SUMMARIZING DISCUSSION

&

FUTURE PERSPECTIVES
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Most common cancers have a large number of genetic alterations, some of which are responsible for their invasive and metastatic phenotype. Genetic alterations can be mutations, chromosomal rearrangements, DNA copy number abnormalities or epigenetic changes, such as methylation or micro RNA regulation. These aberrations can all have an effect on gene expression, and eventually on the expression or function of proteins. This thesis aimed to investigate specific genetic aberrations in patients with Non-Small Cell Lung Cancer (NSCLC). The research described in this thesis is focused on the identification and use of such aberrations as ‘biomarkers’ to predict response to therapy or prognosis, and to identify candidate genes for targeted therapy in NSCLC patients.

The epidermal growth factor receptor (EGFR) is a gene in which mutations and high DNA copy number have been described to be correlated with response to EGFR tyrosine kinase inhibitors (TKIs) [1-3]. In contrast, mutations in the KRAS oncogene have been related with primary resistance to these agents. Secondary resistance to EGFR TKIs has been attributed, at least partially, to a specific T790M EGFR mutation [4]. We have confirmed the correlation between the presence of EGFR and KRAS mutations and correlation with response to Erlotinib (an EGFR TKI) in a clinical phase II study, described in chapter 2. However, in our study as in the literature, the concordance between response and presence of EGFR and KRAS mutations was not perfect [5-7]. The lack of a perfect concordance is further emphasized by a case report that is described in chapter 3. This case report describes a Caucasian, male, former smoker, with NSCLC and squamous cell carcinoma histology, responding to Erlotinib. The clinical characteristics formerly reported to be associated with positive response to EGFR TKIs are female gender, Asian ethnicity, never-smoking status and adenocarcinoma histology [8,9]. As chapter 3 describes there are always exceptions to the rule, highlighting that care should be taken to base treatment with EGFR TKIs on those clinical characteristics only. Recent data [10] cast doubts that these clinical characteristics and EGFR aberrations are specific to EGFR TKI benefit and may be a more general indication of benefit to other treatments as well.

EGFR and KRAS mutation analyses can also be applied in a different clinical situation. Some patients with NSCLC present with multiple lung lesions at time of diagnosis or develop additional lesions during their lifetime. To decide on treatment, it is important to determine the relation between the various lesions, in order to discriminate between second primary tumors or metastasis. Currently, this is done by pathologic examination of histology slides, although this is sometimes inconclusive. A second case report (chapter 4), describes three patients who developed multiple lung lesions. We performed EGFR and KRAS mutation analysis of their
different tumors and, in addition, performed array CGH on the multiple lesions. By combining those techniques we were able to define the clonal relation between the multiple lesions.

Various techniques to determine EGFR mutations have been investigated, although the most commonly used method remains PCR and sequencing [11-15]. The source of the samples, i.e., whether the DNA or RNA was isolated from formalin fixed and paraffin embedded (FFPE) or fresh frozen samples, has been shown to influence outcome of mutation analysis [16]. We have performed a comparison study (chapter 5), in order to determine the effect of using different source, and of using DNA or RNA in the mutation analysis using PCR and sequencing. We showed that RNA extracted from frozen tissue is the preferred source for analysis since all PCRs were successful and, in some cases, EGFR mutations could be more easily identified using RNA compared to DNA. The latter could not have been caused by the percentage of tumor cells present in the sample, since RNA and DNA were derived from the same tissue. It has been reported that in EGFR amplified tumors there is selective amplification of the mutant allele [17]. The fact that we have observed the EGFR mutation more prominently in the RNA sample suggests that the mutant allele may be transcribed more efficiently. Chapter 5 furthermore highlights the challenge to standardize fixation and storage protocols in order to allow molecular studies to be completed without sample related technical difficulties.

As mentioned previously, high EGFR copy number can also be a predictive marker for response of EGFR TKIs [18,19]. The technique used to assess copy number in most studies is fluorescent in situ hybridization (FISH). This technique has some technical limitations since the use of fluorescence requires the need for an expensive fluorescence microscope, and rapid evaluation because of fading of the signal. These limitations can be overcome by a method that uses a chromogenic rather than a fluorescent signal, chromogenic in situ hybridization (CISH). We performed a comparison study, described in chapter 6, in order to evaluate whether CISH could be a useful strategy to determine EGFR copy number in NSCLC samples. We determined the concordance between FISH and CISH, and evaluated the performance of the two techniques in both FFPE and in fresh frozen samples. We show that CISH is a useful strategy to evaluate EGFR copy number and showed high concordance with FISH. This chapter also highlights the challenge to standardize interpretation of in situ hybridization techniques and the urge to determine the appropriate cut-off in defining to what extent EGFR copy number influences response to anti-EGFR TKI treatment.

Various types of EGFR mutations have been identified to date [20], and they are differentially related to response to EGFR TKI treatment. In this regard, it has been shown that deletions in EGFR exon 19 are related with better response to EGFR TKIs than the L858R mutation [21]. Moreover, the T790M mutation is related with acquired resistance to EGFR TKIs [22,23]. Yet another type of EGFR mutations, insertions in EGFR exon 20, have been related to primary
resistance to EGFR TKIs [24]. These data highlight that treatment cannot be simply based on the presence of any type of EGFR mutation, but each mutation should be investigated in more detail. To circumvent the use of complex and time consuming assays [25,26], we describe in chapter 7 a simple assay able to quickly determine the influence of specific mutations on sensitivity to EGFR TKIs. Using mutagenesis of a YFP-tagged fragment of the EGFR intracellular domain, following transfection and immunofluorescence microscopy analysis we were able to determine the effects of Erlotinib treatment. Using this system, we confirmed the sensitivity to Erlotinib of the EGFR deletion mutant and the L858R mutant, and the resistance to Erlotinib of the T790M mutant. Moreover, we show the insensitivity to Erlotinib of some uncommon EGFR mutations that were identified during a mutational screening of NSCLC patients. This system might be important in the clinical management of NSCLC patients that present with uncommon EGFR mutations. Moreover, this assay can be applied in investigations of genes and drugs, other than EGFR and EGFR TKIs.

The EGFR pathway has extensively been studied to identify additional biomarkers for response to EGFR TKIs. In addition to the previously mentioned EGFR and KRAS mutations and EGFR copy number, studies have evaluated the activation status of AKT and STAT signaling pathways [27], and the expression level of HER2 [28,29]. More recently, amplification of the MET receptor [30,31], the expression of epithelial to mesenchymal transition (EMT) markers, such as E-cadherin and vimentin [32,33], and the downregulation of HIF1α have also been linked to responsiveness to EGFR targeted agents [34]. Next to their predictive role for response to EGFR TKIs, some of those markers have also been linked to prognosis. However, conflicting results are often reported by different laboratories [4,35-42], and there is thus no single marker that can be used to predict prognosis of NSCLC patients. In chapter 8 we evaluate whether the combined analysis of several markers provides better prognostic information than the evaluation of single markers or the pTNM staging system, the most reliable prognostic marker so far, in which the size of the tumor and the degree of spread to lymph nodes and other sides in the body is taken into account. We investigated EGFR and KRAS mutational status, EGFR copy number, and the expression of EGFR, HER2, pCMET, pAKT, PTEN, pSTAT3, pSTAT5, pERK, HIF1α, E-CADHERIN and VIMENTIN, using immunohistochemistry on tissue microarrays (TMA) in resected NSCLC patients. Using a statistical model we have compared the performance to predict survival using the molecular markers with the pTNM staging system. We show that using the molecular markers we were able to improve prediction of prognosis by 7-10% as compared to the pTNM staging system alone. Furthermore, this statistical model was able to identify the most important markers, which were pSTAT5, HIF1α, PTEN and pCMET. Although the improvement in prediction is only modest, this study highlights that a combined analysis of several molecular markers and pathological staging can provide better prognostic information than the use of single markers.
Although the EGFR pathway is extensively studied in NSCLC, and well recognized to play an important role in this disease, there is also a subset of patients in which EGFR probably does not play a prominent role. Thus, new genes need to be identified. The use of microarray comparative genomic hybridization (array CGH) has been used to identify that amplification of the gene encoding the MET receptor is partially responsible for developing acquired resistance to EGFR TKIs [30,31]. In chapter 9, we describe a study in which we used array CGH to determine genome-wide copy number changes in NSCLC patients. Using the same patient samples, gene expression profiling was performed. Through the combined analysis of the results obtained with both techniques, and through the use of newly developed algorithms to analyze the data [43,44], we were able to identify genes whose expression was influenced by copy number. We identified 359 genes, mainly residing on chromosomes 3 and 5, showing altered expression as a result of aberrant gene copy number. In this chapter we focus on the gene HSP90 residing on chromosome 14, whose expression showed a clear relation with the survival of patients. This gene was the only gene whose expression was affected by a deletion of this tight region. This study further stresses the importance that specific inhibitors of HSP90, which are already in clinical investigation [45,46], may have in the clinical management of NSCLC patients.

As described in chapter 9, microarray studies to determine gene expression and gene copy number can be used to identify potential targets for therapeutic purposes. Other use of microarray analysis in cancer research is to compare patient groups with known outcome (treatment, relapse, survival) and to determine a signature able to discriminate between groups of patients (supervised analysis). One can also use the gene expression or array CGH data itself to create groups, which could be related with patient outcome (unsupervised analysis). The supervised approach was successfully used in studies that identified gene signatures related with lung cancer patient outcome. An interesting observation was the fact that there were only some genes in these signatures that overlapped [47-52]. A similar phenomenon is observed in breast cancer patients [53,54]. The genes included in the lung cancer signatures, as well as in the breast cancer signatures, were often part of similar pathways, including pathways regulating immune response, cell growth and DNA repair. This highlights another application of microarrays, namely the identification of important pathways in selected groups of patients.

Some important aspects when analyzing microarray data should be considered. Microarray experiments result in a huge amount of information, and care should be taken to analyze the data in the most efficient way without misinterpretation or missing important findings. One important aspect is that the study should be clearly designed and objectives clearly formulated. It is very important to correct for confounding factors such as stage, histologic subtype, age and treatment of the patient. Another important aspect is the use of the appropriate algorithm and, since microarray data will always result in more variables than cases, multiple testing corrections
should always be applied. After having determined a certain signature or genes of interest, validation of the findings needs to be carried out in an independent set of patients. For example, Wigle et al. reported the use of a gene signature to predict recurrence in NSCLC patients [55], and the same group performed a validation study. Although RT PCR was able to confirm the results in the same set, a microarray analysis of an independent set of patients failed to confirm the prognostic significance of the initial microarray results [56]. Our study, described in chapter 9, of this thesis is also currently being validated in an independent set of NSCLC patients.

A nice example of a well validated study is the 70-gene signature for breast cancer [53], of which results have been confirmed in two independent patient sets [57,58], and an update of the original series was recently published [59]. These studies have led to approval by the US Food and Drug administration of the 70-gene signature (Mammaprint®) in breast cancer patients.

The introduction of the publicly available microarray databases has allowed the elaboration of meta-analysis. One such analysis is the study described by Potti et al. in which they constructed genomic signatures to guide the use of chemotherapeutics [60]. They used 60 cell lines derived from various cancer cell types, and developed gene expression signatures that correlate with the response of these cells to various commonly used cytotoxic agents. Using publicly available data, they derived signatures from microarray profiles of the NCI-60 human cancer cell lines with known in vitro sensitivity or resistance to a particular drug. They used these profiles to predict in vivo chemotherapeutic response to seven different drugs. However, a similar analysis was unsuccessfully attempted by another group, who could not reproduce these results [61]. This finding emphasizes that quality control and continued checking of all the bioinformatics and procedures is also extremely important, and highlights that also results from meta-analysis should be treated critically and with caution.

Although great progress has been achieved with the use of microarrays to determine gene expression profiles and copy number aberrations, these techniques do not identify gene regulation by epigenetic changes such as regulation by non-coding RNAs (i.e. micro RNAs) or additional post translational modifications. In this respect, other emerging research fields are micro RNA expression analysis and proteomics. Micro RNAs (miRNAs) are small non coding RNAs, which are expressed in various organisms, including animals, plants, and viruses. These miRNAs are able to regulate several target mRNAs thereby affecting gene expression [62]. Abnormal expression of miRNAs has been shown in various types of cancer [63,64]. In lung cancer, unique miRNA profiles have been linked to patient outcome [65]. The identification of the role of miRNAs represents an area of active investigation [66]. As proteins are the effectors of the information encoded by genes, technologies that look at proteins in an “omic” fashion are also being developed, and proteomic signatures have been described in lung cancer [67-70]. A strategy based on the combined analysis of proteomics and microarrays, and confirmation with
immunohistochemistry on tissue micro arrays appears promising [71,72]. As described in chapter 8 and 9 of this thesis, we have gathered information on the DNA, RNA and protein level simultaneously in several patients and we aim to perform a combined analysis of these parameters in the near future.

In conclusion, the research described in this thesis shows that progress has been made in identifying candidate therapeutic targets and biomarkers for prediction or prognosis purposes in lung cancer. In NSCLC, specifically, EGFR and KRAS mutations have been shown to correlate with response to EGFR TKIs although exceptions remain. We have identified several genes that might have an interesting role in NSCLC, which has to be investigated in more detail in future research. The emerging fields of microarray technology for DNA, RNA and miRNA screening, as well as proteomics, has already given us many new insights into the complexity of human cancers. We are facing many challenges to improve our understanding of cancer and to ultimately improve therapy for cancer patients.
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