Modeling the cancer patient with genetically engineered mice: Prediction of toxicity from molecule-targeted therapies

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Current trends foretell the use of cancer treatments customized to each patient. Genetic and molecular profiling of tumors and an increasing number of molecule-targeted therapies contribute to making this a reality. However, as targets of anticancer therapies become specific proteins or pathways, unanticipated side effects may emerge. In addition, the chronic use of these treatments may contribute to the development of degenerative toxicity not predicted by short-term clinical trials. Here we review and propose how genetically engineered mouse models can serve as valuable tools to predict targeted therapy toxicity, as well as to identify allelic variants that predispose individuals to side effects.

Introduction

The genetically engineered mouse has become an established tool used to both better understand cancer and develop strategies to treat it, as exemplified by the extensive investigations ongoing in the National Cancer Institute-sponsored Mouse Models of Human Cancer Consortium (http://emice.nci.nih.gov). A now standard technique to validate the role of specific genes during tumorigenesis is to engineer mice under- or overexpressing the genes of interest. Intercrossing combinations of engineered alterations is a powerful technique to elucidate genetic interactions contributing to tumorigenesis in vivo.

In a majority of cases, genetically engineered mice display abnormalities in development or homeostasis, defects that in some cases appear to preclude the use of these lines in studies of adult diseases. Nevertheless, such mice are still valuable tools for cancer researchers, as they serve as predictors of the effects on the whole organism induced by pharmacological manipulation of particular gene products. Indeed, intended therapeutic responses of most targeted inhibitors mirror developmental abnormalities in mice with genetic ablation or reduction of the corresponding target (Zambrowicz and Sands, 2003). As the postgenomic era creates a burgeoning wave of molecular targets and combinatorial treatment strategies, approaches to predict possible toxicities should become integral components of the drug development process. Mouse models represent one such tool in that they provide the specificity of a genetic manipulation with a phenotypic readout that involves the whole organism. Additionally, genetic models overcome some aspects of pharmacologic toxicity studies in mice, namely metabolic differences and absence of bioactivity of some agents in mice, including most antibody-based therapies. Likewise, robust phenotypes of genetically engineered mice may be more predictive of chronic and severe toxicity than relatively short-term treatment of mice with pharmacologic inhibitors, modeling the extreme reactions that may be observed in the clinic.

The ERBB story

Overexpression of and/or aberrant signaling by ERBB receptors, particularly EGFR and ERBB2, occurs frequently in human cancer. Consequently, ERBB-targeted therapies consisting of ligand-blocking antibodies or ATP-competitive small molecule tyrosine kinase inhibitors have been developed and are in advanced phases of clinical investigation (Arteaga and Baselga, 2003). Trials with ERBB-targeted drugs have revealed uncanny similarities between treatment side effects in patients and phenotypes in mice with reduced ERBB receptor function (Table 1). The most common side effect seen with EGFR inhibition therapy is an acneiform follicular rash of variable severity (Figure 1). This rash recapitulates the skin and follicle defects seen in engineered mouse models with low or absent EGFR activity. *Egfr*-deficient mice have abnormally dry, flaky, thin skin and extensive hair follicle defects resulting in thin, brittle hair (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Likewise, mice homozygous for the hypomorphic $Egfr^{wa2}$ allele display wavy coats resulting from hair follicle defects, often with skin flaking and alopecia (Luetteke et al., 1994). *Egfr* null skin shows a mixed inflammatory infiltration at the follicle with rupture of the follicular epithelium, hallmarks of skin reactions in patients treated with EGFR antagonists (Baselga et al., 2002; Busam et al., 2001; Hansen et al., 1997).

Gastrointestinal toxicity has also been seen in these clinical trials, likely due to disruption of gut homeostasis as indicated by studies in mice deficient for *Egfr* or its ligands. These mice demonstrate a variety of gastrointestinal phenotypes, including necrotizing enterocolitis, duodenal lesions and perforations, defects in acid secretion, and delays in healing following surgical or chemical damage (Egger et al., 2000; Helmrath et al., 1997; Joshi et al., 1997; Miettinen et al., 1995; Troyer et al., 2001). Similarly, pulmonary fibrosis and interstitial pneumonia reported in lung cancer trials with EGFR inhibitors recapitulate developmental phenotypes from *Egfr* nullizygous mice, with proliferation of nonepithelial cells resulting in thickening of the alveolar walls, reduction of air-filled aveoli, and respiratory distress (Kanemura et al., 2003; Miettinen et al., 1995; Sibilia and Wagner, 1995; Suzuki et al., 2003; Teramoto et al., 2003).

Perhaps the most well known toxicity caused by ERBB inhibition is cardiac dysfunction. Herceptin (trastuzumab, Genentech), a humanized antibody against ERBB2, was the

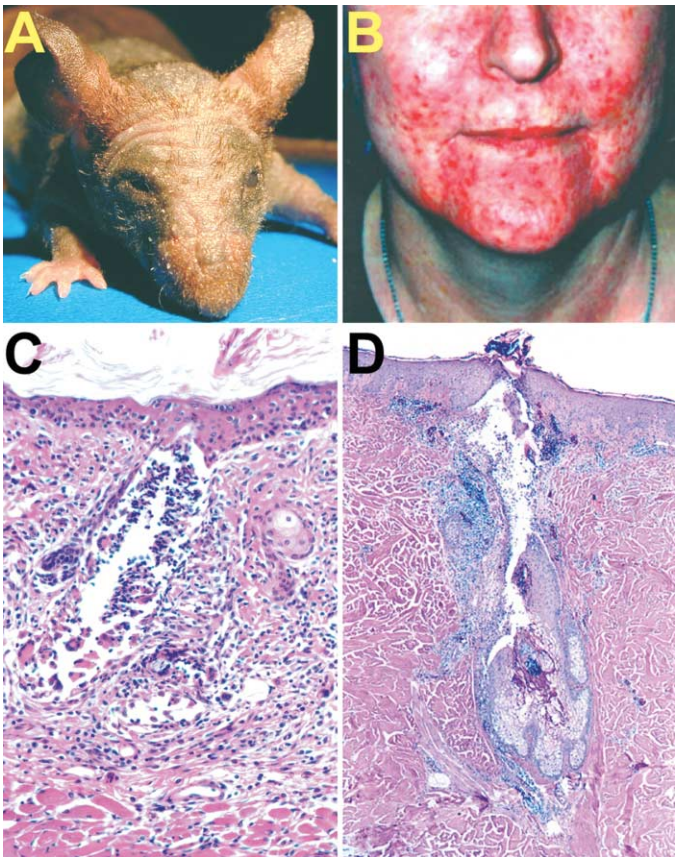

Figure 1. Developmental skin defects in *Egfr*-deficient mice (**A** and **C**) and skin toxicity in patients treated with EGFR inhibitors (**B** and **D**)

Gross images of severe skin and hair pathology in a *Egfr* mutant mouse (**A**) and folliculitis in a patient susceptible to EGFR inhibition therapy induced skin toxicity (**B**). Comparative histology of hair follicle in *Egfr* nullizygous skin (**C**) and follicle from patient with EGFR therapy-induced folliculitis (**D**). Rupture of follicular epithelium and strong mixed inflammatory response are evident in both **C** and **D**. (**B**) reprinted with permission from the American Society of Clinical Oncology (Baselga et al., 2002); (**C**) provided by Laura Hansen (Creighton University); and (**D**) reprinted with permission from Blackwell Publishing (Busam et al., 2001).

Table 1: *Erbb*-deficient mouse phenotypes and ERBB-inhibitor toxicity in humans

Erbb-related defects (mouse)	ERBB inhibition-related toxicity (human)
Inflammation (*Egfr*)	hypersensitivity (EGFR)
Skin/hair follicle (*Egfr*)	acneiform folliculitis (EGFR)
Heart (*Egfr*, *Erbb2,3,4*)	cardiac dysfunction and toxicity (ERBB2)
Gut repair/homeostasis (*Egfr*)	diarrhea, stomatitis, vomiting (EGFR)
Neuronal (*Egfr*, *Erbb2,3,4*)	neuropathy and tremor (EGFR)
Liver (*Egfr*)	hepatotoxicity (EGFR)
Kidney (*Egfr*)	renal effects (EGFR)
Vasoconstriction (*Egfr*)	hypotension (EGFR)
Lung (*Egfr*)	pulmonary fibrosis (EGFR)
Circadian-related activity (*Egfr*)	?
Eye (*Egfr*)	?
Skeletal (*Egfr*)	?
Hearing (*Egfr*)	?
Sensory gating (*Erbb4*)	?
Hyperactivity (*Erbb4*)	?
Skeletal muscle (*Erbb2*)	?
Lactation (*Egfr*, *Erbb4*)	?
Seizure (*Egfr*)	?

?, unknown.

first ERBB-targeted therapy approved for clinical trials. Cardiomyopathy, congestive heart failure, and decreases in left ventricular ejection fraction have been observed, particularly in patients with cardiac risk factors or those who received concurrent anthracyclines (Slamon et al., 2001). Notably, *Erbb2* null mice suffer from a lack of cardiomyocyte differentiation (Lee et al., 1995). Additionally, cardiac-specific conditional *Erbb2* knockouts develop dilated cardiomyopathy prior to the second postnatal month (Crone et al., 2002; Ozcelik et al., 2002); they also have reduced survival following cardiac overload, as well as increased sensitivity to anthracycline-induced cardiomyocyte death. These results are consistent with the observation of clinical toxicity predominantly in patients with cardiac risk factors and/or anthracycline therapy. Cardiac toxicity has yet to be reported following treatment with EGFR-specific antagonists. However, *Egfr* mutant mice do exhibit aortic valve overgrowth, resulting in stenosis and ventricular hypertrophy, the severity of which varies with genetic background (Chen et al., 2000; Jackson et al., 2003), suggesting that cardiac toxicity may occur in some patients on anti-EGFR therapy.

Erbb-deficient mice also exhibit neuronal and sensory deficits, demonstrating that *Erbb* receptors are required for proper development and maintenance of the brain and peripheral nervous system (Britsch et al., 1998; Erickson et al., 1997; Gassmann et al., 1995; Threadgill et al., 1995). Additionally, the enteric nervous system develops normally when *Erbb2* is rendered null via conditional knockout, but is subsequently lost postnatally, resulting in a Hirschsprung's disease-like phenotype (Crone et al., 2003). At present, no major neurological toxicity has been reported from clinical studies of ERBB antagonists, though low-grade peripheral neuropathy has been observed (Fountzilas et al., 2001; Mrsic et al., 2001). Similarly, no sensory damage has been reported in patients given EGFR inhibitors despite the fact that *Egfr* and *Tgfa* mutant mice exhibit developmental eye defects, glaucoma, and hearing defects (Mann et al., 1993; R.B.R. and D.W.T., unpublished data; Thaung et al., 2002). Given the involvement of ERBB receptors in nervous system development and maintenance, neurological side effects would be predicted in some patients treated with ERBB antagonists, particularly those on extended treatment. This suggests that neurological and sensory parameters be followed over the course of treatment to uncover subtle chronic toxicity.

Mice with reduced EGFR activity exhibit follicular defects that vary with strain background (R.B.R. and D.W.T., unpublished data); likewise, patients taking EGFR inhibitors demonstrate folliculitis of varying severity. In this way, phenotypic variation across inbred strains with the same genetic alteration begins to represent the range of toxic responses seen in a population of patients with heterogeneous genetic backgrounds. Similarly, most *Egfr*-related phenotypes, including defects in viability, heart, eye, and sensory response, also exhibit variable penetrance and severity depending on genetic background (R.B.R. and D.W.T., unpublished data; Sibilia and Wagner, 1995;

Threadgill et al., 1995). Strain-based variance of a phenotype serves as a foundation to map modifier genes of the phenotype. In the framework of toxicity studies, such modifiers would enhance resistance or susceptibility to adverse events in patients, and thus may serve as biomarkers to predetermine toxicity and response to therapy. Indeed, the severity of folliculitis induced by EGFR antagonists appears to correlate with patient survival, suggesting that the rash can be used as a biomarker for therapeutic activity (Clark et al., 2003; Saltz et al., 2003). However, many potentially predictable side effects, including lethal ones, occur with rarity that prevents mapping in human populations, and patient numbers in phase I and II clinical trials are usually too low to provide genetic data for modifier mapping. Fortunately, modifier studies in engineered mice should elucidate the underlying molecular biology of these individual patient responses, allowing for the creation of biomarker assays to individually predict disease response and the likelihood of side effects, including cardiac, pulmonary, sensory, and neuronal damage.

Table 2: Toxicity predictions from mouse models for targets of clinical interest

Target	Toxicity predicted from genetically engineered mouse models
COX2	**kidney, heart**
VEGF	**vasculature**, heart, neuronal
PDGF	vasculature, skin, bone
ABL	spleen, bone, immune system, eye, heart, nervous system, sensory, behavior
KIT	**pigmentation, anemia,** sterility
TGFBR	inflammation, spleen, bone, liver, lung, vasculature, heart, thymus, eye, hearing, nervous system, paralysis
SRC	bone
TRP53 (p53)	accelerated ageing/degeneration of all tissues
AKT	diabetes, skin/hair, lung, muscle, bone, reduced lifespan
IGFR	diabetes, bone, lung, muscle, nervous system
PI3K	cancer, diabetes, immune system, liver, muscle, heart
BCL2	spleen, thymus, kidney
ESR	**infertility, bone**, diabetes, eye, nervous system, anxiety, aggression, myeloproliferation, prostate and bladder hyperplasia
RAR	infertility

Boldface, adverse events observed in patients. Only a few of the targets listed are currently in clinical trials.

Developmental complications

The striking similarities between human responses to ERBB inhibitors and engineered mouse models is reflected in other top molecule-based therapies (Table 2). Inhibitors of cyclooxygenase-2 (COX2), in addition to modulation of inflammatory response, can result in renal and cardiovascular toxicity (Ahmad et al., 2002). These toxicities recapitulate lethal heart and kidney defects present in *Ptgs2* (*Cox2*) null mice (Dinchuk et al., 1995; Morham et al., 1995). However, renal disease in the *Ptgs2* knockout mice is the result of developmental dysmorphology, and thus does not truly replicate renal dysfunction following treatment with COX2 inhibitors. Similarly, the variable fibrotic cardiac phenotype seen in *Ptgs2* mouse models differs from thrombotic events seen in patients treated with COX2 inhibitors (Bannwarth et al., 2003). Although the pathologies resulting from genetic and pharmacological insult differ mechanistically, the engineered mouse models accurately predict the tissues sensitive to perturbation. Similarly, while mice heterozygous for a null mutation of *Vegf* die in utero with abnormal vasculature and hearts (Ferrara et al., 1996), they remain predictive of toxicity in adults observed in clinical trials with the VEGF-blocking antibody Avastin (bevacizumab). Some patients on Avastin exsanguinated and died from pulmonary hemorrhage (Johnson et al., 2004), and others developed gut perforations, hypertension, proteinuria, and thrombosis (Hurwitz et al., 2003; Yang et al., 2003). The side effects in Avastin-treated patients can be attributed to vascular abnormalities and leakage, the root cause of *Vegf*-related phenotypes in the mouse. Although mice with hypoxic expression of *Vegf* exhibit a striking phenotype of adult-onset neuronal degeneration, reminiscent of amyotrophic lateral sclerosis (Oosthuyse et al., 2001), no neurological toxicities have been reported in VEGF inhibitor trials, though chronic treatment would likely be necessary to produce such toxicity.

It is clear that some genetically engineered modifications are highly detrimental during development but have little to no effect on cellular maintenance in adult animals. In these cases, simple gene knockouts may not serve as predictors of therapy-related toxicity in adults with terminally developed organs. The minimal toxicity following treatment with Gleevec (imatinib mesylate, Novartis), which inhibits activity of ABL, PDGFR, and KIT kinases, serves as an example. Mice nullizygous for any of these targets are essentially nonviable, with hemorrhagic and anemic phenotypes, and, depending on the mutation, a variety of developmental defects (Schwartzberg et al., 1991; Soriano, 1994, 1997; Tybulewicz et al., 1991). Aside from some reports of anemia, thrombosis, and hair pigmentation in Gleevec-treated patients, all predicted from abnormalities in the genetically engineered mouse models, there have been no other toxicities reported. Interestingly, the small molecule inhibitor of c-Kit, VEGFR, and PDGFR, SU11248 (Pfizer) causes hair depigmentation in patients (Moss et al., 2003; Robert et al., 2003), an obvious prediction from the white spot coat pattern of mice with *Kit* mutations (Little and Cloudman, 1937).

Conditional null alleles overcome both confounding developmental defects and any molecular compensation resulting from development in the absence of a gene product. For example, embryonic fibroblasts from mice homozygous for a knockout of the retinoblastoma (*Rb1*) gene senescence normally, apparently having adapted for lack of RB by upregulating the *Rb1*-like gene p107 (*Rbl1*) during development. Similar cells homozygous for a conditional null *Rb1* allele do not have altered p107 levels, and thus proliferate unchecked following acute removal of the *Rb1* gene (Sage et al., 2003). Use of such temporal null alleles will also allow toxicity-predictive studies of target genes whose traditional knockout would result in pre- or perinatal death, and tissue-specific knockout would allow the modeling of delivery of molecular therapeutics. A study utilizing a temporal tissue-specific knockout strategy removed the TGFβ type II receptor (*Tgfbr2*) gene from hematopoietic cells of adult mice (Leveen et al., 2002). Within two months following *Tgfbr2* removal, mice exhibit massive autoimmune inflammation and multiorgan necrosis, with a paralysis-like phenotype and ultimately death. These results imply that autoimmune phenomena and organ inflammation should be prospectively examined in patients receiving inhibitors of TGFβ signaling currently in development (Dumont and Arteaga, 2003).

Degenerative models

Some genetically engineered mice exhibit degenerative phenotypes without any developmental defects. For example, *Src* null mice are viable but develop osteopetrosis due to defects in osteoclast function (Lowe et al., 1993; Soriano et al., 1991). The *Src* knockout mouse predicts an otherwise nonintuitive phenotypic outcome in patients, and thus the inclusion of bone density measurements is under consideration for trials with SRC inhibitors. Another key example is the *Akt1* knockout mouse, which exhibits increased sensitivity and reduced lifespan in response to genotoxic stress (Chen et al., 2001). Thus, combinations of AKT inhibitors with chemotherapy or radiotherapy may synergize and induce profound toxicities in normal tissues as well. *Akt2* null mice exhibit glucose intolerance (Garofalo et al., 2003), implying that AKT inhibitors may also induce or exacerbate a diabetic condition. Genetic inactivation of other genes involved in glucose homeostasis also leads to a diabetic phenotype, including such potential cancer therapy targets as the insulin-like growth factor receptors and phosphatidylinositol 3-kinase (Fruman et al., 2000; Terauchi et al., 1999; Zhang et al., 2002). Therefore, serum glucose should be monitored in diabetic and nondiabetic patients enrolled in trials with IGF and PI3K antagonists.

Modes of activation and reconstitution of the tumor suppressor p53 (*Trp53*) are currently in development to place uncontrolled cell proliferation in check (Chene, 2003; Moon et al., 2003). Important lessons can be learned from degenerative phenotypes produced in mouse models of p53 hyperactivity to foretell potential complications of such strategies as cancer therapy. Genetically engineered mouse models of p53 hyperactivation produce mice that are less susceptible to both spontaneous and induced cancers (Garcia-Cao et al., 2002; Tyner et al., 2002). While one mouse model of increased p53 activity has phenotypes associated with premature aging, including reduced lifespan and progressive degenerative defects (Tyner et al., 2002), a second model showed a beneficial tumor resistance phenotype without the negative aging-related defects (Garcia-Cao et al., 2002). The degenerative phenotypes were observed when using a modified *Trp53* gene, a C-terminal truncated protein apparently capable of maintaining the wild-type p53 protein in an activated state. In contrast, the mice that appeared to be free of major degenerative defects were created by adding an extra copy of wild-type *Trp53* gene in its genomic context, thereby increasing normal gene dosage while maintaining endogenous expression and feedback regulation of *Trp53* at the transcriptional and protein level. Since the former study more closely models the effects of pharmacological activation of p53, that is, addition of an exogenous activator of p53 that uncouples it from normal cellular regulation, its results throw strong caution at potent activation of p53, especially for chronic courses or for those already afflicted by degenerative or aging-related disease. These studies can also be used to suggest potentially safe parameters for treatment-induced activation of p53.

Targeted toxicity models

Eventually, the risk of adverse side effects from targeted therapy may be greatly lessened by using a low dose combinatorial approach, targeting a set of molecules customized for individual disease and patient. Likewise, targeted therapy may allow preventive or senescent strategies that would necessitate chronic treatment in the span of years to maintain a carcinoma-free state. The use of genetically engineered mice may help predict side effects unique to extended treatment and thus not uncovered during relatively brief clinical trials or treatment regimens. The cost for the creation and phenotypic analysis of a targeted toxicity mouse model pales in comparison to the expense of a single clinical trial, especially if the trial is confounded by unexpected morbidity. Even if a transgenic mouse is engineered to ask a specific question, full phenotypic analysis is essential to uncover unexpected phenotypes that may predict toxicity. As a prime example of a significant, "hidden" phenotype, mice carrying the *Egfr*^{wa2} mutation were maintained for over sixty years before heart valve abnormalities and ventricular hypertrophy were discovered—phenotypes that may prove relevant to clinical treatment with EGFR inhibitors.

As combinatorial targeted therapy becomes a practical reality, the combination of multiple targeted genes in a single mouse line may produce phenotypes that anticipate unique toxicity resulting from additive or synergistic therapeutic effects. For example, EGFR and COX2 inhibitors are in the forefront of targeted therapies for colorectal cancer prevention and treatment, and mice with defects in *Egfr* or *Ptgs2* activity individually demonstrate cardiac and renal abnormalities. Crossing lines carrying conditional null alleles would produce a mouse model of simultaneous ablation of EGFR and COX2 activity during adulthood, potentially uncovering side effects specific to dual inhibition. Similarly, potential toxic synergy can be uncovered by treating mice carrying a mutation in one target with a pharmacologic inhibitor against the other target. This latter method is particularly practical if mice are unavailable or nonviable with a mutation in a gene of interest.

Perspective

In the end, genetic ablation of a target protein may demonstrate potential toxicities not revealed by incomplete or short-term pharmacological inhibition, modeling the worst-case scenario for therapeutic side effects. The defects seen in genetically engineered mice may thus represent expectations in a small subset of patients whose genetic background makes them particularly susceptible to severe adverse effects when targeting a particular molecular pathway. Similarly, the use of mouse models has demonstrated the importance of genetic background to phenotypes resulting from the perturbation of a pathway. Analysis of mouse models of therapy toxicity on multiple inbred backgrounds will add a level of complexity and cost, but with the potential benefit to allow mapping of loci involved in interindividual variation in toxicity susceptibility. Mapping experiments, especially if properly combined with expression and proteome studies, may allow the identification of biomarkers or gene polymorphisms modulating toxicity susceptibility, thus providing an avenue for simple tests to anticipate and detect side effects, such as the *TMPT* gene test for 6-mercaptourine (6MP) toxicity susceptibility (Marshall, 2003).

Analysis of genetically engineered mouse models with forethought to toxicity may greatly reduce the morbidity associated with unexpected toxicities caused by molecule-targeted therapies when introduced into the clinic. More detailed analysis of these mouse models will provide the foundation for dissecting the complexity of individual patients, thus providing strategies for "rational utilization" that will inevitably follow intense efforts put into the "rational design" of targeted therapies. Only following an understanding of the genetic basis of resistance and susceptibility to side effects will truly customized therapy be a reality—maximizing disease response while minimizing toxicity to the patient.

Acknowledgments

We would like to thank members of our labs for suggestions during the development of this review. We also thank Laura Hansen (Creighton University), Blackwell Publishing, and the American Society of Clinical Oncology for providing images for Figure 1. This work was supported by NCI grants CA92479 and CA84239 to D.W.T., CA62212 and CA80195 to C.L.A., and Cancer Center Support Grants CA16086 (UNC-Lineberger Cancer Center) and CA68485 (Vanderbilt-Ingram Cancer Center).

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