

Assessment of tumor response to tyrosine kinase inhibitors

Amanda Lowery, Zhaozhong Han

Department of Radiation Oncology, School of Medicine, Vanderbilt University, Nashville, TN

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1. ABSTRACT

This review briefly summarizes recent developments in the use of non-invasive imaging to assess tumor response to TKI therapy. Receptor tyrosine kinases play important roles in cancer development. A new class of drugs, tyrosine kinase inhibitors (TKI) can induce rapid and dramatic tumor suppression when administered to carefully selected patient groups. Identifying these patients with responding tumors prior to or shortly after the initiation of therapy remains challenging. The gold standard of response assessment has been by invasive biopsies used in biological and biochemical procedures. Advances in non-invasive imaging at the anatomical, functional and molecular level have enabled the early detection of tumor response; sometimes within days of beginning treatment. The growing area of molecular imaging has spurred the discovery of novel targeting peptides to bind TKI responding tumors. The emergence of targeted, quick responding imaging probes advances the field of cancer management towards the goal of personalized medicine.

2. INTRODUCTION

Receptor tyrosine kinases (RTKs) have been identified as therapeutic targets of cancers. Dozens of antagonists against RTKs have been tested in clinical trials and several of them have been approved for clinical treatment of cancers. The fact that only limited portion of patients respond to the treatment highlights the need for selecting the right treatment for the right patients by predictive or real-time assessment of tumor response to the therapeutics. Traditional assessments using biopsies and blood samples are invasive making most repeated assessments unfeasible. Medical imaging of the anatomical, functional and molecular changes can monitor the tumor response with increasing sensitivity, preventing extended inefficient therapies or improper patient selection from the start based upon tumor characterization and therapy responsiveness.

Imaging has changes the way cancer is diagnosed and managed. Advances in imaging technologies and probes promote increasingly specific and selective imaging

Table 1. Comparison of cytotoxic and molecular targeted therapies of cancer

	Cytotoxic therapy	Molecular targeted therapy
Targets	cell Proliferation	dysfunctional pathways
Selectivity	low	high
Toxicity	high	low
Solution	targeting delivery	target specificity
Administration	in a brief period	long-term
Dosing & scheduling	maximum tolerated dose	functional dose
Impact on tumor	shrink	stop progression
assessment	anatomic/functional assays	molecular biomarkers

from the anatomical to molecular level. Computed tomography (CT) and magnetic resonance imaging (MRI) are routinely used in phase II and III clinical trials to detect changes in the size of tumors after treatment (1). For cytotoxic therapies, an anatomical change may be the appropriate indicator, but for many small molecule inhibitors, such as anti-angiogenic tyrosine kinase inhibitors (TKI) that are cytostatic and have no direct toxicity to the cancer cells, an anatomical indicator may not ever be reached even with a significant treatment response (comparison of the cytotoxic and molecular targeted therapies of cancer can be found in Table 1). Functional imaging measures general physiological changes within the tumor, such as cell proliferation, cell death, metabolism or blood flow. This imaging approach can detect a positive response much quicker than anatomical imaging, but the general nature of the monitored changes leaves open the possibility that these changes can be biased by other factors, such as necrosis. With molecular imaging, individual receptors and the direct effect of the targeted therapeutics such as TKI can be tracked.

The benefits of medical imaging and molecular imaging specifically illustrate why they have grown in popularity. In general, imaging enables the repeated evaluation of a single tumor without repeated biopsies. During drug development this decreases the number of animals and costs required for the pre-clinical phase. It also prevents patients from undergoing multiple surgical procedures. Molecular imaging can very quickly, possibly within hours of initial therapy, provide biological evidence of the drug activity, the on-target effects and effective dose. The spatial and temporal resolutions are efficient for use in patient selection and assessing molecular response. The assessment of predictive factors prior to treatment and the early detection of response advance the field of personalized medicine. The imaging modalities used in molecular, functional and anatomical imaging are complimentary and may be combined to provide the fullest understanding of a patient's tumor status (2).

The purpose of this paper is to briefly review many of the available imaging techniques and their

application for monitoring tumor response following treatment with TKIs.

3. RTKS AND TKI

3.1. RTK in cancer development

RTKs compose a large family of proteins that sense and transduce extracellular signals across the cell plasma membrane. The most notable examples include vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF). RTKs contain an extracellular ligand binding domain, a single pass transmembrane domain and an intracellular kinase domain. In most cases, ligand binding causes the dimerization or oligomerization of multiple RTKs, activating cytosolic kinase functions by phosphorylation of tyrosine residues, which then recruits a cascade of proteins and enables downstream cell signaling pathways. Today 59 receptor tyrosine kinases are known from the human genome (3, 4). They play important roles in growth and development throughout life, regulating cell functions such as proliferation, differentiation, and migration (3).

RTKs also play critical roles in cancer development. Approximately 50% of known oncogenes encode protein tyrosine kinases, receptor or non-receptor types (4). Dysfunctions of RTKs associated with tumorigenesis include constitutive activation by multiple mechanisms, such as mutations leading to overexpression of the ligand or receptor, or permanent dimerization of receptors and related proteins. The mutations can be point mutations or larger gene rearrangements. For example, mutations of a single D (aspartic acid) to a V (valine) or N (asparagine) within c-KIT and c-MET, respectively, leads to their constitutive activation in gastrointestinal stromal tumors (GIST) (5), colorectal cancer (6), and renal papillary carcinoma (7). The most common mutation variation of EGFR, known as EGFRvIII, is the deletion of exons 2-7 which causes the EGFR variant to bind constitutively to the Shc adapter protein (8). Dimerization also occurs with a point mutation in fibroblast growth factor 3 (9) as well as with the Xmrk protein tyrosine kinase in the *Xiphophorus* fish model of hereditary melanoma (10). Overexpression of RTKs is a common dysfunction. Some examples are HER2 in 30% of breast cancers, EGFR in 40-80% of non-small cell lung cancer (NSCLC) (11) and 40% of glioma (12), and platelet derived growth factor receptor (PDGFR) in chronic myelomonocytic leukemia (5).

2.2. RTK as a therapeutic target and TKIs as targeted therapies

Due to their role in cell signaling and tumorigenesis, RTKs are becoming attractive targets for cancer therapies. They possess both extracellular and intracellular targetable sites. Monoclonal antibodies, engineered ligand mutants, and synthetic soluble extracellular domains of receptors have been explored to block receptor-ligand interaction and receptor dimerization. Trastuzumab was the first monoclonal antibody that has been approved by the FDA (1998) for treatment of HER2 positive breast cancers. Tyrosine kinase inhibitors (TKIs)

Tumor response to tyrosine kinase inhibitors

Table 2. FDA approved molecularly targeted therapies and some late stage protein tyrosine kinase drugs in development (4, 15)

Molecule	Agent Class	Targets	Tumor Types	FDA Approval
Trastuzumab (Herceptin)	Monoclonal Antibody	HER-2	Breast cancer	1998
Imatinib (Gleevec)	Small Molecule TKI	KIT, PDGFR, BCR-ABL	Gastrointestinal stromal tumors (GIST)	2002
			Chronic myeloid leukemia	
			Chronic myelomonocytic leukemia	
			Chronic eosinophilic leukemia	
Gefitinib (Iressa)	Small Molecule TKI	EGFR	Non-small cell lung cancer (NSCLC)	2003
			Phase I - hepatocellular cancer (HCC)	
Erlotinib (Tarceva)	Small Molecule TKI	EGFR	NSCLC	2004
			Phase II - HCC	
			Pancreatic cancer	2005
Cetuximab (Erbix)	Monoclonal Antibody	EGFR	Colorectal cancer	2004
			Head and neck squamous cell carcinoma (HNSCC)	2006
			Phase III - pancreatic cancer, NSCLC	
			Phase II - HCC	
Bevacizumab (Avastin)	Monoclonal Antibody	VEGF	Colorectal cancer	2004
			NSCLC	2006
			Breast cancer	2008
Sorafenib	Small Molecule TKI	VEGFR, PDGFR, KIT, FLT-3, RAF	Renal cancer	2005
			HCC	2008
Sunitinib (Sutent)	Small Molecule TKI	VEGFR, PDGFR, KIT, FLT-3, RET	Renal cancer	2006
			GIST	2006
Panitumumab (ABX-EGF, Vectibix)	Monoclonal Antibody	EGFR	Colorectal cancer	2006
			Phase I - refractory solid tumors	
Lapatinib (Tykerb)	Small Molecule TKI	EGFR, HER-2	Breast cancer	2007
Temsirolimus	Small Molecule serine/threonine kinase inhibitor	mTOR	Renal cancer	2007
Matuzumab (EMD 72000)	Monoclonal Antibody	EGFR	Phase I/II - NSCLC, ovarian, pancreatic cancer	
EKB-569	Small Molecule TKI	EGFR	Phase II - advanced colorectal cancer, NSCLC	
Cancertinib (CI-1033)	Small Molecule TKI	Pan-erbB	Phase II - SCC, ovarian, metastatic breast cancer	
Semaxanib (SU5416)	Small Molecule TKI	VEGFR, EGFR, KIT	Phase II - metastatic melanoma	
Vatalanib	Small Molecule TKI	VEGFR, PDGFR	Phase III - colorectal cancer	
			Phase II - GIST, prostate and kidney cancer	

are small molecule inhibitors intracellularly aimed at preventing ATP binding and subsequent kinase activity. TKIs often compete with ATP for binding. Natural TKIs identified in the early 1980's were highly toxic and had no specificity for cancerous tissues. However, these natural compounds served as templates for the synthesis of specific, targeted small molecule inhibitors (4). In 1996, the FDA approved the first small molecule inhibitor, imatinib, for treatment of NSCLC. Shortly thereafter, the approval was extended for the use of imatinib in chronic myeloid leukemia (CML, 2001) and in GIST (2002) (3). Today, over 100 protein kinase inhibitors are in clinical trials and 8 small molecule inhibitors are FDA approved (Table 2) (13).

In order to harness the full anti-cancer potential of small molecule TKIs, validated biomarkers and a panel of approved TKIs is needed. Target utilization determines the biological relevance of a specific TKI, and the rationale for the respective assessment approach. As an example, Sunitinib (also known as SU11248, and Sutent commercialized by Pfizer) affects tumor cells as well as

tumor-associated endothelial cells, and thus can be assessed by monitoring tumor cell death or functionality of the tumor-associated blood vessels (Figure 1). A TKI might only make a small change within a web of interacting cell signal pathways. Anti-cancer activity by a specific TKI in one tumor doesn't guarantee activity in another tumor with similar characteristics. Multiple TKIs must be available to build a more effective therapy for a given patient, but patient selection has not been easy. Validated biomarkers are also needed to provide predictive and early response assessment at the initial stages of treatment.

4. PREDICTIVE BIOMARKERS FOR TUMOR SUSCEPTIBILITY TO TKIS

Predictive biomarkers suggest a positive response to TKI therapy before the therapy begins. The success rate for drugs entering clinical trials is near 20%. The tremendous cost of \$800 million associated with the 14.2 years to bring a drug to market illustrates the need to be selective in pursuing clinical application (2). Some failures of very promising TKIs are attributed to the inability to

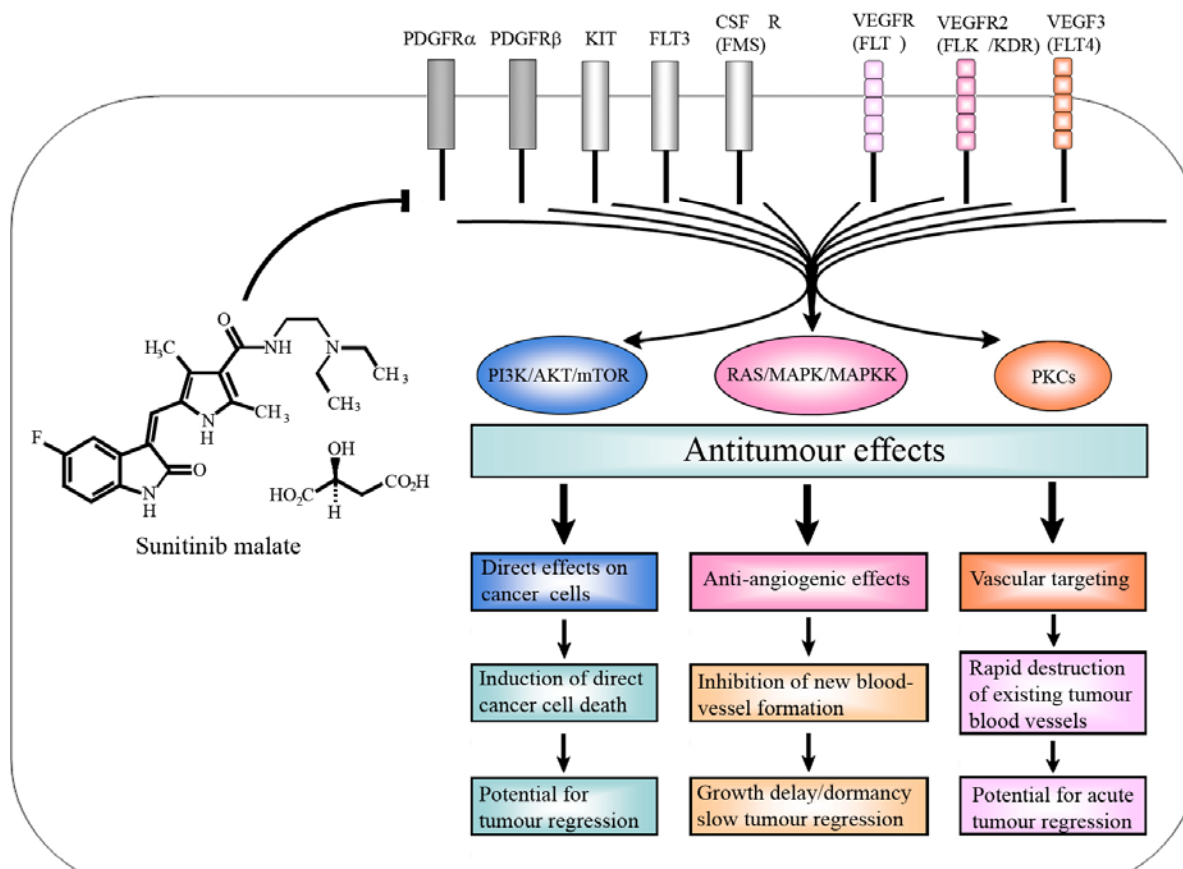


Figure 1. Target utilization and biological relevance of sunitinib in tumor growth control (62). Sunitinib targets multiple tyrosine kinases that are involved in tumor cell proliferation, initiation and maintenance of tumor-associated blood vessels. Therefore, assessment of sunitinib in tumor growth control can be rationally designed by monitoring the respective biological effects. Reproduced with permission from Nature Publishing Group.

appropriately select patients who are likely to benefit from a given treatment. The poor performance of some TKIs has been due to trials with unselected patient groups (14). Stratifying patients based on validated predictive biomarkers may improve the clinical outcomes for patients. Identifying and developing predictive biomarkers will limit the late stage failures in clinical trials, encourage the development of more tailored drugs, and minimize a patient's treatment with ineffective drugs. Many groups are working to identify predictive markers for TKI therapies, but the results have been mixed.

Predictive biomarkers may be in a form of protein expression level, gene copy number, or mutational status, as well as signaling signatures such as activation of the downstream effectors (vertical biomarkers) or parallel signaling pathways (horizontal biomarkers, cross-talk of pathways). (15). It can be derived from retrospective or prospective analyses of tumor biopsies with genetic (microarray, single nucleotide polymorphisms-SNPs, genomic sequencing) proteomic (LC-MS, 2D-differential gel analysis) or biochemical (immunohistochemistry, western blot) approaches, and correlation studies with clinical or experimental outcomes of the treatment. Even though a significant number of reports has been published

on discovery and validation of predictive biomarkers in the past few years, within each class of the predictive markers, the data has been conflicting. High expression of the target protein does not consistently correlate to a clinical benefit. The c-KIT gene is overexpressed in 90% of GIST but does not guarantee a response to c-KIT inhibitors (14). Overexpression of EGFR has implied sensitivity to gefitinib, although the association is not as significant as the gene multiplication in copy numbers where the predictive value has been more consistent (11, 15-17). The predictive value of the mutational status varies as much as using overexpression or gene copy number to predict benefit. Le Tourneau (15) claims mutation status is prognosis not predictive. Garassino (17) agrees that mutations in EGFR have no predictive use, contradictory to some studies reporting a significant predictive value to EGFR mutations (18, 19). The biomarker with the strongest predictive evident might be a K-ras mutation. The K-ras mutation is a negative indicator. It is a vertical biomarker, downstream of EGFR. An activating mutation here enables signaling without the involvement of EGFR. Activating mutations in K-ras predict resistance to EGFR TKIs (15, 17, 20). Horizontal biomarkers also appear to have some predictive roll, especially when expressed in elevated levels in conjunction with the target, such as

EGFR and HER2 (11, 18, 20). Overall, potentially predictive biomarkers are not ready for routine clinical use and need more validation. Recent technology development such as whole genome sequencing, single cell analysis, and systemic information integration would help to identify and validate new predictive biomarkers. More information on this aspect can be found at The Cancer Genome Atlas (TCGA) program host by the National Cancer Institute (<http://cancergenome.nih.gov/>).

5. EX VIVO AND IN VITRO BIOLOGICAL AND BIOCHEMICAL ASSESSMENT OF TKI EFFICACY

Biological and biochemical assessment relies on repeated invasive tissue acquisition. Although it is not always possible in some tumors, this approach provides the most direct and reliable evidence for biological activity of molecular targeted treatments.

5.1. Substrate phosphorylation and downstream signal mediator activation

Classical molecular assessment directly on tumors cells is invasive. It requires biopsy of the tissues, which limits the tumor area that can be assessed and frequency of assessment. Still, molecular analysis of tumor biopsies is the standard by which other assessment methods are compared. Here we will address immunohistochemistry, western blot analysis, and mass spectrometry (MS) imaging for the assessment of TKIs in tumors. Although MS imaging is not a standard analytical technique, its high spatial resolution and sensitivity make it hold potential in biomarker discovery and validation, as well as pharmacokinetic studies.

Immunohistochemistry evaluates tissue expression by antibody staining of tissue sections under microscopic inspection. When looking at the tissue response, three common markers are assessed: cleaved caspase 3 (apoptosis marker), Ki-67 (proliferation marker), and the RTK of interest. Additionally, substrate phosphorylation and downstream signal mediator activation can be assessed depending on the TKI. For example, AKT is phosphorylated by the activity of PDGFR and HER2 (21, 22). In a study by Shah *et al.* (22), treatment of transgenic mice bearing HER2 expressing tumors with trastuzumab resulted in a decrease of phosphorylated AKT (p-AKT) and an increase in cleaved caspase 3. These results indicate that the antibody was able to prevent downstream signaling of HER2 and induce apoptosis within the tumor. Similar results were seen in a glioblastoma multiforme (GBM) model treated with STI571 (Gleevec), a PDGFR inhibitor (21). Immunohistochemistry provides high spatial resolution, but the semi-quantitative nature makes it difficult to be standardized.

Western blots provide similar information to the histology without the spatial information. In a Western blot, the cellular proteins are collected from the cell lysate, separated by electrophoresis, and probed with antibodies. The relative levels of expression can be deduced from the staining intensity. STI571 treatment of GBM tumors lead to a decrease in the pPDGFR and pAKT levels as evident

on the Western blot and in agreement with the histological results (21). Phosphorylation of EGFR decreased after AEE788 treatment of prostate cancer cell lines *in vitro* (23). Also, Cuneo *et al.* (24) observed a transient attenuation of pAKT when SU11248 was given in combination with irradiation therapy. Western blots can make the identification of low expression levels easier because the molecule of interest is concentrated into a single band instead of distributed across a histology section. At the same time, this technique loses the spatial information that allows you to visualize the location of the expression. Often, both Western blots and immunohistology will be presented to provide a more complete picture.

5.2. Pharmacokinetics

Mass spectrometry (MS) imaging uses the spatial distribution of exact masses to identify and locate proteins, peptides, or compounds within a tissue slice. Both the protein level and post-translational modifications can be detected. The qualitative and quantitative data can be converted into images to show relative location and abundance of the targets within the tissue sections. 3-D pictures can be developed by stacking images from continuous tissue sections. The high spatial resolution and sensitivity are the most impressive features of this technology (25). To create a MS image, a laser is rastered over frozen tissue sections. For each laser spot, the MS is analyzed. Then individual masses can be plotted versus the tissue location. Images such as these will have 1000 to 30000 laser spots per tissue sample and typically more than 200 proteins per spot. Although the technical complication limits its broad application in biomarker discovery, a few reports showed great potentials of this technology for biomarker discovery (26, 27), validation and pharmacokinetic study (23).

Circulating metabolites in the blood may also be analyzed by MS to identify predictive biomarkers or monitor drug levels in the serum. In the study by Taguchi *et al.* (28), MS on blood serum was used to identify patients with NSCLC who would most likely benefit from TKI therapy. Using a training cohort, the MS of responders and non-responders were compared. Eight metabolites were identified as predictive markers. In a blinded test cohort, patients were identified as responders or non-responders based on the 8 MS peaks. Those classified as responders had a nearly 3-fold increase in mean survival over those classified as non-responders (28). MS analysis of the serum was also applied to determine pharmacokinetics of drugs within circulation. Haouala *et al.* (29) simultaneously monitored the presence of TKIs in serum using MS. Because TKIs bind to serum proteins and only approximately 1% of the injected dose remains free and bioactive, a large portion of the TKIs are available in the blood stream for assessment (29). Although the pharmacokinetic studies can not provide direct evidence for tumor responsiveness, the simultaneous monitoring of multiple TKIs enables the identification of target serum levels, the evaluation of patient compliance, and the identification of drug interactions. Each of these is important as combination drug therapies become prevalent.

Table 3. Comparison of imaging modalities

Modality	Signal	Resolution	Depth	Acquisition time (sec/min/hours)	Quantification	Cost
CT	iodine	50 μ m	no limit	min	N/A	\$\$
PET	^{18}F , ^{11}C , ^{15}O , ^{68}Ga	1-2 mm	no limit	min	very good	\$\$\$
MRI	gadolinium iron oxide particles dysprosium	10-100 μ m	no limit	min/hours	fair	\$\$\$
SPECT	$^{99\text{m}}\text{Tc}$, ^{111}In , ^{123}I	1-2 mm	no limit	min	good	\$\$
Ultrasound	microbubbles	50 μ m	mm	min	poor	\$\$
Fluorescence	fluorescent proteins, NIR fluorochrome	1-2 mm	<1 cm	sec/min	poor to fair	\$
<i>Bioluminescence imaging (BLI)</i>	luciferin	several mm	cm	min	poor to fair	\$\$

5.3. Metabolic and blood biochemistry changes

Prostate-specific antigen (PSA) in circulation has been used as a biomarker for prostate cancer progression and effectiveness of treatment for many years, although it was recently questioned with contradictory results. For TKIs, many blood-based compounds have been proposed as potential biomarkers for the prediction or the measurement of therapeutic response. Circulating endothelial cells, growth factors, and soluble receptors each show promise and challenges in their development as clinically relevant and reliable biomarkers.

5.3.1. Circulating endothelial cells and cancer cells

The concentration of circulating endothelial cells (CEC) is increased in many cancers including head and neck, breast, colon, esophageal, ovarian, and prostate to name a few (30). Their presence is attributed to poorly formed blood vessels within the tumors releasing the cells into circulation. It is unclear if the increase in CEC indicates a positive response or a progression of disease. In imatinib-resistant GIST treated with Sutent, the CEC concentration increased but did not correlate to a clinical outcome (31). Batchelor (32) reported increased CEC in progressing tumors after AZD2171 therapy. On the other hand, circulating endothelial progenitor cells (CEP) have been shown to decrease in patients with HCC (33). The mechanisms controlling the CEC and CEP release are not understood. Circulating cancer cells contribute to metastasis. Monitoring circulating cancer cells is proved to be sensitive enough to assess biostatic molecular targeted therapeutic agents including TKIs (34, 35).

5.3.2. Growth factors and soluble growth factor receptors

Evaluating response to therapy with blood-based markers is different than using the markers to predict response in pre-treatment. Evaluating the response requires correlating changes in biomarker expression to clinical outcomes. In anti-angiogenic therapies, it may be necessary to track expression levels of multiple markers, such as VEGF (31-33), soluble VEGF receptors (sVEGFR) (31-33, 36), placental growth factor (PIGF), basic fibroblast growth factor (bFGF), and stromal cell-derived factor 1

alpha (SDF1alpha) (32). The direction and extent of the change is agent specific. For example, when targeting VEGFR directly as with Sutent, AZD2171, or Sorafenib, the sVEGFR will decrease (31-33). When VEGFR is affected indirectly as with Bevacizumab, the sVEGFR can increase (33). In GIST, the VEGF increased while the sVEGFR decreased while patients were on Sutent therapy. After the therapy was stopped the levels reversed (31).

6. RESPONSE TO TKI BY NON-INVASIVE IMAGING

The use of imaging to evaluate response to therapies has become routine clinical practice. The non-invasive nature of imaging allows multiple, repeated assessments that are not possible with biopsy based approaches. Additionally, the whole tumor area as well as distant metastases can be evaluated simultaneously, providing a more complete picture of the disease state. Multiple imaging modalities have been explored for clinical and experimental imaging, pros and cons of each are summarized in Table 3. Response to therapy and imaging assessment occur at three structural levels. The anatomical level evaluates gross changes in the tumor, such as tumor size. Techniques including CT, MRI, and ultrasound are at the anatomical level. The functional imaging level focuses on the sub-tissue and microenvironment changes. Here, positron emission tomography (PET) can be employed to evaluate metabolism within the tumor, cell proliferation, and hypoxia. Other tumor states such as apoptosis and vascularity can also be evaluated. The last imaging level is molecular imaging, uncovering the alterations at the cellular and subcellular level. Many of the same functional imaging techniques can be used in molecular imaging by incorporating molecular specific targets. PET and single positron emission computed tomography (SPECT) can be used to image receptor overexpression, receptor-ligand interactions, and apoptosis.

Imaging science has been driven by the technological advances, beginning with large scale anatomical imaging and advancing to the smaller molecular

imaging. In contrast, tumors respond to treatments from the molecular scale first before changes occur at the larger anatomical scale. Often a delay persists between the cellular changes and anatomical changes. In the quest for early response markers, reliable, reproducible molecular imaging is a requirement. Novel biomarkers and imaging probes are needed to enable the best of individualized medicine.

6.1. Anatomical imaging

Anatomical imaging by MRI, CT, and ultrasound (US) has been the standard for determining response to therapy. To help define a response and standardize clinical trial evaluations, the World Health Organization developed a guideline for classifying the response based on 2-dimensional measurements in 1998. Then in 2000, the Response Evaluation Criteria in Solid Tumors (RECIST) was published defining response based on 1-dimensional measurements. The guidelines require macroscopic changes in the tumor volume and size (1, 37). Changes at the anatomical level take time and may not be seen for weeks to months after the initiation of treatment, limiting their usefulness in early response detection (38). Anatomical imaging still plays an important role in clinical diagnosis and monitoring. Advances in MRI, CT, and US when augmented with other imaging modalities such as PET have enhanced the capabilities to provide functional and molecular information. This section will focus on the basic anatomical imaging using MRI, CT, and US. Their uses in functional and molecular imaging will be addressed in later sections.

6.1.1. Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) was developed in the 1970's. It is based on unpaired nuclear spins in a magnetic field. Applying a magnetic field across the tissue, the spins align. When the field is perturbed, the time to realignment can be measured. This time is tissue dependent and can be used to generate an image. The contrast between tissues can be enhanced by the use of contrast agents. MRI may be used to image abnormal anatomy including tumor size, location, metastases, and phenotypes (39). The high maximal image resolution (50-100 μm) is in contrast to the low sensitivity requiring high concentrations of contrast agents (37). MRI anatomical imaging has limited value as an early response indicator. A xenograph HER2 positive breast cancer model showed significant differences in the MRI determined tumor volume 9 days after Gefitinib treatment as compared to the untreated controls, but at day 4 when there was only a small decrease in the tumor volume, MRI could not detect a significant response (40).

6.1.2. Computed tomography (CT)

Computed Tomography (CT) forms an image by the differential absorption of x-rays within tissues. The contrast within soft tissue is poor but can be enhanced by the use of contrast agents. The resolution can be as high as 50-100 μm , but the radiation dose required for those resolutions limited the number of CT scans per patient (37).

The delay in anatomical response is also evident with CT scans. The CT was unable to predict the survival

or time to progression 19 days after imatinib treatment in GIST patients (38). Stroobants *et al.* (41) reported detecting the response on CT scans a median of 8 weeks post treatment, while FDG-PET detected the response at one week. Although as a stand alone imaging modality CT has limited early response detection, it provides important anatomical information for the planning of surgical procedures. When combined with PET, the functional and anatomical information provide a more complete picture of response (39).

6.1.3. Ultrasound (US)

Ultrasound detects the reflection of sound waves (2-10 MHz in humans) to measure the blood flow in tumors. The low tissue penetration of sound waves (within 5 mm of the detector) limits its application even with resolutions near 40 μm . Contrast enhancement by microbubbles is limited to vascular targets. US has detected decreases in the tumor blood flow following treatment with the combination of VEGFR inhibitors and radiation. The decrease was correlated with a delay in the tumor growth and was evident five days after treatment (23, 42).

6.2. Functional imaging

Functional imaging monitors physiological changes such as metabolism, cell proliferation, hypoxia, apoptosis or vascularity within tumors. By comparing these physiological and pathological indications before and after treatment, the functional imaging is dominant in clinical evaluation of treatment efficacy.

6.2.1. Metabolism within tumors

Positron emission tomography (PET) enables the visualization of cellular processes *in vivo* through the use of a radioactive probe. Although the resolution is fairly coarse in the low millimeter range, the sensitivity is extremely high, detecting probes at nanomolar concentrations (4). For functional imaging, PET has been used to evaluate tumor metabolism, cell proliferation, and tumor hypoxia. Proliferation and hypoxia will both be discussed later in this section. ^{18}F -fluorodeoxyglucose (FDG) is the most common PET tracer. FDG, a glucose analog, is taken up by cells but cannot complete glycolysis. It becomes trapped and rapidly accumulates within the cell. FDG-PET is routinely used for the initial staging of tumors, evaluation of response to therapy, and diagnosis of disease (2). It is useful to evaluate cytostatic drugs which have little effect on tumor size but may have a significant effect on glucose uptake. In pre-clinical studies on MMTV/HER2 expressing mouse model, Trastuzumab alone had little effect on the FDG uptake (22), but in combination with rapamycin, an antibiotic with immunosuppressant properties, the FDG uptake decreased within one week of treatment (43). In a clinical evaluation of imatinib on GIST, the FDG uptake decreased within 24 hours of the imatinib dose (2). Other studies have seen less dramatic but equally insightful results. Goerres *et al.* (38) compared FDG-PET to CT scans for patients with GIST treated with imatinib. The PET scans identified fewer tumors than the CT, but the FDG active tumors appeared to be more clinically relevant. After one course of treatment,

patients with decreased FDG went on to have longer mean survival times than those whose FDG uptake continued to increase. FDG uptake was not uniform among multiple lesions and even varied within a single large lesion. Some lesions may be missed with PET due to low uptake or small size. These factors complicate the use of FDG-PET as a measure of tumor response (38).

6.2.2. Cell proliferation

Nucleoside-based imaging probes are gaining popularity for cell proliferation assessment by PET. 3'-deoxy-3'-¹⁸F-fluorothymidine (FLT) is the most promising probe of this type. FLT is a marker for the activity of the thymidine salvage pathway, the way cells obtain deoxyribonucleosides from the extracellular environment. Although this pathway is not required for proliferation, it is typically upregulated in proliferating cells and diminished in quiescent cells. FLT is transported into the cells and phosphorylated by thymidine kinase 1 (TK1). FLT cannot be incorporated into DNA, but once it is phosphorylated it cannot leave the cell either. The accumulation correlated to proliferation (44). FLT-PET can discern a drug's effect on the proliferation of tumors *in vivo*, which may differ from those *in vitro* (45).

6.2.3. Hypoxia

Tumors often develop regions of hypoxia, where the tissue is poorly oxygenated. These nutrient poor areas play an important role in angiogenesis, metastasis, and therapy resistance, both radioresistance and chemoresistance (44, 46). It is also a negative indicator for progression free survival in head and neck cancer, cervical cancer, and sarcomas (46). VEGF is upregulated by hypoxia as well. The PET tracer ¹⁸F-fluoromisonidazole accumulates in hypoxic regions by nitroreductase-dependent binding to intracellular macromolecules when the oxygen level is below 10 mm Hg (46). Although the signal to noise ratio can be low, PET hypoxia imaging is capable of accurate, quantitative measurements that reflect the histological analysis of the tissue (47). Changes in the hypoxia can be seen in as little as two hours following treatment (47).

6.2.4. Apoptosis

Apoptosis is an organized energy dependant cell death pathway. Annexin V is often targeted as the primary apoptosis biomarker. During the apoptosis process, phosphatidylserine (PS) flips from the inner leaflet of the cell membrane to the exterior of the cells. Annexin V binds to the newly exposed PS. Annexin V labeled with a near infrared (NIR) optical probe has demonstrated significant uptake within pre-clinical models of prostate and breast cancers following cetuximab and trastuzumab therapies, respectively (45). Increases in annexin V binding can be seen as early as 1-3 days following treatment in responding tumors. The imaging must be completed before PS positive cells are cleared by macrophages. Because apoptosis is an energy dependant process, a transient increase in glucose consumption will precede the cell death. This can be seen as a "metabolic flare" on a FDG-PET image. Although annexin V is significantly associated with apoptosis, it is not exclusively so. PS can also become externally

presented in necrotic cells due to the breakdown of the cell membrane or stressed cells that then recover and do not proceed with apoptosis (48).

6.2.5. Vascularity

Dynamic contrast-enhanced MRI (DCE-MRI) uses a macromolecule contrast agent along with pharmacokinetic modeling to uncover the functional changes in tumor vasculature. With DCE-MRI protocols, a serial set of images are acquired before and after the injection of a paramagnetic contrast agent. This rapid acquisition of images allows an analysis of the variation of the MR signal intensity over time for each image voxel. As the contrast agent enters the tissue, it changes the MR signal intensity from the tissue by a degree that depends on the local concentration. When the contrast agent leaves the tissue, the MR signal intensity begins to descend toward the baseline value. Using an appropriate pharmacokinetic model, the difference between the two states can be analyzed to determine blood flow, permeability and tissue volume fractions for each voxel within Regions of Interest (ROI). Elevated transendothelial permeability is characteristic of many tumors. Following pulsed high dose Gefitinib plus paclitaxel, Moasser *et al.* (49) reported a transient decrease in transendothelial permeability, increased fractional plasma volume, decreased tumor edema, and decreased hydrostatic pressure. These results are in agreement with DCE-MRI on tumors treated with AZD2171, a VEGF TKI with additional inhibitory effects on c-KIT and PDGFR alpha and beta (18). An U251 cell orthotopic brain tumor model yielded different results. In this model following anti-VEGF therapy with Bevacizumab, the transendothelial permeability increased. The apparent inconsistency was attributed to the stimulation of a secondary pathway (50). Additional studies are still needed to understand the vascular changes occurring in the tumor.

6.3. Molecular imaging

Unlike functional imaging that can use the same imaging probes across a variety of tumor types and locations, at the molecular imaging level each agent is designed to interrogate a specific protein or marker. The targeting agent may be directed to the receptor to determine kinase activity, to caspase 3 to detect apoptosis, or to a new biomarker using novel targeting peptides. The common goal is to obtain information specifically at the molecular level that can then be used to predict both the functional implications and the anatomical consequences.

6.3.1. Receptor imaging

Receptor imaging probes can take the form of a ligand, an antibody, a TKI already in development, or a novel compound. NIR labeled EGF has been used to image the EGFR within xenograph models. The probe bound to cells in an expression dependant manner, with the highest binding being present in the tumor with high EGFR expression (45). The radiolabeled DTPA-PEG-cetuximab antibody has also shown promising results. The relatively large molecular weight of the antibody reduces the non-specific binding that can be seen with lower molecular weight probes (4). Although radiolabeled TKIs currently under investigation have yielded disappointing results as

imaging probes, the PET probe [^{18}F]-ML04 has demonstrated promising results in a glioma xenograph model (51). [^{18}F]-ML04 has high specific binding and can accurately measure the number of EGFR molecules per cell (4). Receptor imaging can be used to determine expression levels and occupation by TKIs.

6.3.2. Caspase 3 imaging

Caspase 3 is the last enzyme of the caspase cascade in apoptosis. It triggers several morphological changes associated with apoptosis and PS externalization. Binding of extracellular PS by annexin V has already been discussed in the functional imaging section. Although PS exposure does occur in apoptosis, it can also be reversibly induced by physiological stress and caspase independent mechanisms (52). Caspase 3 activity is emerging as a more specific marker for apoptosis.

Currently, caspase 3 *in vivo* imaging agents measure the enzyme by an indirect measurement of the cleavage activity. New multimodal fusion molecules are being developed for application in optical, fluorescent, bioluminescent, and nuclear imaging. The fusion probes contain detectable markers that are silenced in the initial state. Upon cleavage by caspase 3, the activity of each single modality agent is restored. Fluorescent resonance energy transfer (FRET) pairs and other quenched fluorescent molecules have been designed in this way (53). Still others have been combine with PET agents to enable both nuclear and fluorescent measurements, which provide both biodistribution and activity information (48, 54, 55). Some fusion probes contain reporter genes where the cleavage activity of caspase 3 restores the transcription and activity of luciferase for bioluminescence. Reporter genes are unlikely to be used as imaging agents in humans but are vital tools for the pre-clinical drug development (56, 57). The downstream activation of caspase 3 can quickly indicate a response to TKIs.

6.3.3. New imaging peptides and antibodies

New molecular targets are needed to improve the specificity and efficiency of imaging agents. Phage display can identify novel specific binding motifs through multiple rounds of biopanning. Phage display has identified peptides that specifically bind within breast cancer (58), bladder cancer (59), or within the vasculature of tumors (42, 60, 61). Our work on phage display yielded a panel of peptides that distinguish treatment-responding tumors from non-responding tumors. These peptides bound specifically within tumors treated with radiation and anti-angiogenic TKIs. One lead peptide with simple structure as HVGGSV showed selectivity to tumors treated with both of ionizing radiation and tyrosine kinase inhibitors. The HVGGSV peptide accumulated less within the tumors treated with radiation or TKI alone, but no accumulation was detected in the untreated tumors, non-responding tumors, or inflamed normal tissues (61). The robust targeting persisted in subcutaneous and orthotopic tumors as well as in tumors treated with one of multiple VEGFR TKIs. Tax Interacting Protein-1 (TIP-1) was identified as the protein on the treated cancer cells that

mediates the HVGGSV peptide binding within treated tumors. The basically intracellular TIP-1 is only translocated onto plasma membrane surface after treatment with radiation and tyrosine kinase inhibitors. The cancer cells with TIP-1 expression on the cell surface are less active in proliferation and more susceptible to subsequent radiation treatment (Wang HL., *et al.* under review). Therefore, *in vivo* phage display technology provides a new tool for probe and biomarker discovery for molecular imaging of cancer response to treatment.

7. CONCLUSIONS

Imaging has revolutionized medical diagnostics. From the macroscopic anatomical sites down to a functional assessment of processes within tumors to the molecular characterization of individual cells, imaging has enabled a better understanding of tumors cells, their environment and how they respond to treatments. It is very clear that not all tumors, not even within the same classifications, respond the same way. The long clinical delay before a response can be seen on anatomical images costs patients valuable time on expensive, potentially ineffective treatments. Functional and molecular imaging can detect therapeutic response within days of beginning effective therapies, but identifying patients who are likely to benefits from targeted therapies has been difficult, leading to inconsistencies in the reported clinical benefits. Better, more reliable molecular targets with reproducible results are needed. The use of new techniques such as MS profiling and *in vivo* phage display, promise to produce molecular biomarkers with predictive value and those that can accurately monitor response to therapy. The advances we are making in molecular targets and imaging move us towards the goal of personalized medicine.

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Abbreviations: ATP: Adenosine-5'-triphosphate; bFGF: basic fibroblast growth factor; BLI: bioluminescence imaging; CEC: circulating endothelial cells; CEP: circulating endothelial progenitor cells; CML: chronic myeloid leukemia ; CT: computer tomography; DCE-MRI: Dynamic contrast-enhanced MRI ; DTPA-PEG: diethylenetriaminepentaacetic acid-poly(ethylene glycol); EGFR: epidermal growth factor receptor; EGF: epidermal growth factor; FDA: United States Food and Drug Administration; FDG-PET: ¹⁸F-fluorodeoxyglucose Positron emission tomography; FLT-PET: 3'-deoxy-3'-¹⁸F-fluorothymidine Positron emission tomography; GBM: glioblastoma multiforme; GIST: gastrointestinal stromal tumors; HCC: hepatocellular cancer; HER2: human epidermal growth factor receptor 2; HNSCC: head and neck squamous cell carcinoma; IGF: insulin-like growth factor; LC-MS: liquid chromatography mass spectroscopy; MMTV: mouse mammary tumor virus; MRI: magnetic resonance imaging; MS: mass spectroscopy; NIR: near infrared; NSCLC: non-small cell lung cancer; PDGFR: platelet derived growth factor receptor; PET: Positron emission tomography; PIGF: placental growth factor; PS: phosphatidylserine; PSA: prostate specific antigen; RECIST: Response Evaluation Criteria in Solid Tumors; RTKs: receptor tyrosine kinases; SDF1alpha: stromal cell-derived factor 1 alpha; SNP: single-nucleotide polymorphism; SPECT: single positron emission computed tomography; TIP-1: Tax Interacting Protein-1; TK1: thymidine kinase 1; TKI: tyrosine kinase inhibitors; US: ultrasound; VEGF: vascular endothelial growth factor

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Send correspondence to: Zhaozhong Han, Department of Radiation Oncology, Vanderbilt University School of Medicine, 1301 Medical Center Drive, B902 TVC, Nashville, TN 37232. Tel: 615-322-1037, Fax: 615-343-3061, E-mail: zhaozhong.han@vanderbilt.edu

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