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# Molecular Diagnostics and Personalized Medicine in Oncology: Challenges and Opportunities

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## ABSTRACT

Increasing evidence demonstrates that target-based agents are active only in molecularly selected populations of patients. Therefore, the identification of predictive biomarkers has become mandatory to improve the clinical development of these novel drugs. Mutations of the epidermal growth factor receptor (EGFR) or rearrangements of the ALK gene in non-small-cell lung cancer, and BRAF mutations in melanoma are clear examples of driver mutations and predictive biomarkers of response to treatment with specific inhibitors. Predictive biomarkers might also identify subgroups of patients that are not likely to respond to specific drugs, as shown for KRAS mutations and anti-EGFR monoclonal antibodies in colorectal carcinoma. The discovery of novel driver molecular alterations and the availability of drugs capable to selectively block such oncogenic mechanisms are leading to a rapid increase in the number of putative biomarkers that need to be assessed in each single patient. In this respect, two different approaches are being developed to introduce a comprehensive molecular characterization in clinical practice: high throughput genotyping platforms, which allow the detection of recognized genetic aberrations in clinical samples, and next generation sequencing that can provide information on all the different types of cancer-causing alterations. The introduction of these techniques in clinical practice will increase the possibility to identify molecular targets in each individual patient, and will also allow to follow the molecular evolution of the disease during the treatment. By using these approaches, the development of personalized medicine for patients with cancer will finally become possible. J. Cell. Biochem. 114: 514–524, 2013. 

**KEY WORDS:** MOLECULAR DIAGNOSTICS; PERSONALIZED MEDICINE; CANCER; NEXT GENERATION SEQUENCING

The discovery of the molecular mechanisms involved in the proliferation, survival, metastatization, and differentiation of cancer cells has provided the knowledge to generate a novel therapeutic approach based on drugs directed against specific molecular targets. A number of target-based agents have been developed in the past few years on the basis of a strong rationale provided by preclinical studies. Nevertheless, the majority of these drugs failed to demonstrate activity when administered to unselected patient populations. These failures have underscored the importance to employ target-based agents in molecularly selected populations of patients and, therefore, to associate their clinical development with the identification of predictive biomarkers (i.e. markers that assess the effectiveness of a specific treatment).

The identification of targets for therapeutic intervention and, at the same time, of predictive biomarkers has been facilitated by the

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discovery that somatic mutations and other genetic aberrations drive human malignancies. Human cancer is characterized by a number of genetic alterations [MacConaill and Garraway, 2010]. However, a peculiar phenomenon that has been defined ''oncogene addiction'' has been demonstrated to occur in selected cancer types. In fact, some cancers that contain multiple genetic, epigenetic, and chromosomal abnormalities are dependent on or ''addicted'' to one or a few genes for both maintenance of the malignant phenotype and cell survival [Weinstein, 2002]. Identification of these genes has led to the development of highly effective anti-tumor drugs for specific molecularly identifiable subgroups of patients (Table I). Mutations of the epidermal growth factor receptor (EGFR) or rearrangements of the ALK gene in non-small-cell lung cancer (NSCLC), and BRAF mutations in melanoma are clear examples of driver mutations and predictive biomarkers of response to treatment with specific inhibitors, as we will discuss in the next paragraphs [De Luca and Normanno, 2010; Gerber and Minna, 2010; Smalley and McArthur, 2012].

Predictive biomarkers might also identify subgroups of patients that are not likely to respond to specific drugs, as shown for KRAS mutations and anti-EGFR monoclonal antibodies (MoAbs) in colorectal carcinoma (CRC) [Normanno et al., 2009b]. In these cases, the biomarkers usually represent mechanisms of drug resistance. This ''negative'' selection is less effective as compared with the ''positive'' selection that is allowed by driver mutations. However, it represents the only possibility to select patients when drugs are directed against targets that do not carry alterations of their gene sequence such as the EGFR in CRC.

Identification of a predictive biomarker is not easy. In fact, it must be emphasized that the presence of a potential driver mutation in a tumor is not sufficient to ensure that the patient will respond to an inhibitor directed against the identified target. An example comes from BRAF mutations: while melanoma patients with BRAF mutations respond to BRAF inhibitors, these drugs showed no effect in BRAF mutant CRC patients [Prahallad et al., 2012]. These findings suggest that potential driver mutations might have a different role in sustaining tumor cell growth and survival, depending on the genetic landscape of the tumor. In addition, the identification of predictive biomarkers should always be performed within a randomized clinical trial. In fact, some molecular alterations might turn to be prognostic (i.e. associated with survival independently of any specific treatment). A negative prognostic factor can be easily mistaken for a negative predictive marker if a population of patients not treated with the drug of interest is not available for the analysis.

In the next paragraphs, we will briefly summarize the current knowledge on predictive biomarkers for target-based agents in human cancer. Next, we will describe the main techniques that can be used for molecular diagnostic in oncology. Finally, we will discuss how we believe that this field will evolve in the next years.

TABLE I. Predictive Biomarkers of Response to Target-Based Agents

| Tumor type | Approved biomarkers (drugs) | Potential novel biomarkers |
|---|---|---|
| NSCLC | EGFR mutations (gefitinib, erlotinib) | ROS1 rearrangements |
| | ALK rearrangements (crizotinib) | RET rearrangements |
| | | KRAS mutations |
| | | ErbB-2 mutations |
| | | BRAF mutations |
| | | PIK3CA mutations |
| | | AKT1 mutations |
| | | MEK1 mutations |
| | | NRAS mutations |
| Breast cancer | Oestrogen receptor expression (tamoxifen, aromatase inhibitors) | BRCA1/2 mutations |
| | ErbB-2 gene amplification (trastuzumab, lapatinib) | |
| Colon cancer | KRAS mutations (cetuximab, panitumumab) | BRAF mutations |
| | | NRAS mutations |
| | | PIK3CA mutations |
| | | PTEN loss of expression |
| | | AREG expression |
| | | EREG expression |
| Melanoma | BRAF mutations (vemurafenib) | NRAS mutations |
| | | c-KIT mutations |
| | | GNAQ mutations |
| | | GNA11 mutations |

NSCLC, non-small-cell lung cancer; EGFR-TKIs, EGFR tyrosine kinase inhibitors.

## PREDICTIVE MARKERS IN ONCOLOGY: CURRENT STATUS

### LUNG CANCER

Lung cancer is the leading cause of tumor mortality in the world, and can be distinguished in two main groups, which are small cell lung carcinomas (SCLC) and NSCLC [Herbst et al., 2008]. NSCLC accounts for approximately 80% of lung cancers and comprehends different histological types, including squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [Herbst et al., 2008].

A number of different molecular subgroups have been identified in the past few years in patients with lung adenocarcinoma [Pao and Hutchinson, 2012]. The lung cancer molecular subtypes are characterized by mutations in specific genes, and at least some of these molecular alterations have been demonstrated to act as driver mutations. More importantly, evidence from clinical trials has led to a shift of the treatment paradigm for NSCLC that is currently based on the preliminary evaluation of genetic alterations that might predict sensitivity to specific target-based agents.

Mutations of the EGFR gene are the first predictive biomarker discovered in lung carcinoma. The EGFR is expressed in up to 80% of human primary NSCLC, and evidence suggests that it is involved in the pathogenesis of lung carcinoma [Normanno et al., 2006]. Activating mutations of the EGFR in NSCLC have been discovered following analysis of the EGFR gene in patients that responded to the EGFR-tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib in early clinical trials. EGFR mutations were detected in almost all patients who responded to EGFR-TKIs. These mutations are usually found in exons 18 through 21 of the TK domain of EGFR, and are either point mutations or in-frame small deletions or insertions. Although more than 250 mutations of the EGFR have been described

up to now, two mutations, a single point mutation in exon 21, the L858R, and a series of small in-frame deletions in exon 19, account for approximately 90% of all EGFR mutations [De Luca and Normanno, 2010]. EGFR mutations are not frequent in unselected Caucasian NSCLC patients. However, a peculiar feature of these mutations is that they are strongly associated with defined clinical and pathological characteristics. In particular, EGFR mutations are far more frequent in female patients as compared with male (38.7% versus 10%); in adenocarcinoma as compared with other histological types (29.4% versus 1.8%); in non-smokers as compared with current smokers or former smokers (45.8% versus 7.1%); and in East-Asian NSCLC patients (33.4%) as compared with Non-East-Asian patients (5.5%) [Normanno et al., 2006].

Although the correlation between EGFR mutations and response to EGFR-TKIs was evident since the early clinical trials with these drugs, several other biomarkers have been hypothesized to be associated with sensitivity (EGFR gene copy number variation, EGFR protein expression, and AKT levels of activation) or resistance (KRAS mutations) to EGFR-TKIs [De Luca and Normanno, 2010]. However, results of randomized phase III clinical trials have clearly demonstrated that EGFR mutations are the only reliable marker that predicts sensitivity to EGFR-TKIs [Fukuoka et al., 2011]. Following the observation that administration of an EGFR-TKI as first line treatment results in a prolonged progression free survival (PFS) as compared with chemotherapy in patients carrying EGFR mutations, treatment with an EGFR-TKI has became the recommended first line therapy for EGFR mutant patients [Mok et al., 2009]. The EGFR-TKI gefitinib has been approved in Europe for treatment of EGFR mutant NSCLC in 2009. As a consequence, assessment of the mutational status of the EGFR has become mandatory in order to choose the most appropriate first-line treatment for NSCLC patients.

The fusion between anaplastic lymphoma kinase (ALK) and echinoderm microtubule-associated protein-like 4 (EML4) genes is a chromosomal translocation recently discovered in NSCLC [Gerber and Minna, 2010; Shaw and Solomon, 2011]. ALK and EML4 are both located in the short arm of chromosome 2. Several ALK fusions have been characterized involving the C-terminal kinase domain of ALK and N-terminal portions of the gene EML4; however, other genes such as KIF5B, TGF, and ROS1 have been also described to form fusion products with ALK [Sasaki et al., 2010]. EML4-ALK fusion oncogene leads to the aberrant activation of ALK tyrosine kinase and to the constitutive activation of downstream signaling pathways. This fusion has been detected in ~5% of NSCLC. Patients with NSCLC harboring ALK rearrangements tend to be younger and have little or no smoking history [Gerber and Minna, 2010; Sasaki et al., 2010]. ALK rearrangements have been found exclusively in adenocarcinomas and are associated with a signet-ring cell histology or with abundant intracellular mucin. EML4-ALK alterations are mutually exclusive of EGFR and KRAS mutations. Although some studies have suggested a correlation with the male sex, this mutation has no clear association with patient ethnicity and gender. Phase I and II trials of the ALK inhibitor crizotinib in patients with ALK-positive advanced NSCLC showed high response rates, and low incidence of side effects [Scagliotti et al., 2012]. Based on the phase I and II clinical trial data, the US Food and Drug Administration granted approval of crizotinib for treatment of NSCLC patients carrying ALK rearrangements in August 2011.

More recently, rearrangements of the ROS1 gene with different partners have been reported to occur in approximately 1% of NSCLC [Bergethon et al., 2012; Takeuchi et al., 2012]. ROS1 is a receptor tyrosine kinase of the insulin receptor family, and the gene coding for ROS1 is on chromosome 6q22. ROS1 fusion has been predominantly identified in young and never-smokers individuals with adenocarcinoma. Importantly, preclinical data suggest that the proliferation of ROS1-positive tumors can be inhibited by crizotinib and a significant response to crizotinib has been observed in an NSCLC patient carrying a ROS1 rearrangement [Bergethon et al., 2012].

Four different studies have described the occurrence of KIF5B-RET fusions in approximately 1% of NSCLC adenocarcinoma [Pao and Hutchinson, 2012]. Intriguingly, a RET TKI, vandetanib, has been recently approved for treatment of patients with medullary thyroid cancer.

A number of other driver mutations have been discovered in NSCLC. KRAS mutations are found in approximately 30% of adenocarcinomas and are more frequent in smoker patients [Subramanian and Govindan, 2008]. Although drugs targeting RAS proteins are not currently available, assessment of KRAS mutations has been suggested to identify patients that are resistant to EGFR-TKIs. Since KRAS, EGFR, and ALK mutations are mutually exclusive, the presence of a KRAS mutation is likely to predict resistance to EGFR-TKIs and crizotinib since it excludes the possibility that the targets of these drugs are present.

Subgroups of NSCLC adenocarcinoma carrying mutations of the genes encoding for ErbB-2, BRAF, PI3K, AKT1, MEK1, and NRAS have been also identified [Pao and Hutchinson, 2012]. However, consistent clinical data demonstrating that the presence of these mutations is correlated with response to specific inhibitors are not available yet.

## BREAST CANCER

Breast cancer (BC) is the most common malignancy in women, with more than one million new cases diagnosed worldwide every year. BC is the first solid tumor for which predictive biomarkers have been made available. In fact, the leading parameters that define treatment recommendations for early and advanced BC are oestrogen-receptor (ER), progesterone-receptor (PgR), and ErbB-2 (HER2) status [Normanno et al., 2009a]. Hormone receptor status dictates the use of endocrine therapy for BC patients, and different types of endocrine agents are now available for treatment of hormone receptor-positive patients. Expression of ErbB-2 is associated with sensitivity to the monoclonal antibody trastuzumab and to the TKI lapatinib. These markers are evaluated by using immunohistochemistry and fluorescent in situ hybridization (FISH), and since they have long been established we will not further discuss their role in BC.

The advent of gene-expression profiling techniques allowed the classification of BC into five main subgroups according to their gene expression pattern [Sotiriou and Pusztai, 2009]. Recently a sixth group, named "claudin-negative", has been identified [Hennessy et al., 2009]. More importantly, these techniques allowed the identification of gene signatures that are associated with notably different clinical outcomes in BC patients [Sotiriou and Pusztai,

2009]. However, these signatures have not been shown up to now to predict response/resistance to specific agents in BC.

BRCA1 and BRCA2 (BC susceptibility gene 1 and 2) are tumor suppressor genes involved in maintenance of genomic integrity [O'Donovan and Livingston, 2010]. Germ-line mutations in these genes identify individuals with an increased risk of developing breast and ovarian cancer. BRCA1 is a gatekeeper of genomic integrity with multiple roles including cell cycle checkpoint control and DNA repair, while BRCA2 is likely to have a crucial role in homologous recombination (HR). BRCA1-associated tumors commonly display a triple negative phenotype and a basal-like morphology, while BRCA2-related BCs are a more heterogeneous group [Carotenuto et al., 2010a].

Novel therapeutic approaches in BRCA-deficient tumors are based on the inhibition of poly(ADP-ribose) polymerase (PARP), an enzyme involved in the repair of single strand breaks. BRCA-related tumors have a defective HR and are unable to repair double strand breaks. The inhibition of alternative mechanisms of DNA repair leads to the accumulation of multiple DNA breaks, so tumor cells lacking homologous repair mechanisms invariably undergo apoptosis whereas normal tissue is unaffected [Farmer et al., 2005]. The introduction of PARP inhibitors in patients with BRCA-related tumors represents the first treatment regimen based on a synthetic lethal approach; this strategy has shown interesting preliminary results [Fong et al., 2009]. There are currently at least five PARP inhibitors in clinical trial development in BC. However, these drugs have not been approved yet for treatment of BRCA-deficient tumors.

Several other signal transduction inhibitors are in clinical development in BC patients, and the number of biomarkers needed to choose the most adequate therapy for BC patients is likely to increase in the next future.

## COLORECTAL CANCER

CRC is the third most common cancer worldwide and represents approximately 10% of all cancer deaths. In the past decade, the clinical development of targeted agents, such as anti-EGFR and anti-angiogenic drugs, has significantly improved the survival of CRC patients. To date, the most relevant data on predictive markers of response concern the anti-EGFR agents.

Preclinical data strongly support the involvement of the EGFR pathway in the pathogenesis and progression of CRC [Normanno et al., 2006]. Cetuximab and panitumumab are two MoAbs that bind and inactivate the extracellular domain of the EGFR, thus leading to inhibition of its downstream signaling [Normanno et al., 2003]. These agents have been approved for the treatment of advanced CRC when used as single agents (panitumumab and cetuximab) or combined with standard chemotherapy (cetuximab) [Normanno et al., 2009b].

Despite the EGFR protein is expressed in approximately 85% of colorectal tumors, only a subgroup of patients with advanced CRC benefits from treatment with anti-EGFR MoAbs [Normanno et al., 2009b]. This observation highlighted the necessity to identify markers of response or resistance to these drugs. In this regard, the role of KRAS mutations in the resistance to anti-EGFR monoclonal antibodies has been investigated in several different studies. KRAS mutations are found in approximately 40% of CRC and are mainly located in the codons 12 and 13 (85–90% of all mutations), although other rare mutations have been described in exons 3 and 4 (codons 61 and 146) [Normanno et al., 2009b]. The presence of these mutations leads a ligand-independent activation of intracellular signaling pathways downstream the EGFR that sustain the proliferation and survival of tumor cells.

A number of retrospective studies have clearly demonstrated that the presence of KRAS mutations is associated with a loss of response to cetuximab and panitumumab or, more generally to a lack of benefit in metastatic CRC patients [Normanno et al., 2009b]. In particular, the negative predictive role of KRAS mutations was confirmed by subgroup analysis of patients enrolled in randomized clinical trials in which CRC patients were treated with anti-EGFR agents alone or in combination with chemotherapy. Based on these results, the American and European health authorities restricted the use of anti-EGFR MoAbs alone or in combination with chemotherapy, only to patients with KRAS wild-type tumors. Intriguingly, recent studies suggest that patients with the G13D KRAS mutation might indeed respond to anti-EGFR MoAbs [De Roock et al., 2010c]. However, contrasting results have been reported up to now, and patients with G13D mutations are currently excluded from treatment with these agents.

Mutations in codons 61 and 146 also lead to activation of KRAS. A large retrospective study of an European Consortium showed that mutations in codon 61 had an adverse effect similar to codon 12 mutations, whereas codon 146 mutations did not affect cetuximab efficacy [De Roock et al., 2010a]. However, these findings have not been confirmed in randomized clinical trials.

A putative role in the resistance to anti-EGFR agents has been hypothesized for other molecular alterations detected in CRC. Retrospective subgroup analysis of patients treated with cetuximanb or panitumumab suggest that patients carrying mutations of NRAS, BRAF, PIK3CA or showing loss of expression of PTEN might indeed be resistant to anti-EGFR agents [De Roock et al., 2010ab]. However, these findings have been obtained by analyzing the outcome of patients that were treated in clinical trials or in clinical practice with EGFR MoAbs as single agent or in combination with irinotecan with the aim to revert the resistance to this drug. In addition, these results have not been confirmed yet in randomized clinical trials. By instance, data from the CRYSTAL study of cetuximab plus FOLFIRI versus FOLFIRI as first line therapy for metastatic CRC patients suggest that BRAF mutant patients might benefit of cetuximab treatment [Van Cutsem et al., 2011]. Therefore, there are no consistent and sufficient data to preclude the use of anti-EGFR agents in these subgroups of patients.

Some somatic mutations might provide important prognostic information. For example, BRAF mutations are strongly associated with a worse outcome in CRC patients [Van Cutsem et al., 2011]. The prognostic role of KRAS mutations is more debated, since they have been found to be associated with a worse prognosis in some studies, but contrasting results have been reported [Normanno et al., 2009b].

Finally, recent studies have shown that high levels of expression of the EGFR ligands amphiregulin (AREG) and epiregulin (EREG) are associated with response to anti-EGFR MoAbs in CRC patients [Khambata-Ford et al., 2007; Jacobs et al., 2009]. These data suggest

that in CRC the regulation of downstream EGFR signaling pathways depends mainly on AREG and EREG binding to ErbB receptors in KRAS wild-type patients. Consequently, higher expression of these ligands results in increased activation of the EGFR pathway and in sensitivity to anti-EGFR MoAbs.

## MELANOMA

Metastatic melanoma is refractory to current therapies and has a very poor prognosis, with a 5 years survival rate of 5–14% [Miller and Mihm, 2006]. Several genetic aberrations are involved in the pathogenesis and development of melanoma. In particular, hyperactivation of the RAS/MEK/MAPK pathway occurs with high frequency in melanoma (up to 90%), and it results from somatic mutations of key genes of this pathway, including BRAF and NRAS mutations [Miller and Mihm, 2006].

Approximately 50% of melanoma patients harbor BRAF mutations, and the BRAF V600E mutation accounts for >90% of BRAF mutations found in melanoma [Ribas and Flaherty, 2011]. The presence of the V600E mutation identifies a subgroup of patients that benefit of treatment with mutant BRAF inhibitors. Vemurafenib (PLX4032) and dabrafenib (GSK2118436) have shown a significant clinical response in melanoma patients carrying BRAF mutations [Chapman et al., 2011; Hauschild et al., 2012], and vemurafenib has been approved for treatment of melanoma patients carrying such mutations. Actually, FDA approved vemurafenib for patients carrying the V600E mutation, whereas EMA approved the drug for patients with BRAF V600 mutations, since in the trial that led to the approval of vemurafenib, patients with V600K and V600D mutations also showed to respond to the BRAF inhibitor [Chapman et al., 2011].

Given the important role of the MEK/ERK pathway in BRAF mutant melanomas, the role of BRAF mutations as predictive marker of response is also under investigation in clinical trials with MEK inhibitors. Recently, results of a Phase III clinical study in metastatic melanoma patients with a V600E or V600K BRAF mutation revealed that the MEK1/2 inhibitor trametinib (GSK1120212) improved progression-free survival (PFS) and overall survival (OS) in this setting of patients, as compared with chemotherapy [Flaherty et al., 2012].

NRAS mutations are observed in 15–20% of cutaneous melanomas and cause RAS-independent activation of the MEK/ERK pathway [Miller and Mihm, 2006]. Although therapeutic strategies to target NRAS have not been developed yet, clinical studies are exploring whether patients carrying NRAS mutations might be sensitive to MEK inhibitors.

Mutations of the type III transmembrane receptor tyrosine kinase c-KIT have been frequently shown (20–25%) in acral and mucosal melanomas [Smalley and McArthur, 2012]. These mutations are expected to promote c-KIT dimerization in the absence of scatter factor (SCF) resulting in its constitutive activation, or to prevent c-KIT from maintaining its auto-inhibited conformation. Preliminary observations suggest that inhibition of c-KIT in melanoma patients harboring mutations of this receptor might result in objective regression and disease control following treatment with imatinib, an inhibitor of BCR-ABL kinase that also blocks the c-KIT receptor tyrosine kinase. In particular, response rate to imatinib was better in cases with mutations affecting recurrent hotspots or with a mutant to wild-type allelic ratio of more than 1 (40% vs. 0%, $P = .05$), indicating positive selection for the mutated allele [Carvajal et al., 2011]. Additional multicenter trials are currently underway to evaluate in melanoma patients' agents that target c-KIT, including imatinib and nilotinib (Phase III first-line NCT01028222 and Phase II second-line NCT01099514). Interestingly, a significant response to dasatinib, a TKI that inhibits c-Src, ABL and c-KIT, has also been reported in two metastatic melanoma patients with the c-KIT$^{L756P}$ mutation. Clinical trials of dasatinib in melanoma are ongoing.

Recently, activating mutations in two highly related G protein-coupled receptor alpha-subunit signaling molecules, guanine nucleotide-binding protein Q (GNAQ) and GNA11, have been described in approximately 70–80% of cases of uveal melanoma [Smalley and McArthur, 2012]. A Phase II trial of the MEK inhibitor AZD6244 as single-agent in patients with metastatic uveal melanoma, to correlate PFS, OS, and overall response rate (RR) with GNAQ and GNA11 mutational status is ongoing (NCT01143402).

## METHODS FOR ASSESSMENT OF BIOMARKERS

The methods used to detect predictive biomarkers depend on the type of molecular alteration that underlies the activation of the specific oncogenic pathway. Expression of hormone or growth factor receptors can be assessed by using immunohistochemistry. FISH analysis is used to evaluate gene amplification and traslocations leading to oncogene activation. We will focus our discussion on the methods that are used to detect point mutations and small deletions or insertions.

Mutational analysis is performed with a variety of techniques that can be subdivided into two main categories (Table II): (1) screening techniques that can identify all mutations present within the amplified DNA fragment, including new mutations and (2) targeted methods that can specifically identify known and pre-defined mutations.

Screening techniques are usually based on sequencing, whereas targeted methods employ genotyping approaches. The different methods employed for mutational analysis present advantages and

TABLE II. Current Methods for Mutational Analysis

| Methods | Limit of detection (i.e., minimum % of mutant alleles in a wild type background required for reliable mutation detection) | Range of mutations detected |
|---|---|---|
| Screening methods | | |
| PCR/sequencing | 20–30 | Comprehensive |
| Pyrosequencing | 1–10 | Comprehensive |
| High-resolution melting (HRM) | 10–20 | Near comprehensive |
| Targeted methods | | |
| PNA-LNA clamp | Up to 0.1 | Limited |
| ARMS (Therascreen) | Up to 1 | Limited |
| Fragment analysis | 5 | Limited |
| Real time PCR (allelic discrimination) | 10 | Limited |

PNA-LNA, peptide nucleic acid-locked nucleic acid; ARMS, allele refractory mutation system.

disadvantages, and the choice of the method depends on several variables (Table II). Conventional direct Sanger sequencing of the PCR product (PCR/sequencing) is the most widely used method for mutational analysis. Pyrosequencing is a method of sequencing via synthesis that has several advantages with respect to traditional methods, including a greater sensitivity and the possibility of analyzing short fragments, overcoming possible problems related to DNA fragmentation. Sequencing-based techniques can detect a larger number of mutations as compared with Real Time PCR-based assays. However, these latter methods have usually an higher sensitivity and can detect somatic mutations even when few cancer cells are diluted in normal cells, a phenomenon that often occurs in lung carcinoma specimens. For example, the Real Time PCR-based Therascreen kits for EGFR and KRAS mutations employ ARMS primers that selectively amplify mutant DNA, allowing detection of as low as 1% mutant DNA diluted in wild-type DNA. Methods based on peptide nucleic acid (PNA) clamp, which uses a PNA probe to block the amplification of the wild-type strand during PCR amplification, may reach a sensitivity of 0.1%. However, highly sensitive methods should be cautiously used in clinical practice since they have not been validated yet.

Several scientific societies have released recommendations on the use of the different techniques for biomarkers' testing [van Krieken et al., 2008; Normanno et al., 2011]. PCR/sequencing techniques should not be used if the specimen contains a percentage of tumor cells <50%. In this regard, we have demonstrated that in CRC samples with a tumor content <30%, PCR/sequencing has a significant rate of false negative results [Carotenuto et al., 2010b]. The outcome of a molecular analysis is also significantly affected by the quality and the quantity of the DNA, in particular when the source of material is represented by formalin-fixed paraffin embedded (FFPE) tissue.

## CHALLENGES FOR BIOMARKERS' ASSESSMENT IN ONCOLOGY

### MOVING FROM A SINGLE BIOMARKER TO A COMPREHENSIVE MOLECULAR CHARACTERIZATION

The field of predictive biomarkers is rapidly expanding. The majority of clinical trials with target-based agents include biomarker analysis. Pharmaceutical companies have indeed realized the necessity to identify the populations of patients that might benefit of treatment with specific drugs, and the experimentation of new drugs is frequently associated with the development of companion diagnostics. In this scenario, we expect that dramatic changes will occur in the coming years in the field of biomarkers as we discuss in the next paragraphs.

Although a restricted number of predictive biomarkers for solid tumors is currently assessed in clinical practice, the need to identify different biomarkers in the same patients' population is rapidly increasing (Table I). As above summarized, different subgroups of NSCLC patients with defined molecular alterations have been identified. Target-based agents are already available for some of these groups (EGFR-TKIs for EGFR mutant patients, crizotinib for patients with ALK rearrangement). Other drugs, such as BRAF or RET inhibitors, have been already approved for treatment of other diseases, or are in advanced phase of clinical development. In melanoma, NRAS and c-KIT mutations may identify patients that are sensitive to specific inhibitors, and these mutations are usually mutually exclusive with BRAF mutations. The only predictive biomarker of resistance to anti-EGFR agents in CRC patients is represented by KRAS mutations. However, a number of other mutations might offer the possibility of therapeutic intervention with targeted agents.

Assessment of different molecular alterations might also provide important prognostic information that might turn useful in the clinical management of patients. For example, BRAF mutations predict a worse prognosis in patients with CRC [Van Cutsem et al., 2011]. In lung cancer, an association between worse prognosis and BRAF V600E mutations has been also demonstrated [Marchetti et al., 2011]. KRAS mutations have been hypothesized to represent negative prognostic factors for colon and lung carcinoma patients [Normanno et al., 2009b; De Luca and Normanno, 2010]. Data from clinical trials also suggest that NSCLC patients with EGFR mutations and metastatic disease have a better prognosis as compared with patients with wild-type EGFR [De Luca and Normanno, 2010].

Finally, almost all patients treated with target-based agents eventually become resistant to these treatment. In this regards, evidence suggests that the mutational profile of the tumors significantly changes during treatment with these drugs, since clones of tumor cells carrying molecular alterations that produce drug-resistance expand and are responsible of the recurrence of the disease. In particular, several different mechanisms of resistance to EGFR-TKI and crizotinib in lung carcinoma; to anti-EGFR MoAb in colon carcinoma; and more recently to BRAF inhibitors in melanoma have been described [De Luca and Normanno, 2010; De Roock et al., 2010b; Ribas and Flaherty, 2011; Sequist et al., 2011; Shaw and Solomon, 2011; Katayama et al., 2012]. Resistance mechanisms are usually represented by mutations that reduce the ability of the drug to bind to and inhibit the function of the target, or by activation of alternative signaling pathways that are able to sustain the proliferation and the survival of tumor cells. Assessment of these resistance mutations is becoming extremely important for the patients, since a numbers of signaling inhibitors that are potentially able to block such mechanisms are in advanced clinical development. Therefore, identification of resistance mechanisms might offer the possibility of different lines of treatment with targeted agents with significant effects on both quality of life and survival.

Taken together, these findings clearly indicate that in several different carcinoma types in the next future it will be necessary to assess the status of a number of different biomarkers.

### NEW TECHNOLOGIES FOR BIOMARKERS' ANALYSIS

Assessment of different biomarkers in each individual patient is difficult to accomplish with the currently available techniques. In fact, the cost of a comprehensive molecular characterization with the current methods would be too high to make possible to use this approach in clinical practice. Since most of the driver mutations are mutually exclusive, it is possible to reduce the cost of the screening by performing consecutive analysis for the different biomarkers, starting with the most frequent. For example, an NSCLC patient might be screened first for EGFR mutations, then for ALK and so on.

However, each method requires at least 2–3 days to obtain the result of the analysis. Therefore, if these analyses are sequentially performed, several weeks might be necessary to analyze the different biomarkers, and this time is not acceptable for patients with advanced disease. An additional limiting factor is represented by the availability of tissue for biomarker analysis. For a fraction of patients with advanced disease small biopsies are only available, and this frequently occurs for NSCLC patients. Analysis of different categories of tumor genomic alterations (translocations, copy number alterations, and point mutations) currently requires techniques for which different samples are necessary in order to allow analysis of protein expression (IHC), gene copy number (FISH), mutations (DNA mutational analysis) or even gene expression. Assessment of a wide range of biomarkers might result feasible only in those patients that have a sufficient amount of available tissue.

Therefore, the possibility to introduce a comprehensive molecular characterization in clinical practice relies on the development of high throughput technologies that allow to detect the different molecular alterations in a cost-effective and timely manner. In this respect, two different approaches are being developed (Table III): high throughput genotyping platforms, which allow the detection of recognized genetic aberrations in clinical samples, and next generation sequencing (NGS) that can provide information on all the different types of cancer-causing alterations.

High-throughput genotyping platforms are usually based on multiplexed assays and microarrays, as detailed in Table III. These platforms can analyze hundreds to millions of selected germline and/or somatic variants simultaneously. In addition, array-based comparative genomic hybridization (aCGH) can detect gene copy numbers with high resolution and high throughput, thus providing information on gene copy number changes including deletions, gains, and amplifications. The combined use of these platforms might allow to detect all the most frequent molecular alterations that have the potential to be predictive biomarkers. Indeed, these platforms have been successfully used for genotyping clinical samples and are currently the most used technologies for screening of molecular alterations in patients with cancer.

The limit of genotyping platforms is that they can only provide information on already known genetic alterations. Within the last years, there has been an increasing interest in mapping human genome to determine mutations and genetic events associated with clonal evolution of cancer. A deeper understanding of these alterations will lead to a further comprehension of tumor evolution and to the identification of new potential targets for therapeutic intervention as well. In this respect, NGS approach presents several potential advantages over traditional methods, including the opportunity of fully sequencing a large number of genes in a single test and detecting at the same time deletions, insertions, exome-wide single nucleotide polymorphisms (SNPs), translocations in several cancer-related genes [Metzker, 2009; Tran et al., 2012]. NGS platforms, also known as second- and third-generation sequencers, can generate millions of reads, each of limited length, by

TABLE III. High Throughput Platforms

| Platform | Method | Application/notes |
|---|---|---|
| Genotyping | | |
| Taqman OpenArray Genotyping System (Life Technologies, Carlsbad, CA) | Uses fluorescence-based PCR reagents to provide qualitative detection of targets by post-PCR (endpoint) analysis. | Somatic mutations; SNPs. |
| MassARRAY (Sequenom, San Diego, CA) | Combines allele-specific PCR with MALDI-TOF mass spectrometry. | Somatic mutations; SNPs; methylation; gene expression. |
| SNaPshot Multiplex Kit (Life Technologies) | Consists of a multiplexed PCR step with labeled nucleotides followed by single-base extension reaction combined with capillary electrophoresis. | Somatic mutations; SNPs; methylation; gene expression. |
| Infinium (Illumina, San Diego, CA) | 50-mer probes hybridize to loci of interest; enzymatic single-base extension incorporates a labeled nucleotide; detection by iScan imaging system. | Somatic mutations; SNPs; methylation; gene expression. |
| aCGH (Agilent, Santa Clara, CA) | Uses 60-mer oligonucleotide microarrays for aCGH analysis. | Gene copy number variation and rearrangements. |
| Next-generation sequencing (NGS) | | |
| HeliScope (Helicos Biosciences, Cambridge, MA, USA) | No clonal amplification. Single molecules of DNA or RNA are sequenced by synthesis. Optical detection. | Lower biased sequence reads; high error rates compared with other NGS technologies. |
| 454 (Roche, Basel, Switzerland) | Clonally amplified beads generated by emulsion PCR serve as sequencing features. Sequencing is performed by pyrosequencing methods. CCD-based signal detection. | Longer read-length than other platforms; high error rates in homo-polymer repeats. Fast run times. |
| HiSeq (Illumina) | Libraries are prepared by bridge PCR and sequenced by cyclic reversible termination. Optical detection. | Low multiplexing capability. |
| SOLiD (Life Technologies) | Libraries are amplified by emulsion PCR and sequencing by synthesis is driven by a DNA ligase. Optical detection. | Discrete percentage of aberrant nucleotide incorporation. The use of the two-bases encoding system enables a more accurate alignment of short reads and a considerable error-rate reduction; run times are long and data analysis complex. |
| PacBio RS (Pacific Biosciences, Menlo Park, CA) | Single-molecule real-time sequencing: detection of the temporal order of enzymatic incorporation of fluorescently labeled nucleotide into a growing DNA strand. Optical detection. | Long read length; higher error rates than other NGS apparatus. |
| Ion Torrent Personal Genome Machine (Life Technologies) | Non-optical detection. Addition of a nucleotide is detected as change in voltage due to release of hydrogen ions. | Short read length. Short run time. |

PCR, polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; aCGH, array-based comparative genomic hybridization; CCD, charge-coupled device; SOLiD, support oligonucleotide ligation and detection.

immobilizing amplified DNA fragments onto solid surfaces and performing the sequencing reaction. For this reason, they result more economical than Sanger sequencing and have higher throughput. Since the launch of first NGS platforms, different improvements have been made, such as improved sequencing chemistry and new signal detection methodologies, although there are still several challenges to face. Disadvantages still include short read length, long run time, complex sample preparation and amplification, and sophisticated data analysis. In fact, the enormous amount of data produced in each experiment makes it essential the development of appropriate bioinformatics approaches to analyze the obtained data.

The characteristics of the main NGS platforms are summarized in Table III. The HiSeq (Illumina, San Diego, CA) is the most widely used NGS platform in the field, but its technology still exhibits different issues such as low multiplexing capability and a discrete percentage of aberrant nucleotide incorporation, due to polymerase errors. The 454 system (Roche, Basel, Switzerland) is based on emulsion PCR and pyrosequencing, has fast run times and provides longer read lengths than other NGS technologies. It is also highly sensitive compared with traditional sequencing but presents contamination risks due to emulsion PCR steps, a poor performance with homopolymer repeated regions and a relatively low throughput. The HeliScope platform (Helicos Biosciences, Cambridge, MA, USA) provides sequencing by synthesis of single molecules of DNA or RNA without a preliminary PCR. The Helicos has been shown to provide the lower biased sequence reads, although it displays a relatively high error rate compared with other NGS technologies. Supported Oligonucletide Ligation and Detection (SOLiD) (Life Technologies, Carlsbad, CA) sequencing is carried out through different round of ligase-mediate oligonucleotide ligation after an emulsion PCR step. The sequence is determinated through a two-base encoding system with color space that enables a more accurate alignment of short reads and a considerable error-rate reduction. Despite this, the SOLiD system presents relatively long run times and complex analysis are required. The PacBio RS (Pacific Biosciences, Menlo Park, CA) uses a process called single-molecule, real-time detection of biologic processes that does not require DNA amplification and results in longer read lengths. Finally, the Ion Torrent, also called Personal Genome Machine (PGM) (Life Technologies), detects nucleotide incorporation through an ion sensor pH changes resulting from the release of hydrogen ions during the nucleotide addition. Although its accuracy is good and run time is very short, the read length is currently quite short.

The majority of these platforms are capable of performing whole genome sequencing (WGS) in few days with costs that are significantly cheaper as compared with Sanger sequencing. However, the complexity and the cost of WGS is still too high to make it feasible in clinical routine diagnostics. Information related to few dozens of genes are sufficient to drive the choice of the most appropriate treatment, at least according to our current knowledge. Therefore, targeted sequencing, a strategy that enriches the input for DNA regions of interest, is likely to represent the main approach through which NGS technology will be applied to cancer diagnostics in the next future. In this latter approach, the target regions are enriched through PCR amplification or hybridization to oligonucleotide arrays that are specially designed. Unfortunately, there are few preliminary data available on the use of NGS apparatus in molecular diagnostics, although this field is rapidly expanding.

## CONCLUSIONS AND FUTURE DIRECTIONS

The identification of driver molecular alterations promoting tumor growth and the development of drugs capable to block such mechanisms will lead in the next few years to a significant improvement of personalized medicine in oncology. However, the progress in this field is limited by the availability of methods to detect such target molecular alterations in the tumor tissue. In this respect, determination of DNA sequence by using the Sanger method has been the only sequencing method used for almost 30 years. However, Sanger methods are limited to single-gene or hot spot mutation analysis, due to the limited sequencing capacity and the difficulties and high costs of multiplexing protocols.

Different methods that can detect somatic mutations with high sensitivity and specificity have been more recently developed. However, cancer somatic variations are not limited to single-nucleotide mutations, but often consist of large deletions, insertions, copy number variations, and rearrangements. In addition, increasing evidence suggests that SNPs of different genes might affect the activity of anti-cancer drugs. In this respect, NGS devices have made the detection of these variations feasible and have surpassed traditional Sanger sequencing in sensitivity, efficacy and time [Tran et al., 2012]. With respect to genotyping approaches, NGS can detect novel sequence variations that cannot be obtained with genotyping. Importantly, NGS applications can also provide information on gene expression and epigenetic regulation of gene expression such as DNA methylation. Therefore, it is conceivable that the introduction of NGS techniques in molecular diagnostics will allow a significant progress in this field.

There are several aspects of molecular diagnostics that will be possible to improve in the next future, thank to this technology advancement. Evidence suggests that some mutations are specifically selected following treatment with anti-cancer agents. For example, the EGFR T790M mutation that produces resistance to EGFR-TKIs in NSCLC can be detected by using highly sensitive techniques in approximately 30% of patients carrying activating mutations of the EGFR [Maheswaran et al., 2008; Su et al., 2012]. Tumor cells carrying the T790M mutations are selected during the treatment with EGFR-TKIs and are responsible of the recurrence of the disease. Identification of this molecular alteration before treatment with EGFR-TKIs might provide information on the duration of the response. In addition, since agents capable to inhibit the T790M mutant EGFR are in advanced clinical development, this information might also allow to treat the patient with additional target-based agents. Similar findings have been recently reported in CRC, where minor clones of KRAS mutant cells seem to be involved in the acquired resistance to anti-EGFR agents [Diaz et al., 2012]. Therefore, it is likely that analysis of genetic variants represented in minor clones of tumor cells might allow to identify before the start of the treatment mechanisms that might lead to drug resistance. Since this phenomenon is mediated by several different molecular

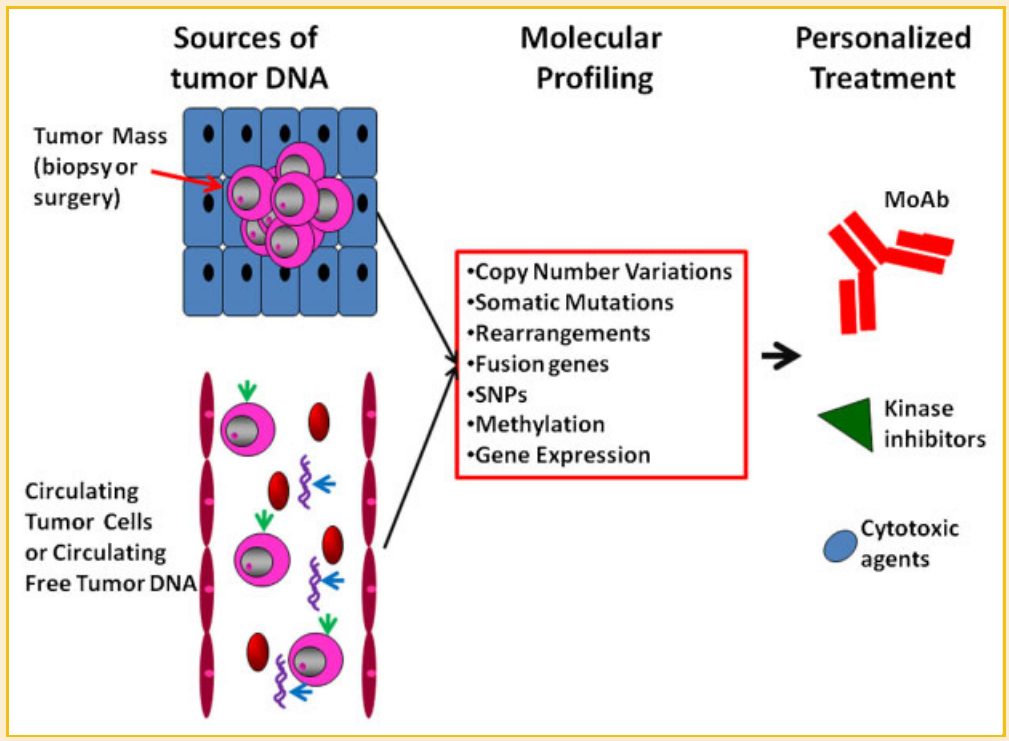


Fig. 1. Tumor DNA can be harvested from tumor specimens obtained by biopsy or surgery. Additional sources of tumor DNA are circulating tumor cells (CTC) (green arrows) or circulating free tumor DNA (cftDNA) (blue arrows) that can be isolated from blood samples. A complete molecular profiling of tumor DNA can be performed with high throughput genotyping and/or next generation sequencing platforms. The identification of predictive biomarkers will allow an individualized treatment with specific target-based (kinase inhibitors and MoAbs, monoclonal antibodies) and conventional agents.

alterations, diagnostic methods that allow to assess the mutational status of different genes in a single analysis will be necessary to identify such genetic variants. In addition, these methods need to have an high sensitivity since the resistance-associated mutations are usually represented in a small fraction of tumor cells and are not detectable by using routine molecular diagnostic approaches. NGS technologies have the features to overcome these methodological problems.

Another important goal of molecular diagnostic in the future will be to move from qualitative to quantitative assessments. Many tumors show an heterogeneous mutational pattern, and driver mutations are not present in all tumor cells. Since qualitative methods have been used in clinical trials to assess predictive biomarkers, we do not know which is the lowest level of mutations that is associated with sensitivity or resistance to specific drugs. Quantitative assessment is hampered by the fact that tumor mass also contain non-neoplastic cells. Nevertheless, quantitative analysis might provide information on the effect of different levels of mutant DNA on the activity of target-based agents. In this regard, NGS systems are single-molecule counting instruments enabled to measure the frequency of mutations in any tissue.

Mutational analysis might not be feasible for patients in advanced stage of disease for which tissue is not available and a new biopsy is not possible, as frequently occurs in lung carcinoma. In addition, as above discussed, the mutational profile of the disease might change during treatment, due to the selection of resistant clones. Since specific inhibitors of intracellular signaling pathways are in advanced phase of clinical development, identification of such resistance mechanisms might allow to assign a personalized treatment to each individual patient with cancer. However, in the majority of the patients, it is not possible to perform repeated biopsies to follow the mutational evolution of the disease. Recently, it has been suggested that somatic mutations can be detected in circulating tumor cells (CTC) or in the circulating free tumor DNA (cftDNA) of patients carrying solid tumors [Maheswaran et al., 2008]. Interestingly, it has been demonstrated that detection of KRAS mutant DNA in the serum of KRAS wild-type CRC patients treated with anti-EGFR MoAbs correlates with the development of resistance to such agents [Diaz et al., 2012; Misale et al., 2012]. In this respect, NGS-based techniques might be able to detect a wide array of mutations in CTCs or serum from patients with advanced solid tumors.

In conclusion, the significant advance in technologies to detect a wide array of genetic alterations with an extremely high sensitivity and specificity is allowing a significant improvement in molecular diagnostic. By using these technologies, the application of a personalized medicine approach to patients with cancer is finally becoming possible (Fig. 1).

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