Original Paper

Multiple Drug Resistance-related Messenger RNA Expression in Archival Formalin-fixed Paraffin-embedded Human Breast Tumour Tissue

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A method is described by which RNA, suitable for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, can be extracted from formalin-fixed paraffin-embedded (FFPE) tissues and subsequently used for detecting the expression of several genes. Using this technique, RNA can be extracted from specimens, quantified, reverse transcribed and regions of interest amplified and analysed within 36 h. The tissue specimens included in this study were from human breast carcinoma, investigating a range of genes associated with the development and/or maintenance of multiple drug resistance (MDR). This technique, applied to archival tissues, offers great potential for increasing our understanding of alterations in expression levels of genes associated with MDR. The method developed is also applicable to studies on expression of other genes in paraffin-embedded tissues.

Key words: formalin-fixed paraffin-embedded (FFPE), paraffin-embedded tissues (PET), reverse transcriptase-polymerase chain reaction (RT-PCR), multiple drug resistance (MDR), breast cancer
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INTRODUCTION

ALTERED EXPRESSION of many genes has been associated with multiple drug resistance (MDR) in cancer [1]. In order to establish if alterations in specific mRNA expression are responsible for the development of MDR in a given tumour type, investigation of a range of genes putatively involved is required for each tumour type. However, the feasibility of such studies is limited by the fact that a long period may be required to obtain the large number of specific specimens and clinical follow-up information necessary for such a study, and also because of the time delay between removal of the biopsy and information on the patient's clinical outcome. These limitations could be overcome if the extensive stocks of paraffin-embedded tissues (PET) (with clinical details on the patient's history, treatment and outcome) stored in pathology archives could be used for retrospective studies.

Polymerase chain reaction (PCR) amplification of DNA extracted from PET can be performed on a fairly routine basis [2, 3]. Although previous studies have indicated the potential for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of RNA recovered from PET [3], techniques reported

to date have been successful in the study of one or two genes which are generally expressed at a high level, e.g. β-actin [4], Aldolase A [5], GAPDH and HPRT [3] and β_2-microglobulin [6]. Because of their ubiquitous expression, the likelihood of extracting intact copies of such mRNAs is increased compared to that, for example, of a gene expressed only in cellular subpopulations of a tumour. To date, RNA analysis of an extensive range of gene products extracted from PET has not been documented.

The aim of this study was to develop a method which would enable RNA, suitable for RT-PCR analysis, to be extracted from formalin-fixed paraffin-embedded (FFPE) archival tissues and subsequently to investigate the possibility of using this RNA for analysis of a range of MDR associated genes.

PATIENTS AND METHODS

9 patients with breast cancer referred to St Vincent's Hospital between 1985 and 1992 were included in this retrospective study. The age of the patients ranged from 25 to 50 years. There was no family history of breast cancer in any of the 9 cases. As indicated (Table 1), some biopsies were taken before chemotherapy treatment and others were taken after treatment. For patient 1, both pre- and post-treatment biopsies were included in this study. All biopsies included had been fixed, dehydrated

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Table 1. Clinical information on patients included in this study

Patient	Age (years)	Chemotherapy	Year specimen removed	Diagnosis	RT-PCR results*
1	41	Pretreatment Post-treatment (CMF)	1987	Infiltrating ductal carcinoma Lymph nodes virtually replaced by metastatic carcinoma	(a(i)) (a(ii))
2	39	Pretreatment	1989	Intraductal and invasive ductal carcinoma; tumour cells invading lymphatic vessels	(b)
3	35	Pretreatment	1985	Intraductal carcinoma; neoplasia of lobules; no definite infiltration	(c)
4	33	Post-treatment (Tamoxifen)	1986	Fatty tissue, tiny node, mild non-specific reactive changes only	(d)
5	49	Pretreatment	1989	Infiltrating ductal carcinoma	(e)
6	25	Post-treatment (CMF)	1988	Lymph nodes involved with metastatic carcinoma from breast primary; lymphatic and vascular spread of tumour cells	(f)
7	50	Pretreatment	1992	Infiltrating carcinoma	(g)
8	50	Post-treatment (CMF plus Tamoxifen)	1986	Tumour cells in blood vessels, dermis and subcutaneous fat; no evidence of epidermal invasion	(h)
9	47	Pretreatment	1990	Infiltrating carcinoma; poorly differentiated grade III; intraductal carcinoma	(i)

CMF, Cyclophosphamide + Methotrexate + 5-Fluorouracil. * Corresponding RT-PCR results, as in Figure 3.

and paraffin-embedded according to the following protocol. All steps prior to infiltration with wax were performed at 35°C. Biopsies were fixed in 10% formalin (made up in tap water) for a period of 5 h (after 1 h, the formalin was changed and the tissues were fixed in fresh 10% formalin for a further 4 h). The tissues were then dehydrated in a series of graded ethanol solutions, i.e. 70% ethanol for 40 min; 94% ethanol for 40 min and 100% ethanol for 2 h (three successive changes; 40 min each). The biopsies were then processed through chloroform (three successive changes; 40 min each), and following this they were infiltrated with wax, by placing them in melted wax at 60°C for a 4 h period (four successive changes; 1 h each). The tissues, embedded in wax, were then allowed to cool at room temperature.

RNA extraction

Sections of tissue (totalling 100 μm in thickness) were cut from the paraffin block and placed in autoclaved microcentrifuge tubes; 200 μl of octane (BDH) was added to this and placed in a 60°C water-bath for 10 min to remove the paraffin. The contents of the microcentrifuge tubes were spun for 10 min at 4°C, at maximum speed in a microcentrifuge (13 000g), resulting in the tissue pelleting at the bottom of the tube and the paraffin forming a wax layer at the top. The wax was removed using a pasteur pipette and the remaining tissue pellet was washed with 70% ethanol. After re-centrifuging at maximum speed in a micro-centrifuge at 4°C and removing the ethanol, the tissue

pellet was resuspended in 200 μl of digestion buffer consisting of 25 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulphate (SDS), 10 mM Tris–HCl, pH 8.0, to which proteinase K (Sigma) was added immediately prior to its use, to result in a concentration of 0.1 μg/μl. The tube was then wrapped in parafilm and incubated at 52°C with constant agitation. After 18 h, a phenol/chloroform/isoamyl alcohol (25:24:1) extraction, followed by chloroform/isoamyl alcohol (24:1) extraction was performed to remove protein degradation products. The nucleic acids were precipitated out of solution using two volumes of cold absolute ethanol and a 1/10 volume of 3 M sodium acetate (pH 5.2) at −80°C for a period of 30 min. The nucleic acids were pelleted by centrifuging and the supernatant discarded. To remove the DNA, the pellet was treated with an RNase-free DNase I enzyme at 37°C, for 30 min. RNA was phenol/chloroform/isoamyl alcohol extracted and precipitated using ethanol and sodium acetate, as above. After centrifuging at 4°C, the pellet was resuspended in diethyl pyrocarbonate-treated water and the RNA concentration and purity were quantified spectrophotometrically by measuring its absorbance at 260 nm and 280 nm (Table 2). The results from this analysis were expressed as μg RNA/100 μm section of FFPE tissue. It must be taken into consideration, however, that the surface areas of all blocks were not equal. This may explain, in part, differences in quantity of RNA obtained. For this reason, a constant amount of RNA (0.5 μg) was used in all reverse transcriptase reactions. The $A_{260/280}$ ratios obtained indicated that the RNA extracted in

Table 2. RNA yields and $A_{260/280}$ ratios of RNA extracted from FFPE breast tumour tissue

Tumour block	1st RNA extraction		2nd RNA extraction	
	RNA yield (μg/100 μm tissue)	$A_{260/280}$	RNA yield (μg/100 μm tissue)	$A_{260/280}$
a(i)	13.2	1.82	14.1	1.79
a(ii)	10.2	1.88	10.9	1.92
b	17.0	1.71	17.2	1.75
c	12.4	1.73	13.2	1.75
d	9.42	2.01	8.88	1.98
e	11.1	1.73	11.6	1.78
f	17.5	1.91	18.1	1.94
g	10.4	1.88	9.01	1.90
h	9.60	1.92	10.5	1.94
i	18.0	1.85	17.1	1.86

Labelling of blocks correspond with RT-PCR results (see Table 1 and Figure 3).

all cases was well within the acceptable range for RT-PCR. The $A_{260/280}$ ratio of pure RNA is approximately 2 [7]. However, RNA preparations with absorbance ratios greater than 1.4 have been shown to be adequate for RT-PCR studies [8].

Reverse transcriptase (RT) reaction

Reverse transcriptase (RT) reactions were performed using oligo (dT) primers or random primers independently, or a combination of oligo (dT) and random primers, as follows (all reactions were kept on ice unless otherwise indicated):

Primers [1 μl oligo $(dT)_{12-18}$ primers (1 μg/μl) (Promega); 1 μl random hexadeoxyribonucleotide primers (0.5 μg/μl) (Promega); or 0.5 μl of each of these in combination], 0.5 μl of extracted RNA (1 μg/μl) and 3 μl of water were placed in microcentrifuge tubes, mixed, heated to 70°C for 10 min, and then chilled on ice. To this, 4 μl of a 5 × buffer (consisting of 250 mM Tris–HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 μl of DTT (100 mM), 1 μl of RNasin (40 U/μl) (Promega), 1 μl of dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP), 6 μl of water and 1 μl of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40 000 U/μl) (Gibco) were added. This mixture was incubated at 37°C for 1 h to allow the MMLV-RT enzyme catalyse the formation of cDNA on the mRNA template. The enzyme was then inactivated and the RNA and cDNA strands separated by heating to 95°C for 2 min.

Once the cDNA copy had been created using the mRNA template, the PCR reaction was conducted immediately, as outlined below. Alternatively, the cDNA was stored at −20°C, until required for analysis.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was set up as described previously [9] with one adjustment. Two sets of primers were used in all reactions to result in amplification of an endogenous control [β-actin (383 bases)] and a specific target gene of interest [MDR 1 (157 bases), MDR 3 (321 bases), MRP (203 bases), GST π (270 bases), Topoisomerase I (180 bases), Topoisomerase II (216 bases), Topoisomerase IIα (139 bases) or Topoisomerase IIβ (118 bases), independently (Table 3)]. However, in this study, only half the concentration (125 ng/μl) of each of the endogenous control primers was added. Following an initial

denaturation step of 94°C for 1.5 min, 30 cycles of PCR amplification were performed—each cycle consisting of a denaturing step of 94°C for 1.5 min, annealing at 54°C for 1 min, and extending at 72°C for 3 min. The amplified fragments were visualised by gel electrophoresis and ethidium bromide staining.

RESULTS

Initial attempts to extract RNA and amplify the resulting cDNA formed using oligo (dT) primers in the RT reaction resulted in amplification of β-actin cDNA only. An example of this is shown in Figure 1. To establish if the inability to amplify the target gene products successfully was due to inefficient extraction of RNA or if it was at the level of the RT reaction, further RT reactions were set up using random primers or a combination of oligo (dT) and random primers. Using random primers independently, a band was frequently produced for β-actin, plus several non-specific bands (Figure 2). Various combinations of these primer types were used; the most successful combination being a 1:1 ratio of oligo (dT) primers:random primers.

Results of the analysis using a 1:1 ratio of oligo (dT) primers:random primers are presented in Figure 3(a–i). Successful amplification of the internal control, β-actin, and many of the other mRNA transcripts including MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ, was achieved to a greater or lesser extent. Table 4 shows the genes which were amplified in each case.

Samples of blocks from six patients (Figure 3a–e) were analysed twice. This involved sectioning the paraffin blocks twice, extracting RNA and repeating the RT and PCR reactions. The two analyses differed only in so far as in the initial study (data not shown), equal concentrations of target and exogenous control (β-actin) primers were used. Although the results produced in the initial run were similar to those shown in Figure 3(a–e), amplification of MDR 3 was not apparent. Because of the intensity of the β-actin band and the relative closeness in size of the MDR 3 band (321 bp) to the β-actin band (383 bp), it may be that the β-actin band masked the MDR 3 band. This was supported by the fact that in subsequent studies, where the concentration of β-actin primers was decreased, a band representing MDR 3 was visible in some specimens, i.e. Figure 3 (a(ii), b, d, g and i).

Low molecular weight bands appeared in some lanes (e.g. Figure 3; patients B and C) but were unlikely to represent primer aggregates as they did not appear in all patients samples for particular primers. While the origin of these bands is unknown, they do not affect the interpretation of the data.

DISCUSSION

A method is reported here which allows RNA to be extracted from FFPE breast carcinoma biopsies and subsequently used for RT-PCR analysis of a range of MDR associated gene products. Successful amplification of MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ (as well as the endogenous control, β-actin) was achieved with FFPEs stored for between 3 and 10 years. The extraction protocol, similar to those previously reported [5, 10] is rapid (requires less than 24 h) and because of its ease, can be performed on many specimens simultaneously. This report, unlike previous studies of formalin-fixed tissues [3], supports the possibility of successfully performing extensive retrospective studies of many gene products using FFPE archival specimens.

Table 3. Primers to amplify cDNA formed by reverse transcriptase on mRNA templates of MDR related factors

Gene	Primer sequences	Amplified mRNA sequence length (bases)	Corresponding DNA length (bases)
MDR 1*	5' CCC ATC ATT GCA ATA GCA GG 3' 5' GTT CAA ACT TCT GCT CCT GA 3'	157	1257
MDR 3	5' ATT AGC AGT TGT TCC AAT TAT TGC 3' 5' TGT CCA TTC AGA ATG AGA TAT GCA 3'	321	N.K.
MRP	5' GTA CAT TAA CAT GAT CTG GTC 3' 5' CGT TCA TCA GCT TGA TCC GAT 3'	203	N.K.
GST π	5' ATG CTG CTG GCA GAT CAG 3' 5' GTA GAT GAG GGA GAT GTA TTT GCA 3'	270	749
Topoisomerase I	5' AGA CGA ATC ATG CCC GAG GAT ATA ATC ATC 3' 5' TCG TGA ACT AGG GTT AAG CAT GAT GTA 3'	180	2280
Topoisomerase II	5' AAC TTT GGC TGT TTC AGG 3' 5' ATC ATT ATC TTC CCA TAA CGA AGC GT 3'	216	N.K.
Topoisomerase IIα	5' ATG CTA GTC CAC CTA AGA CCA 3' 5' TGT GTA GCA GGA GGG CTT GAA GAC AG 3'	139	N.K.
Topoisomerase IIβ	5' TCC TTC ATA TTC TCA GAA GTC AGA AGA TGA 3' 5' ACT TGG AAC TTT ATC TGT CTG TTT CAG A 3'	118	N.K.
β-actin*	5' GAA ATC GTG CGT GAC ATT AAG GAG AAG CT 3' 5' TCA GGA GGA GCA ATG ATC TTG A 3'	383	590

* These primers were not selected in this laboratory, but were published by another research group [12]. N.K., Not known (as gene sequence not available).
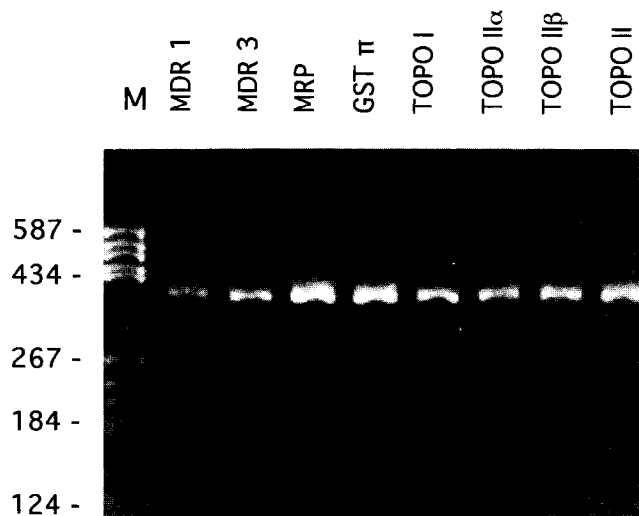

Figure 1. Priming with oligo (dT) primers in the RT reaction and amplifying β-actin (as endogenous control) with MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase IIα, Topoisomerase IIβ and Topoisomerase II, respectively. M indicates molecular weight marker standards (bases). TOPO, Topoisomerase.
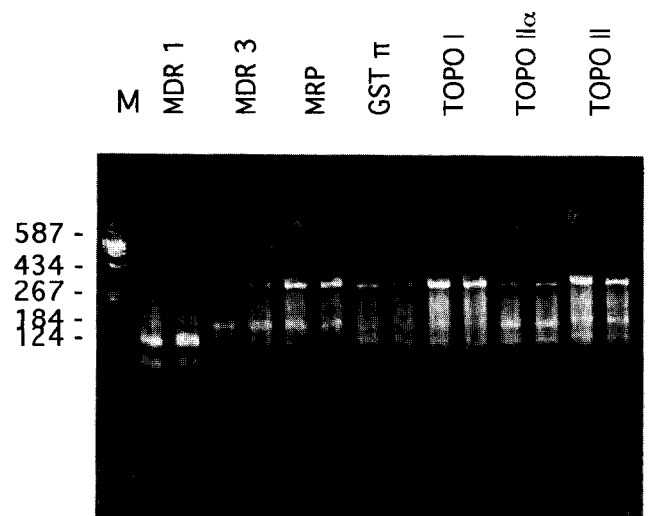

Figure 2. Priming with random primers in the RT reaction and amplifying β-actin (as endogenous control) with MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase IIα and Topoisomerase II, respectively. All reactions were performed in duplicate. M indicates molecular weight marker standards (bases). TOPO, Topoisomerase.

Previous studies have reported that increasing numbers of PCR cycles are required when amplifying increasing lengths of cDNA targets—40 cycles was sufficient when amplifying a 150 bp product, whereas 50 cycles plus additional Taq DNA polymerase enzyme were necessary for successful amplification of a 204 bp target [11]. In this study, 30 cycles of PCR (without additional enzyme) was sufficient for the amplification of fragments ranging from 118 bp to 383 bp (in the case of the endogenous control) and 270 bp (GST π, the largest target sequence under analysis). This not only saves time and resources,

but more importantly, avoids the likelihood of amplifying rare contaminants.

Some previously reported methods for extracting RNA from PET have incorporated an extra step involving guanidinium salts and have achieved only marginal success when this step is eliminated [6]. It was reported that if this step were omitted, even doubling the amount of DNase enzyme used did not remove all DNA contaminating the RNA [5]. The inclusion of this step, however, complicates the procedure as it involves
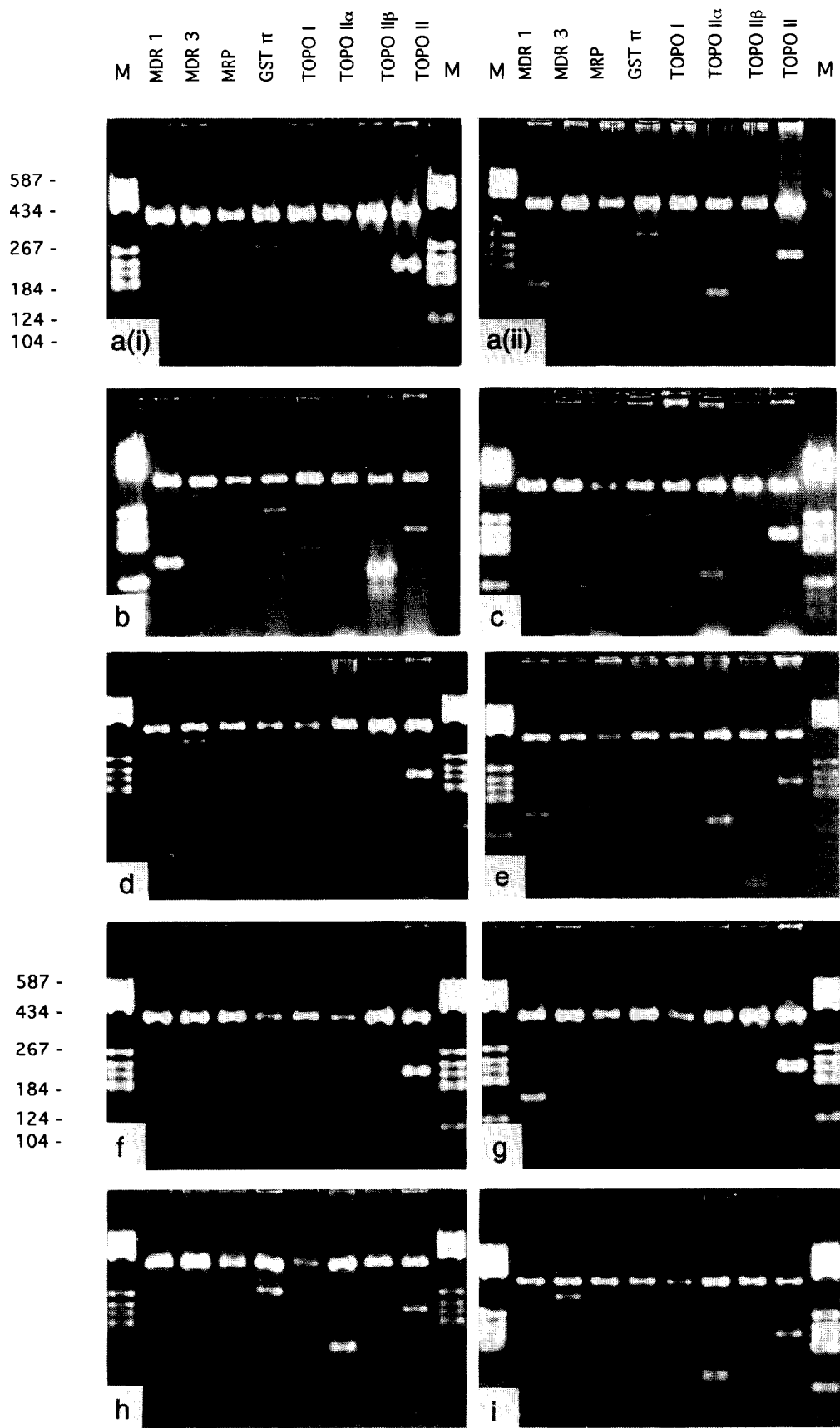
Figure 3. Priming with a 1:1 ratio of oligo (dT):random primers in the RT reaction and amplifying *β-actin* (as endogenous control) with *MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase IIα, Topoisomerase IIβ* and *Topoisomerase II*, respectively. Illustrations (a)–(i) correspond to patients (1–9), as in Table 1. M indicates molecular weight marker standards (bases). TOPO, Topoisomerase.

Table 4. Amplified genes from breast cancer specimens

Patient		MDR 1	MDR 3	MRP	GST π	TOPOI	TOPOIIα	TOPOIIβ	TOPOII
1	Pre	+	−	+	+	+	+	+	+
	Post	+	+	+	+	+	+	+	+
2		+	+	+	+	+	−	+	+
3		−	−	+	+	+	+	+	+
4		±	+	±	±	±	+	±	+
5		+	−	±	−	±	+	−	+
6		+	−	−	+	+	+	+	+
7		+	+	+	−	+	+	+	+
8		+	−	+	+	−	+	−	+
9		−	+	−	−	−	+	−	+

+, gene expression, ±, weak expression, −, no expression.

including an extra phenol/chloroform/isoamyl alcohol extraction and precipitation step. These additional steps not only increase the time required for the extraction procedure by up to 24 h [5], but also increase the likelihood of losing RNA by increasing the handling involved in the technique. In this study, the guanidinium salt step was found to be unnecessary as all DNA was eliminated by treatment with DNase enzyme. This is indicated by the fact that the primers used to amplify the cDNA [which were selected to span introns and so result in a larger band if amplifying DNA (Table 3)] produced only the bands expected from cDNA amplification.

From a range of four potential endogenous controls (β-actin, β_2-microglobulin, GAPDH and esterase D) selected for inclusion in all RT-PCR reactions, β-actin was chosen. Although β-actin has some limitations as a control [3, 9], it was found to be consistently expressed in sensitive and MDR cells and unaffected during, or following, exposure to the anticancer drugs doxorubicin, vincristine and VP-16 (data not shown).

In conclusion, although the number of specimens included in this study was too small to make any correlation between the results produced and the patients' clinical records, the results presented indicate the potential use of archival tissues for extensive retrospective studies detecting MDR associated genes. Furthermore, to ensure that the absence of a signal, whenever it occurs, is real and not due to selective degradation of certain mRNAs, further studies are necessary to establish how results from FFPE tissues and fresh tissues relate quantitatively.

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