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Diagnosing Liver Cancer Through Amplification of Mutational Extracellular mRNA: A Novel Approach

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Abstract

Hepatocellular carcinoma (HCC) remains the predominant cause of cancer-related mortality. Traditional diagnostic methodologies are invasive and exhibit limited sensitivity for early detection. Non-invasive alternatives, particularly liquid biopsies utilizing extracellular vesicles (EVs), have emerged as promising approaches. EVs contain crucial biomarkers, including mRNA, proteins, and nucleic acids. However, the limited abundance of EV mRNA in liquid biopsies has impeded its clinical application. To address this limitation, researchers have developed the Self-amplified and CRISPR-aided Operation to Profile EVs (SCOPE) platform. This innovative system integrates CRISPR-Cas13 for RNA target identification with replication and signal amplification, achieving subattomolar detection sensitivity. SCOPE offers high specificity with single-nucleotide resolution in a single-step assay. Investigators have validated probes targeting key mutations in KRAS, BRAF, EGFR, and IDH1 genes and developed an automated device for multi-sample analysis. SCOPE has demonstrated efficacy in identifying early-stage lung cancer in animal models, monitoring tumor mutational burden in colorectal cancer, and classifying glioblastoma patients. In HCC, EV mRNA exhibits potential for non-invasive detection of recurrence and monitoring disease progression. Current studies indicate that EV-based mRNA profiling holds significant promise for early detection, treatment monitoring, and recurrence prediction in HCC, offering valuable clinical applications. The integration of advanced platforms such as SCOPE with EV analysis could transform liquid biopsies in oncology, providing a rapid, highly sensitive, and non-invasive method for cancer detection and management.

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent form of liver cancer, the sixth most frequently diagnosed cancer, and the third leading cause of cancer-related deaths worldwide (Ferlay *et al.,* 2019). The early detection of HCC, surveillance status, and curative treatment are associated with significant improvements in patients' overall survival (OS) (Kim & Han, 2012). However, the incidence rate is increasing annually, and its early diagnosis and accurate staging remain challenging (Prince *et al.,* 2020). An additional challenge is HCC risk assessment and prevention of cancer recurrence, along with monitoring the patients' postoperative status and treatment response (Singal *et al.,* 2014). Approximately 70–90% of all HCC cases develop due to liver cirrhosis, which, in turn, can be caused by inflammation associated with hepatitis B virus (HBV) or hepatitis C virus (HCV), exposure to toxins such as alcohol abuse and aflatoxin B1 (AFB1), congenital disorders, and metabolic syndrome (Llovet *et al.,* 2021). Since a large proportion of patients with HCC also have cirrhosis, it is considered an important factor in liver injury, which leads to liver cancer. Therefore, the discovery of minimally invasive biomarkers that can enable precise HCC risk prediction and differentiation of HCC from non-HCC diseases is crucial for identifying the early stages of HCC (Moldogazieva *et al.,* 2021). Typically, tissue biopsies are invasive, and for some anatomical sites, they are not easily obtainable. They also provide a limited representation of intratumoral and intermetastatic genetic heterogeneity because tumors are heterogeneous entities containing various subpopulations of cells that feature different lesions *(*Ignatiadis *et al., 2021;* Martins *et al.,* 2021). Furthermore, cancer cells undergo genetic and epigenetic changes over time and can evolve dynamically, guided by microenvironmental stimuli and clonal selection due to therapeutic pressure. This results in further tumor heterogeneity (Martins *et al.,* 2021), thus affecting the accuracy of the examination and the therapeutic decisions made based on it. Additionally, surgical biopsies have limitations in terms of time, repeatability, patient age, and cost and can occasionally cause harmful clinical complications (Martins *et al.,* 2021). Consequently, they are not suitable for highlighting the overall tumor profile, identifying lesions in different locations, or longitudinal monitoring of the disease.

Identifying genomic variability in liquid biopsies can significantly advance precision oncology

Liquid biopsies are emerging as key tools for addressing challenges in the prognosis, diagnosis, and monitoring of disease progression. The SCOPE technique offers several advantages, including reduced invasiveness, lower cost, and the ability to provide up-to-date information on tumor status. In some cases, it can also address the problem of tumor heterogeneity or multiple metastatic changes (Killingsworth *et al.,* 2021). These biopsies involve examination of bodily fluids, primarily blood, but can also include urine, saliva, cerebrospinal fluid (CSF), and bone marrow (Pantel & Alix-Panabières, 2019). In precision oncology, liquid biopsies enable the collection of dynamic molecular data regarding the entire tumor through minimally invasive and repeatable tests (Ignatiadis *et al.,* 2021; Killingsworth *et al.,* 2021). Consequently, both scientists and medical professionals use liquid biopsies to track tumor evolution and heterogeneity (Parikh *et al.,* 2019). The real-time insights gained from these biopsies can influence patient care in various ways, such as early detection of lesions, tracking of minimal residual disease, guiding personalized treatment decisions based on resistance profiles, and monitoring tumor recurrence (Parikh *et al.,* 2019).

Liquid biopsy components, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomes, reflect the phenotypic and genotypic properties of tumor cells. Tumor-derived exosomes transport a diverse array of molecular cargoes, such as microRNAs, long non-coding RNAs, and circular RNAs, which are subsequently delivered to recipient cells. Exosome biogenesis involves invagination of the plasma membrane to form early endosomes, which subsequently mature into late endosomes or multivesicular bodies (MVBs). MVBs either fuse with lysosomes for degradation or merge with the cell membrane to release exosomes into the extracellular milieu.

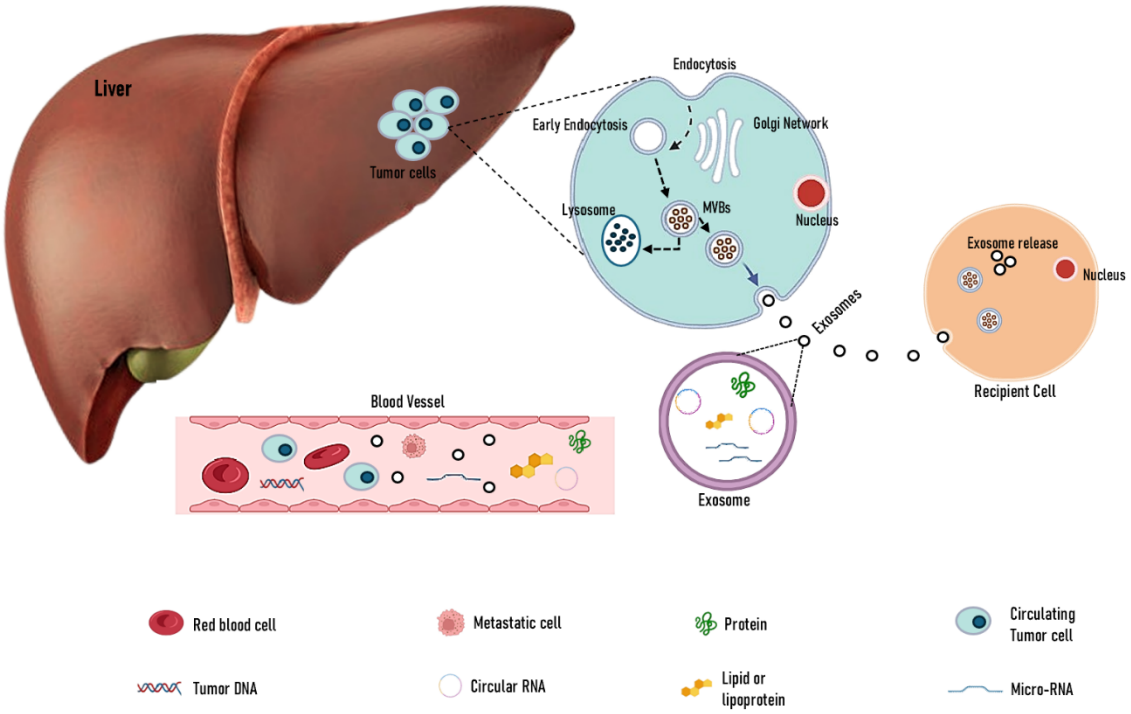

Figure 1. Overview of Liquid Biopsy Components and Exosome Biogenesis in Liver Tumor Cells.

Extracellular vesicles (EVs) constitute a heterogeneous group of membrane-bound structures that are secreted by all cell types into various biological fluids. Encapsulated within a phospholipid bilayer, EVs contain a diverse array of bioactive molecules, including proteins, lipids, and nucleic acids, originating from their parent cells (Ferlay *et al.,* 2019; Kim & Han, 2012). Based on their dimensions, biogenesis mechanisms, and molecular composition, EVs are broadly classified into two primary categories: exosomes and microparticles (MPs). These distinctions are essential for elucidating the multifaceted functions (Prince *et al.,* 2020).

Under physiological conditions, EVs are continuously secreted. However, their release is significantly elevated in pathological states such as inflammation and cancer (Martins *et al.,* 2021). Upon release, EVs can interact with recipient cells through processes such as endocytosis, delivery of functional cargo, and eliciting a diverse array of cellular responses, as explained in Figure 1 (Ignatiadis *et al.,* 2021; Llovet *et al.,* 2021). This capability enables EVs to influence both immunostimulatory and immunoinhibitory pathways, with their effects varying depending on the originating cell type and the specific bioactive content they carry (Singal *et al.,* 2014).

EVs play a crucial role in numerous biological processes, including inflammation, immune signaling, coagulation, vascular reactivity, angiogenesis, and tissue repair (Zarà *et al.,* 2019). They function as essential mediators of intercellular communication, facilitating the transfer of molecular signals between cells (Everaert, 2020). These vesicles transport vascular growth factors, such as VEGF and ANGPT2, to endothelial cells (ECs), modulating their biological characteristics and promoting angiogenesis via pathways such as AKT/eNOS. Hypoxia further enhances EV-mediated angiogenic signaling, with elevated exosomal miR-155 levels correlating with VEGF expression and vascular

density. In addition to ECs, macrophages contribute to angiogenesis through EV-transferred miRNAs, which modulate epithelial-mesenchymal transition (EMT) and vascular permeability. Furthermore, EVs can function as platforms for enzymatic activity, further expanding their range of physiological functions (Block *et al.,* 2022). These distinctive characteristics have led to their recognition as significant factors in health and disease, particularly in the context of liver disease, where they form complex regulatory networks with hepatic macrophages (Cheng *et al.,* 2024).

In pathological conditions, EVs have garnered considerable attention owing to their involvement in various disease mechanisms, including autoimmune disorders. They are being increasingly investigated as potential biomarkers for cell activity or death, offering insights into disease progression. Moreover, EVs show promise as innovative drug delivery vehicles, leveraging their inherent ability to transport molecular cargo across biological barriers (Makrygianni & Chrousos, 2023). EV cargo comprises proteins (e.g., heat shock proteins, adhesion molecules, and growth factors), nucleic acids (notably RNA, including miRNA, mRNA, and ncRNA), and lipids (e.g., ceramides and sphingolipids), which influence recipient cells and serve as potential biomarkers. Proteins such as CD63 and CD81 are particularly significant given their roles in EV formation and cargo sorting (Cheng *et al.,* 2024).

The emerging understanding of EVs underscores their importance in both physiological and pathological contexts, thereby opening new avenues for research and therapeutic development (de Lima *et al.,* 2020; Singal *et al.,* 2014). Their multifaceted roles in disease pathogenesis, cellular communication, and potential clinical applications have rendered them a focus of increasing scientific and medical interest.

Techniques for Isolating Extracellular Vesicles (EVs)

The pre-analytical phase

Successful outcomes in EVs isolation commence with the initial steps of blood extraction and acquisition of bodily fluids. The generation of artifactual EVs becomes more probable during experimental conditions due to multiple factors that particularly affect platelet-derived or red blood cell-derived EVs owing to their high sensitivity. The methodology of blood collection, the specifications of tubes and anticoagulants, transportation protocols, and the duration between sample collection and testing contribute to EVs isolation (Lacroix *et al.,* 2012). The development of standardized procedures is ongoing, which will mitigate pre-analytical variables (Witwer *et al.,* 2013). The isolation process for EVs should be conducted within the first 2 hr after sample collection, during which the samples should undergo minimal movement. Exosome isolation initiates with centrifugation at low speeds, followed by filtration or size-exclusion chromatography (SEC) before high-speed centrifugation at 100,000 g sediments them (Witwer *et al.,* 2013). Ultracentrifugation of density gradient is an efficacious method to enhance purity. The recommendations by ISTH 2010 and Lacroix *et al.,* 2012 indicate that MPs should be isolated through double centrifugation of whole blood at 2500 g for 15 minutes at room temperature.

The Analytical phase

The analysis of EVs is complicated due to their submicron dimensions and heterogeneous characteristics regarding origin, size, and composition. A standardized method to characterize EVs does not currently exist. Flow cytometry has remained the primary technique for EVs characterization for more than two decades due to its ability to analyze EVs composition along with their quantity (György *et al.,* 2011; Horstman *et al.,* 2004; Edwin Van der Pol *et al.,* 2012). The conventional flow cytometry method utilizing light scattering detection enables the detection of only large EVs that measure approximately 1 µm (Arraud *et al.,* 2014; Chandler *et al.,* 2011; E Van Der Pol *et al.,* 2012). The detection of the majority of EVs, except the smallest ones, is feasible through a recent modification of flow cytometry that uses fluorescence intensity-based detection techniques (Arraud *et al.,* 2015; Van Der Vlist *et al.,* 2012).

Improved flow cytometry instrumentation will advance research by enabling the detection of smaller objects. Evolutionary progress in EVs science began with platelet MPs detection by electron microscopy, which provided accurate images of EVs and fundamental information about size and phenotype expression (Aalberts *et al.,* 2012; György *et al.,* 2011; Heijnen *et al.,* 1999; Wolf, 1967). Unprecedented views of EVs in blood plasma and other body fluids have been achieved through recent applications of cryo-electron microscopy, a method that optimally preserves complex objects (Arraud *et al.,* 2014; Zonneveld *et al.,* 2014). Scientists established a comprehensive characterization of pure plasma EVs through their work, which demonstrated spherical EVs between 50-500 nm as the predominant population, while plasma also contains larger tubular EVs and membrane fragments exceeding 500 nm (Arraud *et al.,* 2015). This research found that Phosphatidylserine exposure affected approximately fifty percent of all detected EVs. Cryo-electron microscopy surpasses other techniques for revealing native biological fluid content at nanometer resolution by demonstrating the presence and variety of individual objects along with micrometer-sized immune complexes near EVs within arthritis patient synovial fluids. Electron microscopy remains a time-consuming and expensive analytical method that requires skilled personnel to operate, thus creating immediate limitations for clinical application. Western blotting and ELISA serve as commonly used methods for EVs phenotype examination by measuring intravesicular or membrane protein markers through antibody detection according to (Revenfeld *et al.,* 2014). EVs were examined by conducting RT-qPCR to reveal their RNA content. High-throughput EVs analysis has expanded through the introduction of two recent technologies: nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS). These techniques excel at creating size-related observations for small particles (50 nm) to facilitate the identification of purified exosome preparations. These two methods prove challenging to use for heterogeneous samples such as pure plasma since they lack the ability to distinguish EVs from contaminants such as lipoproteins (Zarà *et al.,* 2019). New performance advancements and improved fluorescent dyes within flow cytometers, along with new technological developments, will enhance EVs analysis capabilities.

Detecting of EVs mRNA revealed key somatic driver mutations essential for tumor initiation and growth.

Extracellular vesicles are emerging as significant targets in liquid biopsy research (Jo *et al.,* 2023; Shao *et al.,* 2018). These minute particles, less than 1 µm in diameter, transport various molecular components, including nucleic acids, proteins, and metabolites, effectively acting as cellular proxies (Dixson *et al.,* 2023). Analysis of EVs messenger RNA (mRNA) can yield valuable clinical insights (Nomura *et al.,* 2009). Extracellular vesicles mRNA can indicate the presence of somatic driver mutations such as KRASG12D and BRAFV600E, which are critical for tumor development (Skog *et al.,* 2008). Additionally, while EVs rarely contain nuclear proteins associated with drug resistance, they carry the corresponding mRNA, providing information about the resistance status (Daane *et al.,* 2022; van de Haar *et al.,* 2023). Vesicular encapsulation of EV mRNAs shields them from nucleases in biofluids, enabling the extraction of intact, high-quality nucleic acids (Park *et al.,* 2021). These characteristics make EVs a promising source of nucleic acids, complementing the advantages of circulating tumor DNA (ctDNA). However, technical constraints have limited the clinical application of EVs. Most EVs RNA is non-coding, and the mRNA levels in EV samples can be extremely low. For example, even abundant mRNA species, such as GAPDH, are detected at a rate of only one copy per 104–106 EVs, in contrast to microRNAs at one copy per 102 EVs (Noerholm *et al.,* 2012; Wei *et al.,* 2017).

This disparity has led most proof-of-concept studies to concentrate on miRNA detection (Park *et al.,* 2021; van de Haar *et al.,* 2023; Wei *et al.,* 2017). Furthermore, tumor-derived EVs comprise a small fraction (<5%) of total circulating EVs (Noerholm *et al.,* 2012). The scarcity of EV mRNAs and low abundance of tumor-derived EVs necessitate large sample volumes (exceeding 2 ml of plasma) and advanced technologies such as droplet-digital polymerase chain reaction (PCR) and next-generation

sequencing. This reduces the competitive edge of EV tests and hinders their incorporation into the standard preclinical and clinical assays.

A novel EVs mRNA test inspired by CRISPR technology was developed. CRISPR systems are increasingly utilized in molecular diagnostics due to their sequence-specific nuclease activity (Kaminski *et al.,* 2021; Pickar-Oliver & Gersbach, 2019). CRISPR-associated (Cas) proteins function as endonucleases when they recognize target nucleic acids (Abudayyeh & Gootenberg, 2021). This property has been exploited to amplify signals through the indiscriminate cleavage of reporter probes, such as single-stranded DNAs tagged with fluorescent dye and quencher pairs. However, applying CRISPR assays to EV mRNA is challenging due to the low abundance of targets, often requiring pre-amplification to replicate mRNA and enhance assay kinetics. This step can introduce replication errors and biases, potentially leading to misleading results (Kebschull & Zador, 2015; Potapov & Ong, 2017). To address this limitation, Cas activity was repurposed to directly recognize and replicate the target mRNA in situ, eliminating the need for pre-amplification and its associated errors. This innovative approach ensures high analytical sensitivity while maintaining sequence specificity, enabling precise detection of low-abundance mRNA targets (Song *et al.,* 2024). This review aimed to elucidate the clinical significance of the SCOPE technique in liquid biopsies for hepatocellular carcinoma (HCC) and examine its potential for prognosis, diagnosis, and monitoring of cancer progression. The mRNA amplification method and its clinical applications will also be analyzed, with a particular emphasis on the development and future prospects of the SCOPE technique.

SCOPE: A CRISPR-enhanced platform for EV mRNA detection

The SCOPE (Self-amplified and CRISPR-aided Operation to Profile EVs) platform is an innovative integrated assay for accurate EV mRNA detection and monitoring. It merges the Cas13a machinery with novel signaling templates, enhancing both specificity and sensitivity. SCOPE operates by Cas13a recognizing target RNA sequences, triggering a dual amplification process that boosts both RNA targets and fluorescent signals, thereby ensuring robust detection.

SCOPE's exceptional selectivity of SCOPE, attributed to Cas13a, allows precise single-nucleotide polymorphism differentiation. The platform achieved high sensitivity and detected subattomolar concentrations through its dual amplification mechanism, which was further refined by systematic optimization. SCOPE's versatility of SCOPE has been demonstrated in various applications, including early-stage lung cancer detection in animal models during preclinical studies. In clinical settings, it has been used to track cancer mutational burdens in patients with colorectal cancer (CRC) undergoing standard treatments, providing valuable insights into disease progression and treatment responses. Moreover, SCOPE has effectively identified crucial mutations in glioblastoma multiforme (GBM), facilitating patient stratification for more targeted treatment approaches (Song *et al.,* 2024).

The implementation of this advanced assay offers significant opportunities in both preclinical and clinical settings. It serves as a tool for understanding cancer progression mechanisms, identifying the emergence of drug resistance, and assessing tumor responses to various therapies. Beyond its scientific applications, SCOPE has the potential to significantly impact clinical workflows and drug trial decision-making processes. By accelerating standard decision making in clinical trials and enhancing the utility of extracellular vesicles (EVs) in liquid biopsy applications, SCOPE bridges the gap between cutting-edge molecular diagnostics and practical clinical use.

In summary, the SCOPE platform introduces a novel approach for incorporating extracellular vesicle profiling in cancer research and clinical practice. Its ability to provide highly accurate, sensitive, and actionable insights into tumor biology makes it an invaluable tool for advancing oncology and personalized medicine.

SCOPE technology setup

The SCOPE technology platform integrates several cutting-edge components to boost the effectiveness of EV mRNA detection (Song *et al.,* 2024). A key aspect of this approach is the use of polymer-coated tubes (pDMAEA-coated PCR tubes) for rapid and efficient nucleic acid extraction.

When an aqueous sample is introduced, the positively charged polymer binds to negatively charged nucleic acids, forming polyplexes that are then isolated through centrifugation. The SCOPE reaction occurred directly in the same tube, eliminating the need for sample transfer and streamlining the process. The system includes a fluorescence detection device with a tray-type heating block, a fluorescent optical detector, and a line scanner capable of analyzing up to 16 samples simultaneously. Separate fluorescent excitation/detection headers allow for one- or two-color measurements with high consistency and uniform temperature regulation, maintaining variations within 0.5°C across samples. Designed for ease of use, the system utilizes standard lab equipment, such as thermal cyclers, and requires minimal sample volumes (e.g., EV isolates from less than 100 µL of plasma). An intuitive graphical interface controls this process and supports its applications in clinical and preclinical molecular diagnostics.

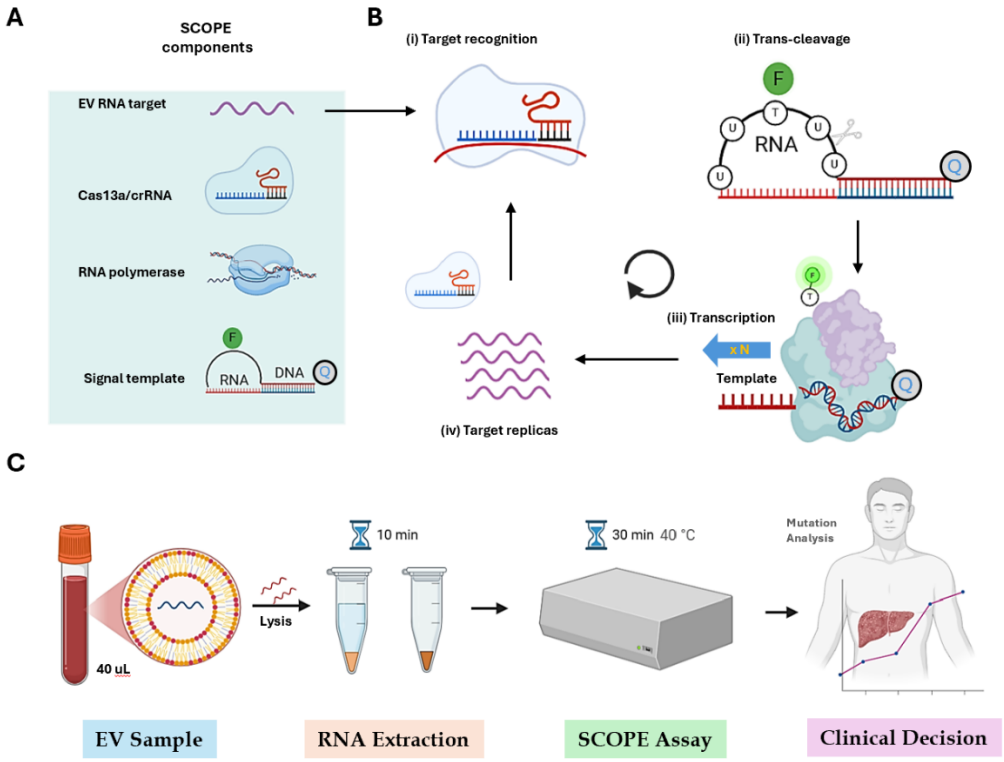

Figure 2: SCOPE workflow for on-site diagnostics. (A) Illustrating diverse elements of SCOPE, such as EV RNA target, Cas13a, RNA polymerase, and signal template. (B) Combined SCOPE components identify the target for replication. (C) The SCOPE system enables rapid on-site molecular testing. Initially, EVs were extracted from the clinical specimens and broken down. The resulting extracts were placed in specially treated containers for RNA isolation, which required approximately 10 min. Subsequently, the SCOPE reaction was performed in a small transportable device for 30 min. In total, the test delivers molecular data within an hour, allowing same-day clinical choices to be made.

SCOPE working principle

The SCOPE operational mechanism integrates CRISPR-Cas13a recognition with RNA amplification. The process begins with a combination of Cas13a, CRISPR RNA (crRNA), T7

polymerase, signal template, and deoxyribonucleotide triphosphates. The signal template, composed of RNA and DNA, plays a vital role in linking the Cas13a/crRNA and T7 polymerase reactions. Initially, Cas13a/crRNA attaches to the target RNA, thereby activating its ribonuclease function. This action cleaves the RNA portion of the signal template, releasing fluorescent dye molecules, and generating a detectable signal. Simultaneously, T7 RNA polymerase multiplies the target RNA, producing numerous copies that are then recognized and cleaved by Cas13a/crRNA, creating a powerful amplification cycle. SCOPE achieved high specificity by incorporating synthetic mismatches in crRNA, allowing Cas13a to distinguish between RNA sequences, even at the single-nucleotide level. Additionally, dual amplification through Cas13a cleavage and RNA replication ensures high sensitivity. The isothermal reaction occurs at 40°C, enabling the completion of the entire process in under an hour within a single tube (Vitale *et al.,* 2021). These features make SCOPE efficient, sensitive, and appropriate for routine laboratory use, with a significant potential for clinical diagnostic applications (Figure 2).

Kinetics of the SCOPE Assay

Researchers have employed analytical modeling to thoroughly examine the kinetics of the SCOPE assay, shedding light on the underlying mechanisms and reaction dynamics (Song *et al.,* 2024). The SCOPE method combines two separate catalytic processes facilitated by a signal template, thereby enabling efficient RNA detection and amplification. The initial process involves the binding of the Cas13a/crRNA complex to the target RNA, triggering fluorescent signaling through the cleavage of the RNA segment within the signal template. The second process uses RNA polymerase to replicate the target RNA using a DNA sequence incorporated into the signal template. When studied independently, these catalytic activities produced linear increases in the reaction products over time, aligning with zeroth-order reaction kinetics under specific assay conditions (Song *et al.,* 2024). However, coupling these processes via the signal template significantly alters the reaction kinetics, approximating a first-order reaction rate and substantially enhancing the efficiency. The SCOPE signals exhibited exponential growth, reaching a plateau within 30 min.

A key feature of the SCOPE assay is its ability to suppress off-target RNA amplification. The amplification process requires the Cas13a/crRNA complex to first recognize and bind to the target RNA, activating Cas13a to cleave the RNA segment in the signal template and initiate RNA polymerase activity. If the RNA segment remains intact, polymerase activity is inhibited, likely because the intact loop configuration of the signal template physically obstructs the polymerase from accessing the promoter region and initiating transcription. To enhance the performance of the SCOPE assay, researchers have refined the signal template design and optimized the reaction conditions for maximum signal intensity (Figure 3A). The validation experiments (Figure 3B) demonstrated that the optimal analytical signal was achieved only when all crucial assay components—target RNA, Cas13a, crRNA, and T7 RNA polymerase—were present (Song *et al.,* 2024). Excluding RNA polymerase from the reaction significantly reduced the signal intensity by halting additional RNA target generation. These findings highlight the robustness and efficiency of the SCOPE assay and confirm its reliability for RNA detection and amplification. The ability of this assay to combine rapid signal generation with high specificity makes it a valuable tool for various molecular diagnostic applications.

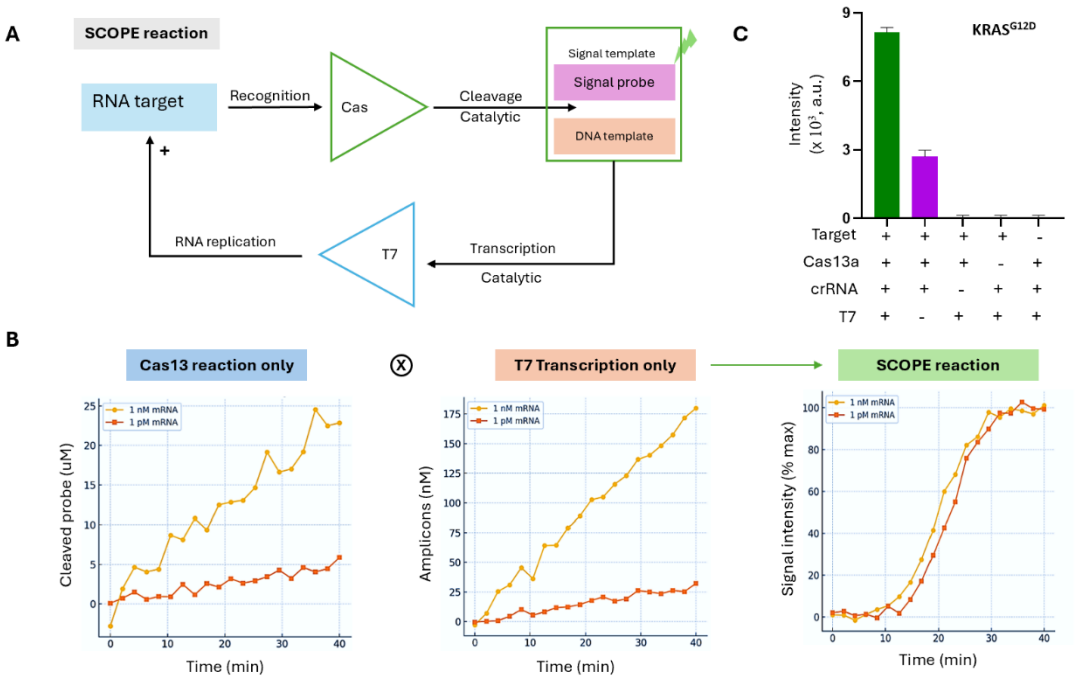

Figure 3: SCOPE assay dynamics. (A) The SCOPE method integrates two enzymatic processes: Cas13a/crRNA produces fluorescence by breaking down RNA in the signal template, whereas T7 polymerase multiplies RNA targets. (B) When operating independently, Cas13a/crRNA and T7 reactions displayed linear product increases over time. However, when combined with SCOPE, they result in exponential signal enhancement, achieving a plateau within 30 min. Experimental confirmation demonstrated a peak signal when all components were present; eliminating T7 polymerase or impeding mRNA recognition diminished signal strength. (C) The starting KRASG12D RNA concentration was 1 nm.

Conclusion and Future Prospects

Self-amplified and CRISPR-aided Operation to Profile EVs (SCOPE) technology represents a significant advancement in liquid biopsy applications and offers exceptional sensitivity, specificity, and accessibility. Its capacity to detect genetic mutations at subattomolar concentrations and differentiate single-nucleotide variations surpasses conventional diagnostic tools. By facilitating the analysis of EV mRNA, SCOPE addresses critical challenges in early cancer detection and monitoring, particularly in malignancies such as liver cancer, where existing diagnostic approaches often fail to identify the disease at an early stage. Furthermore, the versatility of this technology extends to monitoring treatment responses in real time, assessing minimal residual disease, and tumor subtyping. With its low cost (less than $4 per marker), rapid assay times (approximately 30 minutes), and compatibility with standard laboratory equipment, SCOPE has the potential to revolutionize clinical and research practices by rendering liquid biopsy both accessible and practical for routine use (Daane *et al.,* 2022).

While SCOPE shows significant promise, several obstacles must be overcome to fully harness its potential. One major issue is the unintended isolation of other extracellular RNA carriers, including lipoproteins and EVs, from platelets during the sample preparation process using size exclusion chromatography (SEC). These unwanted components can increase background noise, make data analysis more complex, and potentially decrease the diagnostic precision. To tackle this problem, advanced techniques, such as single-vesicle imaging, can be employed. This method involves labelling and monitoring specific proteins on vesicle surfaces, which can greatly improve the accuracy of target identification and enhance the reliability of SCOPE diagnostic results.

Future research should focus on validating the SCOPE across a broader spectrum of cancers and diverse treatment settings. Integrating the analysis of EV mRNA with circulating tumor DNA (ctDNA) could provide a more comprehensive molecular tumor profile by capturing both transcript-level changes and unique genomic alterations, such as promoter mutations and methylation patterns (Everaert, 2020). This dual approach would bridge the gap between transcriptomics and genomics, ensuring improved diagnostic precision and enabling a deeper understanding of tumor biology. Furthermore, optimizing SCOPE for multimodal treatment strategies and establishing cancer-specific timelines for EV analysis after surgery or therapy initiation will enhance its clinical relevance. For instance, EV mutational loads observed in patients with colorectal cancer (CRC) demonstrate fluctuations after surgery and during chemotherapy, highlighting the importance of defining optimal timeframes for sample collection to refine prognostic predictions and guide adjuvant therapy decisions (Daane *et al.,* 2022).

Self-amplified and CRISPR-aided Operation to Profile EVs (SCOPE) exhibits considerable promise in preclinical drug development, offering swift and accurate insights into tumor biology that can accelerate the assessment of therapeutic responses. Its capacity to identify point mutations with minimal interference, even at low variant allele frequencies (such as 0.01%), outperforms many advanced techniques including digital PCR and BEAMing PCR, making it an invaluable tool for drug testing. These attributes can streamline the drug discovery processes, enhance experimental therapies, and promote clinical translation.

The revolutionary aspect of SCOPE lies in its ability to provide same-day results and enable real-time clinical decision-making. This rapid turnaround, combined with its high sensitivity and cost-efficiency, makes it particularly well-suited for applications such as early cancer detection, monitoring recurrence, and personalizing treatment. When integrated with complementary methods, such as the analysis of EVs mRNA and ctDNA, SCOPE offers a comprehensive molecular profile of tumors, ensuring more precise diagnoses and customized treatment plans.

Addressing current limitations, such as improving EVs specificity, expanding the range of cancers analyzed, and exploring its utility in multimodal treatment contexts, will be critical to realizing SCOPE's full potential of SCOPE. For instance, in cases such as EGFRvIII deletion, in which designing a specific ctDNA assay is challenging, the detection of EV mRNA through SCOPE provides a more feasible and effective alternative. Additionally, incorporating new imaging techniques to study the origin of EVs-associated mRNA will further enhance its utility as a diagnostic tool.

Building on these advancements, SCOPE is well-positioned to redefine cancer diagnostics and treatment monitoring. Its ability to provide a reliable, accessible, and comprehensive molecular analysis platform will undoubtedly enhance precision oncology, improve patient outcomes, and establish a new standard for liquid biopsy in both academic and clinical settings.

Data and code accessibility

The authors confirm that the data presented in the article and additional data can be provided by the corresponding author upon request.

Author contributions

A.H., and M.A., have gathered data, outlined and finalized the initial manuscript draft. M.K., G.M., Z.U.A., A.H., M.Q., Y.R., and M.B., helped to analyze data. W.S., provided revisions and finalized the manuscript. All authors have reviewed and approved the final manuscript.

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References

Aalberts, M., van Dissel-Emiliani, F. M., van Adrichem, N. P., van Wijnen, M., Wauben, M. H., Stout, T. A., & Stoorvogel, W. (2012). Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. *Biology of reproduction*, *86*(3), 82, 81-88.

Abudayyeh, O. O., & Gootenberg, J. S. (2021). CRISPR diagnostics. *Science*, *372*(6545), 914-915.

Arraud, N., Gounou, C., Linares, R., & Brisson, A. R. (2015). A simple flow cytometry method improves the detection of phosphatidylserine-exposing extracellular vesicles. *Journal of Thrombosis and Haemostasis*, *13*(2), 237-247.

Arraud, N., Linares, R., Tan, S., Gounou, C., Pasquet, J. M., Mornet, S., & Brisson, A. R. (2014). Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *Journal of Thrombosis and Haemostasis*, *12*(5), 614-627.

Block, T., Zezulinski, D., Kaplan, D. E., Lu, J., Zanine, S., Zhan, T., Doria, C., & Sayeed, A. (2022). Circulating messenger RNA variants as a potential biomarker for surveillance of hepatocellular carcinoma. *Frontiers in oncology*, *12*, 963641.

Chandler, W., Yeung, W., & Tait, J. (2011). A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer. *Journal of Thrombosis and Haemostasis*, *9*(6), 1216-1224.

Cheng, L., Zhang, L., Wang, X., Wang, Y., Yu, J., Li, M., Ma, Z., Ho, P. C.-L., Chen, X., & Wang, L. (2024). Extracellular vesicles in the HCC microenvironment: Implications for therapy and biomarkers. *Pharmacological research*, 107419.

Daane, M., Vigneri, P., Sieuwerts, A., Dits, N., van Royen, M., Jenster, G., Sleijfer, S., Lolkema, M., Martens, J., & PHM, J. M. (2022). Detection of tumor-derived extracellular

vesicles in plasma from patients with solid cancer. *Evaluation of Liquid Biopsy Workflows and ESR1 Alterations in Breast Cancer*, *21*, 85.

de Lima, L. T. F., Broszczak, D., Zhang, X., Bridle, K., Crawford, D., & Punyadeera, C. (2020). The use of minimally invasive biomarkers for the diagnosis and prognosis of hepatocellular carcinoma. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1874*(2), 188451.

Dixson, A. C., Dawson, T. R., Di Vizio, D., & Weaver, A. M. (2023). Context-specific regulation of extracellular vesicle biogenesis and cargo selection. *Nature reviews Molecular cell biology*, *24*(7), 454-476.

Everaert, C. (2020). *Development of extracellular RNA sequencing technologies and applications for cancer diagnosis and monitoring* Ghent University].

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2019). Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *International journal of cancer*, *144*(8), 1941-1953.

György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., László, V., Pállinger, E., Pap, E., & Kittel, A. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and molecular life sciences*, *68*, 2667-2688.

Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J., & Sixma, J. J. (1999). Activated Platelets Release Two Types of Membrane Vesicles: Microvesicles by Surface Shedding and Exosomes Derived From Exocytosis of Multivesicular Bodies and□-Granules. *Blood, The Journal of the American Society of Hematology*, *94*(11), 3791-3799.

Horstman, L. L., Jy, W., Jimenez, J. J., Bidot, C., & Ahn, Y. S. (2004). New horizons in the analysis of circulating cell-derived microparticles. *The Keio journal of medicine*, *53*(4), 210-230.

Ignatiadis, M., Sledge, G. W., & Jeffrey, S. S. (2021). Liquid biopsy enters the clinic—implementation issues and future challenges. *Nature reviews Clinical oncology*, *18*(5), 297-312.

Jo, A., Green, A., Medina, J. E., Iyer, S., Ohman, A. W., McCarthy, E. T., Reinhardt, F., Gerton, T., Demehin, D., & Mishra, R. (2023). Inaugurating High-Throughput Profiling of Extracellular Vesicles for Earlier Ovarian Cancer Detection (Adv. Sci. 27/2023). *Advanced Science*, *10*(27), 2370180.

Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F., & Collins, J. J. (2021). CRISPR-based diagnostics. *Nature Biomedical Engineering*, *5*(7), 643-656.

Kebschull, J. M., & Zador, A. M. (2015). Sources of PCR-induced distortions in high-throughput sequencing data sets. *Nucleic acids research*, *43*(21), e143-e143.

Killingsworth, B., Welsh, J. A., & Jones, J. C. (2021). EV translational horizons as viewed across the complex landscape of liquid biopsies. *Frontiers in cell and developmental biology*, *9*, 556837.

Kim, D. Y., & Han, K.-H. (2012). Epidemiology and surveillance of hepatocellular carcinoma. *Liver cancer*, *1*(1), 2-14.

Lacroix, R., Judicone, C., Poncelet, P., Robert, S., Arnaud, L., Sampol, J., & DIGNAT-GEORGE, F. (2012). Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *Journal of Thrombosis and Haemostasis*, *10*(3), 437-446.

Llovet, J. M., Kelley, R. K., Villanueva, A., Singal, A. G., Pikarsky, E., Roayaie, S., Lencioni, R., Koike, K., Zucman-Rossi, J., & Finn, R. S. (2021). Hepatocellular carcinoma (primer). *Nature Reviews: Disease Primers*, *7*(1), 6.

Makrygianni, E. A., & Chrousos, G. P. (2023). Extracellular vesicles and the stress system. *Neuroendocrinology*, *113*(2), 120-167.

Martins, I., Ribeiro, I. P., Jorge, J., Gonçalves, A. C., Sarmento-Ribeiro, A. B., Melo, J. B., & Carreira, I. M. (2021). Liquid biopsies: applications for cancer diagnosis and monitoring. *Genes*, *12*(3), 349.

Moldogazieva, N. T., Zavadskiy, S. P., & Terentiev, A. A. (2021). Genomic landscape of liquid biopsy for hepatocellular carcinoma personalized medicine. *Cancer Genomics & Proteomics*, *18*(3 Suppl), 369-383.

Noerholm, M., Balaj, L., Limperg, T., Salehi, A., Zhu, L. D., Hochberg, F. H., Breakefield, X. O., Carter, B. S., & Skog, J. (2012). RNA expression patterns in serum microvesicles from patients with glioblastoma multiforme and controls. *BMC cancer*, *12*, 1-11.

Nomura, S., Shouzu, A., Taomoto, K., Togane, Y., Goto, S., Ozaki, Y., Uchiyama, S., & Ikeda, Y. (2009). Assessment of an ELISA kit for platelet-derived microparticles by joint research at many institutes in Japan. *Journal of atherosclerosis and thrombosis*, *16*(6), 878-887.

Pantel, K., & Alix-Panabières, C. (2019). Liquid biopsy and minimal residual disease—latest advances and implications for cure. *Nature reviews Clinical oncology*, *16*(7), 409-424.

Parikh, A. R., Leshchiner, I., Elagina, L., Goyal, L., Levovitz, C., Siravegna, G., Livitz, D., Rhrissorrakrai, K., Martin, E. E., & Van Seventer, E. E. (2019). Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nature medicine*, *25*(9), 1415-1421.

Park, J., Park, J. S., Huang, C.-H., Jo, A., Cook, K., Wang, R., Lin, H.-Y., Van Deun, J., Li, H., & Min, J. (2021). An integrated magneto-electrochemical device for the rapid profiling of tumour extracellular vesicles from blood plasma. *Nature Biomedical Engineering*, *5*(7), 678-689.

Pickar-Oliver, A., & Gersbach, C. A. (2019). The next generation of CRISPR–Cas technologies and applications. *Nature reviews Molecular cell biology*, *20*(8), 490-507.

Potapov, V., & Ong, J. L. (2017). Examining sources of error in PCR by single-molecule sequencing. *PLoS One*, *12*(1), e0169774.

Prince, D., Liu, K., Xu, W., Chen, M., Sun, J.-Y., Lu, X.-J., & Ji, J. (2020). Management of patients with intermediate stage hepatocellular carcinoma. *Therapeutic Advances in Medical Oncology*, *12*, 1758835920970840.

Revenfeld, A. L. S., Bæk, R., Nielsen, M. H., Stensballe, A., Varming, K., & Jørgensen, M. (2014). Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. *Clinical therapeutics*, *36*(6), 830-846.

Shao, H., Im, H., Castro, C. M., Breakefield, X., Weissleder, R., & Lee, H. (2018). New technologies for analysis of extracellular vesicles. *Chemical reviews*, *118*(4), 1917-1950.

Singal, A. G., Pillai, A., & Tiro, J. (2014). Early detection, curative treatment, and survival rates for hepatocellular carcinoma surveillance in patients with cirrhosis: a meta-analysis. *PLoS medicine*, *11*(4), e1001624.

Skog, J., Würdinger, T., Van Rijn, S., Meijer, D. H., Gainche, L., Curry Jr, W. T., Carter, B. S., Krichevsky, A. M., & Breakefield, X. O. (2008). Glioblastoma microvesicles transport RNA

and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature cell biology*, *10*(12), 1470-1476.

Song, J., Cho, M. H., Cho, H., Song, Y., Lee, S. W., Nam, H. C., Yoon, T. H., Shin, J. C., Hong, J.-S., & Kim, Y. (2024). Amplifying mutational profiling of extracellular vesicle mRNA with SCOPE. *Nature Biotechnology*, 1-11.

van de Haar, J., Ma, X., Ooft, S. N., van der Helm, P. W., Hoes, L. R., Mainardi, S., Pinato, D. J., Sun, K., Salvatore, L., & Tortora, G. (2023). Codon-specific KRAS mutations predict survival benefit of trifluridine/tipiracil in metastatic colorectal cancer. *Nature medicine*, *29*(3), 605-614.

Van der Pol, E., Böing, A. N., Harrison, P., Sturk, A., & Nieuwland, R. (2012). Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacological reviews*, *64*(3), 676-705.

Van Der Pol, E., Van Gemert, M., Sturk, A., Nieuwland, R., & Van Leeuwen, T. (2012). Single vs. swarm detection of microparticles and exosomes by flow cytometry. *Journal of Thrombosis and Haemostasis*, *10*(5), 919-930.

Van Der Vlist, E. J., Nolte-'t Hoen, E. N., Stoorvogel, W., Arkesteijn, G. J., & Wauben, M. H. (2012). Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. *Nature protocols*, *7*(7), 1311-1326.

Vitale, S. R., Helmijr, J. A., Gerritsen, M., Coban, H., van Dessel, L. F., Beije, N., van der Vlugt-Daane, M., Vigneri, P., Sieuwerts, A. M., & Dits, N. (2021). Detection of tumor-derived extracellular vesicles in plasma from patients with solid cancer. *BMC cancer*, *21*, 1-17.

Wei, Z., Batagov, A. O., Schinelli, S., Wang, J., Wang, Y., El Fatimy, R., Rabinovsky, R., Balaj, L., Chen, C. C., & Hochberg, F. (2017). Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nature communications*, *8*(1), 1145.

Witwer, K. W., Buzás, E. I., Bemis, L. T., Bora, A., Lässer, C., Lötvall, J., Nolte-'t Hoen, E. N., Piper, M. G., Sivaraman, S., & Skog, J. (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *Journal of extracellular vesicles*, *2*(1), 20360.

Wolf, P. (1967). The nature and significance of platelet products in human plasma. *British journal of haematology*, *13*(3), 269-288.

Zarà, M., Guidetti, G. F., Camera, M., Canobbio, I., Amadio, P., Torti, M., Tremoli, E., & Barbieri, S. S. (2019). Biology and role of extracellular vesicles (EVs) in the pathogenesis of thrombosis. *International journal of molecular sciences*, *20*(11), 2840.

Zonneveld, M. I., Brisson, A. R., van Herwijnen, M. J., Tan, S., van de Lest, C. H., Redegeld, F. A., Garssen, J., Wauben, M. H., & Nolte-'t Hoen, E. N. (2014). Recovery of extracellular vesicles from human breast milk is influenced by sample collection and vesicle isolation procedures. *Journal of extracellular vesicles*, *3*(1), 24215.