKN2A loss in HPV-negative HNSCC. Furthermore, comparable chromosomal aneuploidy profiles are found in HPV-negative and HPV-positive HNSCC, indicating genetic instability in both subgroups (Chapter 2). Analysis of the HNSCC PanCancer cohort [373] comprising 523 molecularly characterized tumors revealed that the mutational load between carcinogen-induced HPV-negative HNSCC (n = 415) and HPV-positive HNSCC (n = 72) was near significantly different (Figure 1, 36 tumors had an unspecified HPV-status, mutational load or gender specification). HPV-positive HNSCC exhibited about a third less mutations than HPV-negative HNSCC (HPV-negative mean = 107 vs HPV-positive mean = 62, two-sided t-test = 0.08). Considering that HPV-induced tumors may have been less exposed to carcinogens, the degradation of p53 and pRb by HPV viral oncoproteins would presumably lead to comparable replication stress as observed in carcinogen-induced HNSCC, which should hypothetically lead to a comparable vulnerability to cell cycle targeting agents such as Chk1 and Wee1 inhibitors in HPV-positive HNSCC. Nonetheless, we did observe reduced susceptibility to targeted S- and G2-phase progression inhibitors. This indicates that these extrapolations are too simple. Further insights in replication stress pathways in the context of HPV-mediated HNSCC are needed to understand the underlying mechanism.

Cell lines from a second subgroup of HPV-negative HNSCC were included in this research, those developing in patients with the inheritable genomic instability syndrome Fanconi anemia (FA). Despite their seemingly different etiology, the FA cell lines seemed to respond similarly to RNA interference and small molecule inhibitors as the sporadic non-FA HNSCC lines except for DNA crosslinkers (Chapters 3, 4, 5) [106]. Since FA-cells carry a homzygous mutation in one of the FA/BRCA genes, they are hypersensitive to interstrand crosslinking agents such as mitomycin C and cisplatin. Consequently, these patients cannot be treated by standard HNSCC treatment protocols as all their somatic cells contain this deficiency and are sensitive to these agents, causing high toxicity [41,54]. To improve clinical management of
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2.0 Further genetic characterization will be necessary to confirm the HPV-status and CN-silent classification of these cell lines.

Evading apoptosis is a common cancer cell mechanism to maintain cell proliferation in the background of genomic instability 133,134, and is likely caused by gene mutations in TP53 or CASP8 in HNSCC 1,486,154 (Chapters 2, 3 and 6). Apoptosis resistance may obviously contribute to resistance to chemotherapy or targeted treatment approaches 135,136. Proteins associated with the mitochondrial membrane maintain the balance between anti-apoptotic and pro-apoptotic signaling 493,492,497. Targeting anti-apoptotic protein Bcl-2 is well studied in hematological malignancies 482,492,537. Recently, another anti-apoptotic Bcl-2 family member, Mcl-1, has been described as a lethal pan-cancer target 367,483, confirming our findings for HNSCC by siRNA genome-wide screening (Chapter 6). Surprisingly, the majority of HNSCC cell lines seem to rely heavily on Mcl-1 expression, and not on Bcl-2. Sensitivity to Mcl-1 inhibition correlated with Mcl-1 protein induction upon treatment with small molecule inhibitor S63845. Ten oral cavity tumor specimens were negative for Bcl-2, but did express both anti-apoptotic proteins Mcl-1 and Bcl-xl.

Targeted treatment approaches have been investigated for over a decade in cancer in general and head and neck cancer specifically, although for HNSCC no major successes were obtained so far in the clinic. It is disappointing that the efforts of the TCGA to comprehensively map the genomic drivers of HNSCC once more predominantly revealed mutations in tumor suppressor genes 1. With the recent successful implementation of PD-(L)1 antibodies in the clinic, the interest shifted towards immunotherapy 127,128,133,136,137. Nonetheless, only a subgroup of (HNSCC) patients benefit from these immunotherapies, potentially due to the lack of neo-antigens or an immune suppressive tumor environment as observed in many cancers as well as HNSCC 96,135,538. Attempts to combine immunotherapy with other treatments increased the overall survival and progression-free survival in non-small cell lung cancer, but also induced more toxicity in the patient 130-132. Functional genomic approaches, such as RNA interference screens and CRISPR-Cas9 genome editing, have indicated that cancer cells as well premalignant cells have acquired many vulnerabilities related to synthetic lethal and collateral lethal interactions that can be exploited (Chapters 3, 4, 5, 6, 7 and 130,137). Particularly for premalignant fields in the head and neck region, targeted treatments are an interesting approach to prevent tumors and recurrences, as immunotherapy might not be a first choice. The research presented in this thesis clearly indicates that multiple targeted treatment approaches deserve further exploring in the clinical setting. The standard chemotherapeutic agent applied in HNSCC is cisplatin, which combined with concomitant radiation therapy is effective, but the survival benefit has been estimated as a mere 6.5% and the treatment comes with significant side effects 493,542. Chemo-radiotherapy induces even more severe adverse effects, and does not seem to have added value for the elderly patient 130,137. In addition, for some patients such as Fanconi anemia patients, cisplatin is contra-indicated. Enabling more precise induction of DNA damage or destabilization of cell cycle control with targeted agents, would be a promising follow-up approach in the treatment of HNSCC. Furthermore, with the new application of immunotherapy in...
HNSCC, further research should focus on combined application of cell cycle targeting agents and PD-(L)1 inhibitors. First reports indicate that PD-L1 expression differs between cell cycle phases, and arresting cells in G1 by CDK4/6 inhibitors might enhance the potency of immunotherapy. Additionally, growing evidence points towards an increased immune response by DNA double strand breaks through ATM activation and signaling, which might lead to new therapeutic possibilities for DNA damage inducing small molecules combined with immunotherapy. Together, this illustrates that the use of targeted agents might not only have potential as monotherapy or in combination with radiotherapy, but there is probably also a role for targeted agents that affect DNA damage and cell cycle regulation in combination with immunotherapy approaches.

Prominent problems at present in HNSCC are the lack of biomarkers for current therapies such as chemo-radiation with cisplatin, bio-radiation with cetuximab, or immunotherapy. Replacing cisplatin for an alternative such as gemcitabine in selected cases becomes particularly interesting when robust biomarkers would be available. Another and perhaps related major challenge in HNSCC in this respect is the molecular heterogeneity between tumors both on the descriptive as the functional genomics level (this thesis), which results in differential responses to applied treatments. Moreover, recent data suggest the existence of major intra-tumoral genetic heterogeneity as well, which likely affects response to chemoradiotherapy, but may also affect targeted treatment efficacy. Molecular distinct sub-clones in tumors may respond very differently to treatment, and a recent observation that half of the recurrences after chemo-radiation for cure are caused by sub-clones that existed in less than 1% of the bulk tumor indicates the major challenges we are still facing.

In terms of functional differences between distinct tumors, the cell cycle control is rewired at many levels and using different molecular strategies. More extensive knowledge of cell cycle progression and especially DNA replication is needed to anticipate on the molecular mechanisms involved in responses to cisplatin and gemcitabine, as well as targeted inhibitors of Chk1, Wee1 and the RNR complex. Better understanding of both the mechanisms and signaling pathways involved would enable biomarker identification to stratify patients and new insights in drug combinations to pursue. Also, the recent developments of proteome-based techniques like iPOND (identification of proteins on nascent DNA) and NCC (nascent chromatin capture), the genomic detection of DNA breaks using sequencing-based methods like BriTl (breaks identified by TdT Labeling) and qDSB-Seq (quantitative double strand break sequencing), and techniques to quantify origin firing and DNA replication progression by Repli-Seq, will soon expand our knowledge of DNA replication dynamics in both malignant and nonmalignant cells. These could also provide crucial insights into the biological phenomena that underlie sensitivity or resistance to targeted molecules of S-phase regulators, and provide footholds for effective targeted combination therapies.

Altogether, ongoing cancer biological research will hopefully contribute to the identification of predictive biomarkers and novel targets, and will enable more clinical trial designs directed at cell cycle therapeutic approaches.