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Developing mRNA-based biomarkers from formalin-fixed paraffin-embedded tissue

Cancer is a complex and heterogeneous disease which is one of the leading causes of death in Western civilisations. Thus, oncology is viewed as a primary focus for personalized medicine. It is recognised that cancer treatment needs to be better tailored in order to improve patient outcome. Patient tumor samples will be required to characterize cancer at a molecular level and identify where there may be disease subgroups that should be treated differently. The use of formalin-fixed paraffin-embedded tissue is important for enabling such studies. In this report, we focus on the challenges that have been faced to date along with the technological developments that have now made this possible. We also highlight the impact this may have on drug and diagnostic development.

KEYWORDS: biomarkers ■ cancer ■ companion diagnostics ■ FFPE ■ formalin-fixed paraffin-embedded ■ predictive tests ■ prognostic tests ■ subtypes

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Cancer is the leading cause of death in Western civilizations. One of the key problems is that tumors from an identical anatomical site demonstrate high levels of heterogeneity. For example, Sørli and Perou's seminal research demonstrated that breast cancer is not one single disease, but a collection of at least five molecular subtypes [1]. Despite this variation, cancer therapies are largely based on a 'one-size-fits-all' approach. As a result, it is rare that more than 25% of tumors respond to single-agent drug [2-4].

The healthcare system at the moment faces challenges from many perspectives. From the healthcare providers' perspective, there is an increasing aging population with an associated increase in chronic diseases. Failed therapies result in more hospital appointments. This increased pressure on limited resources means that therapy needs to be rationed. From a patient's perspective, patients face needless toxicity, failed treatments and poor outcomes owing to the 'one-size-fits-all' approach to therapy and the associated low response rates. The pharmaceutical industry undergoes large expense for drug attrition during clinical trials, with approximately 95% of drugs which enter clinical development currently failing [5]. For those drugs which do succeed, development times are getting longer and ultimately, the window of opportunity for the industry to capitalize on sales before it goes off-patent is reduced. Finally, reimbursers face economic pressure to support and treat an increasing aging population. For this reason, paying for expensive treatments in an unselected manner will no longer be possible in the future.

It is becoming apparent, from all perspectives, that some fundamental changes are needed in the process of drug development and delivery, but for this to happen there are numerous technical challenges that still need to be met.

What is needed?

Cancer is a molecular disease and therefore, a molecular biology approach is required to classify tumors properly. Various approaches can be used to identify biomarkers that help to define a tumor from a defined anatomical site as belonging to a particular molecular subtype. These biomarkers may be a single analyte, such as a protein or gene, or may be multivariate assays, such as multiparametric PCR tests or multigene signatures, as measured by DNA microarrays. In this special report, we will focus on the application of DNA microarrays to biomarker discovery and development from archived formalin-fixed, paraffin-embedded (FFPE) tissue.

Using FFPE tissue for DNA microarray analysis

The biggest issue in discovering novel biomarkers using a DNA microarray approach is the availability of suitable material. In this report, we will focus primarily on the use of RNA. Analysis can also be carried out on a DNA level and this can be useful for detecting germ-line mutations and copy number variations [6]. However, in drug resistance, epigenetic aberrations have also been implicated, particularly in acquired resistance [7]. The analysis of RNA offers insight into the expression

of the genes involved and can be important in understanding both innate and acquired drug-resistance mechanisms.

RNA extracted from tumor cell lines represents the highest quality material. These model systems are often used in the initial phases of biomarker discovery, but their applicability to actual human tumors can be problematic owing to artifacts associated with *in vitro* passage. The second highest quality comes from fresh frozen tumor material collected from patients. This represents the ideal discovery material but can also be problematic. Collection of fresh tissue requires a change in clinical practice with a coordinated approach between surgeons and researchers. Therefore, fresh tissue banks are a limited resource. This makes it very difficult to perform retrospective studies from fresh material, where treatment outcome data from months to years needs to be correlated to DNA microarray profiles. In addition, if a test is developed from fresh tumors, it may only work with this type of material, necessitating a change in standard clinical practice.

Current clinical practice involves fixing tumor tissues in formalin and embedding the samples in paraffin blocks. These samples are stable over time and represent a large resource of biological information. There are a number of advantages to being able to use FFPE blocks in the discovery and development of biomarkers. It is likely that there are millions of FFPE samples worldwide in tissue banks. Access to these samples and the ability to work with FFPE allows the performance of retrospective studies, thereby potentially decreasing biomarker development times.

However, probably the most important consideration is the clinical applicability. As previously mentioned, FFPE is the clinical standard method for storing tissue. As such, when developing a test, it is important that it is usable in standard conditions, that is, it can be carried out on FFPE tissues.

Technical challenges

There are a number of technical challenges when working with this material. Formalin fixation results in the degradation of mRNA, which can make reliable detection difficult with standard DNA microarray protocols and platforms. However, a number of developments have been made in recent years that have made mRNA profiling from FFPE tissue a reality. Specific FFPE-optimized DNA microarray platforms have been developed using a 3' biased probe design [8] or cDNA-mediated annealing, selection, extension and ligation (DASL™) assay (Illumina, CA,

USA) [9]. FFPE-specific RNA extraction methods have been developed which increase the yield and quality of total RNA recovered from FFPE samples [10–12]. Finally, development of linear RNA amplification methods, based on the use of random primers in the cDNA synthesis reaction [13], and the identification of the ribo-single primer isothermal amplification approach to mRNA amplification [14] have been important milestones in enabling RNA profiling from FFPE samples. Early studies involving amplification of mRNA populations using PCR amplification identified what was termed the 'Monte Carlo' effect. This is an artefact in which template concentration affects amplification and rare mRNAs were demonstrated to be more significantly affected [15]. The use of linear methods of amplification, rather than cycles, seems to overcome this issue.

The use of these various platforms and techniques has now been widely published. Many of these studies have involved the direct comparison of fresh frozen and FFPE samples from the same tumor. A high retention of information has been demonstrated on the DASL array [16] and the whole-transcript-based exon and GeneST arrays by Affymetrix (CA, USA) [17]. A range of cancer-specific arrays which we have developed are the Cancer DSA™ research tools (Almac Diagnostics, Craigavon, Northern Ireland) [18]. These are disease-specific Affymetrix 3' arrays which have also been optimized for FFPE profiling by using 3' sequencing in the generation of the array content and design of the array probes at the extreme 3'-end of the transcript [8]. In our own experience, it has been possible to extract good quality transcriptional data from samples as old as 17 years using the Roche (Basel, Switzerland) High Pure FFPE extraction kit and the Nugen (CA, USA) WT-Ovation™ FFPE system.

Another important consideration for FFPE tumor analysis is that handling and fixation protocols may vary between centers and over time. This can be a particular issue for retrospective studies where genetic variation between tumors may be an artefact of the processing rather than true biology. An important factor in the design of a study, whether prospective or retrospective, is the use of samples from multiple centers to ensure that the samples being analyzed fairly represent the population as a whole. However, the introduction of multicenter studies in itself does introduce a potential confounding variable. Careful study design with a balancing of centers for drug response or tumor recurrence and a knowledge of which transcripts are vulnerable to fixation artifacts are needed and could remove this form of bias.

microRNA analysis

In recent years, there has been significant interest in the function of microRNA (miRNA). These are small RNA molecules ranging from 21–24 nucleotides in length, which have been found to regulate the expression of complementary messenger RNAs [19]. It has been suggested that every cellular process may be regulated by miRNAs and their aberrant expression has been implicated in tumor development and progression [20]. Numerous studies have been carried out profiling miRNA extracted from FFPE material. Owing to the small size of the miRNAs, their extraction and recovery has been highly successful from FFPE samples and has been suitable for array-based gene-expression profiling [21,22]. Further studies have gone on to suggest that not only are FFPE-derived miRNAs suitable, but they may be superior analytes to mRNA [23,24]. While miRNAs do appear to be viable candidates, not just for profiling, but for biomarker discovery [25], there is still one major drawback. With most miRNAs their biological function is as yet unknown, which makes them inappropriate for hypothesis-driven biomarker research. However, in the future it is likely that miRNAs will be increasingly used in biomarker strategies.

Application of FFPE profiling to drug development

The ability to mine large archived FFPE datasets for transcriptional data has the potential to result in improvements in the cancer drug-development process. The profiling of hundreds of samples makes it possible to identify and define molecular subtypes for any cancer type that has been archived. In general terms, this involves a process of unsupervised analysis, where tumors are classified according to their molecular similarity rather than their histopathological appearance. As mentioned earlier, Sørli *et al.* have already demonstrated how this approach can be used to

better understand breast cancer at a molecular level [1,26–28]. These molecular subtypes have recently been demonstrated to have clinical significance by predicting outcome from standard chemotherapy [29,30].

To date, the majority of such studies have been carried out using frozen tumor material. As the technology develops, it is becoming apparent that the use of FFPE is indeed a viable option, even when directly compared with fresh frozen material [31]. A recent study undertaken in colon cancer by the Pan European Trial in Adjuvant Colon Cancer (PETACC3) group has involved mRNA isolation for global gene-expression studies and has concluded that FFPE tissue is a viable starting material for biomarker development and that “translational studies based on FFPE should be included in prospective clinical trials” [32].

It is possible that knowledge of molecular subtypes for each cancer type will improve the drug-development pipeline at the following stages, as summarized in **FIGURE 1**.

■ Target discovery

A better understanding of the heterogeneity of a specific cancer type may guide the discovery and development of therapeutics specifically targeted to defined molecular subtypes. This approach has the benefit that the population likely to benefit from treatment is already known, making response in early clinical trials more likely. The best known example of this is HER2. In 1987, the *HER2/neu* oncogene was identified as being amplified in human breast tumors and was demonstrated to be correlated with relapse and survival [33]. A monoclonal antibody was developed against HER2, trastuzumab, which was approved for the treatment of HER2-positive breast cancer patients with remarkable results. Adjuvant trastuzumab therapy in HER2-positive patients has been demonstrated to halve the recurrence rate and to reduce mortality by 30% [34].

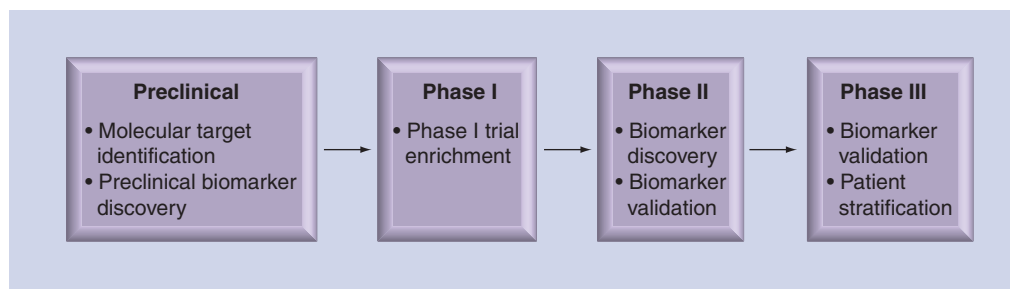


Figure 1. Phases of drug development and the applications of formalin-fixed paraffin-embedded tissue-derived biomarkers.

HER2 tumors have been demonstrated to be a distinct molecular subtype of breast cancer, which is the only population within breast cancer to benefit from trastuzumab treatment [1,35].

■ Phase I clinical trial enrichment

Transcriptional data from large FFPE datasets may allow investigators to identify distinct populations that may benefit from drugs that are already under development. This may allow for the selection of patients for Phase I studies that are likely to demonstrate a clinical response, providing the new drug is active. For example, a target may only be expressed in an active form in a distinct molecular group, such as the estrogen receptor in luminal-like breast cancer. Another example is mismatch repair-deficient colon cancer, which forms a distinct molecular group and may not benefit from specific treatments. [36].

BRCA1 mutant and triple-negative breast tumors have been demonstrated to be predominantly basal-like [37,38]. Recent studies into the benefits of PARP inhibitors have been demonstrated to be beneficial in triple-negative breast cancer and Phase I and II data has demonstrated that these agents have single agent activity in cancers with *BRCA1/2* mutations [39]. Specific Phase I studies have been carried out with an enriched *BRCA1/2* mutant population and antitumor activity has been associated with the *BRCA1* and *BRCA2* mutants [40].

■ Companion diagnostic development

It may be possible that a specific molecular subtype or part of a subtype, defines the population that benefits from a specific drug. This can be discovered prospectively in clinical trials or in a retrospective manner, where large FFPE datasets have been collected as part of a clinical trial, linked with known patient outcomes. These approaches may allow the development of a clinical test that becomes part of the registration of the drug for regulatory purposes. For example, *K-ras* mutant colon tumors are different at a transcriptional level to *K-ras* wild-type [41], and do not benefit from EGFR-targeted therapies [42]. Similarly, drugs have been developed to target *B-Raf* mutant tumors and a companion diagnostic has been developed for patient selection [43].

Although DNA microarrays may be applied to FFPE tissues in the initial discovery of molecular subtypes, the biomarkers developed for Phase I clinical trial enrichment or companion diagnostics may be simpler and

delivered as immunohistochemistry or quantitative reverse transcription (qRT)-PCR-based tests if the transcripts that define a subtype and response are relatively few. For example, it has recently been demonstrated that the DNA microarray signatures that define the molecular subtypes within breast cancer can be simplified to a qRT-PCR [30] or an immunohistochemical test that could be readily applied in a routine histopathological laboratory [29,44]. The use of immunohistochemistry or qRT-PCR may be a lower cost option if available. There are also issues regarding central laboratory testing versus distributed testing. At present, there has not been sufficient uptake of microarray platforms for these tests to be run in a distributed manner and these are all presently run in central laboratories. Immunohistochemistry tests and qRT-PCR tests are more often sold as kits and are run in local hospital laboratories.

Application of FFPE profiling to prognostic test development

The prognosis, as defined by recurrence and survival following surgery with curative intent, varies considerably between patients with apparently identical tumors. Assuming surgery of equal quality, the variation in outcome may be largely explained by the molecular variability between tumors.

Unfortunately, current histopathological techniques are inadequate for accurately determining a prognosis in the majority of cancer patients. This results in overtreatment with adjuvant therapy, such as cytotoxic agents or can result in no further treatment for those who would benefit. Since FFPE samples have been collected for several decades, it is now possible to analyze tumors with known patient outcomes, using DNA microarray technology. This allows for the identification of those mRNA transcripts that indicate a favorable outcome following surgery or those that suggest a high likelihood of recurrence and a requirement for adjuvant therapy. This approach has been taken by Agendia (Amsterdam, The Netherlands) [45], who developed a 70-gene signature that predicts the likelihood of recurrence following surgery for breast cancer. Recent data suggests that this test is also useful as a response predictor for neoadjuvant chemotherapy in breast cancer [46]. However, this test was developed from frozen tissue that may limit its general adoption in the clinic, as discussed earlier.

Genomic Health (CA, USA) have also developed prognostic test for breast cancer, in this case a 21-gene signature [47]. This test has been demonstrated to have both prognostic and predictive value. An important distinction is that the latter test is qRT-PCR based and has been optimized to work with FFPE samples [48,49].

Conclusion

The development of personalized medicine relies on the discovery and validation of adequate biomarkers. The ability to analyze FFPE tissue on DNA microarrays should greatly enhance the discovery process, and for complex biology, may also represent a suitable technology for delivery in the clinic.

The identification and development of predictive and prognostic biomarkers should result in benefits for all stakeholders involved in cancer drug development. From a patient's perspective, there are obvious benefits, with more effective, better targeted treatment, leading to improved survival, less side effects and faster recovery times.

From a healthcare provider's perspective, biomarkers should result in increased efficacy of treatments, reduced needless toxicity and a saving in clinic time and cost. From the perspective of the pharmaceutical companies, they will be able to accelerate drug development, reduce attrition, reduce development costs and benefit from a longer drug patent life. For reimbursers, better targeted therapies will result in a more efficient use of resources. This is increasingly a consideration as more people reach retirement age and therefore, rely on a relatively smaller working population to support healthcare.

Future perspective

In the future, the use of molecular subtyping and companion diagnostics to guide patient care will become standard practice. FFPE remains the standard method for sample storage in routine clinical practice. The ability to work with FFPE samples will therefore be a key component to biomarker discovery, validation and, ultimately, delivery in the clinic.

Executive summary

- Cancer is a complex and heterogeneous disease, but is often treated in a simplistic 'one-drug-fits-all' manner.
- The healthcare system faces a number of challenges from the perspective of patients, pharmaceutical companies and insurance companies.
- From all perspectives, fundamental changes are needed in drug development and delivery, both to improve patient care and reduce the costs of drug development and delivery to the patient.

What is needed?

- The use of molecular biological approaches to analyze large clinical datasets to better understand cancer complexity.
- The use of biomarkers to define subtypes of the disease that will benefit from specific therapies.

Using formalin-fixed paraffin-embedded tissue for DNA microarray analysis

- RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue is the focus of this special report.
- Fresh tissue is difficult to obtain, whilst formalin fixation and paraffin embedding is standard clinical practice and large datasets already exist.
- However, numerous technical challenges exist when working with RNA from FFPE samples.

Technical challenges

- Optimized methods for RNA extraction and amplification have been developed.
- Analysis platforms have been developed for working with RNA extracted from FFPE samples.
- Specific analysis approaches can be taken to overcome sources of bias and variation.

microRNA profiling

- microRNA analysis may be especially informative in FFPE tissue.
- The use of microRNA profiling in biomarker strategies is expected to increase.

Application of FFPE to drug development

- To date, most molecular profiling and subtype analysis has been carried out on fresh tissue.
- Recent technical developments have expanded subtype analysis into FFPE samples.
- Molecular profiling offers significant value for target discovery, clinical trial enrichment and companion diagnostic development.
- DNA microarrays offer an excellent platform for the discovery of biomarkers that can then be applied clinically using alternative platforms, such as immunohistochemistry or quantitative reverse transcriptase PCR.

Application of FFPE profiling to prognostic test development

- microRNA profiling can be used to give indication as to patient prognosis.
- Several tests currently exist in breast cancer for this purpose, namely Genomic Health's (CA, USA) Oncotype Dx® and Agendia's (Amsterdam, The Netherlands) MammaPrint®.

Conclusion

- The development of personalized medicine relies on the identification and validation of prognostic and predictive biomarkers.
- The use of archived patient tissues greatly enhances the likelihood of identifying clinically meaningful biomarkers.
- The development of effective biomarkers will benefit patients, care providers, reimbursers and the pharmaceutical industry.

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