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A Novel Blood-Based Method for Evaluating *KRAS* in Circulating Microvesicles from Colorectal Cancer Patients

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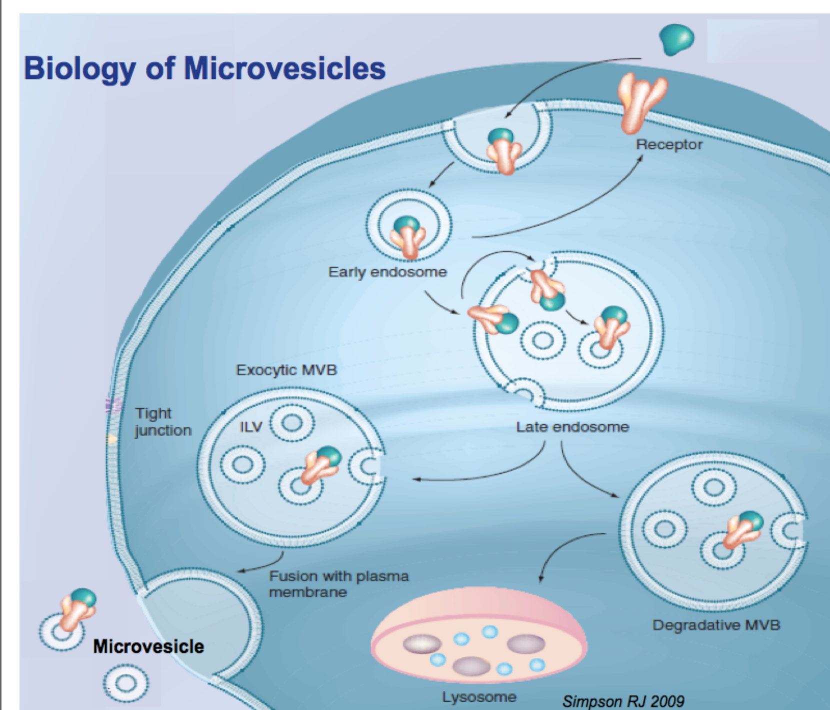
Abstract

Circulating microvesicles (cMV) are lipid-encapsulated bodies that are secreted from various tissues and can be detected in a number of body fluids, including plasma. Once collected from plasma, they can be exploited diagnostically for their protein and RNA signatures. Mutations in *KRAS* are diagnostically important for predicting response to chemotherapy and prognosis. A blood-based method of assessing *KRAS* mutation status would be helpful for patients with colorectal cancer (CRC). Traditional methods of *KRAS* detection examine the genomic DNA sequence. We developed a method to sequence exon 2 of *KRAS mRNA*. The limit of *KRAS* mutation detection was 0.78 ug of mutation- positive exosomes per ml of plasma for both Pyrosequencing and Sanger sequencing. To further enhance mutation detection, we sorted cMV from CRC patients by first capturing cMV with a CRC-associated membrane protein and then sorting for CD63 positive events. A Taqman gene expression assay was not sensitive enough to detect *KRAS* in the sorted samples. However, Pyrosequencing was sufficient to identify mutant and wild-type sequences in patient plasma samples.

Introduction



A major subpopulation of cMV are called exosomes, which are endosome-derived vesicles between 40-100 nm in diameter with a unique cup-shape morphology that are secreted by most cell types (1). These vesicles are formed intracellularly by invagination followed by fusion with the multivesicular body (MVB). The MVB ultimately merges with the plasma membrane, leading to exocytosis of exosomes with a membrane protein composition unique to their cell of origin (1). Exosomes can be distinguished from other cMV by the presence of a characteristic protein composition and their physical morphology.

Exosomes have been detected in a large variety of biological fluids including plasma, urine, serum, cerebrospinal fluid, saliva, breast milk and semen. The number of exosomes in body fluids has been found to be elevated in diseased states, including cancer, suggesting that their levels can be used for disease diagnosis and prognosis. Taylor et al. found levels of circulating tumor-derived exosomes to increase with progressive stages of ovarian cancer compared to control patients (2). Rabinowits et al. discovered that lung cancer patients have elevated serum exosomes (3). Furthermore, it has been shown that exosomal membrane proteins and internal cargo can be used to determine the tissue of origin, facilitating noninvasive molecular profiling as shown by Skog et al. (4) who detected EGFRvIII mutant transcripts in serum microvesicles of glioblastoma patients.

Results

Limit of *KRAS* Mutation Detection in Pyrosequencing and Sanger Sequencing

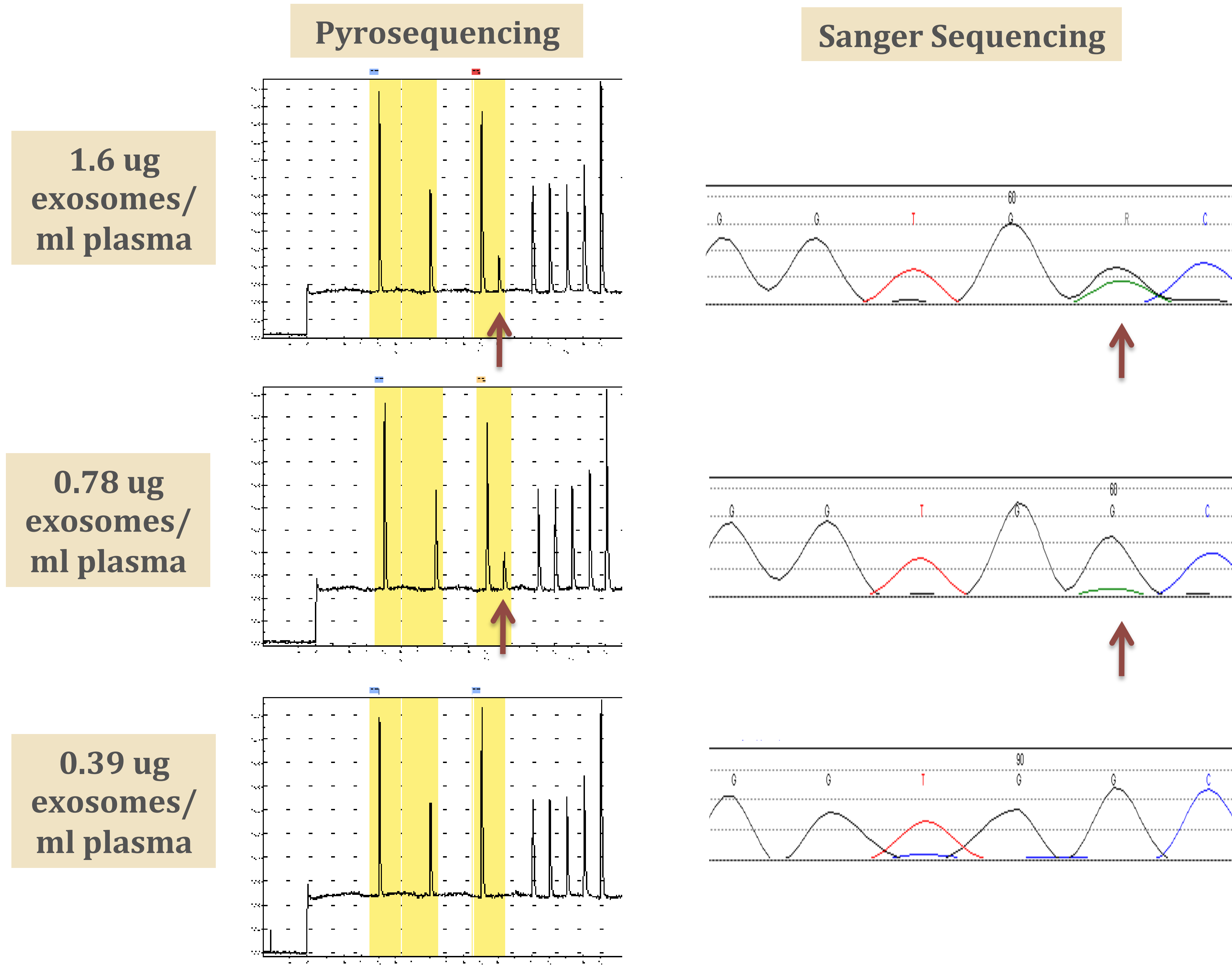


Figure 1. Programs and chromatograms depict the limit of detection for each assay. Red arrows indicate where the c.38G>A mutation is visible.

Membrane Protein-Positive cMVs Sorted for CD63 Events

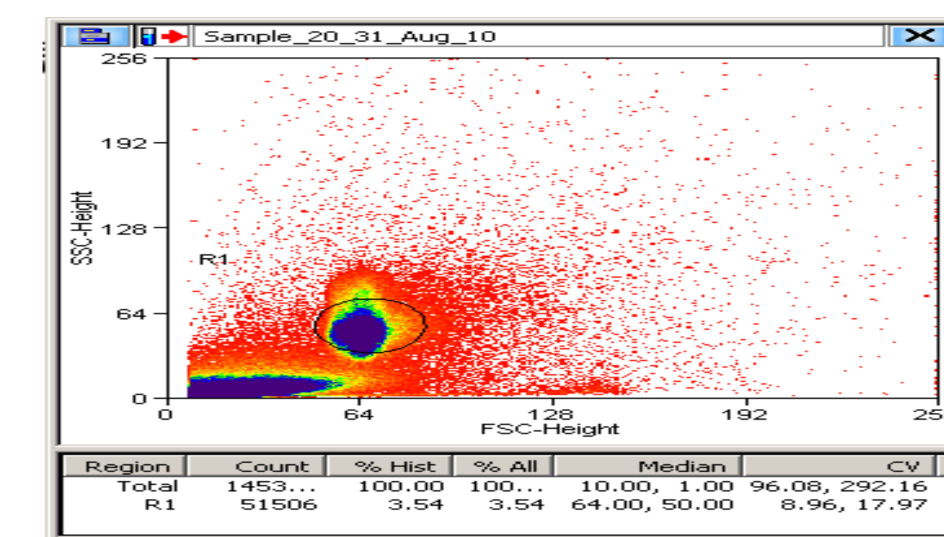


Figure 2. Membrane protein positive-gated events were analyzed for CD63 expression.

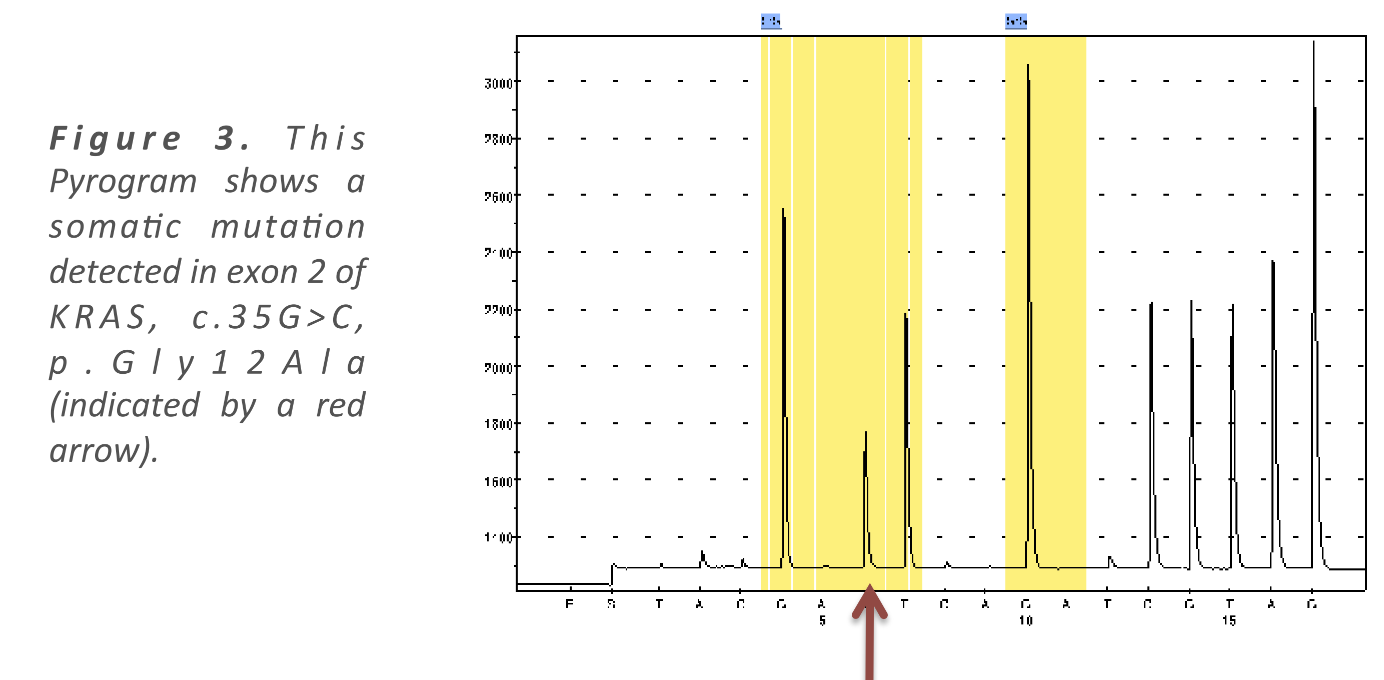


Figure 3. This Pyrogram shows a somatic mutation detected in exon 2 of KRAS, c.35G>C, p.Gly12Ala (indicated by a red arrow).

Methods

Limit of *KRAS* Mutation Detection

Exosomes from a *KRAS* mutation-positive cell-line (HCT116) were serially diluted into normal human plasma, with concentrations ranging from 50 to 0.19 ug of exosomes per ml of plasma. cMV were isolated using Carisome™ technology, and RNA was extracted from cMV using a phenol-based lysis solution. *KRAS* was amplified for Sanger sequencing by traditional methods and analyzed using a 3730xL Genetic Analyzer (Life Technologies). A custom *KRAS* Pyrosequencing assay was designed to amplify and detect mutations from cDNA.

KRAS Mutation Detection from a CRC patient

cMV were first captured with a CRC-associated membrane protein and then sorted for CD63 positive events using a Beckman Coulter MoFlo XDP. RNA was extracted from the cMV-sorted events, then reverse transcribed, and evaluated for somatic mutations using the Pyrosequencing assay.

Conclusions

It has recently been shown that different regions of tumors can have different mutations (5). Thus, extraction of DNA from a particular region of a tumor can result in an incorrect determination of *KRAS* status, resulting in the wrong therapy prescribed. It is possible that a blood-based method could find mutations that may be missed by biopsy tissue, and could offer clinicians more information about the tumors they are treating. The work presented here shows that somatic mutations can be detected in circulation, which is the first step in the development of a robust blood-based assay to identify the presence of *KRAS* mutations.

References

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