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REVIEW PAPER

Preclinical strategies to define predictive biomarkers for therapeutically relevant cancer subtypes

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Abstract Defining key driver mutations in cancer, the resulting aberrations in molecular mechanisms and the subsequent phenotype underpins the development and implementation of novel personalized medicine strategies. The literature is replete with biomarkers of prognosis and therapeutic responsiveness identified in single cohorts of patients that have not been independently validated and as a consequence, not developed. Integrating companion biomarker discovery with therapeutic development at the preclinical stage creates the opportunity to identify candidate biomarkers early, which would significantly facilitate both biomarker and therapeutic development. Advances in "-omic" technologies have led to large-scale efforts in characterizing and cataloguing the full range of aberrations in cancer. These include the International Cancer Genome Consortium and The Cancer Genome Atlas, which aim to comprehensively catalogue the range of genomic aberrations for large numbers of cancers for a progressively increasing range of cancer types and subtypes. The technical challenges associated with achieving these goals in some instances have required the generation of primary xenografts and cell lines. These extensively characterized model systems will provide an unprecedented resource for the discovery of biomarkers of therapeutic responsiveness for established therapies, and the development of companion biomarkers linked with preclinical novel therapeutic development in the future.

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Introduction

The ultimate goal of cancer therapy is to administer optimal treatment that is determined prior to the initiation of therapy and tailored to an individual based on the characteristics of their disease and their metabolism. Stratifying individual patients based on stage and phenotype to guide treatment decisions is the basis of modern clinical oncology practice. This stratification has evolved through clinical observation of the natural history and therapeutic responsiveness of the disease and their relationship to morphological, and more recently, molecular and genetic characteristics. Significant gains have been made through defining molecular phenotypes of individual cancers. First, this aids individualization of therapy, so that optimal treatment is given without delay, minimizing unnecessary adverse effects. Second, this has guided and focused research to identify mechanisms amenable to the development of novel therapeutic strategies for phenotypes that are non-responsive to conventional treatments. Finally, patients are better informed with respect to the likely outcome of their disease and their expected quality of life.

The advent of drugs designed to target a specific molecular mechanism has introduced novel therapeutic approaches for several cancers. The earliest examples include tamoxifen and estrogen receptor (ER) positive breast cancer, the anti-HER2/*neu* monoclonal antibody trastuzumab, which is effective against a significant proportion of HER2/neu amplified breast cancers (Slamon et al. 2001), and the tyrosine kinase inhibitor imatinib, which targets BCR-ABL, a constitutively active tyrosine kinase fusion protein, for the treatment of chronic myeloid leukemia (CML) (Druker et al. 2001a, b). Subsequent identification of post-treatment acquired BCR-ABL mutations, which confer imatinib resistance, promoted the

development of second generation tyrosine kinase inhibitors such as dasatinib and nilotinib. These drugs were designed to bind and inhibit ABL with less stringent conformational requirements and were effective in the treatment of imatinib resistant CML (Kantarjian et al. 2006; Talpaz et al. 2006). Although these model examples show how the discovery and successful inhibition of key molecular mechanisms define biologically and clinically relevant cancer phenotypes, the majority of malignancies are yet to be subclassified in such a meaningful way. Defining these subtypes, their underlying molecular evolution and the molecular mechanisms underpinning de novo or acquired resistance to current therapy is critical for identifying potential targets for the development of novel therapeutic strategies. This article presents some of these challenges and suggests how the development of in vivo systems, which capitalize on novel "-omic" technologies, can potentially be used to accelerate the discovery and implementation of biomarkers with clinical utility.

Defining cancer phenotypes

There is now compelling evidence that the molecular heterogeneity of cancer leads to disparate phenotypes, and as a consequence, differential sensitivity to therapy and outcome occur in histopathologically indistinguishable cancer types. Molecular diagnostics that provide improved accuracy in prognosis and facilitate the selection of the optimal therapeutic regimen for an individual have the potential to both improve patient outcomes and reduce the cost of treatment, and are central to the development of a personalized approach to patient care.

Single-gene biomarkers of therapeutic response

Thus far, single-gene biomarkers useful in therapeutic selection have been successfully developed primarily in instances where the target molecule is known and the molecular mechanism well-characterized, for example, the use of anti-epidermal growth factor receptor (EGFR) antibody, Cetuximab®, for the treatment of EGFR-expressing, KRAS wild-type metastatic colorectal cancer (Bokemeyer et al. 2009) or Gleevec® in BCR-ABL-positive CML and c-kit-positive gastrointestinal stromal tumors (Dagher et al. 2002; Heinrich et al. 2002). In a significant minority of patients with non-small-cell lung cancer (NSCLC) molecular markers (*EGFR, KRAS, EML4-ALK*) have been discovered that can guide treatment decisions, suggesting that EGFR-tyrosine kinase inhibitor therapy should be considered first-line treatment in NSCLC patients with *EGFR* mutations (Pao et al. 2004). Notwithstanding the success of these agents, the majority of cases eventually progress despite initial sensitivity and/or long-lived responses, indicating that for the majority of tumors, therapeutic responsiveness is regulated by multiple factors.

Whilst experimental models have demonstrated that cells can be transformed by as few as 4 or 5 key driver mutations, more recent high-throughput, large-scale genome analyses have demonstrated that the mutational landscape of cancer is complex (Beerenwinkel et al. 2007; Croce 2008), indicating that cancers may evolve through driver mutations in as many as 20 cancer-associated genes. This is further illustrated by the existence of de novo resistance of HER2-positive breast cancers to trastuzumab. In the first-line single-agent setting, only one-third of patients with metastatic HER2-overexpressing breast cancer respond to this therapy (Vogel et al. 2002), indicating that within this subset of patients there are as yet unexplored subtypes of HER2-positive disease, defined by additional molecular aberrations that contribute to therapeutic resistance.

In parallel with biomarkers of therapeutic responsiveness, several assays for germline mutations have successfully been implemented to identify individuals at high risk of developing cancer, with cancers that develop in these situations sometimes having phenotypes and therapeutic responses that differ from sporadic cases. For example, mutations in *BRCA1* or *BRCA2* tumor suppressor genes lead to a dramatically increased risk of developing breast and ovarian cancer (Futreal et al. 1994; Wooster et al. 1995) and mutations in genes whose products are involved in DNA-mismatch repair (such as *MLH1*, *MSH2* or *MSH6*) have been associated with hereditary non-polyposis colorectal cancer (Marra and Boland 1995). Perhaps the most striking cellular hallmark of tumors with mutations in the Fanconi anemia (FA) pathway, is their hypersensitivity to a class of DNA damaging agents that create DNA interstrand cross-links, such as mitomycin C or platinum agents (Xia et al. 2007; Byrski et al. 2009), reviewed in (Moldovan and D'Andrea 2009). Through complete exomic genome sequencing in a patient with familial pancreatic cancer, a germline, truncating mutation in PALB2, a partner and localizer of BRCA2 to DNA, was found to be responsible for this patient's predisposition to the disease (Jones et al. 2009).

Gene signatures

The advent of high-throughput technologies, which interrogate many thousands of molecular variables, has enabled characterization of individual human cancers in an unprecedented level of molecular detail, with potential to identify relevant critical mechanisms that ultimately drive cancer phenotypes. It is expected that a complete atlas of genomic aberrations that occur in individual cancers will

become available in the near future, with the subsequent challenge being to establish which molecular abnormalities drive cancer development and progression and which simply represent 'noise'.

Breast cancer is the most advanced in its molecular taxonomy with the establishment of biomarkers and signatures with potential clinical utility. Several prognostic signatures are under clinical development with prospective clinical trials underway for two independent breast cancer signatures of survival (van't Veer et al. 2002; Paik et al. 2004). The first US Food and Drug Administration (FDA)-approved microarray-based prognostic for breast cancer recurrence, MammaPrint, uses a 70-gene signature to predict the risk of distant metastasis and is designed to guide therapy by indicating which lymph node-negative breast cancer patients are likely to require chemotherapy in addition to surgical intervention (van't Veer et al. 2002; van de Vijver et al. 2002). The second gene expression-based prognostic test for breast cancer in clinical trials, Oncotype DX, analyzes the expression of a panel of 21 genes and quantifies the likelihood of disease-free survival in women with early stage ER-positive, node-negative breast cancer, who will be treated with tamoxifen (Paik et al. 2004). Using similar strategies, gene expression signatures that assess the risk of recurrence in several other cancer types have also been reported.

Recent advances in nucleic acid sequencing technology have made it feasible to rapidly, and exhaustively sequence an entire genome. With sequencing costs continually decreasing, these advances are dramatically changing approaches to cancer research, and in the longer term, will alter clinical practice for many cancers. The landmark study undertaken by The Cancer Genome Atlas (TCGA) Research Network in glioblastoma is one example of the power of combining multi-dimensional datasets. TCGA, launched in 2006, is a comprehensive and coordinated effort to define the key molecular aberrations that drive cancer development, through the application of genome analysis technologies, including large-scale genome sequencing. In an interim integrative analysis of DNA copy number, gene expression and DNA methylation aberrations in 206 glioblastoma tumors, the TCGA program revealed several resistant phenotypes that have the potential to inform decisions on optimal treatment strategies for individual tumors (2008).

Following the launch of comprehensive cancer genome projects in the United Kingdom (Cancer Genome Project) (Dickson 1999) and the United States (TCGA) (Collins and Barker 2007), an International Cancer Genome Consortium (ICGC) was formed to catalogue the full range of aberrations in the genome, epigenome and transcriptome in a large number of cancers, using the latest sequencing approaches to define novel "driver" mutations (2010). Importantly, the examination of the relationship between the data generated by the ICGC with complete clinico-pathological, treatment and outcome variables that will be available for each patient, has potential to lead to the definition of clinically relevant subtypes for prognosis and therapeutic management, and enable the development of novel personalized cancer therapeutic strategies. It is envisaged that mining the cancer genome, transcriptome and epigenome with current high-throughput technologies will provide important clues as to the mechanisms of carcinogenesis to facilitate molecular biomarker discovery and identify molecular events that can be targeted, aiding in the early detection, diagnosis, prognosis and the prediction of patient responses to specific therapies.

Current challenges in defining clinically and biologically relevant phenotypes

The initial enthusiasm for gene expression-based prognostic and therapeutic response markers has been tempered by the reality of translating research findings to the clinic, with at best only incremental changes in diagnostic and treatment strategies in most cancers. Important technical concerns with earlier studies have been the lack of attention to proper study design and quality assurance, as well as questions relating to the robustness of microarray technology (Marshall 2004). Furthermore, as multigene signatures are often generated from retrospective cohorts, their prospective validation is an additional hurdle for their implementation into routine clinical practice. This is primarily due to the need for large cohorts requiring thousands of patients, where results are usually at least 5 years away and have a significant cost.

Predictive biomarkers are increasingly being used in clinical trials in leukemia. However, considerably less progress has been made in stratifying and treating solid tumours, due to the difficulty in repeatedly sampling tumor tissue so that biomarkers can be measured. In contrast to leukemia, where tumor cells are abundant in the peripheral blood and repeated sampling is relatively non-invasive, access to solid tumor tissue is only guaranteed at the time of diagnosis, when the tumor is biopsied or surgically resected. Although this approach may be sufficient to identify prognostic biomarkers, as illustrated by the microarray-based prognostic, MammaPrint (van't Veer et al. 2002), their value for predicting therapeutic responsiveness to particular treatments is less convincing. Moreover, considerable inter- and intra-patient variability in drug pharmacokinetics is an additional obstacle when trying to predict therapeutic responsiveness in the clinic. Finally, the efficacy of novel therapies is often first

determined in patients with late-stage disease, who do not routinely undergo additional tumor biopsies.

An alternative strategy may be to identify candidate predictive signatures or biomarkers in preclinical models, such as cell lines or animal models, with subsequent clinical validation, thereby reducing the number of patients required for tissue collection. Several groups have used gene-expression profiling on selected chemo-sensitive and -resistant human cancer cell lines (e.g., a panel of 60 established cell lines, NCI-60), to identify gene signatures that correlate with treatment response in vitro (Staunton et al. 2001; Lee et al. 2007b), although some challenge the validity of this approach (Coombes et al. 2007). Further limitations include the heterogeneity in the tissue of origin of the NCI-60 cell lines, as such tissue-independent profiles may not be reliable predictors of drug responses for specific malignancies. More recently, in vitro loss-of-function genetic screens have been used as a tool to predict responsiveness to novel therapeutics (Mullenders et al. 2009). The clinical utility of the biomarkers of therapeutic responsiveness identified from in vitro screens remains to be determined as the screening approaches used may not recapitulate the clinical disease accurately enough.

In vivo models for the discovery of predictive biomarkers with clinical utility

Given the technical and logistical difficulties associated with collecting and using patient samples in identifying predictive signatures of therapeutic responsiveness, a potential way forward is to use in vivo models, which more accurately reflect the human disease. Although preclinical models have been extensively used for the development and efficacy testing of novel chemotherapeutic agents, their use in the discovery of predictive biomarkers is in its infancy.

Dissecting the mechanisms underpinning the responsiveness of individual tumors to therapy and secondary resistance mechanisms is likely to lead to improved treatments. Thus far, analysis of tumor samples from patients and of tumor cell lines selected for resistance in vitro has led to the identification of a wide range of resistance mechanisms (Szakacs et al. 2006). Attempts to investigate the relative importance of each of these resistance mechanisms in vivo have been problematic, as there are few realistic animal models of drug resistance.

A large proportion of preclinical testing of novel therapeutics in a variety of solid tumors is based on xenograft models established from tumor cell lines (Johnson et al. 2001; Katz et al. 2004; Larbouret et al. 2010). These models do not represent the full heterogeneity of the human disease, and as a consequence may hamper progress in the development of novel therapies. In vivo models that better recapitulate the human disease and its response to therapy are therefore necessary to identify potential biomarkers of therapeutic responsiveness with clinical utility. The more stable genetic background of mouse models presents a more homogeneous environment and allows examination of tumor-specific factors that may influence therapeutic responsiveness, since several host factors are effectively eliminated, including clinical inter-patient variability in drug disposition. Several in vivo models of cancer are characterized by specific phenotypes, for example, well-defined activating or inactivating lesions that lead to tumor formation in genetically engineered mouse models (GEMMs). These models do not usually reproduce the full genetic complexity of human cancers, which could potentially create issues when using these models for predicting a patient's response to a therapy. The key advantages and disadvantages associated with the mouse models currently used in preclinical testing are summarized in Table 1.

As discussed previously, prognostic biomarker studies utilize patient tissues obtained at diagnosis, whereas studies assessing therapeutic efficacy and chemoresistance are carried out in patients with advanced disease, who do not routinely undergo surgery for additional tissue samples to be collected, thus creating further difficulties in assessing molecular alterations that occur in tumors post-treatment. The use of preclinical models, where pre- and post-treatment samples are readily available for correlative studies and to generate candidate predictive signatures of therapeutic responsiveness using existing and novel technologies, may dramatically accelerate progress in this area.

Genetically engineered mouse models (GEMMs)

Genetically engineered mouse models (GEMMs) for certain types of cancers, including hereditary breast cancer, have proven to be a useful tool for the identification of in vivo chemoresistance mechanisms and to further examine therapeutic benefit of inhibiting novel druggable resistance pathways (Rottenberg et al. 2007, 2008; Pajic et al. 2009; Zander et al. 2010). In 2008, Rottenberg et al. (2008) demonstrated increased sensitivity of *Brca1−/−;p53−/−* mammary tumors in the *K14cre;Brca1F/F;p53F/F* mouse model to the chemical inhibitor of the base excision DNA repair protein Poly(ADP-ribose) polymerase (PARP), illustrating the clinical utility of PARP inhibitors for the treatment of tumors with defects in homology-directed double strand break repair.

The *Pdx1-Cre LSL-Kras^{G12D} Trp53^{R172H}* genetically engineered model for advanced pancreatic ductal adenocarcinoma (PDAC) has proven particularly useful for studying certain aspects of pancreatic cancer development, but also in examining tumor–stroma interactions and its

Table 1 Characteristics of current in vivo models for preclinical testing

Cell line xenografts	Primary xenografts	GEMM
Advantages		
Easy propagation	Heterogeneous tumors	Immunocompetent
Genetic manipulation	Microenvironment (stroma)	Microenvironment (stroma + immune system)
Some characterisation, frequently used, less costly	Potential for characterisation (ICGC)	
Monitoring if heterotopic	Monitoring if heterotopic	
Disadvantages		
Immunocompromised	Immunocompromised	Potentially lack genetic complexity of human tumors
Immunomodulatory agents cannot be examined	Immunomodulatory agents cannot be examined	Disease predisposition model
Poor microenvironment	Some selection in mice inevitable	Differences in mouse versus human
Usually heterotopic (away from tissue of origin)	Usually heterotopic (away from tissue of origin)	Expensive to generate and maintain
Divergent from primary tumor		Monitoring more complex

contribution to drug resistance (Olive et al. 2009). Most recently, a large-scale study has demonstrated that the *Kras*$^{LSL\text{-}G12D}$*Trp53*$^{frt/frt}$ NSCLC GEMM provides insight into the chemotherapeutic responsiveness in the context of mutant *Kras* and wild-type *Egfr* (Singh et al. 2010), which closely reflects clinical findings (Herbst et al. 2005). In contrast, the response to the current standard-of-care in pancreatic cancer, gemcitabine, in the PDAC *Pdx1-Cre LSL-Kras*^{G12D}*p16/p19* $^{fl/fl}$ GEMM was found to be greater than that typically observed in the human patient population (Singh et al. 2010). Furthermore, in the same study, addition of anti-VEGF therapy showed a significant improvement in the overall survival in the PDAC GEMM, whereas there was no survival benefit in the clinic (Kindler et al. 2005), potentially reflecting greater heterogeneity seen among human compared to mouse tumors. Although these GEMMs may therefore be excellent models for studying tumor development and progression and specific resistance mechanisms in the context of mutated oncogenes, such as *Kras*, their use in the identification of predictive signatures and biomarkers of therapeutic responsiveness will largely depend on the model, and may be hindered primarily by the homogeneous genetic background of the murine tumors in comparison with largely heterogeneous sporadic human tumors.

Primary xenografts

Primary xenografts, generated by direct engraftment of cancer samples resected from individual patients may represent improved preclinical models for the discovery of predictive biomarkers and subsequent development of biomarker-guided treatment strategies (Jimeno et al. 2005; Rubio-Viqueira et al. 2006; Kim et al. 2009). Most recent improvements in primary xenograft models have come from the subcutaneous transplantation of primary human tumor tissue into more severely immunocompromised, nonobese diabetic (NOD)/severe combined immunodeficiencies (SCID) IL2rg−/− (NOG) mice, enabling efficient establishment of xenografts (Ito et al. 2002; Quintana et al. 2008), which in the majority of cases appear to faithfully recapitulate the histopathological characteristics and the biological complexity of the primary tumor, including a significant stromal component. Primary xenograft models of childhood acute lymphoblastic leukemia have been successfully used as a preclinical testing tool for novel treatment strategies in this disease (Kang et al. 2007; Lee et al. 2007a; Carol et al. 2010). In neuroblastoma, a rare but aggressive pediatric malignancy, large-scale primary xenograft analyses rapidly translated into promising clinical trials with a topoisomerase I inhibitor, topotecan (Zamboni et al. 1998; Furman et al. 1999). Thus far, primary xenograft models have largely been used to examine the in vivo efficacy of several single-agent targeted therapies with limited characterization of tumor biology pre- and post-treatment, with limited exploration of predictive signatures of therapeutic responsiveness and virtually no examination of secondary resistance mechanisms (Jimeno et al. 2005; Rubio-Viqueira et al. 2006; Messersmith et al. 2009). Most recently, Pitts et al. (2010) demonstrated the importance of primary xenograft models in the generation and preclinical validation of predictive biomarkers for an insulin-like growth factor I receptor (IGFIR) inhibitor, that has potential utility in the treatment of colorectal cancer.

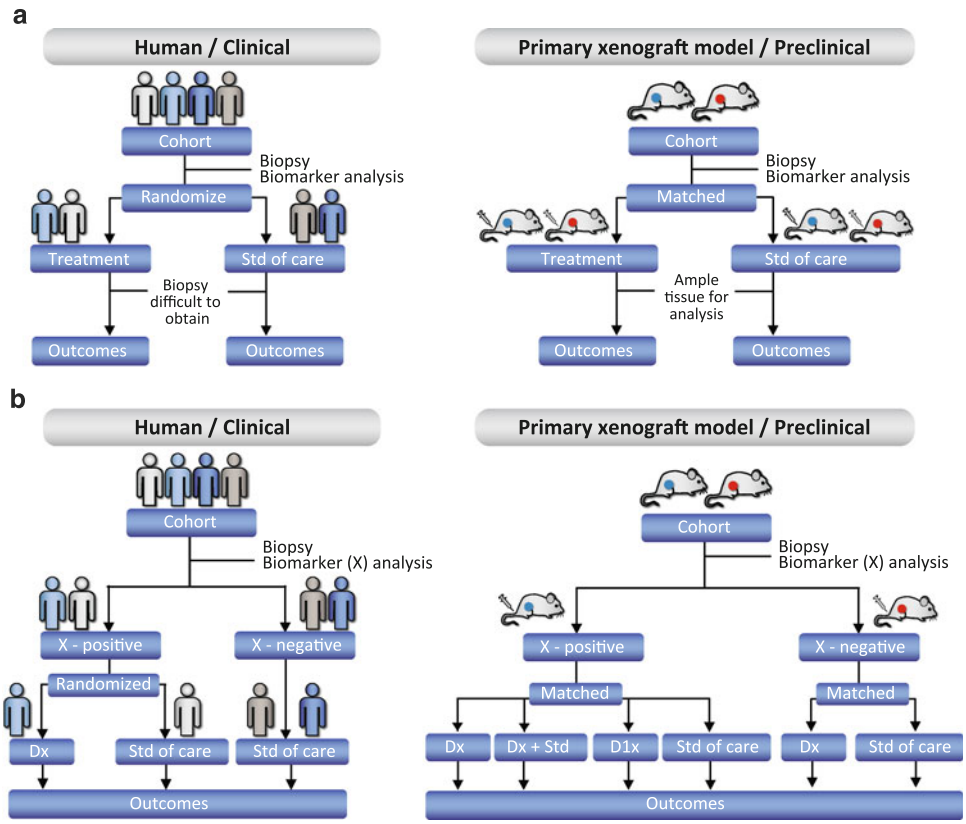

Fig. 1 Trial designs for **a** biomarker identification and **b** targeted trials in a clinical (*left panel*) or preclinical setting (*right panel*). The xenograft studies parallel the design of clinical trials, with the added advantage of treating the same tumor several different ways. *Std of care* standard of care. *Dx, D1x* various novel drugs (*D*) targeting aberration *X*

The generation of primary xenograft models from tumors which will be comprehensively characterized through large-scale sequencing efforts such as the ICGC and TCGA, will provide a valuable resource of renewable material for ongoing experimentation and testing of novel therapeutics linked with discovery of biomarkers of therapeutic responsiveness and molecular mechanisms of therapeutic resistance. Since these extensively characterized xenografts are from individual patients and are renewable from initial stocks, multiple treatments can be examined, providing the opportunity to model and test proposed personalized medicine strategies that are currently intractable in a clinical trial setting, and if the treatment fails, to have the opportunity to essentially re-examine the same tumor. Therefore, the real advantage of using primary xenograft models in the identification of biomarkers of prognosis and therapeutic responsiveness remains to be realized. Such approaches will become increasingly relevant, particularly in cancers such as pancreatic ductal adenocarcinoma, where mortality rates remain extremely high.

The future for preclinical models of cancer

The key applications for animal models in the identification of relevant biomarkers of therapeutic responsiveness and personalized medicine strategies in comparison to human clinical trials are summarized in Figs. 1 and 2. Biomarker discovery can be performed in xenografts in a similar fashion to that in clinical trials with the equivalent of GWAS performed in relation to endpoints such as disease response, PFS and overall survival.

Clinical development of biomarkers, which is discussed in detail elsewhere (Lee et al. 2009), is costly, takes a long time to complete and is associated with considerable ethical, regulatory and cultural challenges. In contrast, prospective testing of biomarker strategies in primary xenografts has distinct advantages compared to clinical trials, as illustrated in Figs. 1 and 2. The major advantage is that each primary tumor is already extensively characterized, and can be reused in different arms of the trial, dramatically altering the numbers required because the heterogeneity between groups is eliminated. As the molecular signature of an individual tumor can be complex and not always clearly defined, involving aberrations in multiple signaling pathways, targeted therapies can be administered to biomarker-negative xenografts to examine potential off-target effects of these compounds and identify secondary resistance mechanisms that can be subsequently targeted (Fig. 2b). The use of relevant and well-characterized preclinical systems should further aid the discovery of biomarkers which predict therapeutic responsiveness to established therapies, enable studies into the molecular mechanisms of chemoresistance and finally, these models will

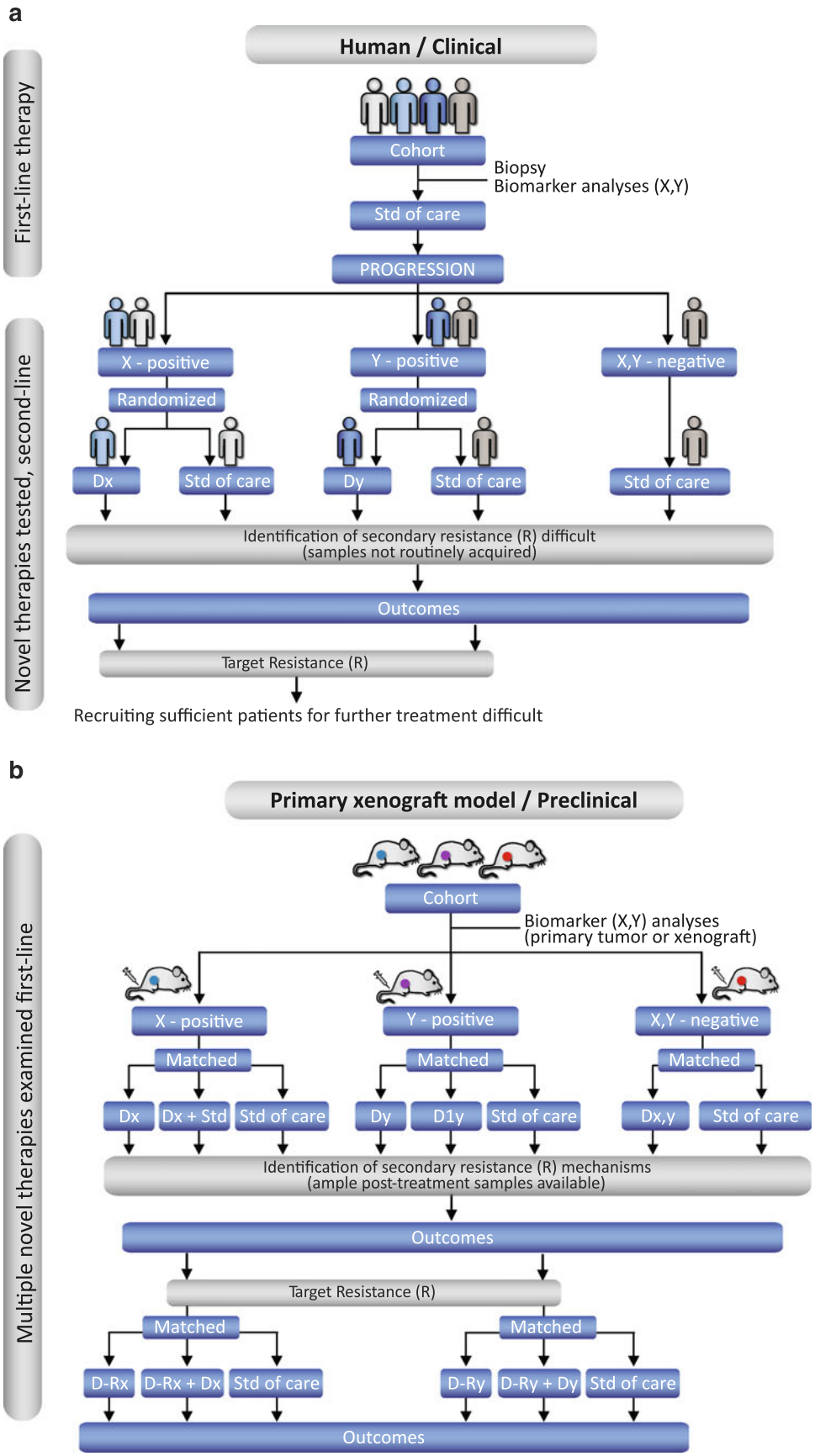

Fig. 2 Personalized medicine trial design in **a** clinical setting and **b** preclinical models. Compared to clinical trials, where efficacy of novel therapeutics can often only be examined second-line, the use of well-characterized xenografts enables simultaneous examination of multiple personalized medicine strategies, administered as single agents or in combination with standard therapy, which is currently intractable in a clinical trial setting. The availability of ample tissues post-treatment allows comprehensive characterization of secondary resistance mechanisms (*R*) and subsequent targeting with novel agents. *D-Rx*, *D-Ry* agents targeting resistance developed after treatment with drugs Dx, Dy, respectively. Moreover, targeted agents can also be administered to xenografts negative for the biomarker-of-interest to study potential off-target effects of selected therapies

enable testing of personalized medicine strategies by administering targeted treatments based on the genomic/molecular signature of each individual tumor.

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