Chapter 11. Summarizing discussion and future perspectives.

Towards TKI treatment selection in cancer

Despite the relative wealth of currently available targeted therapies including TKIs, more accurate predictive biomarkers are warranted to guide therapy selection to improve benefit and prevent ‘unnecessary’ toxicity from these agents. The development of new biomarkers in preclinical settings to guide therapy selection requires sound evaluation and validation in order to establish their value. Stagnated clinical application of personalized medicine to a broader context may be in part explained by the lack of an efficient strategy to evaluate the molecular mechanisms of sensitivity and resistance to targeted agents in patients with cancer (1). For many years, a major obstacle has been the availability and quantity of tumor tissue that could be obtained prior to initiation of treatment (2). Factors contributing hereto included ethical concerns, in general, on and variable attitudes of medical oncologists toward (mandatory) biopsies for research purposes in clinical trials (3,4). Fortunately, the safety of tru-cut (liver) biopsies (5,6), as well as the feasibility and usefulness of needle biopsies in early-and later-phase clinical trials have been improved and demonstrated (7,8). Remarkably, patients are also willing to consent for tumor biopsies for exploratory research purposes only, as we (Chapter 8, this thesis) and others have reported (4,9,10). In this thesis we focus on TKI-treatment selection, although we do realize that for other targeted therapies, including antibody based immunotherapies, similar issues play a significant role in the development of biomarkers for therapy selection. While pre- and, to a lesser extent, on-treatment biopsies are increasingly available, evaluation and interpretation of the biological effect, in a broad sense, of TKIs on tumor tissue is not straight-forward, since their activity is influenced by multiple factors including pharmacokinetics, their affinity and (lack of) selectivity for individual kinases and substrate availability (11-13) as well as signaling network wiring. Therefore, when investigating potential benefit or (primary) resistance to these drugs, aforementioned factors should be taken into consideration. An obvious approach would be to evaluate whether, during treatment, achieved intra-tumor concentrations are actually
sufficient and target inhibition is achieved without induction of alternative kinase signaling. Despite concerns on adequate TKI delivery to cells situated in isolation from functioning blood vessels, efforts to systematically study their tumor tissue penetration are scarcely available (14) (Labots et al, Chapter 8). Furthermore, when considering molecular profiling strategies to predict efficacy of targeted therapies in individual patients, ideally methods that can (also) take their method of action and ‘level’ of activity into account. Essentially all available targeted therapies are directed at signaling molecules, rather than at genes. Although cancer is caused at the genetic level, the resulting abnormal, (hyper) abundant or absent protein expression will alter the normal cellular protein ‘circuitry’ or network and determine its aberrant biological function (15). Proteomics-based approaches to complement genomics-based initiatives to address biomedical questions have been first suggested in the early 2000s (16,17). In 2001, Liotta et al predicted that entire cellular networks or pathways, instead of single proteins, would be the drug targets of the next decade (18). In a recent somewhat provocative perspective, Michael B. Yaffe states that the massive resources spent on genome sequencing of human tumors have produced important datasets for the cancer biology community, but that these studies, paradoxically, have revealed little new biology. Rather than to produce more sequence data by “looking under the sequencing lamppost”, where light is brightest, a better strategy, as argued by Yaffe, would be to analyze signaling networks that underlie cancer development, progression and therapeutic resistance at both a personal and systems-wide level (19). Mass spectrometry-based phosphoproteomics, through the analysis of the cancer signaling networks, has been suggested as a candidate method for selection of protein kinase inhibitors in individual patients (20-22). Thus far, one study has reported on pre- and on-treatment global phosphoproteomics in a single patient with advanced hepatocellular carcinoma treated with sorafenib(23). Tyrosine phosphoproteomics on small clinical samples has not been considered feasible due to the required tissue quantity for this type of analysis until the research described in this thesis.
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The studies described in this thesis have evaluated or applied molecular profiling at the proteomic (Chapter 3, 4, 5, 6, 7, 8) and the genomic (Chapter 9 and 10) intended to optimize efficacy of targeted therapies in patients with advanced solid tumors. For Chapters 9 and 10, this was performed by analysis of available array CGH data from patients with gastric and colorectal cancer. The remaining chapters have been performed to contribute to treatment selection in ‘out-of-option’ patients with advanced solid tumors without specific histopathology. Chapter 4 and 5 hereto, respectively, involve the experimental evaluation and pilot clinical application of a tyrosine kinase peptide substrate microarray, while Chapter 6 and 7 aimed to optimize the experimental workflow for MS-based tyrosine phosphoproteomics (Chapter 6, 7) followed by its evaluation in a clinical study in Chapter 8.

In Chapter 2, we performed a systematic review on mass spectrometry (MS)-based serum and plasma peptide profiling performed in patients with solid tumors, in relation to outcome following systemic and local treatment, and evaluated its appropriateness for clinical application. For several years, matrix-assisted and surface-enhanced laser desorption/ionization time-of-flight MS (MALDI- and SELDI-TOF MS) have been considered a promising method and the serum peptidome a valuable source for cancer detection (“Spinning biologic trash into diagnostic gold”) (24-27). Following early promising results in the early 2000s, this technique was scrutinized after a proteome pattern reported to discriminate patients with ovarian cancer from healthy controls with 100% sensitivity, 95% specificity and a positive predictive value of 94%, could not be reproduced by others (26). Notably, this resulted in improved experimental designs to avoid bias, that, together with technological advancements enhancing reproducibility, caused this approach to mature in later years.

Review of 38 studies using blood-based MALDI- or SELDI-TOF MS showed that the majority of studies were not optimally designed to facilitate clinical decision making. Most studies had limited sample size (median N=59) and applied variable design and outcome parameters, while independent validation efforts were performed in only a subset of studies. We performed a pooled analysis of 9
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studies encompassing more than 1000 patients with non-small cell lung cancer (NSCLC) treated with EGFR TKIs. These studies applied a MS-based 8-peptide signature, ‘Veristrat’, previously shown in these patients to be indicative of progression-free and overall survival (PFS, OS) by Taguchi and coworkers (28). Our analysis revealed a median PFS and OS in patients classified to have ‘poor’ prognosis of only 2.0 and 4.6 months respectively, which is clinically significant short such that it could influence clinical decisions by withholding treatment in these patients. We also found a significantly longer PFS for patients with ‘poor’ classification treated with chemotherapy than with targeted therapy (3.94 vs 1.87 months, \( p = 0.002 \)), while this was not different for patients with ‘good’ classification. These results, as well as those from the Taguchi algorithm-stratified phase III PROSE study (29), in which patients with advanced NSCLC were randomized between erlotinib or chemotherapy, support the proof-of-concept for MS-based pre-treatment profiling to influence clinical decision making in NSCLC. Future MS-based serum peptide profiling studies should be designed to facilitate clinical application which includes validation efforts of the identified putative biomarkers. Moreover, we stated that consensus criteria for the evaluation and clinical implementation of proposed treatment selection tools are needed.

The human genome encodes for more than 500 protein kinases, of which 90 belong to the tyrosine kinase family (30). Kinases are enzymes that transiently phosphorylate specific amino acids on ~30% of all human proteins, by transferring a phosphate group from adenosine triphosphate (ATP) to specific substrates (31,32). Phosphorylation and dephosphorylation catalyze intra and intercellular signal transduction, by influencing enzymatic activity, cellular localization, protein conformation and protein-protein interaction (33). Since deregulation of kinase activity by gene amplification or mutation has been implicated in cancer, kinases and especially tyrosine kinases have become one of the most intensively targeted enzyme classes for therapy. In Chapter 3, we have described peptide array and mass spectrometry-based technologies and new developments for kinome profiling, and we present an outlook towards future implementation of therapy selection based on kinome profiling in clinical practice.
In Chapter 4, we investigated a tyrosine kinase peptide substrate array which was discussed and suggested in the previous chapter as a potential selection tool for TKI therapy in patients with advanced solid tumors. This commercially available chip was considered potentially clinically applicable in aforementioned patient population based on its design allowing high-throughput usability and limited tissue input requirements. We aimed to determine optimal sample preparation and test conditions as well as its reproducibility using lysates derived from cancer cell lines and tumor tissues from patients. We obtained optimal signal intensity with indeed only 7.5-10 µg lysate protein per sample and with ATP concentrations up to 400 µM. Kinase activity profiles of unpertubated lysates revealed to be robust, but, at the same time, this microarray reproducibility raised concerns on its ability to identify patient-specific profiles by the moderate differences found between 14 distinct tumor cell lines and tissues. Incubation of patient-derived tumor lysates with 4 different TKIs predominantly induced inhibition of substrates which were consistently found to be highly phosphorylated in the basal profile analyses. We found specific activity of recombinant kinases to be adequately measured, as also illustrated by this microarray’s use in drug development (34). For its use with more complex samples such as patient samples that contain multiple kinases, we concluded that the array peptides need further optimization for specificity. Ideally, phosphorylation on chip should be exclusively related to a single kinase or pathway, such that inhibition may be directly related to clinical sensitivity. We confirmed that the required protein input, robust specifications and anticipated short turnaround time of this microarray enable clinical implementation as TKI selection tool, yet that this platform could benefit from an improved selection of peptides. We proposed that MS-based phosphoproteomics may provide such an enhancement.

Chapter 5 reports a pilot treatment selection study in patients for whom no standard therapy was available, using the tyrosine kinase peptide array discussed above to select one of 6 protein kinase inhibitors (sunitinib, dasatinib, erlotinib, sorafenib, everolimus, and lapatinib) based on their in vitro inhibition profiles, induced by their ‘spiking’ in lysate of tumor tissue obtained by needle biopsy. We designed this study to improve the clinical benefit rate in this phase I patient population from 10 % to
25 %. Microarray analysis resulted in a selection preference for dasatinib which displayed the most potent inhibition of peptide substrate phosphorylation in 9/12 patients, without subsequent evidence of clinical activity. Hereafter, the study was halted for lack of drug selection and clinical activity of this treatment selection method. We anticipated that the PKI concentrations used in this in vitro assay should be reevaluated based on intra-tumor concentrations of specific inhibitors in patients, while it also caused our focus, for the same personalized treatment selection purpose, to shift to MS-based phosphoproteomics. Meanwhile, we will await results of responder vs non-responder analysis of 121 tumor tissues from sunitinib-treated patients with renal cell cancer utilizing this tyrosine kinase peptide microarray, performed by Van der Zanden et al.(35).

Chapter 6 describes the first optimization step of our MS-based tyrosine phosphoproteomics (pTyr-phosphoproteomics) workflow for aforementioned purpose. The performance of P-Tyr-1000 and 4G10, two commercially available antibodies for capture of tyrosine phosphorylated phosphopeptides, were compared in terms of depth and reproducibility when applied to moderate to high protein input of 10 mg derived from the colorectal cancer cell line HCT116. We found that P-Tyr-1000, compared to 4G10, identified more phoshopeptides per sample, displayed higher identification reproducibility and a lower coefficient of variation. The best performing antibody was then applied in a commonly used cell culture model of the glioma cell line U87 +/- EGFR-mutation, to a) study the effects of the TKI erlotinib on EGFR signaling, and b) demonstrate the potential of this approach to evaluate drug targets and provide insight in the pharmacodynamics effects of TKIs. Incubation of mutant cells with erlotinib resulted in significant reduced phosphorylation of several known (auto) phosphorylation sites at the intracellular domain, while increased phosphorylation of one site was observed. In addition, we found enhanced phosphorylation of other kinases in the EGFR-mutated cell line, including MET, PTK2, EPHA2 and MAPK1. Their co-activation may indicate the potential of pTyr-phosphoproteomics to enhance insight in resistance mechanisms to single agent targeted therapy.

Chapter 7 describes a crucial step for the clinical application of pTyr-phosphoproteomics in patients
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with advanced cancer. Since this method was originally developed for the evaluation of tumor tissue requiring 20 mg of protein input per sample, reflecting the needle-in-haystack search for the tyrosine phosphorylated peptides that constitute only 1% of the phosphoproteome, application of this approach to small clinical samples instead of resection tissue had not been considered feasible. Based on the starting point that needle biopsies from metastatic lesions should provide sufficient material for reproducible profiles and be representative of the individual tumor phosphoproteome, we aimed to downscale protein input to the clinically relevant and achievable level of 1 mg protein. We first showed a downscaled immunoaffinity protocol to 1 mg protein input using HCT116 lysate to be effective, while acceptable reproducibility of phosphopeptide identification and quantitation were maintained. By including high-input protein samples in these experiments, we were able to propagate phosphopeptide identification in the data processing and analysis steps of the small input samples. We then tested this approach in small patient-derived samples. Using 2-4 replicate needle biopsies from multiple readily resected colon tumors, we obtained an average protein yield of ± 1,8 mg per biopsy. Subsequent pTyr phosphoproteomic analysis of only 1 mg identified > 200 phosphopeptides per sample and enabled the identification of patient-specific phosphoproteome profiles. In addition, multiple distinct phosphokinases per individual tumor were found, both measured directly and indirectly through analysis of phosphorylated protein substrates predictive of kinase-substrate relations. Together, these studies demonstrate the feasibility of pTyr-phosphoproteomics at the biopsy level of protein input while suggesting that target identification and, as a consequence, treatment selection, using such small samples might be possible.

In Chapter 8, we performed pTyr-phosphoproteomics in the setting of a clinical (pilot) study in patients with advanced solid tumors. Elaborating on one of the concluding remarks of Chapter 5 and based on the paucity of data on this matter, we aimed to determine intra-tumor drug concentrations after 2 weeks of treatment with a protein kinase inhibitor (PKI) and to evaluate the effect of such treatment on pTyr phosphoproteomic profiles in serial tumor needle biopsies in this patient population. We obtained pre- and on-treatment biopsies from 5 patients for each of the cohorts
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erlotinib, sorafenib, dasatinib, sunitinib, everolimus and vemurafenib; study drugs were administered either as on-label, standard palliative treatment or, off-label, prior to standard palliative (chemo)therapy. First, the design of this study underlines patient willingness to participate in research and to consent for research-only biopsies, consistent with previous findings (4,9,10).

PKI tumor concentrations could be determined in 90% of 31 patients whom underwent a tumor biopsy after 2 weeks of treatment. Median tumor PKI concentrations were found to range between 2-10 µM for the first 5 aforementioned cohorts, while for vemurafenib this was above 1 mM. Achieved plasma and serum concentrations were similar to those found in literature. Importantly, median PKI concentration in tumor tissue was considerably higher than in plasma. This finding may have two important implications. Firstly, these results show that tumor drug concentrations well exceed the concentrations required for inhibition of the assumed drug specific kinase targets (36-39), implicating that their biological activity might be also due to inhibition of other kinases for which these TKIs have lower affinity. Considering both beneficial and potentially harmful consequences of drugs hitting their off-targets, this underlines the need for drugs with rationally designed selectivity profiles (40). Secondly, these results question the relevance for therapeutic drug monitoring (TDM) to optimize the clinical benefit rate or duration of PKIs, also considering the absence of improved relevant clinical efficacy endpoints over fixed dosing (41).

In 84% of 31 patients whom started study treatment, pTyr-phosphoproteomic analysis could be performed, using a median of 1.6 mg of (matched) protein input, on the pre- and on-treatment biopsies. Similar to Chapter 7, tumor biopsies from individual patients tended to cluster, but the clustering of pre- and on-treatment tissues in this study also indicated that PKI-induced changes to the phosphoproteome are smaller than differences between patients. Within individual patients, comparison of pre- and post-treatment biopsies revealed that 10s to 100s of phosphopeptides were up- or downregulated with a fold-change of on-/pre-treatment intensity (Fc) of > 5 or < - 5, respectively. Supervised clustering analysis of phosphopeptides displaying such regulation in at least 3 of 5 (≥ 3/5) patients could separate pre- and on-treatment groups in all drug cohorts. When
applying a much less stringent selection of phosphopeptides, regulated with a Fc cut-off of 1.5 in ≥ 3/5, we found only marginal overlap between drug cohorts, confirming that the 39-74 up- and 4-135 downregulated phosphopeptides per treatment group were drug-specific. This regulation of multiple substrates may either result directly from the drug treatment, or indirectly, through an avalanche-like effect on the downstream signaling cascade. Finally, we identified a number of phosphopeptides that displayed reduced intensity in on- vs pre-treatment samples with increasing tumor concentration in ≥ 3/5 patients. For erlotinib, these (potential pharmacogenomics biomarkers) included peptides related to the target kinase EGFR, as well as PTK2, STAT3 (3/5) and PRKCD (4/5).

In conclusion, in this study, we performed the first large(r)-scale phosphoproteomics analysis of pre- and on-treatment patient tumor biopsies and thereby the potential of this approach for TKI therapy selection. The two main unprecedented findings from this trial were that 1) tumor drug concentrations exceeded concentrations required for inhibition of the assumed drug specific kinase targets and 2) alterations in tyrosine peptide phosphorylation in serial tumor biopsies from patients are drug specific.

Compared to the previous chapters, a biologically ‘upstream’ tumor profiling approach, at the DNA level, has been applied in Chapter 9 and 10 to identify established and potential drug targets in gastric and colorectal cancer, respectively. Genetic alterations are considered targetable if genetic changes cause perturbations in proteins, pathways, or both, that can be specifically intercepted by agents(42). DNA copy number changes, more specifically high-level gains, including amplifications, can lead to mRNA overexpression and consequently overexpression of oncoproteins that are targets of available drugs. The clinical efficacy of trastuzumab in HER2-neu amplified gastric cancer has demonstrated that such proteins can serve as drug targets in this disease (43). As in several other tumor types, the poor outcome of patients with advanced gastric cancer reflects the need for improved treatment options, either by identification of more targets and agents, or by repurposing of existing (potential anticancer) drugs. In Chapter 9, we therefore aimed to establish the prevalence of DNA high-level copy number gains (CNG), including amplifications, of potential drug target genes
in a large cohort of patients with gastric cancer. To this end, genome-wide array comparative
genomic hybridization (array CGH) data from 183 primary gastric cancer samples were analyzed to
assess whether any established or potential anticancer drug target genes showed such high-level
copy number gains. Thirty genes were found that were previously annotated as drug target gene to
show high-level gain in at least 2% of the tumors, including taxane-target genes involved in
microtubule assembly (TUBB3, TUBG1 and TUBG2), as well as the anthracycline target TOP2A, and
primary targets for clinically approved targeted therapies (ERBB2, VEGFA and EGFR). In addition,
targets for drugs with repositioning potential were identified, including HRH3 and DGAT1 which were
amplified in ~10% of patients, suggesting exploration of the potential efficacy of antihistamines and
statins for this disease.

In Chapter 10, in addition to studying the associations of DNA copy number changes to progression-
free survival in 349 patients with metastatic colorectal cancer (mCRC) treated with first-line palliative
chemotherapy in the phase III clinical trial CAIRO or CAIRO2 (44,45), we documented the prevalence
of high-level CNG in this disease based on a similar approach as in Chapter 9. High-level CNGs
identified in this series of CRCs included gene targets for clinically available targeted therapies such
as FLT3, FLT1, SRC and FGFR1. FGFR1 showed high-level gain in 3.7% of patients and may be of
particular ‘treatment selection interest’ since this is a known target receptor for regorafenib, a drug
which has been approved for treatment in non-biomarker selected patients with refractory mCRC,
based on a limited overall survival benefit of 6 weeks (46). For 2 genes from the list of genes with
high-level gained regions, TOP1 (target of irinotecan) and VEGFA (target of bevacizumab), the
relation with outcome to systemic treatment could be assessed, albeit in very small subgroups of
patients treated with one of the 3 treatment regimens: capecitabine monotherapy (CAP; n=14 with
TOP1 high-level CNG; n=3 with VEGFA h-l CNG), capecitabine/irinotecan (CAPIRI; n=8 TOP1, n=2
VEGFA) and capecitabine/oxaliplatin/bevacizumab (CAPOX-B; n=15 TOP1, n=2 VEGFA). However, for
both TOP1 and VEGFA no ‘obvious’ association between high-level CNG and treatment outcome
(PFS) was found.
The prospective molecular screening study SAFIR-01 provided the first proof of concept that genomic profiling, including whole-genome array CGH, could be implemented as treatment selection strategy in patients with advanced breast cancer. However, the actual percentage of patients receiving effective targeted therapies was low, as well as the number of patients experiencing clinical benefit from this profiling approach (47).

Personalized medicine: now, or when and how is the time? Future perspectives

To date, several clinical trials utilizing molecular tumor profiling of fresh tumor biopsies for selection of targeted therapies in patients with cancer have been published, including SAFIR01, SHIVA and MOSCATO 01 (8,47-50), while several are actively accruing patients (MSK-IMPACT, WINTHER, NCI-MATCH, TAPUR (51-54), DRUP (Abstract LBA59, ESMO 2017 Congress) and ProfiLER (J Clin Oncol. 2017;35(suppl; abstr LBA100)). In these studies, mainly genomics-based approaches are applied, such as whole-genome sequencing, array CGH and mutation hot spot cancer panels. The feasibility of tumor profiling using fresh tumor biopsies - even collected, analyzed and reported from > 500 patients across multiple institutions per month with turnaround time of < 21 days (MSK-IMPACT (52) - for clinical application has meanwhile been sufficiently shown. Unfortunately, patient benefit of these sequencing efforts seems thus far to be limited. The SHIVA trial, the only study to randomize between targeted therapy selected by molecular tumor profiling and conventional treatment, did not show an improvement in progression-free (48) and overall survival (J Clin Oncol 35, 2017 (suppl;abstr 11515)).

The foregoing may also implicate that genomics is not the only way to go. Based on its potential to analyze aberrant cellular signaling networks in relation to biological function, together with the expanding therapeutic portfolio of protein kinase inhibitors, we foresee mass spectrometry-based phosphoproteomics to complement genomics to guide selection of targeted therapies in patients with advanced cancer. This requires proof of concept studies showing the clinical feasibility and utility of phosphoproteomics for this purpose in this patient population, as well as algorithms to infer
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kinase or pathway activity that enable prioritization between (combinations of) available drugs in addition to an integrated DNA/protein data analysis pipeline. Improved phosphopeptide enrichment methods and increased sensitivity for detection of low abundant proteins may enable further downscaling of protein input and thereby allow (comprehensive) analysis of small clinical samples.

Conclusions

Based on our translational studies and prior study reports, we conclude that intervention studies using patients’ tissue and/or blood (during treatment) are important to improve selection methods for targeted therapies and impact clinical treatment decision making for individual patients. Patient willingness to contribute to these type of studies is of great importance and should receive most attention. Secondly, we found that tumor concentrations of protein/tyrosine kinase inhibitors during treatment provide more insight in the potential mechanism of action of these agents and should be taken into account for future development of clinically applicable treatment selection tools. In addition, the results of this thesis contribute to the development of pTyr-phosphoproteomics for TKI treatment selection in patients with advanced solid tumors. Based on our finding that these drugs induce a differential phosphoproteomic profile in patients’ tumors, we propose that, along with genomic selection markers, pTyr-phosphoproteomics provides another piece in the puzzle to realize the promise of treatment selection in this high-need patient population.

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