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# Detection of Specific mRNAs in Culture Medium Conditioned by Human Tumour Cells: Potential for New Class of Cancer Biomarkers in Serum

LORRAINE O'DRISCOLL*, ELAINE KENNY*, MAIDER PEREZ DE VILLARREAL and MARTIN CLYNES

*National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland*

**Abstract.** *Background: This study aimed to develop and optimise procedures to enable us to establish, in a well-controlled environment, if cancer cells routinely secrete gene transcripts potentially suitable for monitoring as biomarkers. Materials and Methods: Aliquots of the conditioned media (CM) exposed to cancer cells (including breast, lung and nasal cancer cell lines) were removed at intervals of 24 hours over a 96-hour time period and were passed through 0.45 μm or 0.22 μm filters, to remove cellular material. Methods for subsequent RNA extraction (from CM and cells) were investigated. RT-PCR was performed for a number of mRNAs, including mdr-1, mrp-1, CK-19, HnRNP B1, GST-π, topoisomerase II, bcl-2 and β-actin. Results: Gene transcripts, amplifiable by RT-PCR, were detected in conditioned media from all human cancer cell lines studied. This RNA did not result from the presence of cells in the conditioned media and, as expected, was absent from control medium not exposed to tumour cells. Conclusion: The results from this study indicate that gene transcripts may be secreted from human cancer cells and are detectable in the subsequent cell-free media. The methods developed and optimised here may be suitable for analysis of mRNAs as biomarkers in serum/plasma from cancer patients.*

Biomarkers, defined as biochemical features that may be used to measure the progression of disease or the effects of treatment, have great potential for cancer detection and monitoring. While much of this type of analysis has been done using tumour biopsies (1, 2), the limitations of this approach include the invasive procedures necessary to obtain suitable specimens and the fact that this only allows analysis of gene expression at one particular time point in the history of a cancer.

Effective, clinically useful, biomarkers should be accurately detectable in a readily accessible body fluid, such as serum, saliva or urine. Such analysis would involve the use of minimally/non-invasive procedures to obtain specimens for study and should allow on-going/sequential monitoring of the course of the disease (progression, response to therapy, *etc.*) over time. A small number of recent studies (3-10) have indicated that it is possible to amplify extracellular mRNA from the serum and/or plasma of cancer patients (see Table I), supporting the potential of this route of analysis. The limitations of many of these studies, however, have been the small numbers of serum/plasma specimens included; the limited range of gene transcripts analysed; the fact that in some cases serum/plasma was not filtered or ultra-centrifuged, raising the possibility that cells circulating in the bloodstream may be included in the RNA isolations; and the fact that reproducibility of techniques, in general, could not be addressed.

The presence of RNA in the extracellular medium of eukaryotic cell cultures has also been described. In 1978, Stroun *et al.* (11) reported the presence of an RNA form in a nucleoprotein complex spontaneously released from human blood lymphocyte and frog auricle cultured cell systems, following media centrifugation (for lymphocytes: 20,000rpm for 1 hour, then 50,000 rpm for 2 hours; for frog auricles: 12,000 rpm for 20 minutes, then 50,000 rpm for 2 hours). The resulting isolated RNA was described as not being tRNA and being more highly methylated than ribosomal 28S, 18S and 4-5S RNA. More recently, using a fluorescence-based assay, the detection of cell-associated and cell-free (following 1.2 μm filtration) RNA in growth/culture medium from HeLa (human cervical carcinoma cell line) and A431 (human squamous cell line)

*contributed equally to this work.

*Correspondence to:* Lorraine O'Driscoll, National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland. Tel: +353-1-7005700 / 5402, Fax: +353-1-7005484, e-mail: Lorraine.ODriscoll@dcu.ie

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cells, represented by 100-200 nucleotide long fragments, corresponding to 2.5-5S RNA, has been reported (12). However, the presence of mRNA(s) secreted from cultured human cancer cells and detectable in cell-free culture medium has not been described in any of these studies.

The aims of the study presented here were to: (i) optimise procedures to enable investigation of the presence of secreted mRNA products, representing a broad range of gene transcripts, in cell-free medium from a number of human cancer cell lines; (ii) evaluate the reproducibility of procedures after optimisation; and (iii) determine if any mRNAs detected were likely to be representative of mRNA fragments or full-length transcripts.

## Materials and Methods

*Cell line culture.* DLKP human lung carcinoma cells (13) were grown in DMEM/ Hams F12 with 5% FCS at 37˚C. RPMI-2650 human nasal carcinoma selected variants, RPMI-2650Ml (RPMI-Ml) and RPMI-2650Tx (RPMI-Tx) (14) were cultured in MEM containing 5% FCS, 2mM L-glutamine, 1% sodium pyruvate and 1% non-essential amino acids (NEAA), at 37˚C and 5% $CO_2$. MCF-7 human breast carcinoma cells were grown under similar conditions, but without 1% NEAA. MDA-MB-435S-F (MDA-F) human breast carcinoma (although recent microarray studies have suggested that it has a melanoma-like gene expression profile (15)) and its selected variants MDA-MB-435-F/ADR-10p10p SI (MDA-F-ADR-SI) and MDA-MB-435-F/Taxol-10p4p SI (MDA-F-TAX-SI) (16) were cultured in RPMI medium supplemented with 2mM L-glutamine and 10% FCS at 37˚C and 5% $CO_2$. BT-474 were cultured in DMEM containing 1% sodium pyruvate and 10% FCS. Routine sterility checks, including screening for *Mycoplasma*, indicated that all cells were clear of contamination.

*Conditioned media isolation and filtration.* All cell lines were seeded in T75 $cm^2$ cell culture flasks at a density of $2X10^6$ cells per flask with 8 ml of culture medium. Aliquots (1 ml) of medium were removed 24, 48, 72 and 96 hours post-seeding. After passing through a 0.45 μm or a 0.22 μm filter (Millex), 100 μl and 250 μl aliquots of filtered conditioned medium were added to TriReagent™ (Sigma; Poole, England) (750 μl TriReagent + 150 μl RNAse-free DEPC-treated or 750 μl TriReagent™, respectively) and incubated for 5-10 min on ice prior to snap-freezing/freezing at -80˚C, to ensure complete dissociation of nucleoprotein complexes. These were stored at -80˚C until required for analysis. Cultured cells were trypsinised, counted and centrifuged (900rpm for 5 min) to form a pellet. Following washing in PBS (X2 times), cell pellets were re-suspended in 1ml TriReagent™ and were, subsequently, incubated and stored as above.

*RNA isolation and quantification.* Total RNA was isolated from conditioned media and pelleted cells by extracting with TriReagent™ with modifications/"optimisation" of the manufacturer's instructions, developed during the course of this study. In brief, 0.2 ml of chloroform was added to each sample and this was shaken vigorously for 15 sec, followed by incubation at room temperature for 15 min This was then centrifuged at 12,000rpm for 15 min. at 4˚C, and the aqueous phase containing RNA (upper layer) was removed and transferred into a fresh RNAse-free 1.5 ml Eppendorf tube. Isopropanol (0.5 ml) and glycogen (final concentration 120 μg/ml) were added, incubated at room temperature for 5-10 min and stored at -20˚C over-night, to ensure maximum RNA precipitation. Eppendorf tubes were then centrifuged at 12,000rpm for 30 min at 4˚C to pellet the precipitated RNA. Taking care not to disturb the RNA pellet, the supernatant was removed and the pellet was subsequently washed by the addition of 750 μl of 75% ethanol and vortexing. Following centrifugation at 7,500 rpm for 5 min at 4˚C, the supernatant was removed (this wash step was repeated). The RNA pellet was then allowed to air-dry for 5-10 min, and subsequently was re-suspended in 15-20 μl of DEPC-treated $H_2O$. To facilitate dissolution, the samples were mixed by repeated pipetting and incubated at 55-60˚C for 10-15 min. To assess the quantity and quality of extracted RNA, an aliquot of each was read at 260 nm, 280 nm and 230 nm using a Nanodrop ND-1000 (Labtech International).

*RT-PCR analysis.* First-strand cDNA was synthesised from 1 μg RNA (from cultured cells) and 4 μl RNA (from conditioned media) using oligo dT primers (Sigma). Five μl cDNA from CM or 2.5 μl cDNA from cells was then amplified in a 25 μl PCR reaction solution containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l deoxynucleotide triphosphates, 20 μmol/l oligonucleotide primers and 2.5 U *Taq* polymerase enzyme (Sigma). Forward and reverse primers for all gene transcripts analysed (including *mdr-1, mrp-1, CK-19, HnRNP B1, GST-π, topoisomerase II, bcl-2* and *β-actin*) are detailed in Table II. As indicated in Figure 1, to investigate if any secreted mRNAs detected were likely to be full-length transcripts or fragmented products, the PCR primers were designed in such a way as to amplify regions close to the 3' end (*e.g. bcl-2*), the 5' region (*e.g. GST π* and *HnRNP B1*) or centrally (*e.g. topoisomerase II, CK-19*). Primers amplifying 4 different regions along the length of the β-actin transcript were evaluated. The PCR cycle used was as follows: 95˚C for 2 min; 30-45 cycles of 95˚C for 30 sec, 54˚C for 30 sec, 72˚C for 45 sec; completion step of 72˚C for 10 min. Amplified products were subsequently separated on a 2% agarose gel to determine the presence or absence of expression.

## Results

*Presence of extracellular mRNA in conditioned medium.* To investigate if detectable mRNAs are secreted from human cancer cells in culture, this study evaluated media conditioned by exposure to a range of cell lines, established from carcinomas of lung (DLKP), nasal (RPMI-2650Ml (RPMI-Ml) and RPMI-2650Tx (RPMI-Tx)) and breast (MCF-7, MDA-MB-435S-F (MDA-F), MDA-MB-435-F/ADR-10p10p SI (MDA-F-ADR-SI), MDA-MB-435-F/Taxol-10p4p SI (MDA-F-TAX-SI) and BT-474). After passing through 0.45 μm or 0.22 μm filters, RNA was isolated and quantified as described in Materials & Methods. Nanodrop MD-1000 readings indicated approx. 2.4-5.6 ng/μl (*i.e.* RNA/μl CM) to be achieved.

Many of the gene products investigated were amplified, following 45 cycles of PCR (Figure 2 shows *mdr-1, HnRNP B1*, and *β-actin*, as example results). As illustrated in Figure 1(a), the regions amplified for these gene products are 5', in the case of *HnRNP B1* and *β-actin*; and between central and 3' for *mdr-1*, suggesting that the transcripts secreted are full

Table I. *Example studies of circulating mRNAs as Tumour Markers in Serum/Plasma.*

| mRNA(s) Studied | Cancer type | Serum/Plasma filtration or ultracentrifugation (if any) | PCR cycle No. | No. of positive cases /total number | | Reference |
|---|---|---|---|---|---|---|
| Tyrosinase[1] | malignant melanoma (6)<br>normal (20) | 0.45 μm | nested:55-70 cycles | 4/6<br>0/20 | | (3) |
| | | | | *Serum* | *Plasma* | |
| Tyrosinase[1&2] | malignant melanoma (10) | unknown | unknown | 3/10 | 5/10 | (4) |
| PBDG | | | | 7/10 | 8/10 | |
| gp100 | | | | 0/10 | 0/10 | |
| MART-1 | | | | 0/10 | 0/10 | |
| 5T4[1] | breast cancer (5)<br>NSCLC (14)<br>normal (25) | not filtered | nested:60 cycles | 2/5<br>6/14<br>3/25 | | (5) |
| | | | | *hTR* | *hTERT* | |
| Telomerase *(hRT)*[1] & *hTERT* | breast cancer (18)<br>benign breast disease (2)<br>normal (21) | not filtered | 50 cycles | 5/18<br>0/2<br>0/21 | 4/16<br>0/2<br>0/21 | (6) |
| CK-19[1] | lung (18) | not filtered | 45 cycles | 15/18 | | (7) |
| PGP 9.5 | | | | 1/18 | | |
| Her2/neu | | | | 7/18 | | |
| hnRNP-B1 | | | | 14/18 | | |
| TTF-1 | | | | 0/18 | | |
| MAGE-2 | | | | 0/18 | | |
| Mammaglobin[2] | breast (10)<br>normal (16) | not filtered | nested:60 cycles | 6/10<br>6/16 | | (8) |
| | | | | *Mg* | *CK-19* | |
| Mammaglobin (Mg)[2] | breast (45) | not filtered | nested:80-83 cycles | 27/45 | 22/45 | (9) |
| CK-19 | normal (25) | | | 3/25 | 5/25 | |
| GAPDH[2] | HCC (16)<br>normal (27) | 0.5μm<br>0.45μm<br>0.22mm<br>ultracentrifugation<br>unfiltered | real-time PCR | detected in all cases; levels detected reduced with filtration & ultracentrifugation | | (10) |

Note: [1] = serum analysed; [2] = plasma analysed; normal = blood specimens were taken from volunteers who did not have cancer; HCC = hepatocellular carcinoma; ultracentrifugation = 99,960g for 120 min. at 4˚C.

length; not fragmented products. This is further supported by the successful amplification of *β-actin* at all 4 regions identified by the PCR primers selected (Figure 1(b)).

*Identifying suitable time-point for conditioned media collection and analysis.* Conditioned media (CM) samples were collected at 4 time-points after cell seeding, to identify a suitable time-point at which amplifiable mRNA could be routinely isolated for analysis. As indicated in Figure 3(a), in the case of relatively highly expressed gene transcripts, such as *β-actin*, mRNA could be isolated and amplified at all time points evaluated. However, for gene transcripts where levels expressed by the cell populations were lower (*e.g. CK-19*; Figure 3(b)), amplified product was undetected after 24 hours, but was present when analysed after 48 hours, or more, of culture. To ensure that all CM analysed was from healthy, proliferating, cells, all subsequent analysis was performed using 48 hours CM.

Table II. *Primers to amplify cDNA formed by reverse transcription of mRNA templates.*

| cDNA | Primer sequences | Amplified product (bp) |
|---|---|---|
| *bcl-2* | (F): 5' TCATGTGTGTGGAGAGCGTCAA 3' | |
| | (R): 5' CTACTGCTTTAGTGAACCTTTTGC 3' | 306 |
| *mrp-1* | (F): 5' GTACATTAACATGATCTGGTC 3' | |
| | (R): 5' CGTTCATCAGCTTGATCCGAT 3' | 203 |
| *mdr-1* | (F): 5' GTTCAAACTTCTGCTCCTGA 3' | |
| | (R): 5' CCCATCATTGCAATAGCAGG 3' | 157 |
| *GST-π* | (F): 5' ATGCTGCTGGCAGATCAG 3' | |
| | (R): 5' GTAGATGAGGGAGATGTATTTGCA 3' | 270 |
| *Topo II* | (F): 5' AACTTTGGCTGTTTCAGG 3' | |
| | (R): 5' ATCATTATCTTCCCATAACGAAGCGT 3' | 216 |
| *HnRNP B1* | (F): 5' GGAGAGGAAAAAGAGAGAAAAG 3' | |
| | (R): 5' TTGATCTTTTGCTTGCAGGA 3' | 155 |
| *CK-19* | (F): 5' GCGGGACAAGATTCTTGGTG 3' | |
| | (R): 5' CTTCAGGCCTTCGATCTGCAT 3' | 214 |
| *β-actin* | (F): 5' TGGACATCCGCAAAGACCTGTAC 3' | |
| | (R): 5' TCAGGAGGAGCAATGATCTTGA 3' | 142 |
| *β-actin* | (F): 5' CTTTGCCGATCCGCCGCCCGTC 3' | |
| | (R): 5' CATCACGCCCTGGTGCCTGGGG 3' | 171 |
| *β-actin* | (F): 5' ACGGCTCCGGCATGTGCAAG 3' | |
| | (R): 5' TGACGATGCCGTGCTCGATG 3' | 196 |
| *β-actin* | (F): 5' GAAATCGTGCGTGACATTA AGGAGAAGCT 3' | |
| | (R): 5' TCAGGAGGAGCAATGATCTTGA 3' | 383 |

Note: β-actin primers were designed to amplify a range of products (142, 171, 196 and 383) at a range of locations (5', central, or 3') along the length of the transcript. (All primers except for *cytokeratin 19* (*CK-19*), *HnRNP B1* (R), and *β-actin* (196 bp) were selected in our laboratory; *CK-19, HnRNP B1* (R), and *β-actin* (196 bp) (7)). [*Topo* = *Topoisomerase*].

*Determining the reproducible minimum detection level.* To optimise the procedures used in this study for routine analyses of secreted mRNAs in CM, volumes (100 μl and 250 μl) that could easily be used with TriReagent™ isolation procedures were assessed. As exemplified in Figure 2, in many cases, amplified products were achieved following RNA isolation from both the 100 μl and 250 μl CM samples; in some cases (*e.g. mdr-1*), slightly higher levels of expression were detected from the 250 μl, compared to the 100 μl, samples. To further investigate the minimum detection level of the system, duplicate 100 μl and 250 μl samples of 48 hour CM were isolated and amplified, in parallel, for a range of gene transcripts. As indicated in Figure 4, for the highly expressed *β-actin* mRNA, RNA isolation and amplification from both 100 μl and 250 μl CM aliquots gave reproducible results, whereas for other transcripts (*CK-19* and *HnRNP B1* illustrated), although the duplicate 250 μl CM samples produced in similar results, amplified product was achieved for only one of the duplicate 100 μl samples. This suggests that analysis of 100 μl aliquots is possibly at the limits of detection for the procedures developed and optimised here. For this reason, 250 μl aliquots of CM were routinely evaluated in subsequent studies (unless otherwise indicated).

*Analysis of gene transcripts in RNA isolated from cell lines and conditioned media.* As expected, the ubiquitously expressed "house-keeping" gene, *β-actin*, was detected following 30 cycles of PCR in all cell line extracts analysed. For CM studies, however, 45 cycles of PCR were found to be necessary and under these conditions β-actin gene transcripts were detected following 0.45 μm or 0.22 μm filtration of media conditioned (for 48 hours) by these cells. Similarly, *CK-19* and *HnRNP B1* were detected in RNA isolated from all cell lines and their corresponding CM (see Figure 5: *β-actin* as example results). For all RNA extracted from cells, *β-actin* was successfully co-amplified in association with all other genes of interest. However, although *β-actin* was routinely amplified alone in all CM, attempts to co-amplify *β-actin* inhibited detection of other gene products of interest. For this reason, β-actin was generally not co-amplified with other cDNAs, when analysing CM.

To investigate if specific mRNAs were detectable only in CM from cell lines expressing that product (*i.e.* to ensure results did not include false positives), CM from cell lines expressing *versus* not expressing a particular gene transcript was analysed. As indicated in Figure 6(a), *mdr-1* mRNA was expressed by RPMI-Tx (as well as RPMI-Ml, MDA-F-Tax SI and, to a lesser extent, BT-474 and MDA-F), but not detected in DLKP (MCF-7 or MDA-F-ADR SI) cells. Analysis of RPMI-Tx CM (Figure 6(b)) showed *mdr-1* mRNA to be secreted by *mdr-1*-expressing cells following both 0.45 μm and 0.22 μm filtration. *mdr-1* mRNA, as expected, was undetected in CM from DLKP cells. As shown in Figure 7, GST π was detected in all cell lines studied, except MCF-7 and BT474. However, although GST π was undetected in all media conditioned by BT474 cells, a weak band resulted from analysis of the MCF-7 0.45 μm CM filtrate.

*Topoisomerase II* (Figure 8) mRNA was expressed by all cell lines studied and was amplified following isolation from 0.45 μm filtered CM. However, with the exception of medium conditioned by the nasal carcinoma cell line

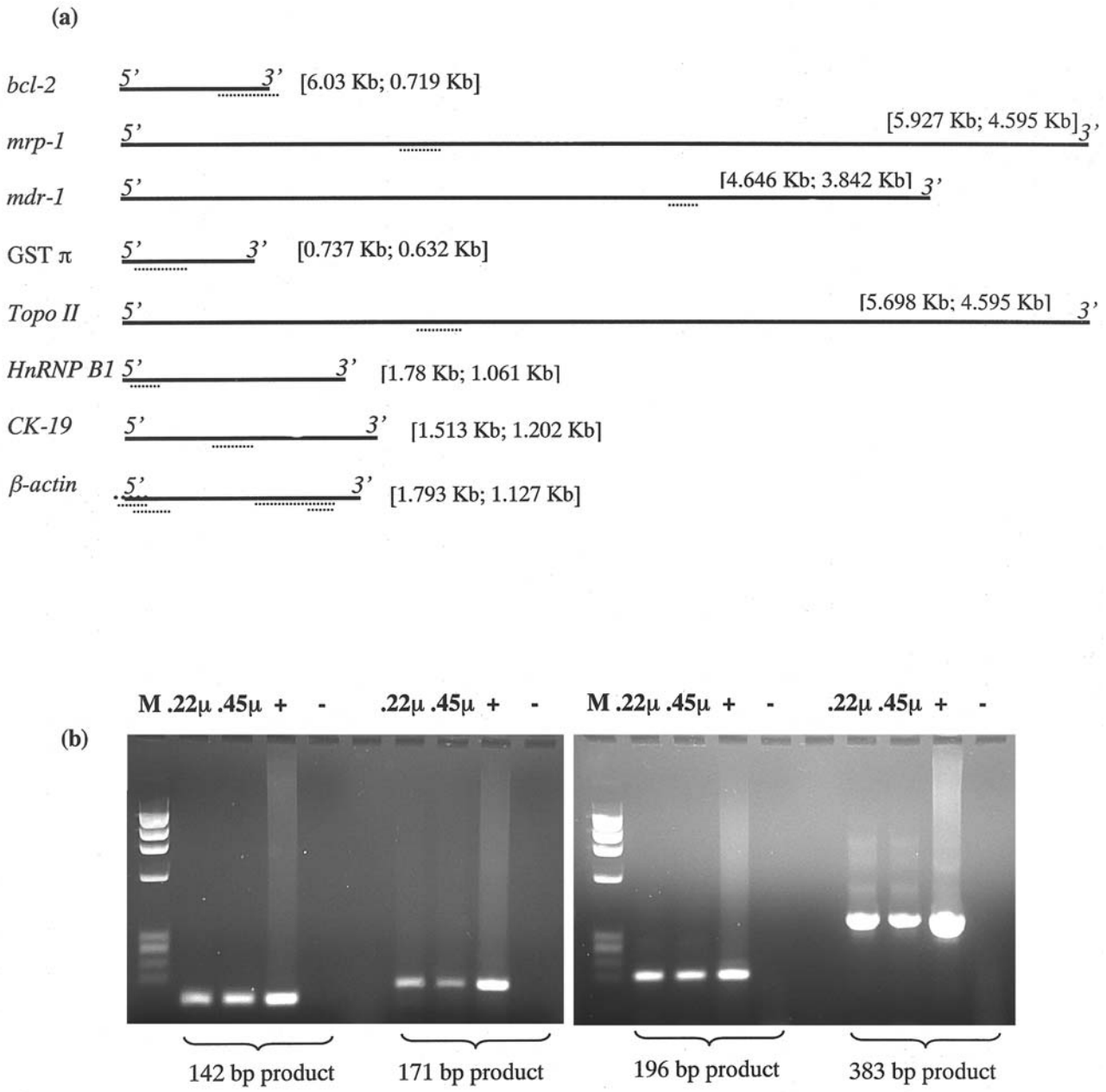


Figure 1. *(a) 5', central or 3' location of the regions selected for amplification (– – – – –) along the coding regions (————) of transcripts analysed, using primers described in Table II. Information on size of full gene transcripts, as well as size of coding regions only – as illustrated here – are included for all gene products [ ] analysed. (b) Example results showing successful amplification of β-actin at all 4 regions analysed along its cDNA length, following RNA isolation from 0.45 μm and 0.22 μm filtered CM (+ = RNA isolated from DLKP cells, as positive control; -RT = RT reaction with $H_2O$ instead of RNA as control).*

variant, RPMI-Tx, and the breast cancer cell lines, MDA-F-TAX-SI (weak band) and BT474, *topoisomerase II* was generally undetected in 0.22 μm filtered CM. *mrp-1* was expressed by all cell lines (Figure 9(a)). It was detected in 5 of the 0.45 μm filtered CM, but in only 2 of the subsequent 0.22 μm filtrates analysed (Figure 9(b+c)).

bcl-2 was expressed (*albeit* at relatively low levels) in all of the 8 cell lines analysed (see Figure 10). However, this gene transcript was apparently not secreted by these cells *i.e.* with the exception of a very weak band resulting following amplification of 0.45 μm filtered RPMI-Tx CM, *bcl-2* mRNA was undetected in all CM.

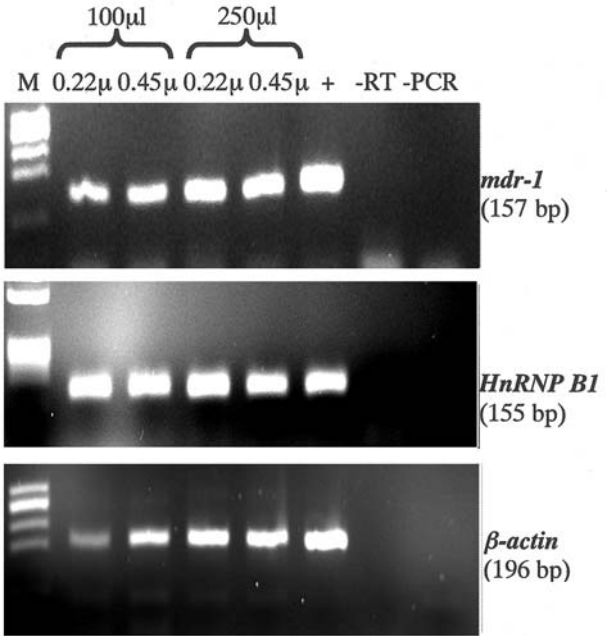


Figure 2. *Conditioned medium (100 µl or 250 µl aliquots) were passed through 0.45 µm or 0.22 µm filters prior to RNA isolation and RT-PCR to investigate if mRNA detected were extracellular. mdr-1, HnRNP B1 and β-actin from RPMI-Tx CM are shown as example results. Differing levels of amplified product were detected, in some cases, between 0.45 µm and 0.22 µm filtrates. (+ = RNA isolated from RPMI-Tx cells, as positive control; -RT = RT reaction with $H_2O$ instead of RNA as control; -PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

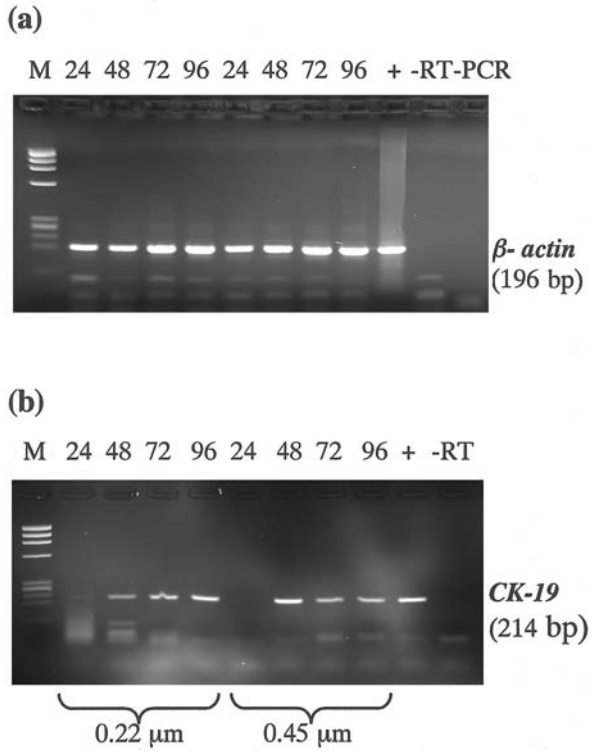


Figure 3. *To establish a suitable time-point for conditioned medium (CM) collection (post cell seeding), CM (250 µl aliquots) was collected at 24, 48, 72, and 96 hrs. and passed through 0.45 µm or 0.22 µm filters prior to RNA isolation and RT-PCR. (a) β-actin and (b) CK-19 amplified products, from RPMI-Tx and RPMI-Ml CM, respectively, are shown as example results. (+ = RNA isolated from DLKP cells, as positive control; -RT = RT reaction with $H_2O$ instead of RNA as control; -PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

## Discussion

Detection of secreted mRNAs in serum from cancer patients has been reported (see Table I as summary). However, presence of amplifiable gene transcripts in human cancer cell line culture supernatants has not previously been studied. Here we report detection of a range of gene transcripts in media conditioned by several cancer cell lines; assess the reproducibility of the procedures employed; and evaluate if secreted products are likely to be full-length or fragmented transcripts.

We show, for the first time, that a range of mRNAs are secreted by human cancer cell lines in culture. The quantity of RNA successfully isolated ranged from 2.4-5.6 ng RNA per µl of medium conditioned by $2X10^6$ cells, for all cell lines included. Although many of the studies assessing mRNA in serum did not report on the quantity of RNA obtained, our results are in the same range as those described by Sisco *et al.* (17) *i.e.* 25.4-144 mg/L of plasma.

Expression of transcripts representing a number of gene products (including *mdr-1, mrp-1, CK-19, HnRNP B1, GST-π, topoisomerase II, bcl-2* and *β-actin*; amplified products ranging between 157-383 bp) was evaluated in cell lines and corresponding CM. Although 30 cycles of PCR were sufficient for amplification of RNA from cultured cells, 45 cycles was generally found to be necessary for CM studies. However, use of nested primers and large numbers (70-83 cycles) of PCR cycles as reported for some of the serum studies (3, 9) were not found to be necessary in our experiments. Results from this study suggest that the secretion of mRNA by human cancer cells may not just be a general "dumping" mechanism, but may be somewhat selective. This is supported by the fact that although *β-actin, CK-19* and *HnRNP B1* are expressed by all cell lines and are detected in all CM analysed, other mRNAs - such as *mrp-1* and *bcl-2* - were present in RNA isolated from most/all cell lines, but were not present in all corresponding CM samples. An alternative explanation to selective secretion of the gene transcripts may be differing stabilities of mRNAs secreted. However, support for the former suggestion is the fact that, for example, RPMI-Ml and

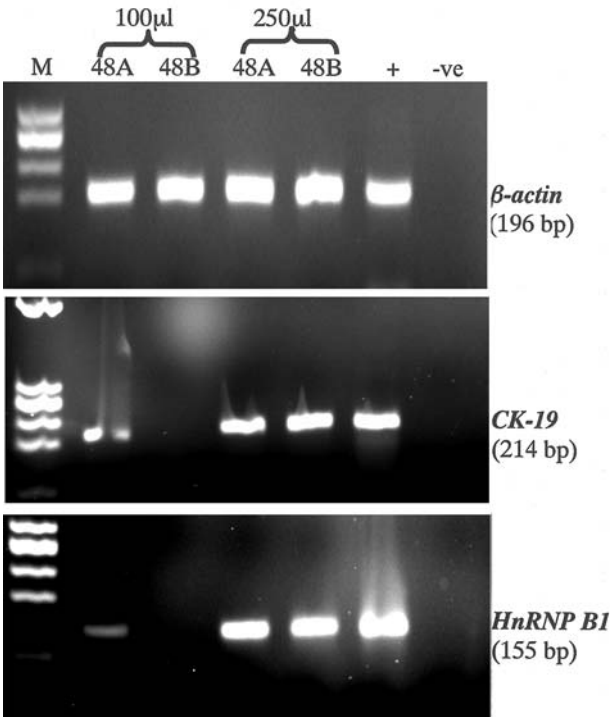


Figure 4. *To establish a suitable volume of CM for collection, 48 hours post seeding 2X10$^6$ cells in 8 ml, 100 µl and 250 µl volumes of CM were collected and passed through a 0.22 µm filters prior to RNA isolation and RT-PCR. A and B represent duplicate samples removed and analysed, in parallel. β-actin, CK-19 and HnRNP B1 amplified products from RPMI-Ml CM are shown as example results. (+ = RNA isolated from RPMI-Tx cells, as positive control; -ve = RT reaction with $H_2O$ instead of RNA as control).*

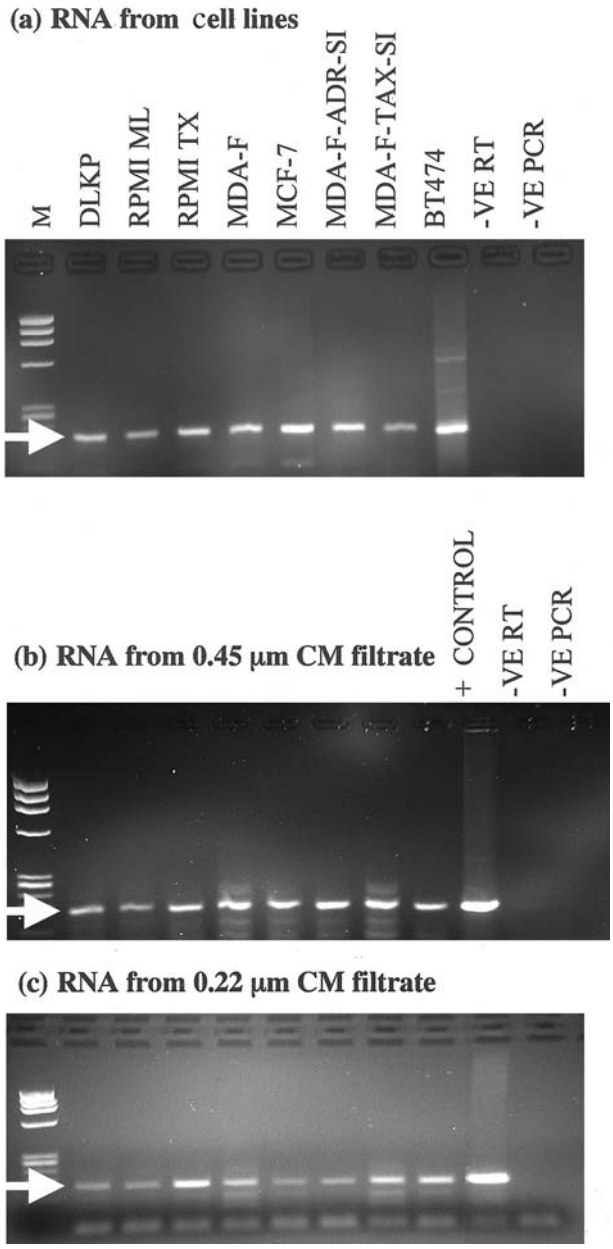


Figure 5. *β-actin gene transcript (arrow) was detected in RNA isolated from (a) all cell lines analysed and in corresponding CM after passing through a (b) 0.45 µm or (c) 0.22 µm filter. Amplified product was detected in the positive control and was undetected in the negative control samples (-ve RT = RT reaction with $H_2O$ instead of RNA as control; -ve PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

RPMI-Tx cells grow in the same medium type, under the same culture conditions; both cell line variants express *mrp-1*; but, although this product is detected in both the 0.45 µm and 0.22 µm CM from RPMI-Tx, it is undetected in all medium conditioned by RPMI-Ml cells.

With one exception, an amplified product was never detected in CM, but not in the corresponding cell line. This exception was the presence of a weak band for GST π in 0.45 µm filtered MCF-7 cell CM. This result was reproducibly found. A possible explanation for this unexpected finding may be the use of 45 cycles of PCR for CM analysis, with only 30 cycles routinely used for amplification of RNA from cell lines.

In some studies of human serum/plasma it has been suggested that the RNA detected is probably present as short fragments (18). Similarly, extracellular RNA isolated from HeLa and A431 growth medium has been suggested to be represented by 100-200 nucleotide-long fragments (12). As indicated in Figure 1, however, we have routinely amplified products ranging from 142-383 bp in length, at various regions (5', central, and 3') along the length of the gene transcripts (full length transcripts ranging from 0.737-6.03 Kb in size), following reverse-transcription of mRNA to cDNA, using oligo (dT) to prime the mRNA's poly (A) tail. Although the amounts of mRNA isolated are too small to confirm that full-length transcripts are secreted, using Northern blotting techniques, successful amplification from 4 regions along the length (1.793

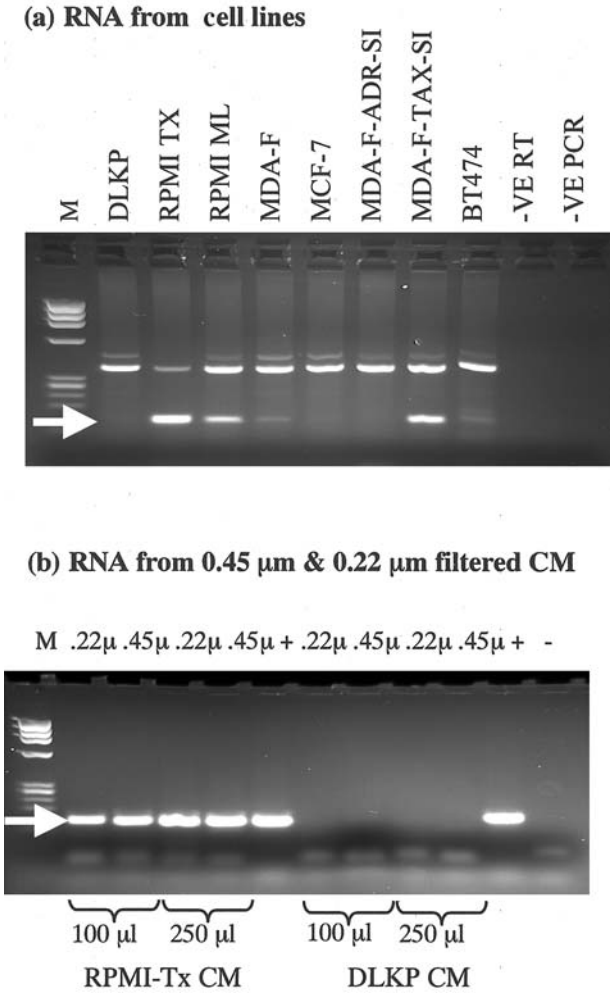


Figure 6. *As expected, expression of mdr-1 mRNA (arrow) was only detected in CM of cell lines expressing this product. β-actin was co-amplified with mdr-1 when analysing RNA from cell lines. Amplified product was detected in the positive control and was undetected in the negative control samples (-ve = RT reaction with $H_2O$ instead of RNA as control; -PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

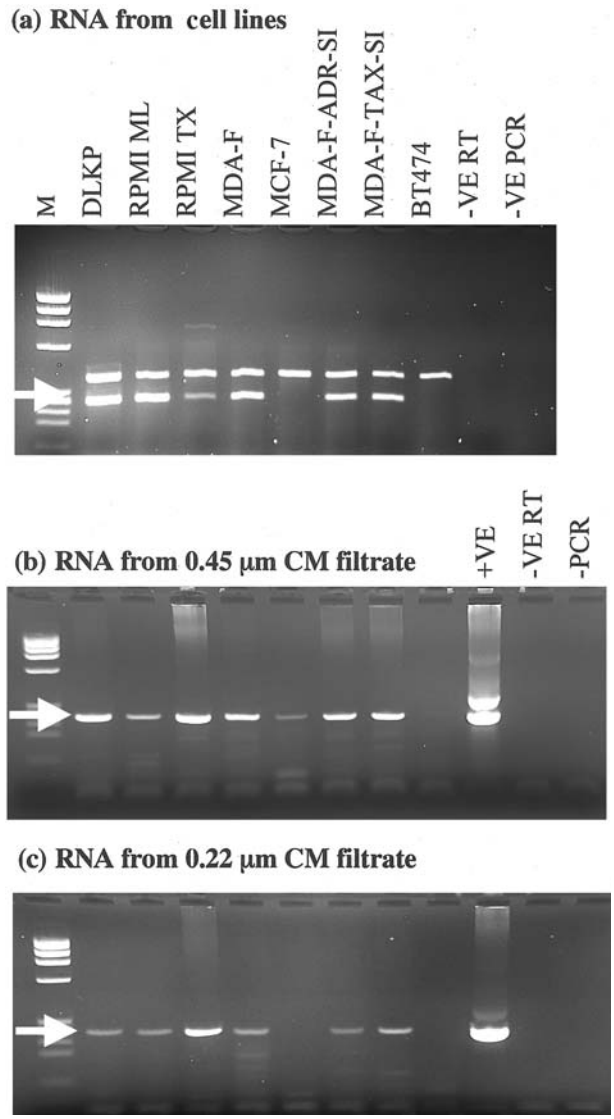


Figure 7. *GST π mRNA (arrow) was expressed by most cell lines and CM. In the case of BT474, no product was detected in cell or CM samples; for MCF-7, GST π was undetected in RNA from cells and 0.22 μm filtered CM, but a weak band was detected in the 0.45 μm filtrate. β-actin was co-amplified with GST π when analysing RNA from cell lines. Amplified product was detected in the positive control and was undetected in the negative control samples (+ = RNA isolated from DLKP cells, as positive control; -RT = RT reaction with $H_2O$ instead of RNA as control; -PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

Kb) of the *β-actin* cDNA, prepared in this way, indicate that the fragments detected here are greater than 200 nucleotides in length and suggests that the mRNAs secreted by these cultured cells, are not fragmented, but are full-length products.

Prior to RNA isolation, samples of medium conditioned by the eight cell lines included were passed through 0.45 μm or 0.22 μm filters, to ensure that intact cells were not included in the isolates. In many cases, although gene transcripts were detected in the CM, the amplified product obtained following 0.22 μm filtration was less intense than that from the corresponding 0.45 μm CM filtrate; indeed, in some cases, an amplified product was no longer detected following 0.22 μm filtration. Similar effects have been reported from studies where plasma/serum has been passed through a 0.22 μm and it has been suggested that some of the mRNA secreted by human cancer cells may be particle-associated, bound to protein/lipoprotein complexes, or sequestered within lipid vesicles (10, 18).

In conclusion, we have demonstrated that a broad range of healthy, proliferating, human cancer cell lines in culture secrete many gene transcripts, which can be reproducibly

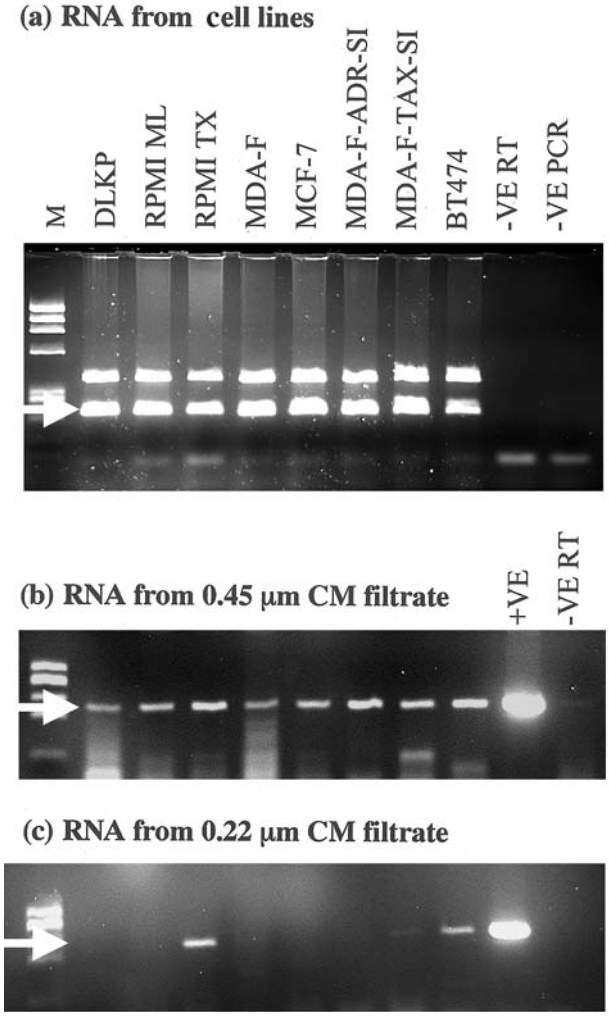


Figure 8. *Topoisomerase II mRNA (arrow) was expressed by all cell lines, detected in all 0.45 μm filtered CM, but in only 3 (RPMI TX, MDA-F-TAX SI and BT474) CM specimens. β-actin was co-amplified with topoisomerase II when analysing RNA from cell lines. Amplified product was detected in the positive control and was undetected in the negative control samples (-ve RT = RT reaction with $H_2O$ instead of RNA as control; -PCR = PCR reaction with $H_2O$ instead of cDNA as control).*

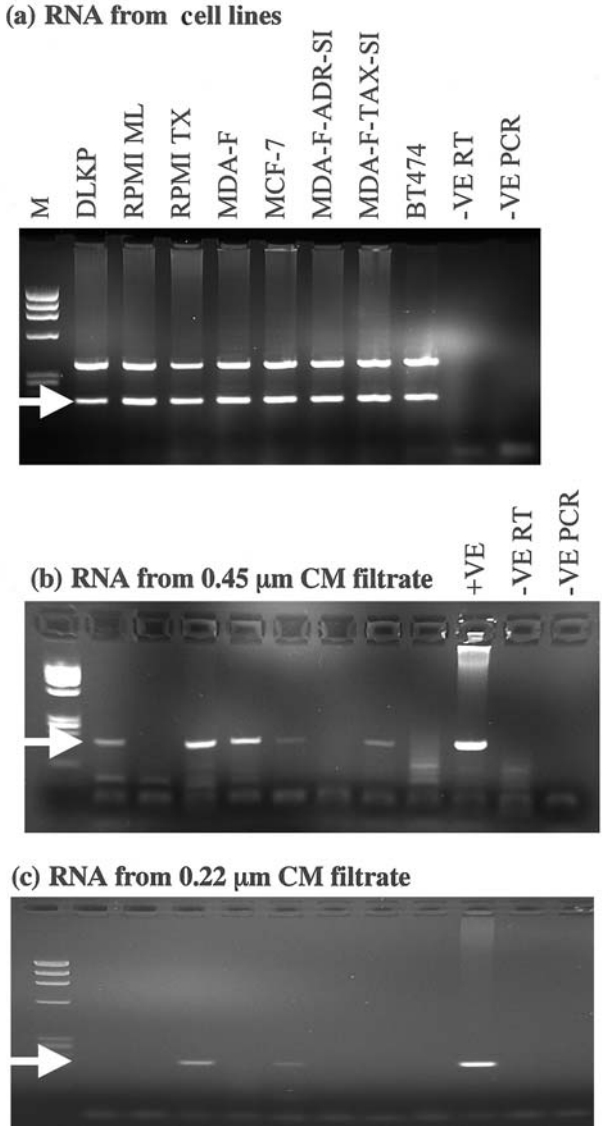


Figure 9. *mrp-1 (arrow) was expressed by all cell lines, detected in 5 of the 0.45 μm filtered CM, but in only 2 of the 0.22 μm CM specimens. β-actin was co-amplified with mrp-1 when analysing RNA from cell lines. (Amplified product was detected in the positive control, DLKP).*

detected using procedures described here. These results suggest that the secretion of particular transcripts by a given cell line may be somewhat selective. The mRNAs secreted by these cancer cells are apparently full-length transcripts; some of which may be particle-bound. Further studies are required to determine the nature of this association.

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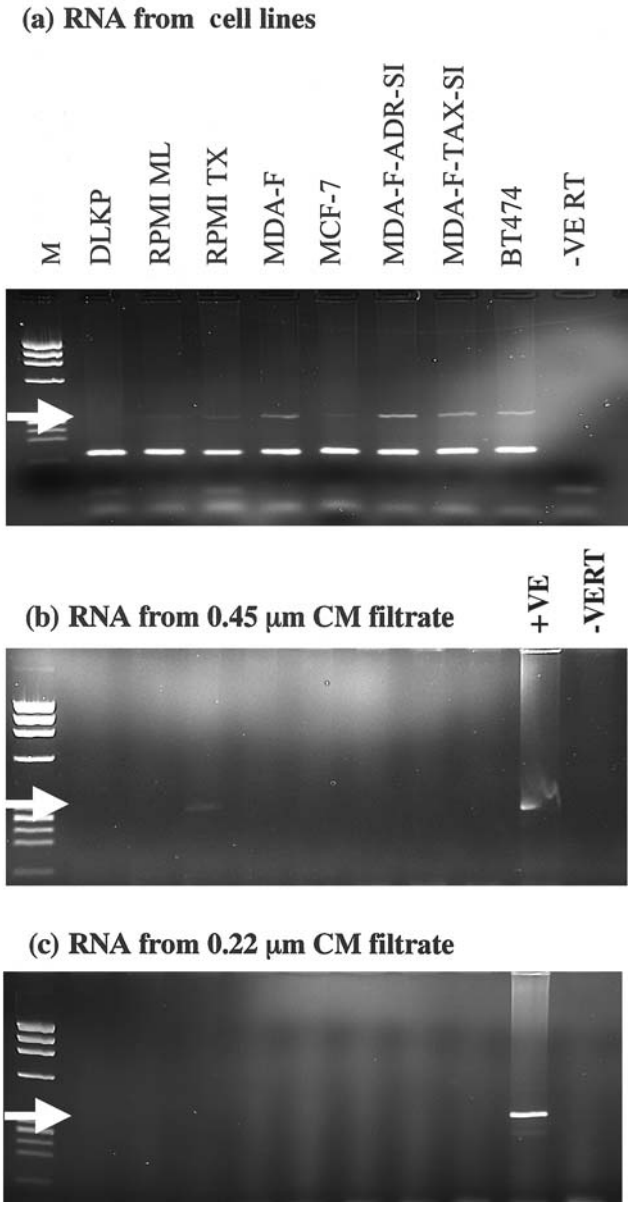


Figure 10. *bcl-2 (arrow) was expressed, to some extent, by all cell lines analysed, but - with the exception of RPMI-Tx 0.45 μm filtrate - was undetected in CM. β-actin was co-amplified with bcl-2 when analysing RNA from cell lines. Amplified product was detected in the positive (+ve) control (DLKP) and was undetected in the negative control samples (-ve RT = RT reaction with $H_2O$ instead of RNA as control).*


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