

*Advances in Brief***Detection of Tumor Messenger RNA in the Serum of Patients with Malignant Melanoma****Michael S. Kopreski,¹ Floyd A. Benko,
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Abstract

Serum RNases are known to be elevated in patients with cancer. Consequently, it is not clear whether human mRNA with sufficient integrity as to permit reverse transcription-PCR (RT-PCR) amplification is detectable in serum. We examined serum from six patients with malignant melanoma for human tyrosinase mRNA using RT-PCR. Serum from 20 normal volunteers served as controls. Tyrosinase mRNA could be demonstrated in serum from four of the six melanoma patients with detection by gel electrophoresis and confirmation by blotting amplified product to a tyrosinase-specific probe. The serum remained tyrosinase mRNA positive, even if passed through a 0.45 μm filter prior to RNA extraction, indicating that the mRNA was extracellular at the time of extraction. Tyrosinase mRNA could not be detected in any control serum (0 of 20 individuals). The presence and integrity of amplifiable RNA was confirmed in all serum specimens (patients and controls) by RT-PCR amplification of *c-abl* mRNA. Amplifiable RNA could be demonstrated regardless of whether serum was freshly drawn or stored frozen for several years. We conclude that human mRNA can be extracted and amplified from serum. The ability to amplify tumor mRNA from serum may have important utility in cancer diagnostics and monitoring.

Introduction

The development of sensitive RNA-based amplification methods has enabled new approaches to diagnosing and monitoring malignancy. One approach under investigation detects circulating cancer cells by extracting and amplifying specific tumor RNA from the cellular fraction of peripheral blood (1, 2). Commonly, cellular tyrosinase mRNA has been assayed in amplification strategies to detect circulating malignant melanoma cells (3, 4). However, circulating cancer cells tend to

correlate with tumor burden, with lower rates of detection seen in those with minimal or early disease (5). The detection of circulating melanoma cells has similarly been shown to correlate with the stage of disease, with low detection rates seen in localized disease (6–8). More sensitive approaches are needed to monitor patients with low tumor burden.

Recently, it has been shown that amplifiable extracellular tumor DNA can be found in plasma and serum (9–15). The extracellular DNA appears detectable, even in the absence of circulating cancer cells (10, 11), and can be detected in those with early disease and low tumor burden (16, 17). However, mRNA is more fragile than DNA and is presumed to be highly susceptible to degradation by blood RNases. Furthermore, blood RNases are known to increase in patients with cancer (18). It has thus been commonly presumed that amplifiable human RNA could not survive extracellularly in bodily fluids. Although several reports have suggested that RNA might be present in plasma or serum (19–22), it remained to be shown whether extracellular RNA exists with sufficient integrity as to allow RT-PCR² amplification. In this preliminary study, we evaluate whether amplifiable tyrosinase mRNA is detectable in serum from patients with malignant melanoma.

Materials and Methods

Serum Preparation. Serum from six patients with metastatic malignant melanoma treated on clinical protocols of the former Biological Response Modifiers Program, National Cancer Institute (Frederick, MD), and serum from 20 normal volunteer controls (rendered anonymous) were assayed. Initially, the sera from melanoma patients were prepared by centrifugation of clotted peripheral blood at $830 \times g$ for 10 min, followed by careful aliquoting and freezing of the serum. Although patient serum specimens were generally prepared and frozen within 4 h of the blood draw, preparation time varied. All melanoma patient sera were initially frozen at -70°C and stored for >2 years prior to use. Control serum was prospectively obtained and prepared in a similar fashion, except that to minimize mRNA degradation, which we found to be associated with freeze-thaw cycles, control serum was not frozen prior to RNA extraction, which was also generally performed within 4 h of blood draw.

RNA was extracted from 50 μl of serum using a commercial kit, Perfect RNA: Total RNA Isolation kit (5 Prime-3 Prime, Inc., Boulder, Colorado), performed according to the manufacturer's direction. The concentration of RNA was then approximated by spectrophotometry.

Amplification. Comparable quantities of RNA per serum aliquot corresponding to 10–45% of the RNA extracted from 50 μl of serum were reverse transcribed by a method

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² The abbreviation used is: RT-PCR, reverse transcription-PCR.

adapted from Kawasaki (23). A mixture was prepared consisting of the RNA, 3 μ l of 0.1 M DTT, 3 μ l of $\times 10$ Taq buffer (Fisher, Pittsburgh, PA), 100 pmol of random hexamer primers (Promega Corp., Madison, WI), 3 μ l of deoxynucleotides (10 mM each; New England BioLabs, Beverly, MA), 4.8 μ l (25 mM) magnesium chloride (Fisher), 1 μ l RNAsin (25 units/ μ l; Promega), 3 μ l of AMV reverse transcriptase (3 units/ μ l; Promega) diluted 1:10 in water, for a total volume of 30 μ l. This was incubated at room temperature for 10 min and then maintained in a heat block at 42°C for 60 min. Fifteen μ l of cDNA were then used in the amplification reaction.

Human tyrosinase cDNA was amplified by nested PCR as adapted from Smith *et al.* (3). Tyrosinase primers were: TYR 1 (outer, sense), 5'-TTGGCAGATTGTCTGTAGCC; TYR 2 (outer, antisense), 5'-AGGCATTGTGCATGCTGCTT; TYR 3 (nested, sense), 5'-GTCTTTATGCAATGGAACGC; and TYR 4 (nested, antisense), 5'-GCTATCCCAAGTAAGTGGACT.

A reaction mixture was prepared consisting of 1 \times reaction buffer (Fisher), 1.6 mM magnesium chloride (Fisher), 200 μ M each dATP, dCTP, dGTP, and dTTP (New England BioLabs), 2.5 pmol each of primer TYR-1 and TYR-2, 1 unit Taq polymerase (Fisher), and distilled water for a total volume of 50 μ l. This reaction mixture was overlaid with mineral oil and amplified for 15 cycles in a Ericomp Delta Cyclor 1, with denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min. Reamplification with nested primers TYR 3 and TYR 4 was then performed, in which the first amplification product was diluted 1:100 in distilled water, with 5 μ l of dilution used as template for the second amplification step. The reaction mixture was identical to that in the first amplification step except that primers consisted of 25 pmol each of TYR 3 and TYR 4. This mixture was cycled for 40 cycles at the same parameters, with a final extension at 72°C for 8 min.

To verify the presence and integrity of serum RNA, all sera were additionally assayed for human c-abl mRNA, a mRNA expected to be expressed in all individuals. RT-PCR for c-abl was performed using 15 μ l of the cDNA in a reaction mixture with 1 \times PCR buffer, 1.5 mM magnesium chloride, 200 μ M each deoxynucleotide triphosphates, 50 pmol of Abl-1 primer (Oncogene Science, Uniondale, NY), 50 pmol of Abl-3 primer (Oncogene Science), and 2.5 units of Taq polymerase in a 50- μ l volume. This mixture was cycled 35 times with an initial denaturing temperature of 94°C for 4 min, followed by a denaturing temperature of 94°C for 60 s, annealing temperature of 65°C for 60 s, and extension temperature of 72°C for 90 s. A nested PCR reaction was then prepared using the same constituents as above, except that 4 μ l of the first-round product was used in place of the 15 μ l of cDNA, the water was reduced by 1 μ l, and primers used were Abl-2 and Abl-4 (Oncogene Science). The mixture was then cycled 35 times under the same cycling parameters (manufacturer's Abl protocol, CML primer set; Oncogene Science).

All RT-PCR amplifications were performed with particular attention paid to prevention of contamination. Amplifications included a tyrosinase-positive control consisting of tyrosinase-positive melanoma tissue and a negative control lacking cDNA. The risk of contamination yielding falsely positive results was further minimized by repeating the assay on the melanoma

patient sera using additional, separately prepared serum aliquots.

Detection. Amplified product was electrophoresed through a 4% agarose gel (2:1 NuSieve GTG; FMC Bioproducts, Rockland, Maine) in 1 \times TBE buffer (pH 8.0) at 100 V for 2 h and stained with ethidium bromide. The nested tyrosinase primers amplify a PCR product of 207 bp. The c-abl mRNA yields an amplified product 267 bp long.

To further verify tyrosinase results, PCR products from the gel were transferred by Southern blot onto a nylon membrane (MSI, Westboro, MA). Replicate blots were then probed by TYR-5, a tyrosinase internal probe (bp 920–934 of cDNA; 5'-CCAGAACCCCAAGGC). Hybridization and wash conditions were as specified by the membrane's manufacturer.

RNA Stability and Abundance. To evaluate whether RNA extracted from serum was extracellular at the time of extraction, sera from two melanoma patients were reassayed by first passing the thawed centrifuged serum through a 0.45 μ m cellulose acetate filter (Nalgene, Rochester, NY) prior to RNA extraction. Reverse transcription, amplification, and detection of both tyrosinase and c-abl mRNA were then carried out as described previously.

The stability of the serum RNA under different conditions was evaluated. To test the stability through several freeze-thaw cycles, identical aliquots of serum were repeatedly frozen to –20°C and rapidly thawed prior to extracting RNA as described above. To determine the rapidity of degradation of the RNA, thawed serum was placed at 4°C with aliquots drawn off for extraction at 15-min intervals. The effect of adding RNase inhibitors to serum prior to freezing was assayed by adding 25 units of RNAsin (Promega) or 50 μ l of 2 M guanidinium thiocyanate (United States Biochemical Corp., Cleveland, OH) to 50- μ l aliquots of serum. Finally, a semiquantitative assessment of RNA abundance was made by preparing 10-fold dilutions of both freshly obtained (three patients) and frozen-thawed serum (five patients), extracting, and testing for c-abl mRNA and (in one melanoma patient serum) tyrosinase mRNA.

Results

Serial dilutions into water of RNA extracted from melanoma tissue demonstrated the ability of the assay to detect tyrosinase mRNA in a 0.4-pg sample (Fig. 1). c-abl mRNA could be demonstrated in the serum of all patients and all normal volunteers, confirming both the presence and integrity of RNA in serum (Figs. 1 and 2). Tyrosinase mRNA was detected in serum from four of the six (67%) malignant melanoma patients assayed (Fig. 1). Results were reproducible upon retesting of additional serum aliquots. In contrast, none of the 20 normal control sera tested positive for tyrosinase mRNA (Fig. 2A). In all amplification assays, the tyrosinase-positive melanoma tissue and cDNA-absent sample tested appropriately. Southern blotting of the amplified product confirmed detection of tyrosinase cDNA in all four gel-positive melanoma patients (Fig. 1B).

To further evaluate characteristics of serum mRNA, serum from two patients who tested tyrosinase positive were reassayed by passing the serum through a 0.45 μ m cellulose acetate filter prior to the initial RNA extraction step (Fig. 3). This size filter

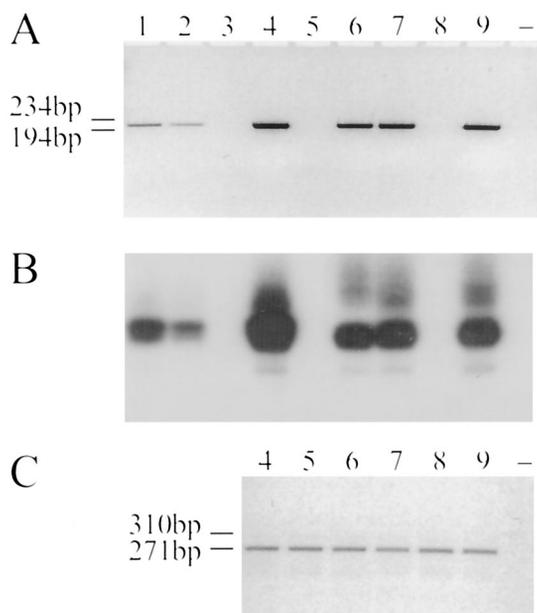


Fig. 1 Tyrosinase mRNA in melanoma patient serum. **A**, RT-PCR of serum from melanoma patients and positive controls using nested tyrosinase primers yields the expected 207-bp product. **B**, Southern blot with internal probe TYR-5. **C**, RT-PCR of serum from melanoma patients using nested c-abl primers. Lane 1, 4 pg of RNA extracted from melanoma tissue; Lane 2, 0.4 pg; Lane 3, 0.04 pg; Lanes 4–9, sera from six melanoma patients; Lane –, water blank.

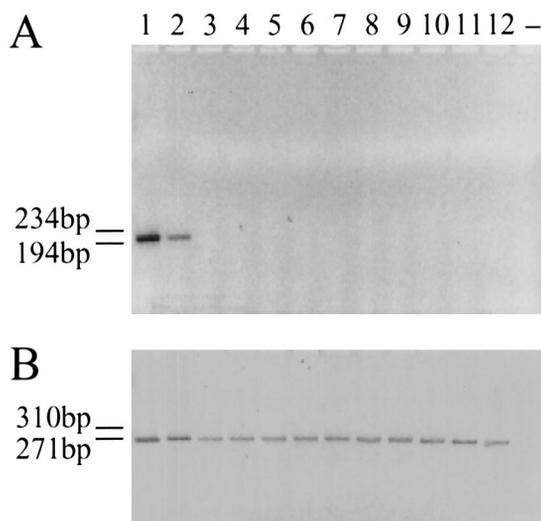


Fig. 2 Absence of tyrosinase mRNA in normal control sera. **A**, tyrosinase RT-PCR of control sera. **B**, c-abl RT-PCR. Lane 1, 4 pg of RNA extracted from melanoma tissue; Lane 2, 0.4 pg; Lanes 3–12, sera from 10 normal donors; Lane –, water blank.

should be sufficient to exclude any intact cells that might have contaminated the serum. In both patients, the filtered serum again tested tyrosinase mRNA positive. Similarly, c-abl mRNA remained detectable in the filtered serum of both patients (not shown). These findings are consistent with extracellular mRNA being present in serum.

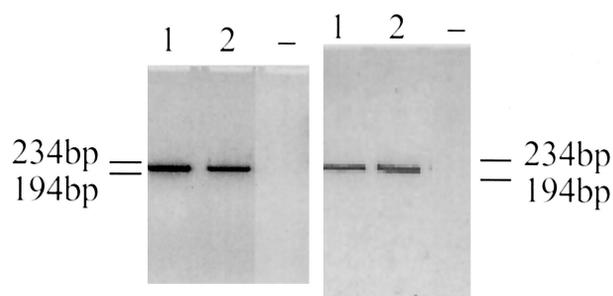


Fig. 3 The filtering of serum does not eliminate tyrosinase mRNA. Sera from two patients (Lanes 1 and 2) were assayed by RT-PCR for tyrosinase mRNA before (left panel) and after (right panel) passage through a 0.45 μm filter. Lane –, water blank.

The stability of the mRNA was further investigated. Serum mRNA was amplifiable, despite an interval of several hours between blood draw and extraction of serum RNA, and despite being maintained frozen several years. However, we found that freezing and thawing of patient serum apparently promotes rapid degradation of both tyrosinase and c-abl mRNA. The RNA was always detectable after the first freeze-thaw cycle. Both tyrosinase and c-abl mRNA remained detectable when extracted at 15 min but not 30 min after the first thaw (Fig. 4A). In one melanoma patient, a reduction in the tyrosinase amplification product was noted after the second freeze-thaw cycle. After the third and subsequent freeze-thaw cycles, amplifiable RNA could no longer be demonstrated, despite rapid extraction of RNA from the thawed samples (Fig. 4B). Addition of RNasin to the twice-thawed serum prevented degradation of the RNA for one additional freeze-thaw cycle, but the protective effect was not maintained after subsequent freeze-thaw cycles (not shown). Addition of guanidinium thiocyanate offered no protective effect. It remains undetermined whether this progressive degradation of mRNA is due to the successive freeze-thaw cycles themselves, or whether elapsed time which occurs during the thawing, is solely responsible. Comparison of c-abl mRNA in freeze-thawed *versus* never-frozen matched serum aliquots (three patients) suggests that a single freeze-thaw cycle results in a 10–100-fold reduction of amplifiable mRNA (Fig. 4, C and D).

Semiquantitative evaluation of RNA abundance found ~ 10 -fold less tyrosinase mRNA than c-abl mRNA was present in the serum of one melanoma patient, as assessed by testing serial dilutions of once-frozen serum (Fig. 5).

Discussion

It has long been known that RNases are present in blood plasma and serum, and further recognized that serum RNase levels increase in patients with cancer (18). In view of the sensitivity of mRNA to degradation by RNase, it was not clear whether RNA could exist in plasma or serum with sufficient integrity to allow amplification. Others have found that upon adding free RNA into blood, the RNA was nonamplifiable (24). Detection of specific tumor mRNA requires an intact and recognizable or amplifiable sequence.

In this study, we demonstrate that amplifiable human mRNA is present in serum. Human tyrosinase mRNA was detected in the

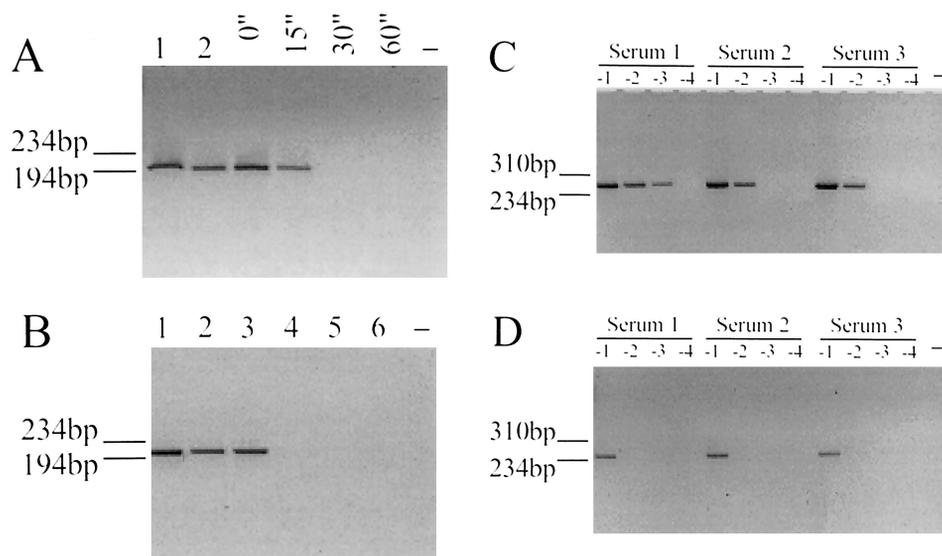


Fig. 4 Serum mRNA stability studies. **A**, time course experiment. Aliquots of a melanoma patient's serum were thawed and incubated on ice for 0, 15, 30, or 60 min, followed by RNA extraction and RT-PCR for tyrosinase mRNA. *Lane 1*, 4 pg of RNA extracted from melanoma tissue; *Lane 2*, 0.4 pg. *Lane -*, water blank. **B**, effect of freeze-thaw cycles on signal. Tyrosinase RT-PCR of melanoma patient serum extracted after two freeze-thaw cycles (*Lane 3*), three freeze-thaw cycles (*Lane 4*), four freeze-thaw cycles (*Lane 5*), and five freeze-thaw cycles (*Lane 6*) is shown. Other lanes as above. **C**, PCR for c-abl mRNA from three never-frozen normal control sera, each serially diluted 10-fold after reverse transcription. **D**, PCR for c-abl mRNA from the same sera, frozen and thawed once, then serially diluted after reverse transcription. *Lane -*, water blank.

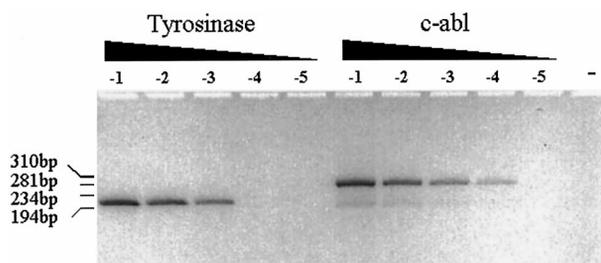


Fig. 5 Relative abundance of tyrosinase and c-abl mRNA in serum from a patient with melanoma. cDNA was serially diluted 10-fold in water (dilution indicated above each lane) and then assayed in parallel for tyrosinase or c-abl. *Lane -*, water blank.

serum of four of six patients with malignant melanoma. In addition, c-abl mRNA was detected in the serum of all individuals tested. The RNA was detectable, even when centrifuged serum was passed through a filter, indicating that the mRNA was extracellular at the time of extraction from serum. Of interest, tyrosinase mRNA was detectable in serum, although the serum had been stored frozen over several years, suggesting that stored serum banks may be used in RT-PCR-based investigations, although the integrity of the RNA is affected during multiple freeze-thaw cycles as noted. We have also found that human mRNA may be amplified from plasma (results not shown).

At the present time, it is not known how serum RNA is protected from blood RNases. It is possible that extracellular mRNA could circulate bound to protein or phospholipid, thus being protected from nucleases. RNA has been found on the cell surface of cancer cells (25, 26) and could be shed within vesicles (27, 28). Rosi *et al.* (29), using nuclear magnetic resonance spectroscopy,

described RNA-lipid vesicles shed *in vitro* from a human colon adenocarcinoma cell line. Further characterization of the RNA-lipid vesicles indicated the presence of triglycerides, cholesterol esters, lipids, oligopeptides, and phospholipids (30). Mountford *et al.* (20) identified a similar proteolipid in the plasma of a patient with an ovarian neoplasm using magnetic resonance spectroscopy. Evaluation of the proteolipid with the orcinol method suggested RNA to be present, although this could not be confirmed using other methods. Furthermore, Stroun *et al.* (31) found that RNA-DNA nucleoprotein complexes were actively released from normal cells in culture, and Wiczorek *et al.* (21, 22) described a RNA-proteolipid complex in the sera of cancer patients that appeared to be an actively secreted product of tumor cells. It is not known whether extracellular tyrosinase mRNA circulates within analogous RNA-proteolipid complexes. RNA could similarly circulate within apoptotic bodies or nuclear fragments. Nuclear RNA-protein complexes, possibly representing functional nuclear suborganellar elements, have been described (32). Alternatively, it is possible that clearance of free RNA *in vivo* by RNase is not as rapid as believed previously. Free RNA could potentially be released after *in vivo* or *in vitro* lysis of tumor cells found in the blood. Further investigation is needed to clarify the etiological and pathophysiological nature of extracellular RNA. Within this context, one may consider the effect that one or more freeze-thaw cycles have upon extracellular RNA stability. In addition to prolonging the potential exposure of RNA to nucleases, it is possible that during the freeze-thaw process, vesicles or apoptotic bodies are disrupted or protein-RNA interaction otherwise affected, thereby rendering the RNA more susceptible to nucleases.

Although the observations presented in this study must be considered preliminary, the finding that tumor mRNA is amplifiable from serum may offer a new approach to cancer diagnos-

tics, monitoring, and pharmacogenomic evaluation. Similar to tyrosinase mRNA, other tumor mRNA should be demonstrable in serum and plasma in other malignancies. One could further anticipate finding extracellular RNA in other bodily fluids. The present study was limited to patients with metastatic cancer. Whether serum or plasma-based tumor mRNA assays will prove sensitive in diagnosing and monitoring early or limited disease remains to be clarified. However, the demonstration that amplifiable tumor mRNA is present in serum offers a new avenue of exploration. Future clinical trials are needed to address the potential of this approach.

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