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REVIEW

RNA expression analysis from formalin fixed paraffin embedded tissues

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Abstract Formalin fixation and paraffin embedding (FFPE) is the most commonly used method worldwide for tissue storage. This method preserves the tissue integrity but causes extensive damage to nucleic acids stored within the tissue. As methods for measuring gene expression such as RT-PCR and microarray are adopted into clinical practice there is an increasing necessity to access the wealth of information locked in the Formalin fixation and paraffin embedding archives. This paper reviews the progress in this field and discusses the unique opportunities that exist for the application of these techniques in the development of personalized medicine.

Keywords FFPE · RNA profiling · Microarray · Gene expression · Personalized medicine

Introduction

Advances in analysis of gene expression have revolutionised our ability to evaluate and understand the mechanisms of disease and therapeutic response. In recent years our molecular understanding of disease has been greatly advanced by the use of modern mRNA profiling techniques such as quantitative real-time PCR (qRT-PCR) and microarray analysis. Several recent reports show that qRT-PCR is a highly sensitive and specific method which can provide valuable gene expression data from clinical specimens (Abrahamsen et al. 2003; Cronin et al. 2004), however only a limited number of genes can be measured at one time with this technique. In contrast microarray technology is not as sensitive or as specific but is widely used to investigate gene expression and allows a snapshot of transcriptional activity on a global scale. Reports from several disease studies have shown that DNA microarray technology allowed for the identification of many different subtypes of diseases (Perou et al. 2000; Golub et al. 1999; Bhattacharjee et al. 2001) and that these subtypes often have important clinical implications (Sørlie et al. 2001; Shipp et al. 2002). However, applying these methods into the clinical setting has been limited due to its reliance on fresh tissue. The majority of studies to date have used high quality RNA from frozen samples however these studies have been restricted due to the small number of samples in these collections. On the other hand, there is a huge resource of FFPE tissues specimens held in histopathology departments around the world. These samples provide an invaluable resource for studying the molecular basis of disease, making it possible to perform large retrospective studies correlating molecular features with therapeutic response and clinical outcome. However extraction of RNA from these tissues has proved to be problematic due to the detrimental effects of formalin-fixation. Over recent years, researchers attempting to understand these adverse effects on RNA and identify solutions to remove or diminish it have published many reports on the subject. As a result of this work RNA

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of sufficient quality for gene expression can now be extracted and used for both qRT-PCR and microarray analysis. Because of these recent advances in gene expression profiling from formalin fixed paraffin embedded tissue, there is considerable awareness within the scientific community regarding the need to use these tissues together with the appropriate gene expression technology for the development of improved prognostic assays and for use in predicting response to therapy.

Challenges in working with FFPE

To date gene expression profiling from FFPE tissues has been problematic, as the retrieval of RNA from FFPE material is challenging (Krafft et al. 1997). Although tissue architecture and proteins are preserved with paraffin embedding this method does not preserve nucleic acids very well resulting in RNA that is often significantly degraded (Bresters et al. 1994) (Fig. 1). Moreover, formalin fixation causes cross-linkage between nucleic acids and proteins (Finke et al. 1993; Park et al. 1996) and covalently modifies RNA by the addition of monomethylol groups to the bases, making subsequent RNA extraction, reverse transcription and quantitation problematic (Feldman et al. 1973; Auerbach et al. 1977). Nearly 40% of adenines as opposed to 4% of uracils acquire monomethylol additions following incubation in buffered formalin (Masuda et al. 1999). As a result it has been speculated that the poly A tail of fixed mRNA is heavily modified (McGhee et al. 1977) inhibiting oligo (dT) primer annealing to the polyA tail and consequently the reverse transcription reaction. In addition, the degraded mRNA may not contain a poly A tail for substrate binding by oligo (dT) (Srinivasan et al. 2002).

Consequently, significant efforts to improve extraction of RNA from formalin-fixed tissue have been made by introducing various modifications to the extraction steps. Masuda et al. showed that solubilization of FFPE tissue was not possible by chaotropic agents such as guanidinium thiocyanate, which is used in the classic method for RNA extraction from fresh tissue. Instead solubilization of FFPE tissue using proteinase K enabled the release of RNA from the cross-linked matrix and resulted in almost the same RNA recovery as from a fresh tissue. However following extraction these RNAs were still poor substrates for reverse transcription and subsequent PCR was limited to the amplification of small targets. This poor result was attributed to the formalin induced monomethylol additions to the nucleic acid bases preventing successful priming of oligo (dT) for cDNA synthesis. It was found that approximately half of the monomethylol groups could be removed simply by incubating the RNA in formalin-free buffer (Tris, pH 8.5) at 70°C resulting in more 'free' RNA that could act as

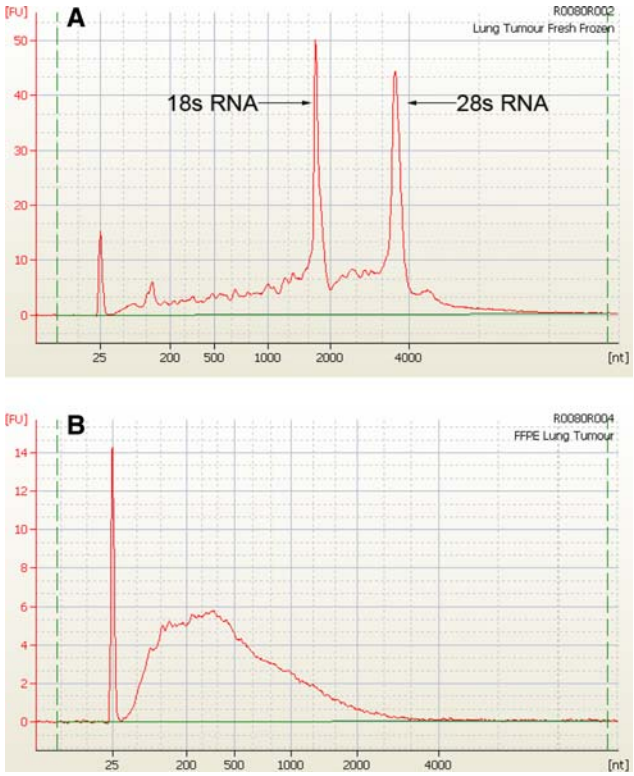

Fig. 1 Analysis of integrity of RNA extracted from (**a**) frozen and (**b**) FFPE matched Lung tumour tissues using the agilent bioanalyzer. Electropherogram A shows RNA of high quality as denoted by the presence of well-defined 18 and 28 s ribosomal peaks. Electropherogram B shows degraded RNA as shown by the complete absence of Ribosomal peaks. The majority of the RNA transcripts from the FFPE sample are approximately 300 base pairs long

template in reverse transcription or PCR. Extended incubation of the sample in proteinase K for up to 5 days can also be attributable to augmented removal of these groups over this time period (Jackson et al. 1990). There has been a great deal of research into modifying methods for successfully extracting usable RNA from FFPE samples (Lewis et al. 2001) and these modifications have been implemented in almost all commercially available FFPE extraction methods today.

However this is not the end of the problem, RNA can degrade naturally in the tissue prior to fixation and many hospitals do not fix the removed tissue immediately. This suggests a change in clinical practise for the development of a standardised, consistent and short time interval between removal of specimen and fixation. Additionally, degradation of RNA over time continues whilst the RNA is stored in the paraffin block through processes such as oxidation (Cronin et al. 2004; Ribeiro-Silva et al. 2007). A number of studies have established parameters aimed at limiting the effects of formalin fixation. In the first instance it has been suggested that, the pre-fixation time (time between surgical incision to fixation) should be as quick as possible to reduce degradation of RNA (Gruber et al. 1994;

Srinivasan et al. 2002). Secondly, it has been recommended that the fixation time should be kept within 12–48 h as longer fixation times can cause further degradation of the samples (Abrahamsen et al. 2003). Finally it was suggested that formalin should be buffered to a neutral pH as prolonged tissue hypoxia reduces pH in tissues locally, decreasing the yield of nucleic acids. This reduction in pH has been reported to cause degradation of nucleic acids (Srinivasan et al. 2002). A number of studies suggested that fixation in neutral buffered formalin (NBF) should occur a 4°C as this causes the least amount of degradation of nucleic acids (Yagi et al. 1996; Noguchi et al. 1997; Tokuda et al. 1990; Srinivasan et al. 2002). It is clear therefore that the adoption of standard operating procedures, at least within the confines of defined clinical trials and preferably in all cases, may markedly improve the quality of RNA extracted from FFPE tissue.

Real-time PCR for expression analysis of FFPE tissues

Successful extraction and amplification of RNA from FFPE tissue has been reported since the late 1980's (Jackson et al. 1989; Stanta et al. 1991; von Weizsäcker et al. 1991; Finke et al. 1993). von Weisäcker and colleagues described a protocol to retrospectively analyse gene expression in fixed tissues. Subsequently Finke and colleagues reported on the successful extraction of RNA suitable for PCR amplification and identified useful housekeeping genes. The development of real-time quantitative PCR (qRT-PCR) has had a significant impact on the study of the molecular basis of disease (Holland et al. 1991; Gibson et al. 1996; Heid et al. 1996). This technique has all the advantages of conventional PCR such as high specificity and sensitivity, but has the added advantage of enabling quantification as well as detection, thus enabling gene expression to be measured quantitatively.

Quantification of gene expression from FFPE tissue is well suited to qRT-PCR interrogation. Many groups have reported the ability to amplify small amounts of RNA from FFPE tissues using this approach (Specht et al. 2001; Godfrey et al. 2000; Cronin et al. 2004). The results from these studies can be used to establish practical recommendations for gene expression analysis from FFPE derived RNA using qRT-PCR (Godfrey et al. 2000; Lewis et al. 2001). The major advantage of qRT-PCR is the ability to use very short RNA fragments for amplification, which suggests that this method is well suited to the amplification of highly degraded RNA in FFPE tissue. RNA fragments extracted from FFPE tissue can be as short as 200 bp and it is therefore suggested that amplification of fragments longer than this should not be attempted. Several studies have shown that amplicons of less than ~130 bp are optimal and show very high success rates (Godfrey et al. 2000; Abrahamsen et al. 2003). Reverse transcription is the most important part of the qRT-PCR process and success at this stage is essential for the generation of cDNA of sufficient yield and quality. Unfortunately, monomethylol groups are never removed completely following RNA extraction and purification, and these groups, which preferentially bind to adenines, can inhibit substrate annealing of oligo (dT) to the poly A tail. In addition, in a fragmented mRNA molecule the polyA tail may not even be present having being fractured from the molecule during the formalin-fixation paraffin embedding process. This loss of priming is the main reason for unsuccessful reverse transcription. Consequently, it is recommended, that the reverse transcription step is primed either with a gene specific primer or random hexamers to ensure detection of a transcript of interest. Normalising results to reference genes is also extremely important to ensure reliability of gene expression results and as a result selection of suitable housekeeping genes is mandatory. A model housekeeping gene is one that is constantly expressed at moderate levels across all samples in the study. It must also have the same amplification efficiency (produce the same amount of amplification product per cycle) and when amplified it must be of similar length to the gene it is being normalised against. However, despite these optimised procedures RT-PCR from FFPE tissue consistently obtains lower Ct values (~5 Cts lower) than for matched fresh frozen tissues with the same input RNA (Gruber et al. 1994; Godfrey et al. 2000; Li et al. 2008). Indicating that a large portion of the RNA is still not accessible for cDNA amplification despite improvements to RNA extraction methods from FFPE tissue. However the following studies show that the reduced portion of accessible RNA appears to be sufficient to obtain accurate, specific gene expression results from this difficult tissue.

One of the early concerns with measuring gene expression from fixed tissue was the uncertainty as to whether gene expression levels would accurately reflect the levels prior to fixation. A study by Godfrey and colleagues utilizing qRT-PCR addressed this question by examining the expression levels of 9 mRNA species with regard to RNA half life, pre-fixation time, and differential effect of fixation on RNA populations in fresh frozen prostate tissue and the same tissue fixed in 10% buffered formalin. Subsequent qRT-PCR gave similar mRNA expression levels from both approaches, despite the observation of significant mRNA degradation in the FFPE samples. This data suggests that mRNA expression levels derived from FFPE tissue reflect the actual expression level in the original tissue samples regardless of the variable effects of fixation (Godfrey et al. 2000).

To examine this in more detail, Specht and colleagues used qRT-PCR to analyze the expression of a panel of cancer-relevant genes, including of EGF-R, HER-2/neu, FGF-

R4, p21/WAF1/Cip1, and MDM2 in matched frozen and FFPE xenograft sections. The authors reported no significant differences between gene expression levels obtained with both approaches and neither fixation time nor grade adversely affected these results. Subsequently the comparability of gene expression analysis results from matched frozen and FFPE tissue was confirmed with colorectal carcinoma samples and lymph node metastases from four patients, further demonstrating the value of the qRT-PCR from FFPE tissues for clinical research (Specht et al. 2001). The same authors analysed the expression level of HER-2/neu in 26 esophageal Barrett's adenocarcinoma FFPE samples by qRT-PCR and IHC. It was concluded that HER-2/neu status assessed by FISH analysis and immunohistochemistry closely correlated with HER-2/neu gene overexpression measured by TaqMan qRT-PCR.

In 2004 Genomic Health (http://www.genomichealth.com) published a paper that took one step further towards the clinical application of this approach. Cronin and colleagues assessed the potential for using breast cancer FFPE material for qPCR based testing using multianalyte assays, one measuring 48 genes and one 92 genes. In this experiment FFPE and frozen samples were used that had been prepared from the same breast tumor in 1995. In the first case, all 48 genes profiled in FFPE RNA yielded measurable values that were similar to that generated from the matched frozen sample. In the second experiment using a 92-gene assay only one of the tested genes failed to yield a signal. In an extension of the study the authors compared the mRNA levels of the estrogen receptor (ER), progesterone receptor (PR), and HER2, to their respective protein levels as determined by standard diagnostic assays based on IHC, for 62 primary breast cancer FFPE samples. Consistent with the reproducibility of the qRT-PCR approach approximately 90% concordance was obtained between the two approaches (Cronin et al. 2004).

DNA microarray analysis of FFPE samples

Recent advances in oligonucleotide microarray technology have transformed our ability to comprehensively evaluate the genome of diseased tissues. Unlike qRT-PCR assays that focus on a small selected numbers of genes, microarray analysis allows interrogation of the expression level of tens of thousands of genes in a single experiment and has quickly become the method of choice for retrospective predictive and prognostic gene profiling. However, the preferred source of mRNA for microarray profiling has always been snap frozen tissue material (Elkahloun et al. 2002). And the poor quality associated with RNA from FFPE has often been cited as a primary reason for the continued use of fresh frozen tissues, in the clinical application of microarray technology (van't Veer and Bernards 2008).

However in spite of these difficulties, there have been a few recent reports of microarray analysis of RNA extracted from FFPE tissues using T7 in-vitro transcription (IVT) oligo (dT) methodologies which have generated valid, reproducible biological data. One such study carried out as a collaboration between Arcturis Bioscience and the Fox Chase Cancer Centre investigated the quality of data that could be retrieved from matched fresh frozen and FFPE material obtained by laser capture microdissection (LCM) and achieved excellent retention of genes between the two storage mediums (Coudry et al. 2007). This study used mRNA amplification and labelling kit developed by Arcturis and a 22,000-oligonucleotide array from Agilent Technologies. The samples had been stored for up to 5 years, and demonstrated that short pre-fixation time resulted in the best quality mRNA. The observation that time to fixation affects mRNA quality has also been reported in a number of other studies (Start et al. 1992; Lukiw and Bazan 1997; Mizuno et al. 1998; Fitzpatrick et al. 2002). The concordance that was seen between frozen and FFPE samples was extremely high, with the Pearson correlation coefficient ranging from 0.8 in the lowest quality sample to 0.97 in the highest quality. However in this study for a number of matched tissues higher percent presents were obtained from FFPE tissue than their matched frozen counterpart. This is an interesting result, considering the understanding implicit in FFPE RNA studies that formalin-fixed paraffin embedding of tissues has a detrimental effect on RNA stored in those tissues. This increase in percentage present calls could be due to cross-hybridisation inferring probe detection where it was not in fact present. We have found in our own studies that FFPE extracted mRNA is more susceptible to cross hybridisation than high quality RNA (Winter in preparation). Also, efforts to optimise probe length found that longer probes such as the 60-mers used by Agilent enjoy stronger signal intensity but also suffer from increased tendency to cross-hybridisation (Relógio et al. 2002). The lack of mismatch (MM) probes on Agilent arrays means this cross-hybridisation is not readily detectable. Another recent study, comparing the same sample stored as FFPE and fresh tissue achieved excellent retention of genes between the two storage mediums, although percentage present calls were very low for both frozen and FFPE samples (~20%) (Frank et al. 2007).

Penland and colleagues proved that reliable transcript information is retained in FFPE tissues by correctly classifying tumour type and subtype from a cohort of 157 FFPE tumours ranging from 2 to 8 years old. In this study, the researchers found that only 25% of the unselected FFPE samples provided mRNA of sufficient quality for successful expression analysis, citing both fixation itself and heterogeneity in fixation techniques and storage conditions as the

primary reason for this. The authors suggested that around 40% of those transcripts that pass standard bioinformatic filtering in frozen samples would not pass similar filters when extracted from FFPE tissue. Despite these limitations, unsupervised clustering of the data was able to correctly classify tumours and identified tumour tissue of origin in three unclassified carcinomas (Penland et al. 2007).

In a recent microarray study, Linton and colleagues studied 34 FFPE samples from extremity soft tissue sarcoma (STS). Samples were again amplified using an oligo (dT) primed IVT amplification method. The aim of this study was to identify if biologically relevant data could be obtained from the FFPE material, with a secondary aim of potentially identifying prognostic markers in this disease. In this study, two sets of matched fixed and unfixed samples were analysed, and a high correlation was obtained between the tissue storage methods as 95–96% of the transcripts detected in FFPE were also detected in unfixed tissue. The sensitivity was markedly lower within FFPE tissue with only ~50% of transcripts from unfixed tissue being detected in the matched frozen sample (Linton et al. 2008). In order to look at the potential prognostic value of the data, a 50 gene expression signature was generated which showed a high predictive value for detection of metastatic recurrence within 3 years of diagnosis. qRT-PCR validation was carried out for 24 candidate genes from the 50-gene signature and was successful for 19 of these. The preliminary findings of this paper suggested reliable microarray data could be generated using FFPE tissue and that this could be used for the identification of prognostic genes. The authors predicted that the sensitivity would be improved in the future by using improved mRNA extraction and amplification methods and by the use of new microarray platforms specifically designed for the interrogation of FFPE tissue.

In recent years, there has been a significant drive to develop tools and protocols that enable mRNA profiling from more readily available FFPE tissue. It has been demonstrated that the addition of random primers to the cDNA synthesis reaction will give higher detection rates from FFPE than oligo (dT) priming alone (Xiang et al. 2003) (Fig. 2). As a result, other commercial amplification kits are becoming available specifically for use with degraded RNA or RNA extracted from FFPE tissues. These kits utilize not only the oligo (dT) primer in the reverse transcription reaction but also random primers to ensure the amplification of RNAs where it has been previously impossible due to the lack of a poly A tail or chemical modifications of this sequence. One of these protocols is the recently released NuGEN WT-Ovation FFPE system, which has been especially developed by Nugen Technologies Inc for use with RNA extracted from FFPE tissue. This method uses WT-RiboSPIA, which is a whole transcriptome amplification system using an isothermal linear nucleic acid amplification method (Fig. 3). This whole transcriptome amplification strategy was previously used to obtain good correlation in gene expression profiles between paraffin-embedded and fresh frozen cell pellets (Scicchitano et al. 2006). In 2006, GlaxoSmithKline directly compared microarray data obtained from matched fixed and unfixed materials using this methodology. The study demonstrated isolation of high-quality mRNA from FFPE samples that was successfully used for microarray based gene expression analysis. One major limitations of this study was that the material used was not from human tumour samples. Cell lines were fixed and embedded for the study, and extraction was carried out immediately after this process. Thus, the study assesses the damage that occurs as a result of fixation, but does not measure natural degradation over time. It is also

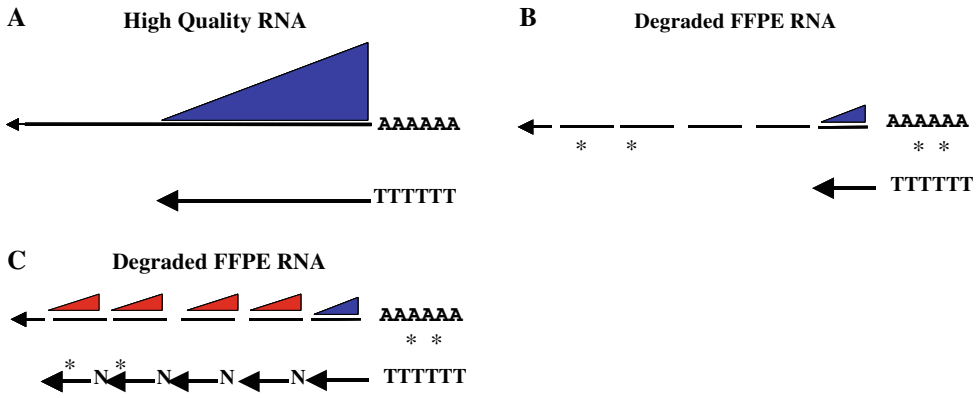

Fig. 2 Schematic diagram showing rationale for using random primers (**N**) in the reverse transcription reaction when amplifying from FFPE derived RNA. Diagram A shows how priming on high quality RNA without chemical modifications (methylol groups are denoted as *) ensures the generation of a long cDNA molecule. However when the mRNA is degraded and chemically modified as in B, amplifications based on oligo (dT) priming are sensitive to RNA degradation and short discontinuous fragments are generated. However when priming is performed with both random primers and oligo (dT) short fragments are generated but they are continuous and are representative of the entire transcript. This results in higher detection rates of transcripts in FFPE tissues than with oligo (dT) priming alone

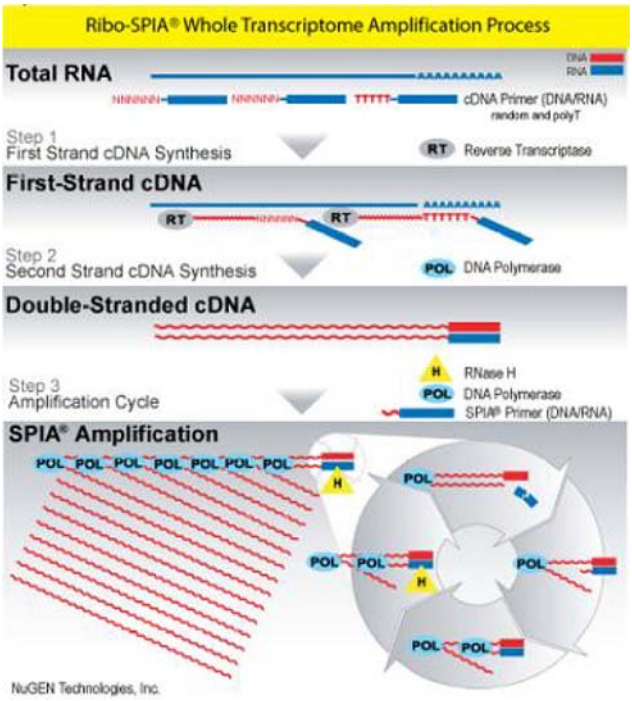

Fig. 3 Overview of the Nugen whole transcriptome RiboSPIA (WT-RiboSPIA) amplification process used in the WT-Ovation™ FFPE System. Note that both oligo (dT) and random primers are used in the reverse transcription step

important to note that the cell pellets used in this study were small and fixed rapidly, which likely contributed to the relative high quality of the mRNA (Scicchitano et al. 2006). In our in-house studies we have consistently found the signal intensities for FFPE samples are lower when analysing gene expression from FFPE samples than their corresponding fresh sample on microarrays. One of the advantages we have identified of using the WT-RiboSPIA technology when compared to IVT for amplifying RNA from FFPE tissues is the lower background obtained. This lower background means that there is more signal above background for gene detection, which is particularly important when identifying differentially, regulated genes.

Another technology used in measuring gene expression from FFPE samples is the DASL (cDNA-mediated annealing, selection, extension and ligation) assay developed by Illumina (Illumina Inc., San Diego, CA, USA). The mechanism behind the DASL assay also uses priming with random hexamers in the cDNA synthesis stage but with no oligo-d(T) priming. The assay works from as little as 50 ng of total RNA to analyse 300–400 transcripts. This is a significant increase over what is readily achievable by multiplex RT-PCR, but is still 10–20 fold less than can be achieved by using microarray analysis. An initial study by Bibikova et al. found that 90% of the genes detected in fresh frozen samples were detected in FFPE tissue; however, the gene expression profiles from FFPE did not correlate exactly with the profiles from fresh samples (R^2 = 0.69) (Bibikova et al. 2004). Subsequently Bibikova and colleagues were able to identify a 16-gene signature that was able to predict prostate cancer relapse after radical prostatectomy and correlated well with Gleason score (Bibikova et al. 2007). The specificity and sensitivity of this gene signature, however, remains to be reported and the authors aim for this test to be validated in a future larger study. However, this result demonstrates the clinical and prognostic value of measuring gene expression from FFPE derived RNA. With the development of high-density microarray platforms that work with FFPE tissue the DASL assay may not be an ideal discovery tool. However, while not directly applicable in the biomarker discovery process, the DASL assay may have a role in the validation of transcripts previously identified by high-density microarray platforms.

Almac Diagnostics (http://www.almacgroup.com), have adopted a different approach to extract robust data from FFPE derived RNA. Almac have developed a range of high density Disease Specific MicroArrays (DSAs™) manufactured on the gold standard Affymetrix platform, which capture as completely as possible all transcripts transcribed in a specific disease setting such as breast, colorectal or non-small cell lung cancer. The actual content of the DSA is derived using a combination of high throughput 3′-based sequencing, data mining and expression analysis (Tanney et al. 2008) (Fig. 4). Because Almac Diagnostics utilised a 3′-based sequencing approach it was possible to design probes at the actual 3′-end of each transcript. Technically this is extremely important as most linear amplification technologies include a reverse transcription step using an oligo (dT) primer. This has the effect of enriching for 3′ information, particularly from degraded RNA typically derived from FFPE. It has previously been suggested that a more 3′ biased design may improve the identification of deregulated genes from FFPE samples (Scicchitano et al. 2006). The combination of extra disease specific content and actual 3′ design results in substantially greater detection of robust gene expression data from FFPE samples.

An example study was performed in-house to demonstrate the utility of this array. The Lung Cancer DSA research tool was used to study a Quad Set™ (Asterand) from a single patient (Fig. 5), representing matched NSCLC tumour and normal lung tissues, both of which were snap frozen and formalin-fixed paraffin embedded. For each of the four samples (FF-tumour, FF-normal, FFPE-tumour, FFPE-normal), five technical replicates were profiled. For RNA amplification and labelling, the WT-Ovation™ FFPE RNA Amplification System was used (Nugen Technologies, CA, USA). This system uses a combination of random hexamer priming in addition to oligo (dT) priming. The coefficients of variance and correlation computed for the expression indices of probesets called

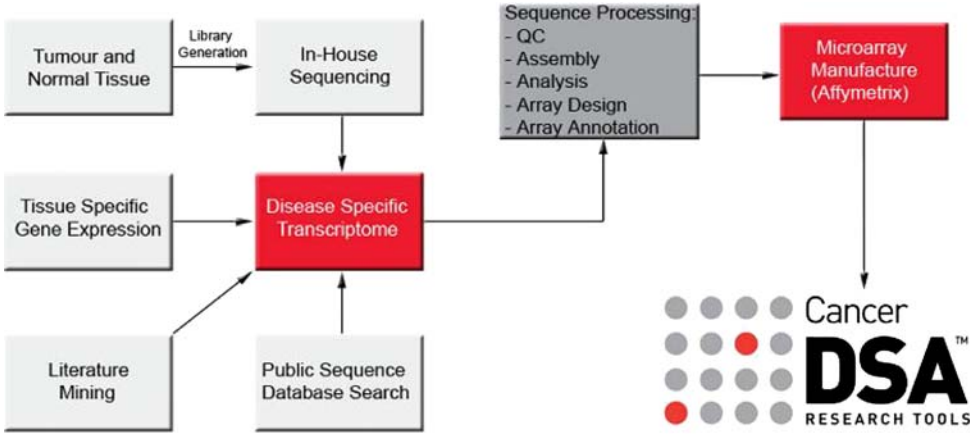

Fig. 4 Schematic representation of the generation of the Cancer DSA research tools showing parallel approaches (in house sequencing, public sequence database mining and gene expression profiling) used to characterise the NSCLC transcriptome, from which the Lung Cancer DSA was designed

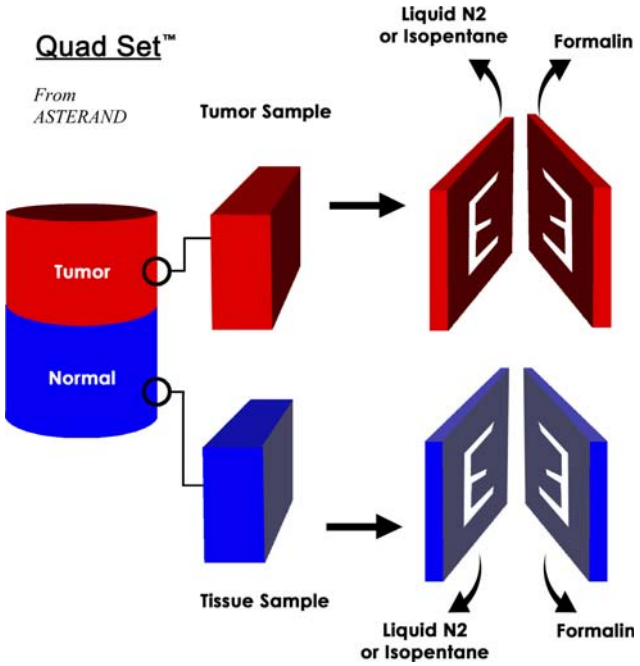

Fig. 5 Schematic representation of the process used to generate the Lung Quad Set. Matched normal and tumour lung tissues pieces are removed from a single patient. The tumour and normal tissue pieces are then cut in two, to form mirror images of the other. One half is stored in liquid nitrogen and the other half is paraffin embedded

consistently present among technical replicates demonstrated similar and very low levels of noise in the data and equally high levels of correlation on both arrays (Table 1). When the present calls were analysed in this experiment, we observed a remarkably high level of present calls in both frozen and FFPE samples. When we examined the overlap between fresh frozen and FFPE tissue expression data, we observed that despite the inevitable loss of information in FFPE tissue, both the specificity and the sensitivity were very high. The sensitivity was 71%, indicating that the transcripts detected in frozen material could be detected in FFPE material and the specificity was 96% with the vast majority of the transcripts detected in FFPE also detected in frozen samples (Fig. 6). These figures were significantly higher than previously reported (Linton et al. 2008; Scicchitano et al. 2006) and provided confidence that reliable high quality data can be derived from FFPE tissue by the Lung Cancer DSA research tool. In order to apply the DSA technology to a more biological relevant question, Hosey et al., utilized the Breast Cancer DSA to carry out FFPE-based profiling comparing 17 BRCA1 mutant and 14 matched sporadic breast cancers. A total of 636 probesets were identified as being differentially expressed between the two cohorts. One of the transcripts identified as down-regulated in the BRCA1 mutant tumours was estrogen receptor 1 (ESR1) that codes for the estrogen receptor alpha (ERα). The authors went on to confirm that BRCA1 modulates response to antiestrogens by transcriptional regulation of ESR1 in an OCT1 dependent manner (Hosey et al. 2007). The study was significant because it provided a model to explain the observation that BRCA1 deficient tumours are typically ERα negative.

Clinical application of mRNA expression analysis

To date, the majority of predictive tests that are available are DNA or protein-based rather than mRNA-based. While there is currently a great deal of focus on developing

Table 1 Reproducibility of data from frozen and FFPE samples profiled on the Lung Cancer DSA™ research tool

Storage method	Coefficient of variation in normal tissue (%)	Coefficient of variation in tumour tissue (%)	Correlation coefficient normal–normal (%)	Correlation coefficient tumour–tumour (%)
FFPE	5.2	5.3	98.59	98.70
Frozen	5.6	5.2	98.10	98.52

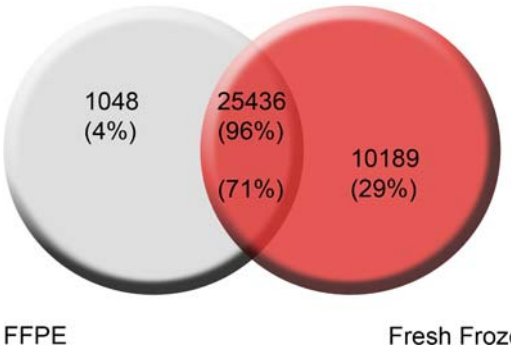

Fig. 6 Venn diagram showing retention of probesets consistently called present between frozen and FFPE samples on the Lung Cancer DSA. Sensitivity was 71% indicating that 71% of the genes from the fresh frozen sample was detected in FFPE. Specificity was extremely high with 96% of the FFPE genes detected being also detected in the fresh frozen sample

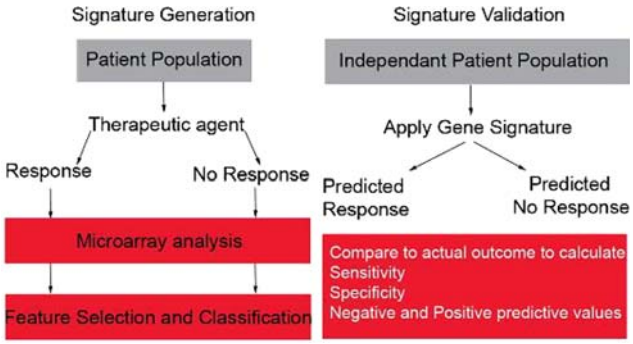

Fig. 7 Schematic outlining a typical design required to generate and validate a predictive signature of response to a specific therapeutic agent. The predictive signature is generated from a suitable powered patient cohort and validated in an independent patient population

mRNA based predictive tests or companion diagnostics, there are currently none CLIA or FDA approved. It is in the field of prognostic testing where mRNA based tests are already available, with breast cancer being the major focus. The three prognostic tests developed so far predict the likelihood of metastatic recurrence of breast cancer following surgery. The first of these was developed by Genomic Health and is marketed as the Oncotype DX test. This is a CLIA certified 21-gene signature using qRT-PCR from FFPE tissue (Paik et al. 2004; Sparano and Paik 2008). The 21-gene signature predicts the likelihood of distant recurrence in patients with node-negative tamoxifen treated breast cancer. The 21-gene signature is composed of 16 cancer specific transcripts and five reference transcripts. The assay gives a 'recurrence score' that provides a better indication of prognosis for ER-positive tumours in node-negative patients than age, tumour size or histologic grade (Paik et al. 2005). It is important to note that this test was developed in and validated for patients treated with tamoxifen monotherapy. As such, the test incorporates an element of prediction of response to tamoxifen. A second CLIA certified qRT-PCR based test has been developed by AviaraDx in collaboration with researchers at Harvard Medical School. The test termed Aviara H/I^{SM} is based on the ratio of the homeobox 13 (HOXB13) to interleukin-17B receptor (IL-17BR) expression. A high HOXB13/IL-17BR expression ratio is associated with increased relapse and death in patients with resected node-negative, ER-positive breast cancer treated with tamoxifen (Goetz et al. 2006). The HOXB13/IL-17BR ratio index has subsequently been validated in an independent patients cohort (Ma et al. 2006).

The first FDA approved microarray-based mRNA expression test was initially developed at the Netherlands Cancer Institute and subsequently commercialised by Agendia (http://www.agendia.com). However the test was developed from the analysis of RNA extracted from fresh frozen tumour material rather than RNA derived from FFPE material. A 70 gene prognostic signature was generated using a supervised classification technique from a total of 117 lymph node negative young breast cancer patients (van't Veer et al. 2002). The test was subsequently retrospectively validated in an independent cohort of 295 breast cancer patients and shown to outperform the traditional clinical prognostic factors. The estimated hazard ratio for recurrence (distant metastases) in the poor prognosis group was reported as 5.1 with a 95% confidence interval ranging from 2.9 to 9 ($p < 0.001$) (van de Vijver et al. 2002). A further retrospective multicentre validation was carried out by the TRANSBIG consortium in 307 patients from five European centres, 137 of which had recurred within 13.6 years. The 70-gene signature outperformed the standard prognostic factors across all endpoints. Interestingly, the hazard ratio for time to distant metastases was estimated as 2.32 (95%CI = 1.35–4.0), which is much lower than the previous study (Buyse et al. 2006). It is not clear why there should be such a difference and may reflect the increased heterogeneity in using samples from multiple sites for the validation. The 70-gene signature is currently being validated in a prospective trial termed MINDACT. The trial started in 2007 and is expecting to recruit approximately 6,000 patients (Cardoso et al. 2008).

The advances in mRNA expression analysis and the emerging clinical application outlined above have raised considerable awareness within the scientific community regarding the need to adopt these technologies in both retrospective studies and prospective clinical trial design. The driving force behind the future acceptance of genomic technologies in clinical trial design and routine patient management is likely to stem from a number of sources. These are likely to include the increasing economic pressures from within the pharmaceutical industry based on the need to bring effective treatments to the market and avoid high profile failures at a late stage in the drug development process. However pressure is also likely to stem from regulatory

bodies such as the Food and Drug Agency who have compiled guidelines regarding the submission of multiplex tests such as gene expression signatures that may be use as a corollary to support the submission of traditional clinical trial data (http://www.fda.gov/cdrh/oivd/guidance/1210.html). Furthermore, there is an increasing awareness within the industry that the traditional approach to clinical trial design will have to change as new therapeutics are likely to provide minimal advantages over current therapies unless targeted to specifically responding populations. The net effect of this is that in the future clinical trials will need to incorporate an effective biomarker strategy to help select the population likely to benefit from the new treatment.

The application of real time qRT-PCR and microarray-based approaches to analyse mRNA expression from FFPE tissue will be instrumental in the development of both prognostic and predictive assays in the future. Developing new prognostic assays requires many years of patient follow up to ensure that appropriate recurrence free survival (RFS) data is available. Performing this type of study prospectively therefore takes many years. The ability to use archived tissue in a retrospective study significantly reduces the amount of time required. In addition, one of the major hurdles in developing proper genomic prognostic tests has been poor study design and lack of proper validation, Simon (2005). Again, retrospective studies using archived materials can reduce this problem. The ideal study design can be formulated, and the samples can be sourced from archives to ensure that the correct samples are profiled. A fully independent validation set of samples can then be sourced to ensure that the test is correctly and properly validated (Fig. 7). It is also possible to develop predictive assays to existing therapies from FFPE material in a retrospective manner from phase III studies in which FFPE blocks have been collected. Predictive molecular signatures can then be validated in independent studies. This is possible since multiple phase III studies are typically carried out for any approved therapeutic. In terms of new therapies it will be essential to properly power phase II studies to develop robust biomarkers of response in a prospective manner. This is likely to require the inclusion of an adaptive trial design to facilitate biomarker development in the most efficient and cost effective manner. Predictive biomarkers developed by this approach will subsequently need to be validated in either a phase IIB or phase III studies.

Conclusion

FFPE tissue archives are a valuable resource of molecular information that has, until recently, been inaccessible to researchers. With recent developments in technology and protocols, it is possible to access this wealth of information. As we move towards an era of personalised medicine, being able to work with FFPE tissue has clear advantages. It enables retrospective studies, thus vastly reducing the development time of tests and facilitates clinical uptake by utilising standard clinical samples.

The research that has been published in recent years has taken us from a point where mRNA profiling from FFPE was not possible, to a point where it is now accepted that gene expression measurements can be performed from FFPE tissue using both qRT-PCR methods and by microarray profiling. It has become clear, that the implementation of a standardised approach to fixation and storage of FFPE tissues and the further improvements in technology and techniques to interrogate this tissue is required if we are to fully embrace the utility of this resource. As our understanding of the molecular basis of cancer changes, so does the way that we treat the disease. With the advent of targeted biological treatments that work on selected sub-sections of the population, it is likely that in the near future, almost all such therapeutic agents will require a "companion diagnostic" for their use. The FDA published their draft guidelines on the co-development of therapeutics and companion diagnostics in 2005 and stated that this is the preferred route for drug development. At the moment, there are only a small number of therapies that are guided by a predictive test, but as the reproducibility and reliability of gene expression profiling from FFPE tissue grows, we are likely to see a marked increase in the number mRNA based companion diagnostics in routine clinical use.

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