The Pharmacogenomics Journal (2012), 1–12

www.nature.com/tpj

REVIEW

Array-based pharmacogenomics of molecular-targeted therapies in oncology

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The advent of microarrays over the past decade has transformed the way genome-wide studies are designed and conducted, leading to an unprecedented speed of acquisition and amount of new knowledge. Microarray data have led to the identification of molecular subclasses of solid tumors characterized by distinct oncogenic pathways, as well as the development of multigene prognostic or predictive models equivalent or superior to those of established clinical parameters. In the field of molecular-targeted therapy for cancer, in particular, the application of array-based methodologies has enabled the identification of molecular targets with 'key' roles in neoplastic transformation or tumor progression and the subsequent development of targeted agents, which are most likely to be active in the specific molecular setting. Herein, we present a summary of the main applications of whole-genome expression microarrays in the field of molecular-targeted therapies for solid tumors and we discuss their potential in the clinical setting. An emphasis is given on deciphering the molecular mechanisms of drug action, identifying novel therapeutic targets and suitable agents to target them with, and discovering molecular markers/signatures that predict response to therapy or optimal drug dose for each patient.
The Pharmacogenomics Journal advance online publication, 17 January 2012; doi:10.1038/tpj.2011.53

Keywords: cetuximab; erlotinib; gefitinib; molecular-targeted agents; trastuzumab

Introduction

Pharmacogenomics, a term sometimes used interchangeably with the term pharmacogenetics, refers predominantly to the application of genomic technologies to further characterize existing drugs, design new ones and understand the drug–genome relation. In contrast to pharmacogenetics,[1] pharmacogenomics use 'genome-wide approaches', they are hypothesis-generating and can be powerful even when little is known regarding specific gene–drug interactions. Therefore, these studies are not biased toward current knowledge of gene function or drug action, and have the potential to identify the molecular signatures associated with complex therapeutic responses.

The advent of microarrays over the past decade has transformed the way genome-wide studies are designed and conducted, leading to an unprecedented speed of acquisition and amount of new knowledge. The applications of microarrays include the study of DNA polymorphisms and mutations, DNA methylation, gene expression, splice variants and more recently microRNA expression. In oncology alone, microarrays have been used in over 14 700 published studies to date (PubMed keywords: 'cancer microarray', September 2011), representing

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Received 17 February 2011; revised 14 September 2011; accepted 7 November 2011

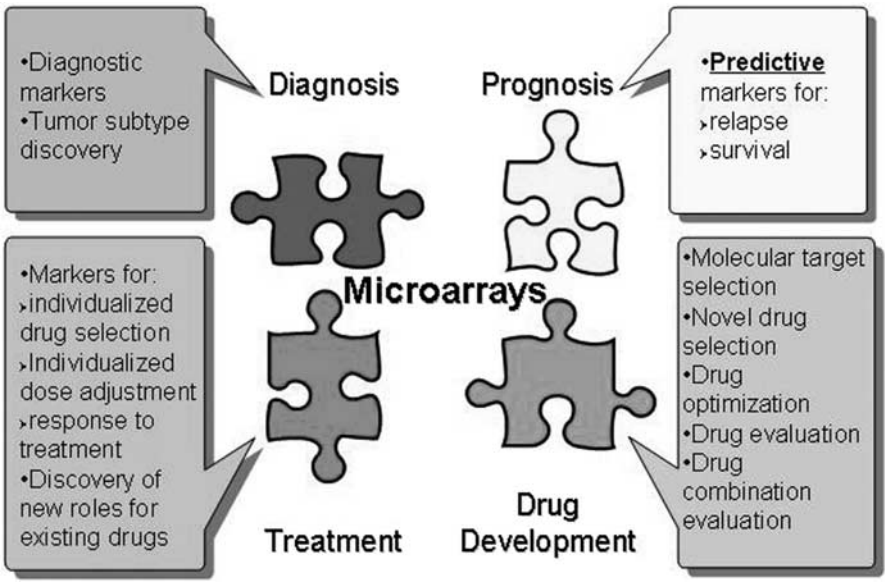

Figure 1 Microarrays are making a significant contribution to clinical oncology via genomics toward improved diagnosis and prognosis, and via pharmacogenomics toward optimized use of existing drugs and development of new ones.

approximately one-third of all published microarray studies (PubMed keyword: 'microarray', September 2011). Among the major contributions of this technology to clinical oncology are the identification of diagnostic and prognostic markers,[2–5] and of molecularly distinct subclasses of tumors.[6–8] Furthermore, microarrays have identified multigene models predictive of disease recurrence and overall survival in early breast cancer,[9–12] as well as chemotherapy sensitivity[13–16] in a variety of human malignancies (Figure 1).

Nowadays, molecular oncology has evolved to a point where a substantial number of targeted agents are available in the clinician's therapeutic armamentarium. Nevertheless, as the complete molecular effects of many of these drugs remain to be elucidated, the selection of the appropriate patient population that is most likely to respond to a specific molecular agent remains a challenge. It was this clear need to identify subjects whose tumors harbor a specific genetic profile associated with higher probability of therapeutic response that led to the development of clinical pharmacogenomics in oncology. The advent of microarrays and the introduction of whole-genome analysis technologies into routine clinical practice are expected to enable the use of pharmacogenomics in treatment decision making in the molecular oncology era. In the current review we aimed to provide a comprehensive overview of the applications of whole-genome expression microarrays in the field of molecular-targeted therapies in solid tumors and to discuss the main methodological challenges encountered with such approaches. The main molecular pathways involving genes implicated in neoplastic transformation and tumor progression are presented and the relevant findings from array-based studies with predictive value for response to molecular-targeted therapies in a variety of solid tumors are discussed.

Pharmacogenomics of EGFR (HER1) molecular-targeted therapies

The epidermal growth factor receptor (EGFR or HER1), represents the first member of the human epidermal growth factor receptors (HER), and is expressed in a number of human tissues. EGFR is overexpressed and shown to have an important role in the development of a number of solid tumors, among which non small-cell lung cancer (NSCLC), colorectal cancer and head and neck cancer.[17] This crucial role of the EGFR pathway renders it an appealing target for the development of new anticancer agents. The two main categories of molecular agents targeting EGFR are: (1) monoclonal antibodies that interact directly with the receptor, and (2) tyrosine kinase inhibitors (TKIs), that interact with the intracellular domain of the receptor and then modify or block completely its TK activity and thus signal transduction (Figure 2).

Gefitinib

The low molecular weight EGFR-TKI gefitinib is currently indicated for the first-line treatment of patients with NSCLC whose tumors harbor somatic mutations of the TK domain of the *EGFR* gene, based on the results of large-scale clinical observations that associated therapeutic response to the agent with the presence of these mutations.[18–20] It should be emphasized that in this setting, it is the presence of mutations and not the immunohistochemical expression or gene amplification that indicate responsiveness to gefitinib. Consequently, for the first time in the history of medical oncology, molecular detection of the mutation in the tumor specimen is a requirement prior to administration of the drug. Gene expression profiling of cancer cell lines and tumors following treatment with gefitinib has proven a

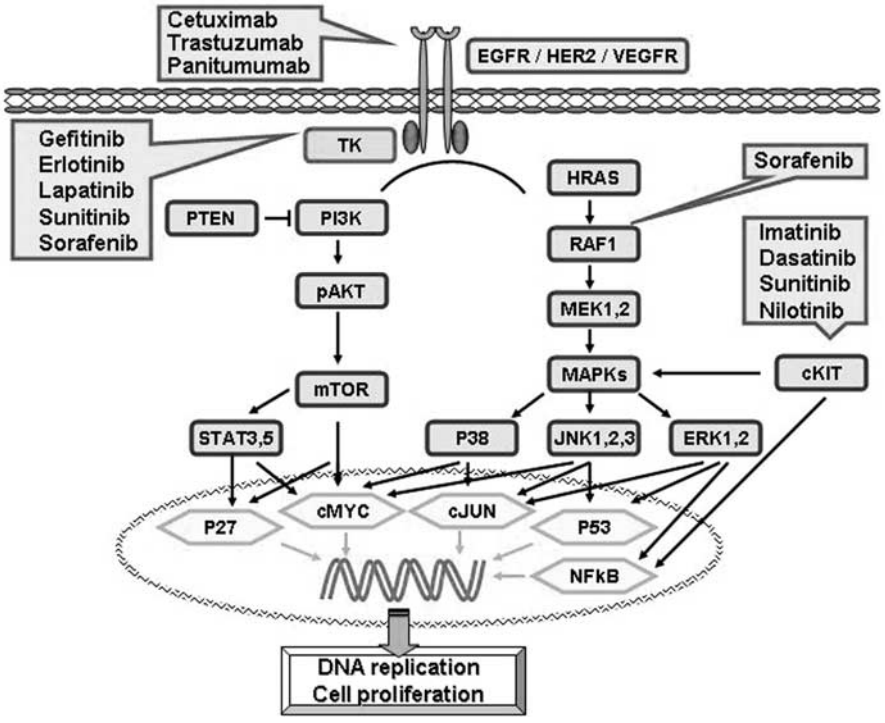

Figure 2 Simplified illustration of the fundamental pathways of the signal transduction process from the cell membrane to the nucleus along with the corresponding levels of pharmaceutical interference by molecular-targeting agents: the proliferate signal is usually engaged by a cell-surface receptor, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and HER2 that carries an extracellular component and an intracellular compartment with tyrosine kinase (TK) activity. The extracellular domain may be targeted by large-molecule monoclonal antibodies, such as cetuximab and panitumumab (for EGFR) and trastuzumab (for HER2). Molecular agents such as gefitinib, erlotinib, lapatinib, sunitinib and sorafenib function as small-molecule inhibitors of the TK domain of the receptor. Two main molecular pathways have been reported to transduce oncogenic signal to the nucleus: The RAS/RAF/mitogen-activated protein kinase (MAPK) pathway and the phosphatidyl-inostitol-3 kinase (PI3K)/AKT/mammalian target of rapamycin and STAT pathway. Both pathways involve intermediate effectors called 'second messengers' that are able to transduce the signal to downstream molecules through a process of phosphorylation and dephosphorylation, mediated by kinases and phosphatases, respectively. Several small-molecule inhibitors, such as sorafenib (a Raf kinase inhibitor), also function in this level of signal transduction. c-KIT represents another significant pathway with oncogenic potential characterized by unique regulation, as it is mainly activated by mutations in the c-KIT proto-oncogene and can be targeted by specific TK inhibitors, such as imatinib, or inhibitors of multiple TKs, including sunitinib, dasatinib and nilotinib. AKT: protein kinase B (PKB); c-KIT: v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog; JNK: c-jun kinase; MEK: dual specificity mitogen-activated protein kinase kinase 1 (MAPKK1); NF-κB: nuclear factor kappa-B; PTEN: phosphatase and tensin homolog; RAS: Kirsten rat sarcoma viral oncogene homolog.

useful means of characterizing its effectiveness at the molecular level. Toward this direction, human insulinoma, bladder cancer and colon cancer cell lines, cultured in the presence of gefitinib, were analyzed by microarrays.[21–23] All three studies demonstrated a significant repression of cell cycle and apoptosis-related genes. The gefitinib-mediated apoptosis appeared to be caspase-3-dependent in the insulinoma cells, and the downregulation of the transcription factor YY1 and E-cadherin may account for the efficacy of gefitinib in bladder cancer. At the clinical level, gefitinib induced gene expression changes when studied in a phase II study of gefitinib monotherapy in advanced esophageal adenocarcinoma, providing evidence of a 'transcriptional response' to treatment.[24]

Similarly to other molecular-targeted therapies, patient response to gefitinib is variable with a significant portion of patients being resistant to it. In NSCLC, squamous-cell carcinoma of the head and neck and hepatocellular carcinoma studies, the gene expression signatures characterizing sensitive and resistant cell lines were shown to predict gefitinib activity.[25–27] Interestingly, some of the genes identified in NSCLC are associated with the HER pathway signaling, which renders them promising targets for pharmacological interventions to overcome primary resistance. Gene expression profiling of sensitive and resistant biliary tract carcinoma cell lines treated with gefitinib or erlotinib (another potent EGFR-TKI), revealed multiple RAS/RAF/mitogen-activated protein kinase (MAPK) pathway genes overexpression and led to the consideration of a combined EGFR and MAPK inhibitory treatment strategy that was more effective than either single agent alone.[28] In a parallel study of *in vivo* gefitinib-resistant mouse models, the epithelial membrane protein-1 emerged as a surface biomarker whose expression correlated with acquisition of gefitinib

resistance in lung cancer.[29] Importantly, gene expression signatures of NSCLC tumors following gefitinib treatment led to the establishment of a gefitinib response score that accurately predicted response to treatment in NSCLC patients.[30]

Array-based genomics have been also used to evaluate prognosis of tumors treated with combinations of gefitinib with cytotoxic chemotherapy: characterization of the gene expression signatures of different tumor subtypes following combinatorial treatment with EGFR-targeting agents (gefitinib and the monoclonal antibody cetuximab) and cytotoxic chemotherapy *in vitro*, identified multiple EGFR-associated profiles that were of prognostic significance *in vivo*.[31] Overall, *in vitro* and *in vivo* gene expression studies following gefitinib treatment have enabled the characterization of the molecular mechanisms of action, as well as molecular signatures associated with various responses to this drug. These findings are expected to have direct implications in treatment selection and prognosis in the foreseeable future.

Erlotinib

Erlotinib is another EGFR-TKI that has been proven effective against metastatic NSCLC and is currently indicated for the second-line treatment of patients with NSCLC that progressed after platinum-based chemotherapy.[32] Erlotinib has also shown a small but statistically significant overall survival benefit when combined with the antimetabolite gemcitabine in the treatment of patients of inoperable pancreatic adenocarcinoma.[33] Its molecular mode of action has been investigated *in vivo* and *in vitro*: whole-genome expression analysis of metastatic breast tumor biopsies after 1 month of treatment with erlotinib, revealed predominantly EGFR pathway-related changes in EGFR-positive tumors (as assessed by immunohistochemistry) and multiple signal transduction-related changes in EGFR-negative tumors.[34] A similar analysis of a human colonic adenocarcinoma xenograft mouse model pointed to significant changes in the expression of cell proliferation-related genes following erlotinib exposure. Interestingly, the gene expression profiles related to combination of erlotinib with the cyclooxygenase-2 inhibitor celecoxib showed only a partial overlap with those of erlotinib alone, thus suggesting a substantial benefit from the synergistic effect of the two agents.[35] The effectiveness of erlotinib in modulating radiation response has been analyzed in human squamous-cell carcinoma of the head and neck cell lines, revealing an enhancement of cell cycle arrest, apoptosis, accelerated cellular repopulation and DNA damage repair.[36]

Notably, gene expression signatures of NSCLC cell lines with differential sensitivity to erlotinib have enabled the development of a model for prediction of response to therapy.[37] However, validation of the accuracy of this predictive model in human NSCLC tumors is difficult as erlotinib is currently indicated as second- or third-line therapy in this setting. Consequently, availability of tissue from patients with NSCLC after erlotinib therapy is unlikely, as biopsy is rarely performed once the diagnosis of lung cancer has been established.

Cetuximab

The chimeric monoclonal antibody IgG1 cetuximab, which targets EGFR, has been approved by the Federal Food and Drug Administration (FDA) of the United States and the European Medicines Agency (EMA) for the treatment of patients with metastatic colorectal cancer whose tumors do not harbor mutations of the *KRAS* proto-oncogene (a key downstream effector of EGFR activation), as the presence of such mutations renders the signal independent of EGFR inhibition.[38] This indication represents a hallmark in the history of molecular-targeted therapy in oncology, as for the first time a targeted agent receives FDA and EMA approval for use in a selected patient population, based on the results of pharmacogenetic analysis; the absence of *KRAS* codon 12 or 13 mutations is required for the administration of the drug. Cetuximab has also been approved for the treatment of locally advanced head and neck cancer in combination with radiotherapy,[39] whereas recently the same antibody was successfully combined with platinum-based chemotherapy to increase overall survival in patients with locally advanced or metastatic NSCLC.[40]

Similarly to EGFR-TKIs, cetuximab has also been evaluated at the whole-genome level yielding largely similar molecular results: In colorectal cancer the molecular mechanisms of cetuximab action were recently investigated by microarrays, revealing significant changes in tumor proliferation- and inflammation-related genes, even after a single drug dose.[41] Additionally, the effectiveness of combination treatment with cetuximab, the antimetabolite capecitabine and radiotherapy was assessed in patients with rectal cancer by combining genomic and proteomic measurements: Through the development of new bioinformatic tools these data led to a predictive model based on five genes and 10 proteins that could predict the rectal cancer regression grade with an accuracy of 91.7%, sensitivity of 96.2% and specificity of 80%.[42]

In metastatic colorectal cancer, even some patients with *KRAS* 'wild-type' tumors are reported to be resistant to cetuximab.[43] In a meta-analysis of microarray data from cetuximab-treated patients with metastatic colorectal cancer, a 26-gene predictive model was developed that could distinguish sensitive and resistant tumors, and therefore select the *KRAS* wild-type patients who would benefit from cetuximab treatment.[44] By improving patient stratification, it is hoped to maximize the clinical benefit from different therapies.

Pharmacogenomics of c-ERBB2 (HER2) molecular-targeted therapies

Trastuzumab

The v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 and neuro/glioblastoma derived oncogene homolog (c-ERBB2 or HER2) receptor has been found to be expressed in approximately 15–20% of breast cancer cases to which it confers a poor prognosis.[45] Trastuzumab is a humanized monoclonal antibody directed against HER2-overexpressing

cancer cells that is currently indicated for the adjuvant or first-line treatment of breast cancer patients whose tumors overexpress HER2 as assessed by either immunohistochemistry or fluorescent/comparative *in-situ* hybridization.[46] Trastuzumab has also been evaluated by a series of whole-genome expression analyses in an effort to characterize its molecular mode of action. Through the study of breast cancer cells overexpressing HER2, whole-genome expression analysis revealed a molecular cross-talk between the HER2 receptor and fatty acid synthase signaling pathways, with pharmacological inhibition of fatty acid synthase preferentially killing HER2-overexpressing breast epithelial cells.[47] Furthermore, trastuzumab repressed fatty acid synthase expression, which could be contributing to its anti-tumorigenic effect. Importantly, these findings raise the possibility that measurement of fatty acid synthase activity post-HER2-targeted therapy could have a predictive potential in the clinical setting.[48] In a different study, breast cancer cells treated *in vitro* with trastuzumab were screened at the transcriptome level, revealing the molecular pathways through which this drug inhibits proliferation and functions in concert with the mitotic spindle-targeting chemotherapeutic agent docetaxel to decrease tumor growth.[49] Specifically, trastuzumab was shown to function primarily not only by affecting genes involved in the progression of the G2–M cell cycle phase but also by inducing cell cycle G1 arrest through the inhibition of the PI3K–AKT signaling pathway. The growth inhibition achieved through the combination of trastuzumab and docetaxel appeared to be associated with the downregulation of two G2–M phase genes, *HEC* and *DEEPEST*.[48]

Transitioning from bench to bedside, it is critical to develop the means for distinguishing the patient subgroups, which are likely to benefit from trastuzumab treatment, either in the adjuvant or in the metastatic setting. In early breast cancer, *HER2* gene amplification and/or immunohistochemical overexpression have been associated with prognosis or response to anticancer therapies, and trastuzumab therapy is well known to benefit patients with metastatic disease too.[49] However, HER2 status alone is not always accurate or sufficient for identifying the patients who will benefit from trastuzumab treatment, and clinical outcome following therapy may vary among patients with the same HER2 status.[50] As a consequence, numerous studies used microarrays in an effort to determine gene expression signatures specific of HER2-overexpressing tumors, and reported repeated and reproducible breast tumor molecular subtypes in independent gene expression data sets with distinct patterns of response to trastuzumab therapy.[51–53] In one of these studies,[53] a total of 115 malignant breast tumors were analyzed by hierarchical clustering based on patterns of expression of 534 genes and were shown to subdivide into one basal-like, one *ERBB2*-overexpressing, two luminal-like and one normal breast tissue-like subgroup. These patterns provide a distinctive molecular portrait for each tumor category, reflect the different oncogenic pathways that contribute to neoplastic transformation and progression, and support the use of individualized therapeutic approaches directed to the specific molecular characteristics of each tumor subtype, including the use of molecular-targeted agents.[54]

Another important question regarding the use of trastuzumab in the clinic involves the identification of markers able to predict HER2-overexpressing breast cancer patients' response. Recently, a 28-gene expression signature was identified that could predict response to trastuzumab- and docetaxel-based treatment with 92% accuracy, 89% specificity and 100% sensitivity.[55] The development of resistance to trastuzumab among patients who initially respond to treatment poses another important clinical challenge. Microarray analysis of BT/Her(R) and BT474 cell lines now points to activation of protein kinase A signaling as one critical mechanism contributing to trastuzumab resistance in HER2-overexpressing breast tumors.[56]

Lapatinib

Lapatinib, a dual TKI of EGFR and HER2, has been shown to significantly inhibit both *in vitro* and *in vivo* the proliferation of cancer cells overexpressing EGFR and/or HER2.[57] Lapatinib blocks ligand-activated signaling from multiple receptor combinations, including homo- and heterodimers of EGFR and HER2.[58] In preclinical models, it inhibits the proliferation of trastuzumab-resistant breast cancer cells,[59,60] and has proven effective in the treatment of phosphatase and tensin homolog-deficient breast cancer.[61] Lapatinib, in combination with the orally available antimetabolite capecitabine, is currently indicated for the treatment of patients with advanced, recurrent or metastatic breast cancer that overexpresses HER2 (assessed by immunohistochemistry and/or fluorescent/comparative *in-situ* hybridization) after failure of treatment with anthracyclines, taxanes and trastuzumab.[62] Characterization of the molecular implications of lapatinib exposure in breast cancer cell lines at the whole-genome level showed strong differential effects on multiple genes in the AKT pathway and a consequent upregulation of the proapoptotic gene *FOXO3A*, which is negatively regulated by AKT.[63] Furthermore, lapatinib stimulated the expression of estrogen and progesterone receptors and modulated the expression of genes involved in cell cycle control, glycolysis, and fatty acid metabolism. Among its different effects, there is evidence to show that lapatinib significantly inhibits the growth of HER2-amplified gastric cancer cells and, in combination with fluoropyrimidines, results in a synergistic growth-inhibitory effect *in vitro*.[64] Microarray analysis of gastric cancer cell lines indicated that lapatinib inhibits the nuclear translocation of EGFR and HER2, and downregulates thymidylate synthase, which is frequently overexpressed in fluoropyrimidine-resistant cancer cells, thus sensitizing cancer cells to fluoropyrimidine.[65] As compared with gefitinib, lapatinib treatment induced higher fold reductions of all of the downregulated genes, suggesting that the inhibition of both EGFR and HER2 might be more effective from the perspective of gene regulation.[65]

Although lapatinib has shown promise in clinical trials in breast and other cancers,[66] and expression of HER2 has been

established as a minimal requirement for patient inclusion in breast cancer trials,[67] biomarkers with greater predictive accuracy for lapatinib sensitivity are needed. Toward this goal an elaborate transcriptome analysis of 11 sensitive and 16 resistant bladder cancer cell lines led to a 33-gene predictive model that achieved a maximum of 98% mean accuracy.[68] It is noteworthy that this model substantially outperformed models based on the EGFR pathway alone, whether it was interrogated through protein microarrays (mean accuracies, 55, 55 and 61%) or as a subset of the microarray data (mean accuracy, 86%).[68] These findings combined provide strategies as well as new information that will allow the consideration of lapatinib for the treatment of additional cancers, and the development of a personalized approach that will enable the administration of the agent only to those patients who are most likely to derive substantial clinical benefit.

Pharmacogenomics of the c-KIT receptor molecular-targeted therapies

Imatinib

Among molecular-targeted therapies imatinib mesylate appears to be the most studied in microarray analyses, which predominantly aimed at identifying the molecular mechanisms of drug action[69] and the gene expression patterns associated with response to treatment in hematological malignancies. Imatinib was initially developed as a specific inhibitor of the BCR-ABL TK, and results from clinical trials have demonstrated that it is highly effective in the treatment of Philadelphia chromosome positive chronic myeloid leukemia patients; however, response to treatment is variable and resistance can develop in all phases of this disease. Using whole-genome expression arrays, a number of studies have identified gene expression signatures (consisting of 15, 30 and 31 genes, respectively) that distinguish imatinib responders and non-responders among cohorts of patients with chronic myeloid leukemia,[70,71] and others that correlate with relapse after initial successful treatment with imatinib[72] or development of imatinib resistance.[73] These findings emphasize the importance of evaluating molecular-targeted therapies at the genomic level across a wide range of human malignancies and demonstrate the clinical relevance of gene expression signatures in pretreatment tumor assessment.

In the field of solid tumors, approximately 95% of gastrointestinal stromal tumors (GISTs) of the alimentary tract are immunohistochemically positive for expression of the c-KIT protein, coded by the *c-KIT* proto-oncogene. Imatinib mesylate is a potent selective inhibitor of the TK domain of the c-KIT protein. Patients with GISTs who received imatinib and are also carrying *c-KIT* exon 11 mutations (usually deletions or substitutions), tend to have a substantially higher response rate, reduced risk of progression and longer median survival, compared with those with GISTs carrying wild-type or exon 9 mutations.[74] Therefore, molecular identification of the specific mutation type is currently recommended for optimal use of the drug. In this context, microarrays have recently started to fulfill their potential in deciphering the pharmacogenomics of imatinib: The agent was studied by whole-genome expression arrays in patients with localized prostate cancer in order to determine its molecular mechanism of action. The findings demonstrated that the MAP kinase cascade and the thrombospondin-1-induced apoptosis in microvascular endothelial cells are the top two pathways upregulated in response to imatinib, supporting the hypothesis that the main mechanism of action is likely through inhibiting platelet-derived growth factor receptor-β activity in pericyte and/or endothelial cells in tumor microvasculature.[75] *In vitro*, human neuroblastoma cell lines were investigated using a transcriptome-wide analysis to characterize imatinib molecular effects, and focused on the *SLUG* gene.[76] The significant downregulation of *SLUG* was proposed to facilitate apoptosis in these cells following treatment and to decrease their invasion capability *in vitro* and *in vivo*. These results point to *SLUG* inhibition as an additional promising therapeutic target that could potentially be combined with imatinib treatment.

Using advanced bioinformatic tools and array gene expression data from GIST cell lines, the molecular off-target effects of imatinib were also determined and appeared to implicate, among else, the p53 signaling pathway.[77] In a reverse approach, the gene expression profiling of an *in vitro* Kaposi's sarcoma model, led to the identification of the genes induced by Kaposi's sarcoma-associated herpes virus, which included c-KIT. Inhibition of c-KIT activity with imatinib reversed the Kaposi's sarcoma-associated herpes virus-induced transformation of infected cells, indicating that c-KIT has a central role in this disease and suggesting imatinib as a potential therapeutic agent.[78]

At the *in vivo* level, in GISTs and leiomyosarcomas, which present with remarkably similar phenotypic features, yet very different response to imatinib treatment (>50% for GIST patients compared with no benefit for leiomyosarcoma patients), whole-genome expression analysis led to the identification of a highly accurate two-gene (obscurin and C9orf65) classifier differentiating GIST from leiomyosarcoma tumors that will help in diagnosing and treating these patients.[79]

Dasatinib

Dasatinib is a potent, orally available small-molecule inhibitor that targets multiple cytosolic or membrane-bound TKs, including Src-family kinases, BCR-ABL, c-KIT, platelet-derived growth factor receptor-β and EPHA2.[80–82] Owing to its potency against leukemic cancer cell lines harboring *BCR-ABL* mutations,[83] the clear and imminent need for overcoming imatinib resistance, and the profound clinical benefit demonstrated in phase II clinical trials, dasatinib was recently approved for use in chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia that are resistant or intolerant to imatinib.[84] The involvement of Src-family kinases in a number of cellular processes, such as cell migration, adhesion and

angiogenesis, as well as participation of Src-family kinases in a number of clinically relevant pathways (for example, the EGFR pathway)[85,86] have prompted investigations into the potential use of dasatinib in solid tumors.[87] Genomic studies of 838 samples from NSCLC and SCLC pointed to the 'leukocyte extravasation' and the 'Wnt–β-catenin' signaling pathways as promising therapeutic targets in both tumor types.[88] On the basis of these predictions, dasatinib would be a well-suited agent for these molecular targets.[89] In a reverse approach, whole-genome expression profiling was used for the identification and stratification of efficacy biomarkers for prostate cancer in a preclinical model. Following exposure of 16 prostate cancer cell lines to dasatinib, an expression signature of 174 genes was selected, and 10 highly and significantly changed genes were proposed as efficacy markers.[90] Studies such as these are increasingly providing the basis for clinical evaluation of molecular-targeted agents in different cancers.

Applications of array-based pharmacogenomics in identifying new targets, targeting agents and therapeutic doses

As exemplified in the aforementioned studies of molecular-targeted therapies, global gene expression screening might not only unveil the molecular pathways affected by specific drugs but may also help to define the pathogenic molecular effectors that need to be targeted by appropriate drugs. Following this approach, Wang *et al.*[91] extracted all PubMed-published genetic associations for the development of cholangiocellular carcinoma and integrated related microarray data, demonstrating that the MAPK pathway was a relevant therapeutic target, and that sorafenib, a selective Raf kinase inhibitor, could be a potential treatment. Another approach for identifying promising therapeutic targets and developing new therapies involves determining the mode of action of empirically used drugs. Along these lines, microarrays were used to determine the molecular mechanisms regulated by all-*trans* retinoic acid for the treatment of non-melanoma skin cancer, and the B-Raf/Mek/extracellular-regulated protein kinase pathway emerged as a significant regulator.[92] In turn, sorafenib was used to target this pathway in tumor-bearing SENCAR mice treated with a tumor-promoting agent, and a favorable outcome was observed. This suggests that sorafenib could potentially function as a tumor suppressor in squamous cell carcinoma of the skin through induction of squamous differentiation and the accompanying exit from the cell cycle that results from blocking B-Raf/Mek/extracellular-regulated protein kinase signaling.[92] A genomic approach has also been applied in characterizing the molecular implications of the combination treatment with sorafenib and the mammalian target of rapamycin inhibitor rapamycin in hepatocellular carcinoma cell lines and xenografts.[93] The findings indicated RAS activation as the key molecular change resulting from a number of genetic alterations, such as methylation of tumor suppressors and amplification of oncogenes. Sorafenib blocks signaling and synergizes with rapamycin *in vivo*, preventing tumor progression, thus providing a basis for testing this combination in clinical studies.[93]

Moving away from the single drug/single target-oriented approach, recent studies are offering a global genome perspective, across a wide variety of cell lines and importantly, in relation to multiple molecular-targeted agents. In a milestone study for the pharmacogenomics field, Sos *et al.*[94] analyzed the global gene copy number alterations and mutations associated with 84 human NSCLC cell lines and analyzed drug activity as a function of genomic alterations in a systematic manner. All the cell lines were profiled against 12 molecular inhibitors (namely dasatinib, erlotinib, lapatinib, vandetanib, sunitinib, purvalanol A, rapamycin, PD168393, VX-680, 17-AAG, UO126 and SU11274) revealing that although some of the compounds exhibited a pronounced cytotoxic activity in a small subset of cell lines (for example, erlotinib, vandetanib, VX-680), others were active in most of them, with only a minority being resistant (for example, 17-AAG). To discover genetic markers for predicting responsiveness to the 12 inhibitors, the investigators proceeded to identify therapeutically relevant genetic alterations using two different bioinformatic approaches. Among the numerous findings, the response predictor for lapatinib involved amplifications of the *EGFR* or the *HER2* genes, and for dasatinib copy number gain of gene family members of ephrin receptor kinases, SRC kinases and ABL2. Overall, this genomically, phenotypically and functionally validated tool serves as a powerful strategy for preclinical molecular drug target validation on the basis of genetic characteristics of individual tumors that will greatly facilitate the transition from preclinical drug discovery to clinical trials.[94]

Toward this goal, a follow-up study utilized these NSCLC data as well as a series of other tumor cell lines and *in vivo* cancer models to identify common downstream oncogenic pathways whose inhibition would have a therapeutic benefit for a broad range of tumors. This represents an important step away from the 'specific therapies for specific mutations' principle. By systematically linking drug response to genomic aberrations, the same authors demonstrated that tumors with genetically activated receptor TKs depend on PI3K signaling, whereas tumors with mutations in the RAS/RAF axis depend on MAPK signaling.[95] However, the efficacy of inhibition of these downstream pathways was limited due to the release of negative feedback loops that activated alternate pathways. Combined inhibition of both PI3K and MAPK signaling potently suppressed the activation of negative feedback loops, resulting in enhanced induction of apoptosis in tumor cells and tumor shrinkage *in vivo*.[95] Furthermore, it was suggested that intermittent high dosing of downstream signaling pathway inhibitors might be better tolerated and allow for more potent target inhibition, induction of apoptosis and tumor control than continuous administration of lower doses of such combinations.

A novel application of gene expression microarrays relates to the identification of transcripts with a close correlation to the dose–response curve of a drug, and is likely to benefit all

the points of drug development where expression profiling is used, from early discovery to evaluation at the clinical setting. In the first study of this kind, imatinib, dasatinib, nilotinib (similar to imatinib but more potent second-generation compound) and PD0325901 (MEK inhibitor) were evaluated at the transcriptional level across a series of different doses.[96] The transcript responses proved a powerful means to characterize and compare the compounds, linking specific targets to EC50 values and identifying dose-dependent effects on cellular processes. For example, a single-dose experiment would depict dasatinib's impact on both DNA replication and p53 signaling, whereas this dose–response design revealed that the impact on the p53 pathway only occurs at micromolar doses, and is therefore unrelated to the nanomolar anti-proliferative effects of dasatinib. In another example, increasing doses of imatinib were associated with the development of additional transcriptional changes, indicating that evaluation of the molecular effects at different dosing levels is critical in the preclinical studies of new molecular-targeted therapies.

Current limitations and perspectives

Advancements in bioinformatic analysis tools in combination with the rapidly increasing numbers of publicly available microarray data sets will allow the performance of powerful meta-analyses and through them the more refined characterization of existing molecular-targeted therapies and the fast development of new and improved ones. Importantly, better characterization of the different tumors, individuals, genders and populations are anticipated to drastically change the way molecular-targeted therapies are developed, evaluated and administered to patients. For example, a recent meta-analysis[89] of the gene expression signatures from 725 NSCLC samples, not only revealed promising therapeutic targets but also pointed to multiple molecular pathway differences between Eastern and Western populations. These molecular differences are consistent with recent trials for advanced NSCLC showing improved results with the combined use of cetuximab and first-line chemotherapy in Western population, but better results with erlotinib in Eastern populations.[39,97]

Despite the major impact of high-throughput genomics approaches on basic and clinical research, several limitations remain to be overcome. Key improvements needed in microarray-based approaches and studies to make them more robust and clinically more relevant involve three general areas as follows: the experimental/methodological aspect, the clinical research study designs and implementation and the mode of 'translation' of research findings to the clinic. Specifically, the microarray-generated experimental and clinical research findings need to be validated across microarray platforms, across molecular methods, across laboratories and across ethnic backgrounds, to avoid technical, methodological, environmental and biological bias. A marked example of the heightened need for validation of microarray findings comes from the work of Potti *et al*.[98–101] originally presenting gene expression signatures predictive of NSCLC recurrence risk and, in a separate study, signatures predictive of individual sensitivity to chemotherapeutic drugs, which were both later retracted for failure to reproduce results validating these signatures. For similar reasons the work of Bonnefoi *et al*.[102,103] on expression profiles predicting response of breast cancer to neoadjuvant chemotherapy had to be retracted in 2011.

Given the hundreds of thousands of measurements obtained in each microarray experiment, as well as the multiple environmental parameters affecting gene expression and drug action, it is imperative that the composition and size of patient cohorts is sufficiently large to provide statistically significant and biologically meaningful data. Appropriate guidelines are still missing regarding the design, conduction, interpretation and translation to the clinic, of pharmacogenomic studies.

At the technical level, the microarray laboratory and analysis protocols need to be standardized for clinical use, ensuring high sensitivity, specificity and reproducibility, while minimizing the chances for environmentally introduced variability. Along these lines, the microarray protocols for routine clinical use should become easier to set up and perform, involving fewer steps and well established, widely accepted, robust quality controls to be applied along each step of the sample handling/preparation. An additional challenge is the extensive use of formalin-fixed paraffin-embedded tissues in the oncology clinics, with thousands of such specimens available in large cancer tissue banks worldwide. As different formalin-fixed paraffin-embedded procedures have varying effects on RNA quality, to date only few studies have systematically characterized microarray gene expression signal performance with degraded RNA from formalin-fixed paraffin-embedded in comparison with intact RNA from unfixed fresh-frozen specimens. Recent developments in the field are now giving rise to methodologies that could help address this problem, although more research toward this direction is still needed.[104–108]

Similarly, the software to be used for the analysis of diagnostic/prognostic/pharmacogenomic microarray-based tests will need to provide easily applicable analysis 'pipelines' for non-bioinformaticians. Importantly, these 'pipelines' will need to include appropriate multigene classifiers that will use carefully developed mathematical forms of combining the individual gene expression measurements, their different 'weights', and thresholds, all of which has to be established before the release of such a microarray test to the clinical setting.[109] As per standard procedures in microarray analysis software, an automatically generated detailed report of the quality control results in each experiment would be critical. An independent, nevertheless significant, parameter toward making the routine clinical application of these molecular tests a financially realistic scenario is the reduction of the hardware, software and consumables' cost. For cases in which the microarray-discovered signatures of clinical value involve a limited number of genes, alternative methods such as quantitative

reverse transcriptase polymerase chain reaction could be used for clinical testing, instead of microarrays. These alternative methods should ideally be simpler technically, cheaper, easy-to-interpret, compatible with fresh/frozen as well as archived (for example, formalin-fixed paraffin-embedded) biological specimens, and at least as accurate, sensitive and specific as microarrays. Ultimately, carefully designed and internationally accepted guidelines will need to be established for the clinical application of microarrays.

Although microarrays have made a significant contribution in the basic and clinical research settings, and as they are steadily gaining ground in the clinical (diagnostic/prognostic/pharmacogenomic) setting, a new technology with similar applications is rapidly evolving, namely next-generation sequencing. The hype created around this technology is reminiscent of that hype around microarrays in the late 1990s. Theoretically, next-generation sequencing should be more accurate in identifying single-nucleotide polmorphisms and mutations, as well as quantifying the number of transcripts present in a given sample than microarrays. The increased affordability of comprehensive sequence-based genomic analysis will enable new questions to be addressed in many areas of biology. It is inevitable that massively parallel sequencing platforms will supersede microarrays for many applications, and several publications are debating whether this is indeed 'the beginning of the end for microarrays'.[110] However, next-generation sequencing is still in its infancy, with many issues to be resolved including accuracy, data analysis, ease of use, cost and so on. Although it is tempting to debate, which technology will ultimately prevail, most likely, as next-generation sequencing matures methodologically, a symbiotic relationship will be established between the two technologies—each one prevailing for certain applications.

In conclusion, pharmacogenomics of molecularly targeted agents has the potential to directly affect the patients, the pharmaceutical industry and the healthcare systems worldwide[111]—a contribution which can be at least partly attributed to microarray findings. Medical oncology has benefited the most to date, as microarray-based pharmacogenomics have led to several major contributions. These include the construction of multigene models with predictive or prognostic significance that may outweigh well-established clinical prognosticators, and the identification of molecularly distinct subclasses of tumors that may bear the same histology but originate through the activation of completely different oncogenic pathways. In the field of molecular-targeted therapy, in particular, elucidation of the molecular mechanisms that provoke neoplastic transformation in each tumor subtype may point to the appropriate molecular target and consequently to the most suitable molecular agent specific for that target. Provided that wide clinical application will be economically feasible and that methodological limitations will be addressed, array-based approaches have the potential to render individualized therapy in the oncology setting a feasible strategy in the near future.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

DS is supported by Grants from the European Community's Seventh Framework Programme FP7/2007–2013 under grant agreement #HEALTH-F2-2009-241526, 'EUTrigTreat', the European Community's Sixth Framework Programme FP6 under grant agreement #LSHG-CT-2006-037277, 'VALAPODYN', the Hellenic Cardiological Society and the John S Latsis Public Benefit Foundation.

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