Development of a molecular diagnostic test from archival material using optimised methods for RNA isolation and gene expression measurement

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Summary

The current classification of breast cancer is based chiefly on clinical and histological parameters which do not reflect the full complexity and heterogeneity of the disease. Highly specific drugs are being developed and it is very likely that subtypes of tumours respond differently to these drugs. Optimal and appropriate therapy depends on more specific tumour classifications. With the recently developed chip technology it is possible to describe additional subtypes and also to test novel hypotheses. At present these methods depend on fresh material collected from patients during surgery. A method based on archival material, usually formalin-fixed, paraffin-embedded (FFPE) blocks, would greatly facilitate and speed up research in this area, since large numbers of such samples exist in pathology departments worldwide. Standard methods for gene expression analysis do not work for FFPE-derived RNA, since such RNA is considerably degraded and chemically modified. We have developed a method which makes it possible to isolate moderately degraded and partially de-modified RNA from archival material. This RNA is suitable for gene expression measurements by TaqMan analysis.

Abbreviations:

ACTB	β-actin
cDNA	complementary DNA
Ct	threshold cycle
FFPE	formalin-fixed, paraffin-embedded
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GUS	glucuronidase
LNA	locked nucleic acid
PCR	polymerase chain reaction
QPCR	quantitative PCR
RNAlater	RNA Stabilisation Solution
RS	recurrence score
RPLP0	ribosomal protein, large protein 0
TFRC	transferrin receptor

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Introduction

Classification of breast cancer is based chiefly on clinical and histological parameters, supplemented by hormone receptor status and ERBB2 levels. This classification into a few subgroups does not reflect the physiological, cellular and molecular heterogeneity of breast cancer. An increasing number of reports indicate that molecular parameters may improve classification in breast and other cancers [1–5]. These reports are based principally on genome-wide gene expression analysis using DNA chips representing thousands of genes. Detailed analysis of these data has shown that in tumours expression levels from relatively few genes quite often correlate closely (positively or negatively) with one or more clinical parameters (e.g. the risk of recurrence in 5 years). The same information can be obtained by measuring the expression levels which are rendered comparable between individual samples by normalisation against expression levels of internal control or reference genes (genes expressed at relatively constant levels in all tumours of the same type).

In a next step, normalised expression values are subjected to an empirically determined algorithm to compute a score. The term «recurrence score» (RS), introduced by Paik and co-workers for breast cancer [6], predicts risk of recurrence within 5 years. RS is calculated from the expression levels of 16 test and 5 reference genes. The test genes described by Paik and coworkers comprise several groups of functionally related genes («proliferation», «invasion», «Her2/ERBB2», «oestrogen» and «reference» or «control genes»). Each gene is assigned a certain weight (0.1 to 1.2) and scores are calculated for each group. Finally, these group scores are integrated into RS. The algorithm for calculating RS and the selection of test and reference genes was originally developed from 675 tumours and several hundred genes (6). RS ranges from 0–100, 0<RS<17 corresponds to «low risk», 18–31 to «intermediate risk», and 31>RS>100 to «high risk».

Technically, gene expression can be determined by quantitative real time PCR (QPCR) or by DNA chip analysis. Both procedures are well established and relatively uncritical when good quality RNA is available. This is usually the case for RNA derived from fresh frozen or RNAlater preserved tumour material. Unfortunately, such material is not generated routinely and tumour material is directly processed for histological examination by formalin fixation and paraffin embedding. Histological material is regularly archived for 10 or more years and a huge number of archival samples exist as FFPE tissue blocks. RNA derived from such material is considerably degraded. In addition, fixation with formalin extensively modifies macromolecules (RNA, DNA, proteins), e.g. by the addition of methylol groups on free amino groups of bases or amino acids [7] and by the formation of intra- and intermolecular cross-links between nucleic acids and proteins. As a result, RNA is chemically locked in the tissue and extensive

enzymatic digestion with protease is necessary to render it accessible for isolation. We have observed that a separate de-modification step which removes at least part of these modifications improves the recovery and quality of RNA for subsequent downstream applications such as reverse transcription (both QPCR and chip analysis are based on cDNAs generated from RNA, and reverse transcription seems to be highly sensitive to such RNA modifications). Finally, gene expression measurement by quantitative PCR (QPCR) depends not only on the specificity of primers used during amplification but also very much on the size of the amplicon given by the position of forward and reverse primers [8]. Ideally, the size of the amplicon should be considerably below that of RNA and cDNA fragments, otherwise the efficiency of PCR amplification declines dramatically, resulting in increased Ct values (threshold cycle values).

Results

We established our own robust protocol, RNAready (FFPE), for isolation of RNA from archival FFPE material (Fig. 1, lane D) and compared it with RNA isolated from the same FFPE blocks but according to the ncLysis buffer/protocol described by Applied Biosystems (lane C) and the RNeasy FFPE protocol of Qiagen (lane B). For comparison, RNA was isolated from fresh cryopreserved or RNAlater treated pieces of the same tumours which yielded high quality RNA (Fig. 1, lane A). RNA isolated according to RNAready (FFPE) results in high recovery (Fig. 1a) and the RNA is less degraded than RNA isolated by one of the other two protocols for FFPE (Fig. 1b). RNA isolated according to the RNAready (FFPE) protocol involves a separate de-modification step and several protocols were empirically tested to optimise de-modification. Here we demonstrate the influence of the time of de-modfication on subsequent QPCR (Fig. 1c). The size of the amplicon is another independent parameter which influences real time PCR (Fig. 1c). As expected, a short amplicon of 54bp results in lower Cts than an amplicon of 83bp or 103bp (Fig. 1c).

Total RNA from each of 14 tumours was reverse transcribed into cDNA and expression levels were determined by quantitative PCR for 26 genes (including 16 test and 5 control genes used for the RS) [6, 7]. Quantitative PCR was performed on an ABI 7500 FAST system using TaqMan probes from Applied Biosystems (https://products.appliedbiosystems.com) and TaqMan probes from Roche (http://www.universalprobelibrary.com/). As expected, high quality RNA consistently resulted in lowest Ct values (average Ct for the 5 control genes was 23.2, Fig. 2a). Interestingly, considerable differences in Ct values were observed between RNAs isolated from FFPE according to the RNAready (FFPE) protocol (average Ct 28.7, Fig. 2b) and RNA prepared by any of the other two protocols for FFPE material (average Ct 32.5 for ncLysis and 34.6 for Qiagen, data not shown). The overall quality of the RNA can also be estimated by measuring the correlation between Ct values from high quality RNA and RNA isolated from FFPE. Correlation coefficients were determined between Cts of high quality RNA and Cts of FFPE-derived RNA for all the 26 genes and 14 tumours. While intact RNA and RNA isolated from FFPE according to RNAready (FFPE) gave a correlation coefficient of 0.81 (Pearson correlation), RNA prepared from the same blocks but according to the ncLysis or the Qiagen protocol gave only correlation coefficients of 0.50 and 0.48 respectively. The relatively close correlation between good quality RNA and RNA isolated according to the RNAready (FFPE) protocol can also be visualised by comparing Ct values for individual genes. Shown are Cts for ESR1, the gene coding for the oestrogen receptor, and the mean of 5 control genes (ACTB, GAPDH, GUS, RPLP0 and TFRC) for each of the 14 tumours

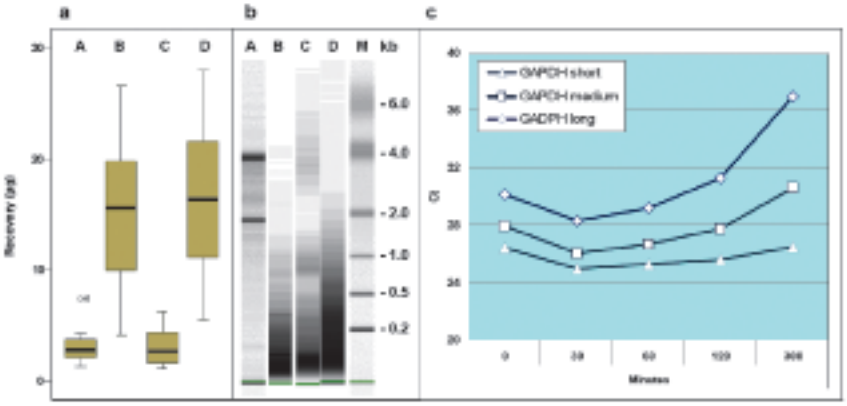

Figure 1. Comparison of RNA isolated according to different protocols and with various kits. High quality RNA was prepared from four serial sections (each 25 µm thick) of snap frozen breast cancer samples, tissue was homogenised by mixer mill (Qiagen) in lysis buffer and RNA extracted according to a standard protocol (Ambion, RecoverAll). RNA was isolated from ten 10 µm thick sections of formalin-fixed, paraffin-embedded tissue according to the RNeasy FFPE (B, Qiagen), ncLysis (C, Applied Biosystems) or RNAready (FFPE) protocol (D, own). RNA recovery (µg) was determined (Nanodrop, panel a) and quality was assessed for one representative tumour on a Bioanalyzer (Agilent Technologies) (panel b). RNA isolated according to the RNAready (FFPE) procedure was de-modified for 0 to 300 minutes at elevated temperature. The RNA from each isolation was reverse transcribed into cDNA using random primers (cDNA Archive kit, Applied Biosystems) and expression levels determined by quantitative PCR on an ABI 7500 Fast cycler (Applied Biosystems) (panel c). Shown are the effects of various de-modification times on Ct values for three amplicons coding for GAPDH. The size of the amplicon was 54 bp (short), 83 bp (medium) and 103 bp (long). De-modification for 30 minutes and a short amplicon resulted in lowest Ct values corresponding to highest QPCR efficiency.

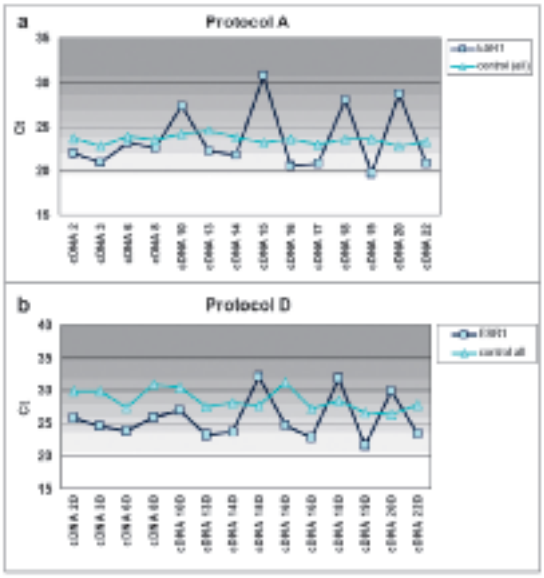

Figure 2. Total RNA isolated from snap frozen material (panel a, protocol A) and RNA isolated from FFPE according to the RNA ready (FFPE) (panel b, protocol D) was reverse transcribed using random primer. TaqMan assays were performed for the estrogen receptor (ESR1) and five control genes (ACTB, GAPDH, GUS, RPLP0, TFRC). Shown are Ct values (for ESR1 and the mean of the five control genes). PCR reactions were performed in triplicates. Low Ct values corresponded to tumours which stained positively for the oestrogen receptor (ER) by immunohistochemistry and high Ct values corresponded to ER negative tumours (tumour 15, 18, 20).

(Fig. 2). Panel a shows the data for high quality RNA and panel b the results with RNA prepared from FFPE according to the RNAready (FFPE) protocol. RNA isolated with ncLysis buffer gave similar results to RNAready (FFPE) (data not shown), but only a poor correlation was observed when ESR1 expression from RNA isolated according to the Qiagen protocol was compared to ESR1 expression levels derived from high quality RNA (data not shown).

Discussion

We document that our own procedure for RNA isolation from FFPE material outperforms the two commercial protocols for RNA isolation from such material. Special precautions are taken to avoid RNA fragmentation during enzymatic treatment of de-paraffinised sections and a separate de-modification step contributes to improved efficiency of this RNA in real time PCR. Our own protocol improves QPCR by more than 3 Ct values (this corresponds to a >10fold greater efficiency than the two commercial protocols for FFPE). We have previously documented that primers and probes coding for short amplicons result in lower and more stable Ct values than long amplicons [8]. TaqMan® Gene Expression Assays (primers and probes) were kindly provided by Applied Biosystems and amplicon sizes ranged between 55 and 122 bp for the 26 genes. QPCR reactions were performed in triplicates and each real time PCR was repeated with LNA assays (Universal Probe Library, Roche) to confirm that Ct values are independent of the assay system (data not shown).

In a next step we intend to apply our optimised protocol to a larger series of formalin-fixed, paraffin-embedded samples derived from a clinically controlled study started in 1998 (Big 01-98). Patients in this study were randomised and subjected to various protocols of tamoxifen, letrozole (an aromatase inhibitor) or sequential administration of the two drugs. At present it is known that about 84% of all patients treated with letrozole and 81% of patients treated with tamoxifen are disease-free after 5 years. Letrozole-treated patients therefore enjoy a relatively small but significant benefit over patients treated with tamoxifen. It will be of great interest to investigate whether subgroups of patients can be identified who benefit preferentially from either tamoxifen or letrozole. A new and more specific classification which helps to distinguish between patients who are likely to respond to tamoxifen or letrozole, or both, would be of great interest. Appropriate treatment with tamoxifen or letrozole could start from the very beginning and not only after tumour recurrence. The initial goal of our study, however, is to confirm that stable and robust expression measurements can also be generated from archival material collected at different time points, in different centres and with similar but not fully identical protocols. As a second step we plan to analyse more samples, in order to determine whether a molecular classification can be established which helps to discriminate between patients who respond to tamoxifen and letrozole. A method which renders such studies possible from archival FFPE material without the need for additional clinical tests would represent a major step forward.

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References

1 Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumour subclasses with clinical implications. Proc Natl Acad Sci USA 2001;98:10869-74.
2 Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci USA 2003;100:10393-8.
3 van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530-6.
4 van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999-2009.
5 Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, et al. Identification of molecular apocrine breast tumours by microarray analysis. Oncogene 2005;24:4660-71.
6 Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817-26.
7 Paik S. Molecular profiling of breast cancer. Curr Opin Obstet Gynecol 2006;18:59-63.
8 Antonov J, Goldstein DR, Oberli A, Baltzer A, Pirotta M, Fleischmann A, et al. Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization. Lab Invest 2005;85:1040-50.