



Chapter 8
General discussion
and future perspectives.

There are several treatment protocols available for advanced stage head and neck cancer, including surgery combined with postoperative radiotherapy, chemoradiation (the concomitant application of systemic cisplatin and radiotherapy), and bioradiation, the combination of systemic cetuximab with radiotherapy. Unfortunately, all treatment protocols come at cost of marked toxicity and impact on quality of life, and tumors respond differently to the various treatment modalities. For this reason, research is focused for many years on the personalization of cancer treatment. The idea is that by examining specific molecular properties of the tumor, a patient would receive only the treatment that might successfully eradicate his/her tumor. A lot of effort has been put in marker identification to predict treatment response upfront. These studies have also been performed for head and neck squamous cell carcinoma (HNSCC)⁽¹⁻⁴⁾, mainly using expression microarrays. Unfortunately, this has not yet led to the discovery of molecular profiles that help to personalize HNSCC therapy.

A shortcoming of techniques that search for genetic profiles in a panel of tumors, like microarray analysis, is that differences among genes related to tumor biology may dominate over the few genes that correlate to therapy response. In addition, most studies focus on patients who were treated with multimodality therapy (chemotherapy and radiation). Microarray analysis of multimodality treated tumors might therefore mix up and mask the genes of interest. In addition, resulting profiles are a reflection of the gene expression of the majority of tumor cells, while a specific subclone or subpopulation with special characteristics, i.e. the cancer stem cells (CSCs), determines treatment response. This is possibly the reason that validated predictive profiles have not been found. Therefore we chose to investigate the genes that are involved in only one treatment (cisplatin) with a technique that analyzes the effect of every gene separately (siRNA transfection).

LESSONS LEARNED FROM HIGH-THROUGHPUT SCREENS

Small interfering RNA screens

siRNA screens have proven to be a successful and unbiased approach to elucidate genes that modulate drug response⁽⁵⁻¹⁴⁾. However, a carefully designed assay is crucial for a successful screen. It is known that siRNA transfections are accompanied with off-target effects, which might be the result of the shared processing machinery with miRNAs, which naturally target multiple mRNA transcripts. In addition, the efficiency of gene knockdown might vary. Finally, there is always noise in the data resulting from large screens. Together, this may lead to a high number of false-positive and false-negative results, especially in large genome-wide siRNA screens. One approach to diminish the problem of false-positives and false-negatives is to use pools of siRNAs that target different sequences within the same target gene. This might on one hand increase the knockdown and on the other hand might also help to make the effect on the gene of interest rule over a weak off-target effect of a single siRNA. Validation of the phenotype can thereafter be done using the separate siRNAs (i.e. deconvolution of the siRNA pool) and by association of the mRNA knockdown with the phenotype. The problem of false-negative results by inappropriate knockdown is harder to tackle, and could only be solved by using newly designed siRNAs. Also the noise in the data will cause false-positive and false-negative results. This problem was demonstrated when we combined the results from genome-wide siRNA screens obtained in different tumor types (Chapter 4). The accumulation of hits pointed to the same biological processes, but many more genes were identified. Moreover, in later validation experiments we could confirm siRNA hits that were missed in the initial genome-wide screen (unpublished data). A simple solution might be to decrease the number of siRNAs in a screen, but this will also reduce the possibility for interesting unexpected findings. Another simple solution is to increase the number of replicates. We used duplicate screens, and triplicates will already increase the statistical power. Because of the workload this requires a set-up in 384 well plates, and we recently succeeded in doing so (De Lange *et al.* Manuscript in preparation).

Data analysis

Another problem implemented with high-throughput screens is the massive amount of data that is produced. Besides extraction of the desired hit list, it is of importance to diminish all sources of noise, including technical errors. Data normalization reduces variations caused by medium evaporation at the edges of plates (within-plate normalization) and systemic plate-to-plate variation (across-plate normalization). Many statistical methods and data normalization procedures have been published⁽¹⁵⁻²¹⁾, but the interpretation of the researcher eventually decides which genes are of interest, and these are not necessarily in the top-20. Especially with the appearance of freely available online gene classification databases (like KEGG, STRING, DAVID, and PANTHER) interpretation of siRNA screen results tend to focus more and more on pathways and biological processes, instead of a single gene. By doing so, false-negative results can be retrieved by examining all genes that are annotated to a pathway of interest. In addition, new genes might be linked to existing pathways or cellular processes, as we noted for SHFM1 and its important role in cisplatin-induced DNA repair (Chapter 3).

MicroRNAs

The siRNA technique is very useful when investigating the cellular effect of one particular gene under certain conditions. However, it is very well possible that not just one gene is responsible for a specific phenotype^(22,23). Moreover, multiple genes may have redundant functions, and knockdown of only one gene may not elicit a phenotype. The application of miRNAs makes it possible to examine the inhibition of a multitude of genes at the same time, and this might result in a lethal phenotype that is very specific for tumor cells. This gene-cluster approach is unfortunately also the limitation of the technique, as inhibition might not be as effective for every gene and it is not immediately clear which genes are responsible for the observed phenotype. MiRNA target identification is difficult, time consuming, and might severely delay the identification of possible drug targets. Furthermore, high expression of ectopically introduced miRNA genes might block processing of endogenous miRNAs, which results in off-target effects. For this reason it might be preferred not to use miRNA gene libraries in viral vectors but to use synthetic miRNAs, since these only require processing by Dicer, like siRNAs.

FUTURE PERSPECTIVES

Biomarkers of response

In our search for determinants of cisplatin sensitivity (Chapter 2), we found that the only factor that significantly correlated with the IC_{50} value was the amount of platinum-DNA adducts. In addition, our genome-wide siRNA screen showed that knockdown of multiple members of the Fanconi anemia/BRCA DNA repair pathway (FA/BRCA pathway), which is responsible for the repair of cisplatin-induced DNA crosslinks, sensitized HNSCC cells to cisplatin (Chapter 3). These results imply that effective cisplatin treatment is characterized by the appearance of many platinum-DNA adducts and the inability to repair this damage.

In light of personalizing chemoradiation, it might be an option to determine the amount of platinum-DNA adducts in a 'pilot cisplatin treatment' prior to starting the actual chemoradiation scheme. This might be possible by exposing patients to a single dose of radiolabeled cisplatin. The label makes it possible to trace the platinum compound and to study, based on the amount of DNA-bound platinum in the tumor, whether or not a patient would benefit from chemoradiation or the addition of cisplatin as such. Furthermore, it might be valuable to examine if the FA/BRCA pathway is functional and at what level, but this is not easy in HNSCC, since mutations in genes that are involved in this pathway are infrequent (0-3.6% according to the COSMIC database (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic>)). In addition, in Chapter 2 we found that there is a broad range in *BRCA1* and *BRCA2* mRNA expression between HNSCC cell

lines, but this did not correlate to cisplatin sensitivity and could therefore not be used as a biomarker for cisplatin sensitivity. This shows the necessity for the identification of biomarkers that disclose whether the FA/BRCA pathway and/or the involved genes are functional. Such biomarkers might for example be genes that are transcribed by transcriptional co-regulator BRCA1⁽²⁴⁾, and possibly BRCA2^(25,26).

In this thesis, we focused on cisplatin sensitivity as part of the response to chemoradiation. As stated before, the DNA damage response elicited by cisplatin seems to be completely distinct from that following radiation⁽²⁷⁻³⁰⁾. In our lab we also used the genome-wide siRNA screen approach to investigate which genes are involved in radiation response. This screen yielded a list of very interesting hits, which showed hardly any overlap with the hit list obtained in the cisplatin screen. This suggests that the cisplatin- and radiation DNA repair response pathways might be distinct, but we should also take into account that the cisplatin screen was performed in HNSCC cells, whereas the radiation screen was carried out in non-small cell lung cancer cells of the adenocarcinoma type. This means that, in order to find novel biomarkers of (chemo) radiation response in HNSCC, the hits from the radiation screen should be confirmed in HNSCC cell lines or even that a complete new genome-wide siRNA screen for radiation response in HNSCC should be executed.

Novel treatment options

Besides genes that relate to cisplatin response, our genome-wide siRNA screen also yielded a list of siRNAs that were lethal to the tumor cells (Chapter 4). The genes targeted by these siRNAs might be interesting targets for the development of new anti-cancer treatments. This is of particular interest for Fanconi anemia (FA) patients suffering from HNSCC. Available treatment options are limited for FA patients as the non-cancerous cells of these patients are also extremely sensitive to DNA crosslinking agents, like cisplatin. Surgical resection is the best option for FA patients, but this is inappropriate as a single modality to treat the more advanced stages of disease. Early diagnosis is crucial and treatment of precancerous fields with targeted drugs that are not toxic to healthy cells would be highly preferable. With the identification of the siRNAs that induce a tumor cell killing phenotype, novel potential drug targets are elucidated. The development of effective therapies for these drug targets should lead to a reduction of cancer incidence in FA patients, as well as reduced morbidity and mortality.

Drug development

One uncertainty in the tumor-lethal siRNA hit list is the possibility that these genes might also harm healthy somatic cells. Future experiments in normal keratinocytes and/or fibroblasts should determine whether the hit list contains potential cancer specific drug targets. However, keratinocyte transfections are difficult, and the short lifespan of these cells contributes to a complicated optimization procedure. In the case of *KIF11*, we were lucky to find a potent small molecule inhibitor (Chapter 4) with excellent pharmacokinetic properties. The availability of targeted drugs makes it easy to test a broad range of cell types, both cancerous and non-cancerous. However, most of the times it is challenging to find a drug or small molecule that is able to specifically target the protein of interest, and pharmaceutical output in terms of new drugs is declining⁽³¹⁾. Although logical, a limitation of switching to drugs is that off-target effects of the drug might hamper further *in vivo* development while the drug target is valid.

Cancer stem cells

CSCs are thought to be highly resistant to general anticancer treatments⁽³²⁾. Studies using HNSCC CSC marker CD44 showed that a high frequency of CSC-like cells, reflected in CD44+ cells, correlates with factors associated with poor prognosis⁽³³⁾ and radiotherapy resistance⁽³⁴⁾. This leads to the assumption that tumors that show good response to anticancer treatments might harbor less CSCs than their therapy-resistant counterparts. We hypothesized that the

good survival outcome of HPV-positive oropharyngeal cancers (OPSCCs) might be the result of elevated therapy response due to low levels of CSCs. Indeed, we found that HPV-positive OPSCCs that did express low levels of CD98-positive cells (<50%) had a better clinical outcome than HPV-positive OPSCCs with higher levels of CD98. This finding is particularly interesting in the light of the recent discussion on de-escalating cancer treatment for HPV-positive OPSCCs. It is argued that HPV-positive OPSCCs might be treated with a less-intensive treatment schedule, assuming that the favorable survival outcome will not be reduced. Our results imply that CD98 expression, in addition to HPV status, might be a useful tool to stratify OPSCC patients to a less intensive, but effective treatment that causes the least side-effects. The performance of CD98 should first be validated in prospective clinical trials.

Concluding

The research described in this thesis focused on finding ways to enhance the efficiency of cisplatin therapy and the identification of new tumor drug targets. Already several potential anticancer drugs were explored *in vitro* and *in vivo*. Research during the coming years will tell whether these new drugs will eventually be suitable for the treatment of HNSCC. Taken together, this work has provided new input for the improvement of head and neck cancer treatment.

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