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Co-development of a companion diagnostic for targeted cancer therapy

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Oncology drug development is a long and costly process associated with a success rate of 5–10%. The parallel development of companion diagnostic tests that will identify patients most likely to receive benefit has the potential to increase the success rate for oncology drugs and decrease development time and associated costs.

Metastatic melanoma is a challenging disease that has been associated with poor survival. Identification of a mutated BRAF kinase gene in many cases of melanoma provided a promising therapeutic target. Here we describe the successful co-development of vemurafenib, a first-in-class selective inhibitor of oncogenic BRAF kinase, and its companion diagnostic, the cobas® 4800 BRAF V600 Mutation Test. Key success factors in the development process included early identification of the BRAF V600E biomarker, early development of the diagnostic test, and early and close collaboration between the pharmaceutical and diagnostic development teams. This focused and integrated process resulted in the first personalized medicine for the treatment of metastatic melanoma less than five years after the Investigational New Drug Application, a remarkably short time.

Introduction

Increased understanding of the cellular and molecular biology of cancer has revealed that cancer is not one disease but rather many diseases which are differentiated not only by their underlying molecular etiology, but also by natural disease course, as well as response to therapeutic interventions. There is no single process that underlies the transformation of cells from a normal to a tumorigenic state and consequently, there is no single way to treat cancer. The complexity of the disease has been reflected in the challenge of developing oncology therapies; the overall success rate of ~5–10% is due in part to insufficient understanding of intended drug targets and the potential influence of upstream and downstream factors [1–3]. Insufficient selectivity for the drug target in cancer cells, resulting in deleterious effects in normal cells, also contributes to the high failure rate [2,3]. Recent successes from ongoing efforts to identify key drivers of tumorigenesis provide opportunities to develop therapies that effectively target specific molecules involved in tumor growth and progression [4]. These targeted approaches should also reduce the widespread toxicity associated with less specific approaches, such as chemotherapy or radiation, which are commonly used to preferentially destroy rapidly growing cancerous cells. Using validated biomarkers and companion diagnostic tests to identify subgroups of patients most likely to respond to treatment can further improve the drug development process, reducing overall development costs and enabling efficacious therapies to reach patients more quickly.

Targeted therapy – the future for oncology

Although the molecular characterization of cancers has revealed the presence of multiple somatic mutations in genomic DNA, as well as chromosomal aberrations, increased understanding of these changes has shown that not all genomic alterations are causally involved in cancer development. A key challenge for cancer biologists has therefore been to distinguish the 'driver mutations,' which fuel unbridled cellular growth of a particular

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cancer type, from the larger number of 'passenger mutations', which arise as a consequence of general genomic instability in cancer cells but play no role in the disease phenotype [5–9]. Decades of research have identified a number of proto-oncogenes and tumor suppressor genes that are frequently mutated in several types of cancers originating from different tissues, including the KRAS, BRAF, EGFR, PIK3CA and TP53 genes. Characterization of such genes has shown that many of the encoded proteins act within shared cellular growth signaling and regulatory pathways. A frequent theme in tumorigenesis is overexpression or constitutive activation resulting from particular point mutations in genes encoding protein kinases, and the identification of selective kinase inhibitors is one of the most active areas in oncology drug discovery [4,10].

The approach of targeting specific growth signaling factors is perhaps best exemplified by two classes of drugs developed to treat breast cancer. Tamoxifen – a selective estrogen receptor modulator – has arguably been the most successful targeted therapy for breast cancer since its introduction for this use in the mid-1970s. Tamoxifen and some of its metabolites bind to the estrogen receptor, thereby blocking the proliferative effect of the hormone estrogen on mammary epithelium. The drug was initially available for non-targeted treatment of breast cancer in post-menopausal women, but was subsequently found to be more effective in patients with estrogen receptor-positive breast cancers [11]. Trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of the HER2 receptor, represents a second example of a successful targeted therapy for breast cancer. The HER2 gene is amplified in ~20–30% of breast cancers, leading to overexpression of the HER2 receptor tyrosine kinase [12]. By binding to the HER2 receptor, trastuzumab inhibits cell proliferation through numerous pathways, including blockade of downstream signaling and increased antibody dependent cell-mediated cytotoxicity. As would be anticipated from its mechanism of action, trastuzumab is effective only in HER2-overexpressing (HER2-positive) breast cancers [13,14].

Imatinib represents another example of a successful therapy that targets an oncogenic tyrosine kinase. This revolutionary molecule was developed by rational drug design in the early 1990s following the discovery that the aberrant Philadelphia chromosome arises by a translocation between chromosomes 9 and 22, which leads to constitutive expression of the BCR–ABL fusion protein in most cases of chronic myeloid leukemia and some cases of acute lymphocytic leukemia [15–17]. As an orally available inhibitor of the BCR–ABL tyrosine kinase, imatinib inhibits proliferation and induces apoptosis in Philadelphia chromosome positive chronic myeloid leukemia and acute lymphocytic leukemia [15–17].

The growth in the discovery and use of targeted therapies, particularly in oncology, has heralded the development of companion diagnostic tests that can be used to stratify patients into potential responders and non-responders based upon predictive biomarkers [18]. For trastuzumab and imatinib, the presence of the intended drug target was indicated by overexpression of the HER2 receptor and presence of the Philadelphia chromosome, respectively – biomarkers that could be used to define the patient populations in which the drugs were to be used. For these two drugs, the target patient populations were defined from the outset. For other agents, however, the development path has been more complex, with biomarkers being identified during development or following initial launch of the drug in unrestricted populations. For example, gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was approved by the US Food and Drug Administration (FDA) in 2003 for unselected patients with non-small cell lung cancer. Retrospective analyses of clinical trials indicated that the drug was actually most effective in patients whose tumors carried activating EGFR mutations [19], and the European labeling granted in 2009 defined the target population as those patients with tumors harboring activating EGFR mutations. Similarly, following approval of the EGFR monoclonal antibodies cetuximab and panitumumab for metastatic colorectal cancer, it was discovered that patients whose tumors had activating mutations in the downstream KRAS gene gained no benefit from the therapies [20,21]. As a consequence, the labels for these agents now define the target population as patients with wild-type KRAS tumors. By ensuring treatment of only those patients expected to gain the greatest clinical benefit from a targeted therapy, companion diagnostic tests can reduce the number of patients who are exposed to potential toxicities without a reasonable chance of benefit. By treating patients in this way, cancer treatment is moving from an empiric 'one drug fits all' treatment paradigm to a more focused personalized approach. It is anticipated that applying the principles of personalized medicine – including the discovery and application of biomarkers and companion diagnostics – will significantly improve the process of drug discovery and development through improved efficacy and safety profile, which will be reflected in reduced costs and decreased time to approval.

BRAF and metastatic melanoma

Melanoma is a challenging disease and its incidence is increasing worldwide despite widespread efforts to improve primary and secondary prevention [22]. Patients diagnosed with metastatic disease have a poor prognosis [23]. Until recently, treatment options for metastatic melanoma were extremely limited. Dacarbazine was approved in 1975 and remains the only chemotherapy approved for melanoma, but treatment is associated with a response rate of only 7–12% and a median overall survival of six to eight months (reviewed in Chapman *et al.* [24]). Immunotherapy with interleukin-2 has resulted in prolonged responses, but only in a small subset of patients [25]. Ipilimumab – a recently approved monoclonal antibody that blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on lymphocytes – improved overall survival in patients, but was associated with a response rate of 11–15% and immune-related toxicities that could be life threatening unless carefully managed [26,27]. As described below, the more recent approval of the small molecule vemurafenib, a first-in-class selective inhibitor of oncogenic BRAF kinase, represents not only a key advance in the treatment of metastatic melanoma, but also a clear example of the integral use of companion diagnostic co-development to achieve the best outcomes for patients and rapid regulatory approval.

Identification of BRAF as a proto-oncogene was an early success of the Cancer Genome Project at the Wellcome Trust Sanger Institute. DNA sequencing of cancer cell lines, primary cancers and short-term cultures revealed mutations in the gene encoding the BRAF kinase in 66% of the melanomas and at lower frequencies in other cancer types [28]. These mutations resulted in constitutive

activation of downstream signaling through the mitogen-activated protein kinase (MAPK) pathway [28]. The observation that BRAF mutations frequently occur in benign nevi suggests that they may be an early event in melanoma formation [29]. Subsequent analyses have shown that BRAF mutations are most common in melanomas developing on skin intermittently exposed to sun [30].

Among the BRAF mutations observed in melanoma, over 90% are at codon 600, and among these, over 90% are a single nucleotide mutation resulting in substitution of glutamic acid for valine (BRAF V600E: nucleotide 1799T > A; codon GTG > GAG) [31]. Among the other activating mutations at codon 600 that have been reported, the second most common mutation is BRAF V600K (GTG > AAG), followed by BRAF V600R (GTG > AGG), an infrequent two-nucleotide variation of the predominant mutation which we distinguish as the BRAF V600 'E2' mutation (GTG > GAA), and BRAF V600D (GTG > GAT) [31]. As of 2011, BRAF V600K represented 5–6% of the melanomas with annotated mutations at codon 600 in the public Catalogue of Somatic Mutations in Cancer (COSMIC) database [31], although the prevalence of BRAF V600K may be higher in some populations [32].

The identification of a frequent kinase mutation in melanoma provided a promising therapeutic target in a disease associated with poor survival [33]. A kinase inhibitor targeted specifically towards the BRAF V600E gene product would potentially suppress tumor growth, while sparing the important biological functions mediated by wild-type BRAF kinase. A highly selective compound would probably also enable higher safe exposures, thereby improving efficacy.

Vemurafenib – a first-in-class inhibitor of mutant BRAF

Vemurafenib (RG7204/PLX4032) is a first-in-class selective inhibitor of oncogenic BRAF kinase. It was identified by Plexxikon, Inc. (a member of the Daiichi Sankyo Group since April 2011) using its proprietary Scaffold-Based Drug Discovery™ platform – a crystallography-guided approach [34]. In preclinical studies using *in vitro* and *in vivo* models of malignant melanoma, vemurafenib inhibited downstream signaling through the MAPK pathway and proliferation of tumor cell lines harboring various BRAF V600 mutations, but not in cell lines expressing wild-type BRAF or non-V600 mutations [35,36]. Treatment also resulted in dose-dependent regression of xenograft tumors and improved survival in animal models [36]. Plexxikon filed an Investigational New Drug Application (IND) with the Center for Drug Evaluation and Research at the US FDA in September 2006 and shortly thereafter initiated a Phase I trial in patients with solid tumors, in partnership with Roche Pharmaceuticals.

The selectivity of Plexxikon's compound pointed to the need for an assay to test for the presence of the predominant BRAF V600E mutation. Early recognition of the need for an accurate, rapid, and robust assay had led Plexxikon to an agreement with Roche Molecular Systems, Inc. in 2005 to co-develop a DNA-based assay to detect the BRAF V600E mutation in cancer tissue specimens. The underlying single nucleotide mutation facilitated development of a polymerase chain reaction (PCR)-based assay, and a prototype test was ready for exploratory use in 2007.

Roche Molecular Systems transitioned to companion diagnostic test development in mid-2007. To align the drug and device development processes, Roche Molecular Systems continued to optimize and then validate the companion diagnostic test in parallel with the drug's progression through clinical development, with the goal of achieving co-ordinated regulatory approval of the drug and device. The test had to undergo rigorous analytical, manufacturing, and clinical validation for approval as an *in vitro* diagnostic test to ensure safety, effectiveness, and reliability in identifying appropriate patients for treatment with vemurafenib.

Companion diagnostic development

The cobas® 4800 BRAF V600 Mutation Test (Fig. 1) involves two main procedures: (1) manual specimen preparation to obtain genomic DNA from formalin-fixed, paraffin-embedded tissue (FFPET) specimens of malignant melanoma; and (2) PCR amplification of the target DNA and real-time detection of mutation status using two TaqMan® probes labeled with different fluorescent dyes. One probe is designed to detect the wild-type BRAF V600 sequence (GTG) and one is designed to detect the BRAF V600E mutation sequence (GAG). Two external run controls are provided and the wild-type allele serves as an internal full-process control. The cobas® 4800 BRAF V600 Mutation Test is a complete system of reagents, hardware and software which provides automated data analysis and reporting of mutation status [37].

One of the first challenges that Roche Molecular Systems faced was that FFPET specimens, the standard specimen type for diagnosis and confirmation in oncology, contain highly fragmented and cross-linked nucleic acids that interfere with DNA recovery and DNA-based analyses (reviewed in [38]). Methods had to be developed to ensure reliable DNA recovery and subsequent analysis of the fragmented DNA for the BRAF V600E mutation. Roche Molecular Systems conducted extensive non-clinical studies to evaluate the impact of variations in tumor size, tumor content and mutation levels; to rigorously establish the limits of detection, analytical sensitivity and specificity, assay reproducibility; and to assess the effects of potential interfering substances, including melanin [37]. Test performance was assessed using DNA from cell lines as well as melanoma FFPET specimens with varying percentages of mutant BRAF V600E alleles [37].

Another challenge was that while validated bi-directional Sanger sequencing was the comparator of choice for the US FDA, this method has a limit of detection of ~20% mutant alleles for FFPET specimens [39–42] and was therefore not sufficiently sensitive to establish the sensitivity of an assay that has a limit of detection of ≤5% mutant alleles. Sanger sequencing was also observed to have a high failure rate with FFPET-derived DNA [43]. If Sanger sequencing gave invalid test results, identified non-V600E mutations, or gave results that were discordant from the cobas® BRAF test result, then the samples were re-analyzed by 454 sequencing (GS FLX Titanium, 454 Life Sciences, Branford, CT). 454 sequencing is a quantitative method that involves clonal amplification of target sequences by emulsion PCR, followed by massively parallel pyrosequencing [44], and had been validated to detect levels as low as 1% BRAF V600E alleles [37]. The 454 sequencing results indicated that the cobas® BRAF test was more sensitive and specific for BRAF V600 mutations than Sanger sequencing [37].

Co-development of the diagnostic and drug offered advantages over traditional standalone *in vitro* diagnostic development by providing clinically relevant specimens and mechanisms for collecting clinical and demographic data, as well as providing clear

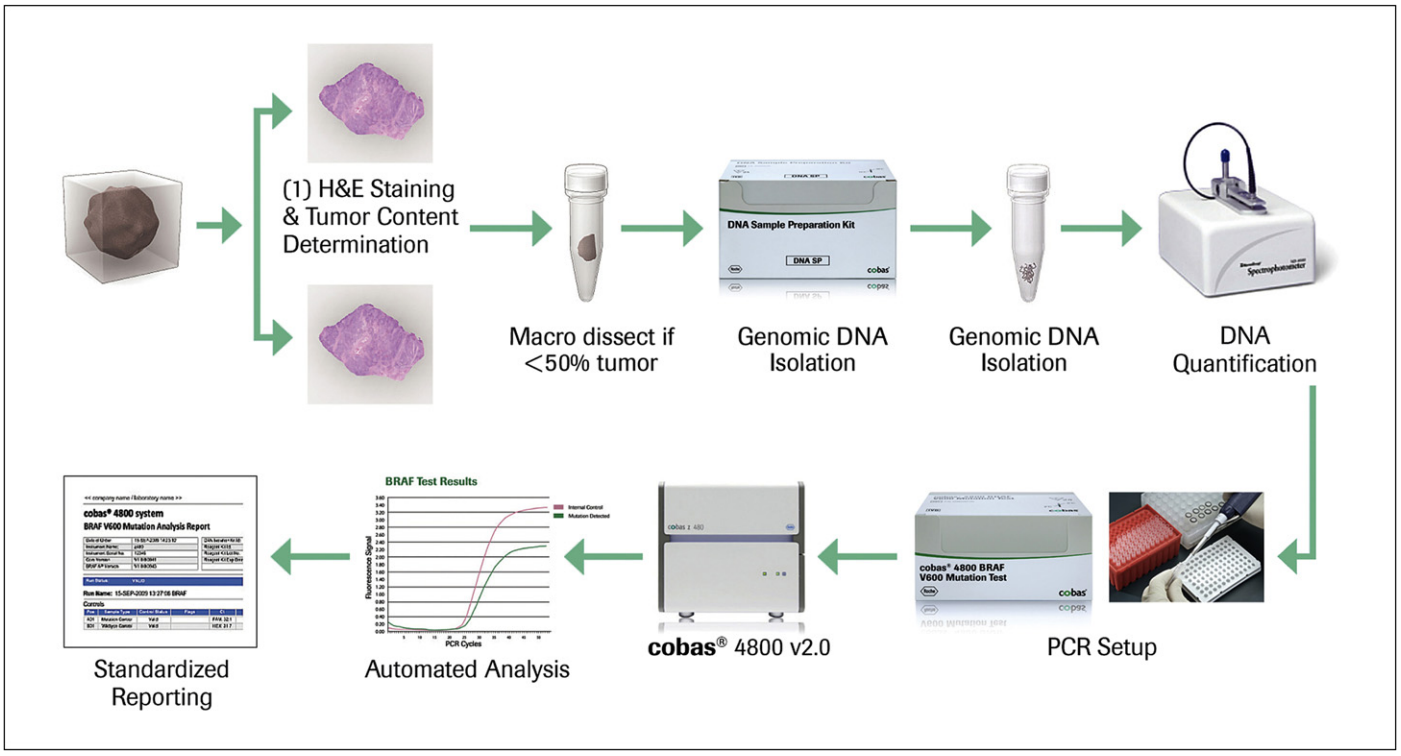

FIGURE 1

cobas® 4800 BRAF V600 Mutation Test workflow. Specimen analysis from DNA isolation to generation of the mutation report can be completed within eight hours [37].

clinical utility as defined by the outcome of the registrational drug trial. To support clinical validation of the cobas® BRAF test, Roche Molecular Systems sequenced specimens from patients screened for the Phase II and Phase III vemurafenib trials to establish the analytical accuracy of mutation calls [43]. Sanger sequencing had a failure rate of 9.2% among 477 specimens, all of which had valid cobas® BRAF test results. Further analysis by 454 sequencing indicated that the cobas® BRAF test was more sensitive than Sanger sequencing in detecting BRAF V600E and also identified some non-V600E mutations. For example, the majority of BRAF V600K mutations among the study samples were detected as V600-mutant although the test had not been designed to detect the V600K mutation. The cobas® BRAF test was also shown to be robust and highly reproducible across three clinical testing sites [43]. As described below, clinical utility was established by the safety and efficacy results from the Phase III trial.

Vemurafenib and the cobas® BRAF test – path to approval

The Phase I vemurafenib dose-escalation study was conducted in patients with solid tumors without selection based upon BRAF mutation status. When the initial pharmaceutical formulation proved to have insufficient bioavailability, dose escalation was suspended while a new formulation was prepared [45]. A recommended Phase II dose was identified using the new formulation and an extension cohort at this dose level was opened [45]. The prototype BRAF test from Roche Molecular Systems was used at a central laboratory to prospectively identify patients whose tumors harbored the BRAF V600E mutation for enrollment into the extension cohort.

Early clinical data from the Phase I study suggested remarkable efficacy with the new pharmaceutical formulation [45]. As Phase II and Phase III clinical study plans were being finalized, the prototype BRAF test was transitioned to the cobas® 4800 platform which had been newly identified as the final *in vitro* diagnostic platform for this test. Although anecdotal evidence suggested that patients with tumors harboring the BRAF V600K mutation would also respond to treatment [34], the rapid pace of the clinical development program precluded modifications to the test to enable detection of multiple mutations at codon 600 with similar sensitivity. Preliminary studies demonstrating the cobas® BRAF test's analytical sensitivity, specificity, and accuracy relative to sequencing, and reproducibility of results were submitted to the FDA in July 2009 to support an Investigational Device Exemption. A standardized method to enrich for patients most appropriate for treatment with the targeted therapy was therefore available on the intended commercial platform before initiation of the Phase II and Phase III clinical studies.

The investigational cobas® BRAF test was placed at designated laboratories in the US, Europe and Australia to centralize identification of patients with BRAF V600E-positive melanoma for the Phase II and Phase III studies. Three testing sites were used for the Phase II BRAF Inhibitor in Melanoma (BRIM)-2 trial of vemurafenib, which enrolled its first patient in September 2009. An additional two testing sites were activated to screen patients with previously untreated melanoma for the Phase III trial, which enrolled its first patient in January 2010. The Phase II BRIM-2 trial was initiated to confirm the anti-tumor activity that was observed in the Phase I trial, and enrolled only previously treated patients with BRAF mutation-positive metastatic melanoma as determined by the cobas® BRAF test. Retrospective sequencing of tumor specimens indicated that 7.6% of the patients enrolled had V600K-positive tumors; all others had V600E-positive tumors [46]. In

total, 132 patients were enrolled and the overall response rate was 53% [46]. The efficacy of vemurafenib was further established by the BRIM-3 trial in patients with previously untreated unresectable stage IIIC or stage IV BRAF mutation-positive metastatic melanoma, as determined by the cobas® BRAF test. In this Phase III trial, 675 patients were randomly assigned to receive either vemurafenib or dacarbazine. Vemurafenib treatment was associated with a significantly better response rate compared with dacarbazine (48% versus 5%, respectively) and with increased rates of both progression-free and overall survival (Fig. 2) [24].

The US FDA has taken a lead in developing specific guidance for companion diagnostics [47] and Roche Pharmaceuticals, Plexxikon, and Roche Molecular Systems actively engaged the Center for Devices and Radiological Health (CDRH) and Center for Drug Evaluation and Research (CDER) at the FDA, as well as the Committee for Medicinal Products for Human Use at the European Medicines Agency (EMA), regarding the co-development program. Roche Molecular Systems submitted a Premarket Approval Application to CDRH at the US FDA in parallel with Roche Pharmaceuticals' submission of a New Drug Application to CDER on April 27, 2011. On August 17, 2011, the US FDA approved vemurafenib (Zelboraf™) to treat patients with late-stage or unresectable melanoma whose tumors express the BRAF V600E gene mutation and approved the cobas® 4800 BRAF V600 Mutation Test at the same time. The drug label specifies that Zelboraf™ is a BRAF kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with the BRAF V600E mutation as detected by an FDA-approved test, and use of the cobas® 4800 BRAF V600 Mutation Test is described in the clinical studies section [48]. The device label specifies that the cobas® 4800 BRAF V600 Mutation Test is a real-time PCR test on the cobas® 4800 system, and is intended to be used as an aid in selecting patients whose melanoma carries the BRAF V600E mutation for treatment with vemurafenib [49]. Both vemurafenib and the cobas® BRAF test were approved well ahead of the FDA action date that had been set following inclusion of vemurafenib in the priority review program at the FDA. It is noteworthy that vemurafenib was approved by the FDA in less than five years from the IND filing, indicating a significant opportunity for the development of targeted therapies with companion diagnostics.

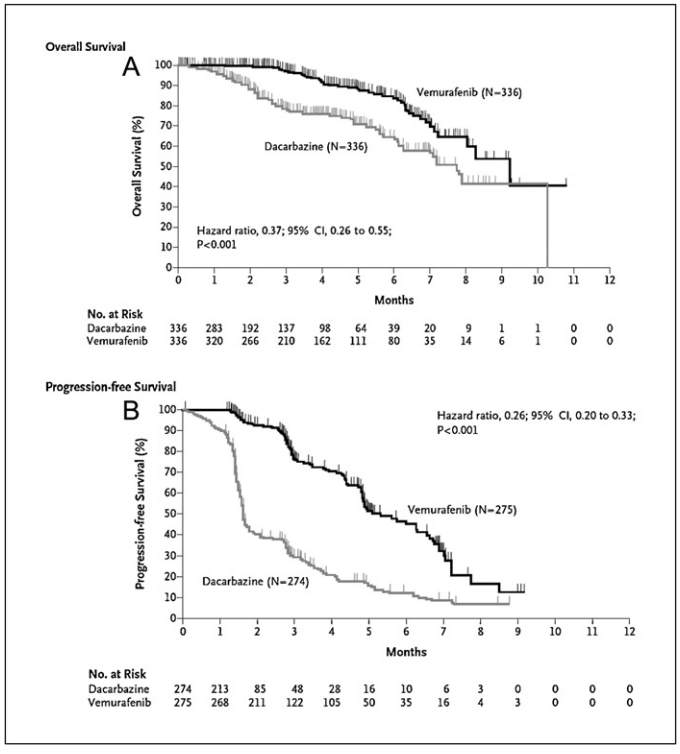

FIGURE 2

Overall survival and progression-free survival in the Phase III analysis of vemurafenib [24].

At present, the EU does not formally define a separate category for companion diagnostics. Roche Molecular Systems obtained CE-marking for the cobas® BRAF test under the EU *In Vitro* Diagnostics Directive in August 2011. On February 17, 2012, the European Commission approved Zelboraf™ as a monotherapy for the treatment of adult patients with BRAF V600 mutation-positive unresectable or metastatic melanoma. Clinical trial use of the cobas® 4800 BRAF V600 Mutation Test to determine mutation-positive tumor status is described in the drug label [50].

Discussion

The development and approval of the cobas® 4800 BRAF V600 Mutation Test alongside vemurafenib, a first-in-class selective inhibitor of oncogenic BRAF kinase, represents an excellent example of the successful co-development of a companion diagnostic for targeted cancer therapy. Key factors in bringing vemurafenib from bench to bedside included the early identification of the BRAF V600E mutation and preclinical evidence of its role as a driver mutation in melanoma, the early development of the diagnostic test – enabling it to be used prospectively in both Phase II and Phase III trials – and the early and close collaboration between the pharmaceutical and diagnostic development teams. Co-development was further aided by the interest that health authorities had in the program. This focused and integrated process has not only resulted in the first personalized medicine for the treatment of metastatic melanoma, but also has led to its approval in a remarkably short time – less than five years after the IND was filed (Fig. 3).

One key challenge for co-development programs is the need to align drug and diagnostic device development timelines, particularly if the biomarker is not identified until later in the drug development process. In the case of vemurafenib and the cobas BRAF test, although the predominant BRAF V600E mutation had been previously identified, melanomas with the more rare V600 mutations represented a challenge for both drug and device development. Ascertaining sufficient numbers of tumors with rare variants to establish efficacy and safety may not be feasible and changing the scope of a companion diagnostic late in clinical development will significantly impact timelines. The challenge is finding a path to address the unmet medical need in a timely manner. Partners also need to understand each other's data requirements to support regulatory filings.

Regulatory challenges remain for manufacturers of *in vitro* companion diagnostic tests. Global harmonization of companion diagnostics regulation is an ongoing effort, and the role of laboratory-developed tests will probably need to be clarified in this context. Laboratory-developed tests offer the clinical community

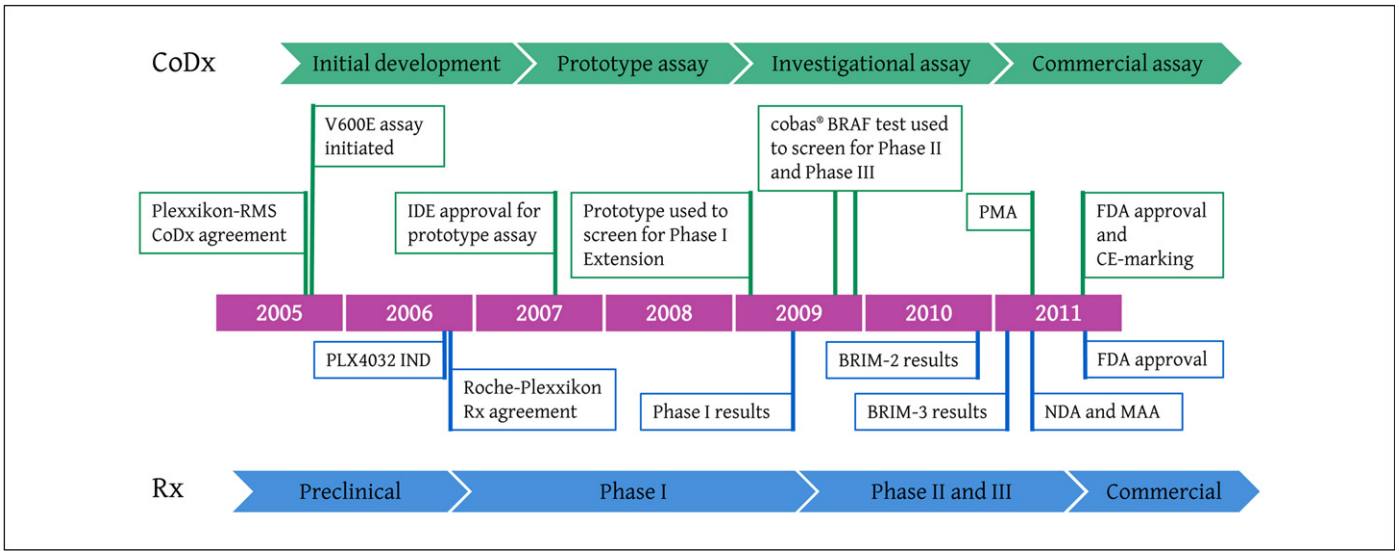

FIGURE 3

Key milestones in the co-development of vemurafenib and the cobas® 4800 BRAF V600 Mutation Test, with milestones and phases of companion diagnostic (CoDx) development in green, drug (Rx) development in blue. (IDE, Investigational Device Exemption; IND, Investigational New Drug Application; MAA, Marketing Authorization Application; NDA, New Drug Application; PLX, Plexxikon, Inc.; PMA, Premarket Approval Application; RMS, Roche Molecular Systems, Inc.).

the flexibility to rapidly introduce new biomarkers, but validation is limited to the corresponding laboratory. Co-development of the cobas® BRAF test provided a standardized test that could be used at sites globally to ensure consistent identification of patients for vemurafenib clinical studies, and subsequently provided a validated test for routine clinical practice worldwide. Furthermore, the US FDA notes that the results from an *in vitro* companion diagnostic device provide 'information that is essential for the safe and effective use of a corresponding therapeutic product' [47], whereas the EU *In Vitro* Diagnostics Directive does not currently define a separate category for companion diagnostic tests and does not require clinical validation for CE marking. Finally, although use of a companion diagnostic test may be mentioned in the clinical section of the corresponding drug label globally, there is no mechanism enforcing use of the test before treatment with the drug. Global harmonization efforts are needed to ensure appropriate support of rigorously validated companion diagnostic tests.

As remarkable as the advent of targeted agents such as vemurafenib, imatinib and trastuzumab has been, the emergence of acquired resistance to single-agent treatments points to the need to identify and test for alterations that underlie drug resistance, allowing rational combination therapies to be selected [51–53]. Single-agent companion diagnostic tests will consequently need to be combined into test panels. Looking further into the future, cancers will be characterized based on the underlying molecular pathology (e.g. driver mutations, oncogene addiction), allowing the selection of appropriate single-agent and combination therapies from an ever-growing arsenal. Highly multiplexed, targeted sequencing of tumor DNA will likely play a major role in subtyping cancers for therapy selection in future generations of personalized medicine.

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