Addendum

Discussion and future perspective

Summary

Nederlandse samenvatting

Reference list

Curriculum Vitae

Dankwoord
SUMMARY

Head and neck squamous cell carcinoma (HNSCC) arises in the mucosal epithelium lining the upper aero-digestive tract. The known risk factors for HNSCC development are carcinogen exposure from tobacco and alcohol, or an infection with a high risk type of the human papillomavirus (hrHPV). In addition, individuals born with the inherited genomic instability syndrome Fanconi anemia (FA) are at high risk to develop HNSCC even as a young adult. Standard treatment protocols are based on tumor stage, site and histology findings and comprise in more advanced stages upfront platinum-based chemotherapy combined with concomitant radiotherapy, also coined as chemo-radiation, with surgical salvage when needed as well as upfront surgery with postoperative (chemo-)radiotherapy. Chemo-radiation induces DNA damage particularly when administered concurrently, which also causes grade 3-4 toxicity to the patient. The added value of cisplatin to radiotherapy alone on the 5-years survival is on average 6.5% and at the cost of serious toxicity. Taken into account that the 5-years survival of HNSCC is about 60%, it is clear that novel therapeutic approaches are awaited, aimed to be more effective in targeting the tumor while being less toxic to the patient. Previous sequencing efforts by the TCGA consortium revealed that there are no actionable mutations in HNSCC, except for PIK3CA mutations in a small subgroup. Hence, other vulnerabilities of HNSCC have to be identified by focusing on the known perturbed cellular functions such as cell cycle regulation, or by unbiased genome-wide functional genomics.

In this thesis, multiple genes involved in cell cycle regulation and DNA damage repair have been identified as putative therapeutic targets for the treatment of HNSCC. The carcinogenesis of HNSCC is typically characterized by the loss of function of two important cell cycle regulators, TP53 and CDKN2A, either by genomic mutations, chromosomal loss, or by a transforming infection with HPV. In Chapter 2, genetic characterization of several commonly used HNSCC cell lines confirmed that these established cell lines are genetically very comparable to the HNSCC tumors characterized by the TCGA consortium. In the majority of HPV-negative HNSCC cell lines, TP53 is mutated. In addition, CDKN2A is frequently mutated, lost or methylated. Furthermore, based on genetic characteristics, FA-derived cell lines showed large similarities with sporadic HPV-negative HNSCC, although these tumors are caused by genomic instability induced by a dysfunctional FA/BRCA pathway rather than by exposure to carcinogenic compounds.

Furthermore, an unclassical subgroup of HPV-negative HNSCC tumors is described in literature with low copy number alterations and with a seemingly functional p53-pathway, and for the first time a cell line model was identified of this subgroup; VU-SCC-040 (Chapter 2). By functional assays it was concluded that the canonical p53-pathway seems intact, although p53 has many functions. It is assumed that p53 wild-type cells are more sensitive to cisplatin and less sensitive to inhibitors targeting Chk1 and Wee1. Surprisingly, this cell line was found sensitive to both cisplatin, Chk1 and Wee1 inhibitors, and sensitivity was not altered when the TP53 gene was knocked-out using CRISPR/Cas9. This indicates that
other alterations such as loss of CDKN2A might already induce sufficient cell cycle rewiring to become vulnerable to these targeted cell cycle inhibitors. In *Chapters 3, 4, 5 and 7*, hits identified in previously performed array-based genome-wide RNA interference screens were selected for further investigation, with emphasis on regulators of different phases of the cell cycle. Chk1 (*Chapter 3*) regulates S-phase progression, and knockdown of RNA expression with siRNAs or kinase inhibitors induced DNA damage accumulation in all tested HNSCC cell lines, but not in non-transformed oral fibroblasts and keratinocytes. However, the vulnerability to Chk1 inhibition was not equal for all tested cell lines, which seemed to correlate with basal CDK1 expression, and remarkably, also the initiated mechanism of cell death. Chk1 inhibition causes DNA damage induction in S-phase, whereupon cells accumulated in S-phase. Sensitive cell lines with low CDK1 protein levels activated the apoptotic cell death cascade through activation of caspase 2/3/7. In contrast, more resistant cell lines with high CDK1 progressed towards G2 and subsequent mitosis, but the obtained DNA damage caused chromosomal breakage followed by mitotic catastrophe. Although prominent CDK1 expression levels seemed to correlate with resistance, combined targeting of CDK1 and Chk1 reversed the effects of Chk1 inhibition alone, as cells became arrested in G2 due to low CDK1 activity. Contrariwise, progressing the cell cycle by adding a Wee1 inhibitor increased sensitivity to Chk1 inhibition as expected. Wee1 is involved in the inhibition of CDK1 and thereby is the major regulator of G2-to-M transition (*Chapter 4*). However, it is now well accepted that in the context of DNA replication stress and genomic instability, Wee1 becomes important for prevention of mitotic entry by reassurance of the inhibitory CDK1 phosphorylation. In case of stalled replication forks, CDK1 is slightly dephosphorylated to enable late origin firing. Inhibition of Wee1 was very potent in all tested HNSCC cell lines as well as premalignant cells. HNSCC cell lines were generally even more vulnerable to Wee1 inhibition as compared to the tested ovarian cancer cell lines, of which great vulnerability to Wee1 inhibition is known and shown in clinical trials. The functional consequence of Wee1 inhibition in (pre-)HNSCC is complete deregulation of the cell cycle, and analysis of mitotic spindle formation during metaphase revealed spindle exhaustion in both premalignant and malignant cells, causing severe mitotic problems and death in mitosis. Besides inhibition of cell cycle regulators, the knockdown of the RNR complex was also found to be lethal in a subset of HNSCC cell lines (*Chapter 5*). The clinically used chemotherapeutic gemcitabine is both known as a RNR complex inhibitor and a nucleotide analogue, and although pre-clinical studies showed responses to gemcitabine in at least a subgroup of HNSCC patients, it was never approved for application in HNSCC. Several Fanconi anemia (FA)-derived HNSCC cell lines were vulnerable to gemcitabine treatment while FA-derived fibroblasts were resistant, indicating that there is no synthetic lethal interaction between FA/BRCA mutations and interference with RRMI and RRM2. Further research in FA-deficient murine models is needed to monitor toxicity, but this indicates a potential application of gemcitabine in FA-patients, since cisplatin treatment is contra-indicated for these patients. The major factor that might hamper the clinical utility of gemcitabine at present, is the lack of a biomarker to predict response. The results in *Chapters 3 to 5* suggested a high vulnerability of HNSCC cells for cell cycle perturbation, especially when S-phase progression and G2/M regulation becomes disturbed. Therefore, we investigated the suitability of other CDKs and Cyclins, important drivers of cell cycle progression, as druggable targets in *Chapter 7*. The RNA interference of these other CDKs and Cyclins did not result in lethality of HNSCC cells. Moreover, (pre-)HNSCC cell lines were also not susceptible to combined CDK4/6 inhibition, which might relate to the high functional redundancy between different CDKs. Furthermore, several mitotic regulators shown to be essential in a previously conducted genome-wide siRNA screen, were indeed CKAP5, SGO1 and CDCA5 resulted in decreased cell viability in HPV-negative cell lines (*Chapter 7*). CKAP5 and its association with microtubule assembly and bi-polar spindle formation, the involvement of SGO1 and CDCA5 in sister chromatid cohesion might shed more light on mitotic regulation in a background of genomic instability. A limitation at present is that there are no clinically relevant small molecule inhibitors available targeting these proteins. HNSCC and other cancers often display apoptotic resistance. Therefore, we mined data of the existing array-based genome-wide screens for vulnerability to interference of anti-apoptotic genes, and revealed BCL2-family member MCL1 as an essential protein for HNSCC (*Chapter 6*). Inhibition of Mcl-1 induced high levels of apoptosis in a subset of HNSCC cell lines, which related to the initiation of increased Mcl-1 protein expression after treatment. Resistant cell lines had a lower induction of Mcl-1 after treatment, suggesting that sensitivity to Mcl-1 inhibition is associated with a positive feedback loop. In ten oral cavity specimen, BCL2-family members Mcl-1 and Bcl-xL were prevalently expressed, however, all tumors were negative for Bcl-2 expression. Artificial overexpression of Bcl-2 expression. Artificial overexpression of Bcl-2 induced resistance to Mcl-1 treatment, indicating a potential resistance mechanism. Together, the results presented in this thesis demonstrate the high dependency of HNSCC and premalignant cells on DNA replication in S-phase, in a background of DNA replication stress and genomic instability. However, tumor heterogeneity and the lack of clinically applicable biomarkers have complicated preclinical studies and subsequent clinical implementation so far. Future research should aim at better understanding of the cell cycle rewiring and DNA replication in the various cancer cells in general and HNSCC particularly. Mechanistic insight in cell cycle regulation and DNA replication will be required for the identification and development of new therapeutic approaches as the head and neck cancer drivers are mainly tumor suppressive genes.

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