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SHORT COMMUNICATION

EV-ADD, a database for EV-associated DNA in human liquid biopsy samples

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Abstract

Extracellular vesicles (EVs) play a key role in cellular communication both in physiological conditions and in pathologies such as cancer. Emerging evidence has shown that EVs are active carriers of molecular cargo (e.g. protein and nucleic acids) and a powerful source of biomarkers and targets. While recent studies on EV-associated DNA (EV-DNA) in human biofluids have generated a large amount of data, there is currently no database that catalogues information on EV-DNA. To fill this gap, we have manually curated a database of EV-DNA data derived from human biofluids (liquid biopsy) and *in-vitro* studies, called the Extracellular Vesicle-Associated DNA Database (EV-ADD). This database contains validated experimental details and data extracted from peer-reviewed published literature. It can be easily queried to search for EV isolation methods and characterization, EV-DNA isolation techniques, quality validation, DNA fragment size, volume of starting material, gene names and disease context. Currently, our database contains samples representing 23 diseases, with 13 different types of EV isolation techniques applied on eight different human biofluids (e.g. blood, saliva). In addition, EV-ADD encompasses EV-DNA data both representing the whole genome and specifically including oncogenes, such as *KRAS, EGFR, BRAF, MYC*, and mitochondrial DNA (mtDNA). An EV-ADD data metric system was also integrated to assign a compliancy score to the MISEV guidelines based on experimental parameters reported in each study. While currently available databases document the presence of proteins, lipids, RNA and metabolites in EVs (e.g. Vesiclepedia, ExoCarta, ExoBCD, EVpedia, and EV-TRACK), to the best of our knowledge, EV-ADD is the first of its kind to compile all available EV-DNA datasets derived from human biofluid samples. We believe that this database provides an important reference resource on EV-DNA-based liquid biopsy research, serving as a learning tool and to showcase the latest developments in the EV-DNA field. EV-ADD will be updated yearly as newly published EV-DNA data becomes available and it is freely available at www.evdnadatabase.com.

KEYWORDS
cfDNA, database, EV-ADD, EV-DNA, extracellular vesicles, liquid biopsy

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1 | BACKGROUND

Extracellular material, such as extracellular vesicles (EVs), proteins and nucleic acids, emitted from cells are now recognized as important mediators in normal physiology and in disease states. Such molecules can be easily isolated and purified from biofluids, such as amniotic fluid, ascites fluid, bile, blood, breast milk, cerebrospinal fluid, pleural effusion, saliva, semen, and urine, making them important candidates as biomarkers in many contexts, including cellular homeostasis, infectious diseases, neonatal screening, and most studied: cancer. Cell-free DNA (cfDNA) (Bustamante et al., 2021; Keller et al., 2021; Osumi et al., 2019) has been identified and isolated from liquid biopsies, and shows great potential to screen, detect and monitor various cancers. This is reflected in the growing number of liquid biopsy tests approved by the Food and Drug Administration (FDA), (i.e. Epi proColon (Nian et al., 2017), cobas EGFR test v2 (Kwapisz, 2017)). cfDNA is emitted during senescence, necrosis, cell death (Rostami et al., 2020), and recently evidence has emerged of active secretion (Bronkhorst et al., 2016; Jeppesen et al., 2019; Stroun et al., 2001; Yokoi et al., 2019). Moreover, studies have shown that DNA can be associated with EVs, which has also garnered a lot of attention in the field of liquid biopsy biomarkers (Malkin & Bratman, 2020).

EVs are a highly heterogeneous group of nanosized phospholipid bilayered membrane entities emitted by virtually all tissue cells. Aside from being isolated from the tissue of origin, these particles are shed and can be isolated with relatively high purity from various human biofluids (Crescitelli et al., 2021; Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015). They act as messengers between cells and tissues by carrying molecular cargo, such as RNA, DNA, membrane-anchored and cytosolic proteins, lipids, and metabolites inside and on the surface of the vesicle (Colombo et al., 2014; Neuberger et al., 2021). EVs are categorized as large/medium and small structures based on their biogenesis, size and cargo. Large/medium EVs include oncosomes, apoptotic bodies, exopheres, migrasomes, and microvesicles, while small EVs include exosomes (exosome-large, 90–120 nm and exosome-small, 60–80 nm) and small EV clusters (sEVC) (Ma et al., 2015; Malkin & Bratman, 2020; Théry et al., 2018; Valcz et al., 2019; Witwer & Théry, 2019; Zhang et al., 2018). The list of EV subtypes continues to grow as the field progresses and the above list is not exhaustive, but it provides evidence of EV heterogeneity. Recently, distinct nanoparticles named 'exomeres' (size < 50 nm), have been discovered using asymmetric-flow field-flow fractionation (AF4) (Zhang et al., 2018, 2019). Exomeres are not the only small non-EV nanoparticles. More recently, supermeres (Clancy et al., 2021; Zhang et al., 2021) and chromatimeres were also discovered (Choi et al., 2019). More comprehensive review articles on EV subtypes are reported elsewhere (Doyle & Wang, 2019; György et al., 2011; Margolis & Sadovsky, 2019; Yáñez-Mó et al., 2015; Zaborowski et al., 2015). In this manuscript, we will use EVs as a generic terminology for any nano-sized particles emitted naturally from cells (Théry et al., 2018).

EVs can entrap biomolecules and mediate intercellular communication under various physiological and pathological conditions (Yáñez-Mó et al., 2015). While much of the literature has focused on proteins and RNA cargo in EVs, numerous studies have reported the presence of DNA either associated with the surface of EVs or within their lumen. To our knowledge, the first report of EV carrying genomic DNA and mitochondrial DNA (mtDNA) in human plasma was published in 2013 (Cai et al., 2013), followed by another report describing the presence of mutant *KRAS* and *TP53* DNA in exosomes from cancer patient serum (Kahlert et al., 2014). Since then, numerous studies have published on EV-associated DNA (EV-DNA) in cell culture (Lee et al., 2014; Thakur et al., 2014) and biological fluids (Allenson et al., 2017; Fernando et al., 2018; Garcia-Silva et al., 2019; Jin et al., 2016; San Lucas et al., 2016), the latter making EV-DNA a promising candidate for liquid biopsy (Chang et al., 2020; Garcia-Silva et al., 2021; Malkin & Bratman, 2020). EVs are ubiquitously present in biological fluids and carry large fragments of intact DNA due to lipid encapsulation providing protection against DNase-induced degradation (Cai et al., 2013; Degli Esposti et al., 2021; Fernando et al., 2018; Kahlert et al., 2014; Vagner et al., 2018). This suggests that EV-DNA presents advantages compared to cfDNA (Garcia-Silva et al., 2021), therefore the combination of EV-DNA and cfDNA analysis may improve assay sensitivity and specificity (Castellanos-rizaldos et al., 2018; Zocco et al., 2020). EV-DNA is being investigated in a number of applications. Recent studies suggested that exosome DNA isolated from maternal plasma can be used to predict fetal sex and Rhesus D (*RHD*) genotype (Yaşa et al., 2021). In cancer patients, bioactive DNA from EVs is being investigated as a biomarker, as a means to monitor disease and treatment response in liquid biopsy (Choi et al., 2019; Yokoi et al., 2019) and to detect mutations to differentiate cancer patients from non-cancer patients (Allenson et al., 2017; Bernard et al., 2019; Yang et al., 2017). Data has shown that DNA contained in human serum-derived EVs spans the entire genome and reflects the mutational status of the parental tumor (Bart et al., 2021; Degli Esposti et al., 2021; Kahlert et al., 2014; Wang et al., 2018). Moreover, studies have demonstrated the utility of EV-DNA as a biomarker in cancer. Indeed, next-generation sequencing of EV-DNA for common hotspot mutations (*BRAF, EGFR* and *KRAS*) has shown higher sensitivity compared to tumor and cfDNA derived from plasma. Fernando et al., reported that more than 93% of total cfDNA in plasma is located in exosomes (Fernando et al., 2017). In another study, ddPCR of EV-DNA outperformed cfDNA for the detection of *KRAS* mutant copies in pancreatic cancer patients (Allenson et al., 2017). Moreover, EVs isolated from early-stage pancreatic cancer plasma demonstrated that *KRAS* mutant copies were significantly higher in small EVs compared to the other seven fractions of blood (red blood cells, white blood cells, platelets, apoptotic bodies,

large EVs, soluble proteins, and flowthrough/EV free-supernatant) (Hagey et al., 2021). Lastly, studies have shown that as little as 0.2–1 ml of plasma can be used for the detection of hotspot mutations in EV-DNA (Allenson et al., 2017; Möhrmann et al., 2018). EV-DNA, therefore, allows promising liquid biopsy approaches for diagnosis (Castellanos-rizaldos et al., 2018; Helmig et al., 2015; Keserű et al., 2019; Sansone et al., 2017), prognosis (Allenson et al., 2017; Bernard et al., 2019) and monitoring of treatment response in many cancers (Möhrmann et al., 2018), as well as real-time evaluation of disease development (Wang et al., 2021).

The topology of EV-DNA is also under investigation, and the true nature of DNA packaging and localization is not yet known (Malkin & Bratman, 2020). While the early EV studies mainly reported EV-DNA inside EV lumens (Kahlert et al., 2014; Lazaro-Ibanez et al., 2014; Lee et al., 2014; Takahashi et al., 2017; Thakur et al., 2014), recent studies found EV-DNA was predominantly on the surface of EVs, particularly in the small EVs (Lázaro-Ibáñez et al., 2019; Liu et al., 2022; Maire et al., 2021), and internal DNA was mainly in large EVs (Vagner et al., 2018). Using high-resolution iodixanol density fractionation, Lázaro-Ibáñez and colleagues divided small EVs into high density (HD) and low density (LD) and found that most of the DNA was associated with the HD fraction (Lázaro-Ibáñez et al., 2019), which was considered as non-canonical exosomes or non-vesicular materials that originated from subcellular organelles (Liu et al., 2022). HD fractions also contained larger DNA fragments than LD fractions. Whether the majority of EV-DNA is ssDNA or dsDNA is still under debate (Balaj et al., 2011; Lázaro-Ibáñez et al., 2019; Liu et al., 2022; Thakur et al., 2014). Atomic force microscopy or specific enzymatic treatment are recommended for more precise determination of ssDNA vs dsDNA ratio (Lázaro-Ibáñez et al., 2019; Liu et al., 2022). Furthermore, genotoxic drug treatment (Choi et al., 2019; Liu et al., 2022) and antibiotics may influence EV-DNA emission. For example, *in-vitro* studies showed that Jurkat and MiaPaCa cell lines treated with antibiotics (ciprofloxacin) emit chromosomal DNA and mtDNA on the surface of exosomes (CD63+ and floating density 1.09 g/ml) (Németh et al., 2017). The surface-bound chromosomal DNA and DNA binding proteins (histones H2A and H3) mediate exosomal adhesion to extracellular matrix protein (fibronectin) (Németh et al., 2017) and binding to the recipient cell surface (Gladys et al., 2014). The topology of EV-DNA is associated with specific EV origin, EV size, EV nomenclature, DNA size, DNA type, histone and DNA-binding proteins. Therefore, taking EV-DNA topology into account and protecting EV surface DNA from nucleases during EV isolation and characterization is fundamental for yielding accurate results in EV-DNA research (Lázaro-Ibáñez et al., 2019; Liu et al., 2022). Finally, a consensus-building approach is necessary (Malkin & Bratman, 2020) in order to achieve standardization of the above-mentioned techniques to guarantee the reproducibility of EV cargo characterization, which is especially important in the clinical setting.

Despite the growing interest in EV-DNA based liquid biopsy, the functional significance of EV-DNA derived from biofluids is largely unknown. As EV-DNA is largely a result of cell death mechanisms, it potentially plays a role in maintaining physiological hemostasis by acting as a mechanism to expel damaged DNA (Takahashi et al., 2017). Studies from our group and others have suggested that cancer EVs can exert effects on recipient cells through transfer of cargo (Abdouh et al., 2019; Cai et al., 2013). Reports have shown DNA integration into the genomes of recipient cells. Lee et al. demonstrated the transfer of full-length double-stranded oncogenic *H-RAS* DNA via EVs, resulting in changes to recipient cell behaviour (Lee et al., 2014). However, the biological role of EVs is still under investigation. A seminal work from the David Lyden group demonstrated that exosomes derived from pancreatic cancer cells induce pre-metastatic niche formation in an *in-vivo* model and facilitate tumor progression (Costa-Silva et al., 2015).

The potential of EV-based liquid biopsy is still being explored, and the number of review articles and experimental data published on EVs has increased in multiple scientific fields, particularly in the field of cancer biology (Supplementary Figure 1). EV-associated miRNA, mRNA and proteins are already established and considered as promising candidates to serve as biomarkers (reviewed in (Janas et al., 2015; Shen et al., 2020; Tamura et al., 2021)). This has led to the development of EV databases such as ExoCarta (Keerthikumar et al., 2016), Vesiclepedia (Kalra et al., 2012; Pathan et al., 2018), EVpedia (Kim et al., 2013), and EV-TRACK (EV-TRACK Consortium et al., 2017) that have been developed to uniformize and facilitate research on EV-associated proteins, RNA, lipids, and metabolites. In contrast, EV-DNA experimental data remains buried in published literature. As more data is generated on EV-DNA, it will be ever more imperative that available datasets are rendered computer indexable, to allow for rapid tracking and facilitate access to users. Currently, there are no online resources on the collection of EV-DNA information based on manually curated literature. To address this need, we have compiled all currently published EV-DNA data from human liquid biopsy samples into a publicly available online database, named the Extracellular Vesicle - Associated DNA Database (EV-ADD), which will serve as a repository of EV-associated DNA data derived from human biofluids. The database was complemented with studies addressing the presence of EV-DNA in *in-vitro* set-ups. Data were collected using Web of Science and NCBI's PubMed system and literature reviews published in the EV-DNA field to assure adequate and efficient coverage, and then manually curated into EV-ADD. Currently, EV-ADD comprises 13 different types of EV isolation techniques and more than 10 methods of DNA isolation, five assays for EV-DNA quantification, eight human biofluids types, 23 diseases and healthy control data (Table 1). The database will be expanded as more data is added by EV communities.

TABLE 1 Data summary of EV-DNA samples currently included in EV-ADD

Diseases	Acute myeloid leukemia (AML)
	Autism spectrum disorder (ASD)
	Bladder cancer
	Breast cancer
	Coronary artery disease
	Colorectal cancer
	Dermatomyositis
	Glioma
	Hepatocellular carcinoma
	Human Immunodeficiency Virus (HIV)
	Late-stage ovarian cancer
	Lung adenocarcinoma
	Metastatic colorectal cancer (mCRC)
	Metastatic melanoma
	Multiple sclerosis
	Neuroblastoma
	Non-small cell lung cancer (NSCLC)
	Osteosarcoma
	Pancreatic ductal adenocarcinoma (PDAC)
	Prostate cancer
	Sepsis
	Squamous cell carcinoma
	Urothelial bladder carcinoma (UBC)
Human biofluids	Ascites
	Bronchoalveolar lavage fluid (BALF)
	Lymphatic drainage
	Plasma
	Pleural effusion
	Serum
	Sweat
	Urine
EV isolation techniques	exoEasy kit
	ExoLution™ Plus
	ExoQuick Exosome Precipitation Solution
	Immunocapture
	Lipid nanoprobe functionalized nanostructured silica platform
	miRCURY™ Exosome isolation kit
	MITEV (Microfluidic Isolation of Tumor-derived Extracellular Vesicles)
	Rapid magnetic beads isolation with lipids based nanoprobes
	Size exclusion chromatography
	Sucrose density gradient
	Total Exosome isolation kit
	Ultracentrifugation
	Vn96 ME Kit

(Continues)

TABLE 1 (Continued)

EV-DNA isolation methods	DNeasy blood & Tissue Kit
	ExoLution Plus™
	MagAttract High Molecular Weight DNA kit
	Maxwell® RSC ccfDNA Plasma Kit
	miRNeasy Micro kit
	Phenol-choloroform, ethanol precipitation
	QIAamp Circulating Nucleic Acid Kit
	QIAamp DNA Mini Kit
	SeleCTEV™ DNA sample-prep kit
	TIANamp Genomic DNA Kit
DNA detection methods	Bioanalyzer
	Droplet digital PCR
	Nanodrop
	Next-generation sequencing
	Quantitative PCR
	Qubit
	Taqman Copy Number RNase P Detection kit

2 | METHODS

2.1 | Database content compilation

To obtain comprehensive information on EV-DNA isolated from *in-vitro* (cell culture supernatants) and biofluids of patients and healthy controls, a combination of keywords (Supplementary Table 1) '(((extracellular vesicles) OR (exosomes)) OR (microvesicles)) AND (DNA)' derived from Medical Subject Headings (MeSH) and non-MeSH terms were searched in the Web of Science and PubMed from January 1980 until June 2022. Of note, human biofluid samples including ascites, bronchoalveolar lavage fluid, cerebrospinal fluids, lymphatic drainage, plasma, pleural effusion, saliva, sweat, and urine were searched individually in both search engines. This filter returned 734 and 428 (total = 963) search results from Web of Science and PubMed, respectively, for the biofluids database. Articles with duplicated PubMed IDs were excluded, and the remaining articles were carefully examined using the title, abstract and full text of each publication. A total of 887 publications were eliminated because they were reviews or did not address human biofluids samples. Finally, we also eliminated an article that was not published in the English language (Figure 1). The list of articles obtained was manually verified, and only those reporting EV-DNA in human biofluids were included (total = 76 studies). We then manually extracted critical information, particularly the reported methods and variables that may affect the experimental outcome, including volume of biofluids, EV isolation method, EV characterization strategy, EV-DNA isolation and detection technique, assays used to determine EV-DNA fragment size, genes used for EV-DNA detection, enzymatic and detergent treatments (if any), DNA type, disease type and reference to the original paper. These data are then provided as tabular archives in EV-ADD. If the data was not reported or is missing, EV-ADD reports 'Not reported' or 'Not tested', respectively. Moreover, if exosomes were not purified and characterized as per MISEV guidelines, EV-ADD reports 'unspecified' under the EV subtypes column. For the *in-vitro* data mining, we used the same keywords '(((extracellular vesicles) OR (exosomes)) OR (microvesicles)) AND (DNA)' without biofluids, and we obtained 1844 (PubMed) and 2152 (Web of Science) articles. To date, we have reviewed 500 *in-vitro* studies. From these, we identified 52 eligible studies on EV-DNA from cell culture supernatant, and these 52 studies have been added to EV-ADD. In the next update (January 2023), *in-vitro* studies will be updated, and *in-vivo* studies will be added into the EV-ADD platform. Lastly, we have integrated a metric system in our database in order to validate and report experimental parameters (EV-TRACK Consortium et al., 2017), as well as compliancy to the MISEV guidelines (Théry et al., 2018). This system calculates and assigns a percentage designed to score a study on its reporting of experimental procedures and EV characterizations. This system is intended to help the user identify the level of compliance with MISEV 2018 guidelines (Théry et al., 2018). A percentage is generated by assigning different weights to several binary categories (yes/no) that include experimental procedures recommended by MISEV 2018 (Théry et al., 2018). In brief, each study is scored on the reporting of biofluid processing, EV purification and EV-DNA isolation, and EV characterization. The greater the compliance with the experimental recommendations, the higher the score. A table with the experimental parameters and their weight on the overall score is shown in Supplementary Tables 2, Table 3A and B. Parameters were chosen based on their potential

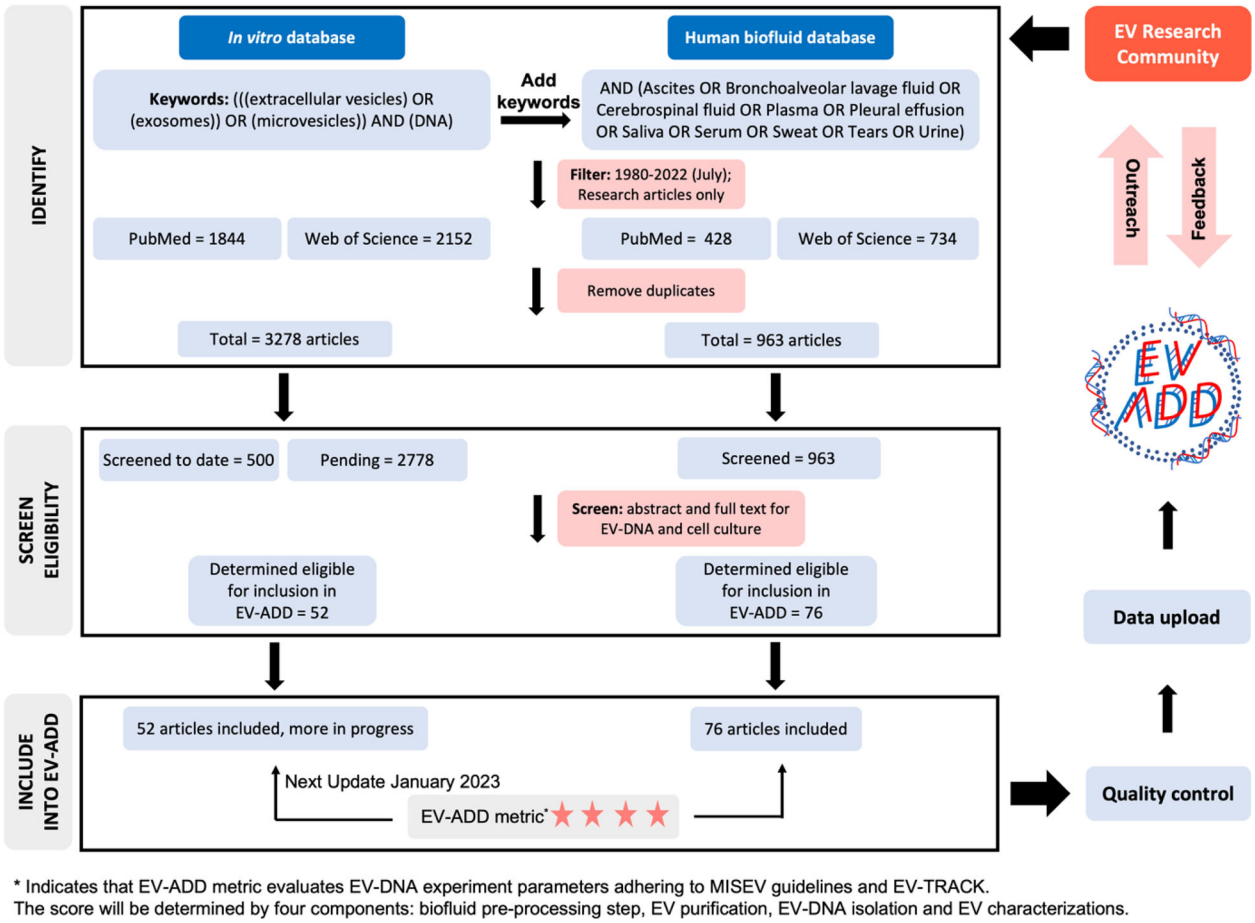

FIGURE 1 **Simplified schematic workflow of EV-ADD**. Workflow is presented in three steps: (1) identification of studies on EV-DNA isolated from *in-vitro* and human biofluids samples; (2) screening of data for eligibility in EV-ADD; and finally (3) inclusion and upload of EV-DNA data to EV-ADD. As the EV-ADD relies on publications from the EV community, clear communication and feedback between researchers and database curators is essential to maintain the EV-DNA lifecycle.

influence on experimental results. We believe that the EV-ADD scoring system will allow users with an additional screening tool for transparency on EV isolation and characterization protocols. In the future, the EV-ADD scoring system will continue to evolve and be adapted to the most recent and updated MISEV guidelines (Witwer et al., 2021) (for example, MISEV 2022).

2.2 | Website design

The EV-ADD website is hosted on an Amazon Web Services (AWS) Lightsail server and it was built using WordPress (WP) as the frontend, and an open-source content management system written in PHP. MySQL serves as the database to store EV-DNA information at the backend. The connection between the frontend and the backend is through React.js (an open-source JavaScript library for graphic components) and WP Data Access. The former provides a user-friendly graphical search menu and sends user requests to WP Data Access. Upon user request, WP Data Access will filter the database and show the result as a downloadable table. Summarized studies and search results can be downloaded to registered users.

3 | RESULTS

3.1 | The EV-ADD user interface allows for searchable criteria of all published EV-DNA data

EV-ADD displays data extracted from peer-reviewed publications on EV-DNA. Manually extracted from these publications and included in the database are data on the following experimental criteria: type of disease, number of patients enrolled in the study, volume of biofluids, EV isolation method, EV characterization, EV-DNA isolation technique, EV-DNA type, EV-DNA detection method, EV-DNA fragment size, targeted gene(s), enzymatic treatment, results, applications of the EV-DNA, and an EV-ADD

score based on the compliancy with MISEV guidelines. These appear on the user interface as filterable and searchable columns (example shown in Figure 2). Moreover, EV-ADD offers search tools based on keywords such as authors, gene symbols and mutations. We have also included a linked reference to the original paper for each entry.

All the data contained in EV-ADD are available for free to download to a spreadsheet in Excel or CSV file format. In addition, users can also upload their published data in the database by submitting a form. https://www.evdnadatabase.com/form/. The Form page provides an interactive way of contributing to our datasets by submitting any newly published studies or missing studies. Importantly, before submission of EV-DNA data, users are encouraged to follow the ISEV and MISEV guidelines and their study should meet the minimum requirements, which include EV biophysical characterization (transmission electron microscope) and EV biochemical characterization (Alix and TSG101), surface tetraspanin marker expression analysis (CD63, CD9 and CD81)), EV quantification (NanoSight), EV negative markers (plasma/serum; APOA1/2, APO B and albumin, urine, Tamm-Horsfall Protein) (Théry et al., 2018), enzymatic treatment (DNase, RNase and proteinase) and detergent (Triton X-100 and NP-40).

3.2 | The EV-ADD currently houses data extracted from 76 papers from eight types of biofluids, spanning 23 different diseases and 52 *in-vitro* papers

Up to June 2022, evidence of EV-DNA from 76 studies performed on human biofluids were deposited in EV-ADD. Moreover, we have expanded our database with 52 *in-vitro* studies and additional studies will be added in the next update (January 2023). Of the more than 50 genes used to detect cfDNA, *BRAF*, *EGFR*, *KRAS*, and *TP53* mutations were the most commonly observed gene mutations identified in EV-DNA isolated from patient liquid biopsy samples (Supplementary Figure 2). This may be explained by the association between hot spot mutations in these genes and various common cancer types (colon cancer, lung cancer and pancreatic cancer). Furthermore, the majority of gene mutations studied are acquired mutations and are involved in cell signalling, oncogenes, tumor suppressors and DNA damage repair. EV-DNA was detected in ascites, bronchoalveolar lavage fluid (BALF), lymphatic drainage, plasma, pleural effusion, serum, sweat and urine and quantified using Bioanalyzer, NanoDrop, quantitative PCR (qPCR), Qubit and Reverse Transcription PCR (RT-PCR). Finally, single nucleotide polymorphism, copy number variation and genomic DNA was detected using droplet digital PCR (ddPCR), qPCR, RT-PCR, targeted tumor gene panel sequencing and next-generation sequencing (NGS) (Figure 3).

3.3 | EV-DNA fragment size profile

Studies have shown distinct cfDNA fragmentation patterns between healthy people and cancer patients (Cristiano et al., 2019). Moreover, cfDNA fragment size has emerged as an important tool to increase the sensitivity for detecting circulating tumor DNA (Mouliere et al., 2018) and it may have prognostic (Chen et al., 2021) and diagnostic (Mathios et al., 2021) value in advanced cancer patients. EV-DNA size distribution profile is reported in 24 of the 76 studies in the EV-ADD with high fragment size variance between studies (Supplementary Table 4). For example, a study demonstrated that the length of urine EV-DNA (1593–16,295 bp) and serum EV-DNA (1508–29,640 bp) are significantly larger than that reported for cfDNA (Zhou et al., 2021). Similar studies have indicated the presence of larger DNA fragments within EVs (Mao et al., 2019; Nguyen et al., 2020; Ruhen et al., 2021). It has been reported that longer EV-DNA fragments may improve the detection of single nucleotide variations, copy number variations (San Lucas et al., 2016) and insertions/deletions during NGS sequencing performance and bioinformatic analysis (Waldenmaier et al., 2022).

However, other studies reported the presence of shorter DNA fragments (152.4 bp, 160 bp) within EVs, with larger DNA fragments possibly resulting from contamination with apoptotic bodies (Sun et al., 2021; Zhang et al., 2019). The comparison between the above-mentioned studies is difficult due to different EV isolation methods resulting in purification of various EV subtypes. This problem is further confounded by the use of different biofluids from various cancer types. Moreover, anti-cancer treatment may also impact EV-DNA fragment length. In one study captured in EV-ADD, EV-DNA from the plasma of an acute myeloid leukemia (AML) patient showed four distinct peaks at 188 bp, 377 bp, 561 bp, and 705 bp before treatment. These peaks disappeared following treatment, with the EV-DNA length profile resembling that of healthy donors (Kontopoulou et al., 2020). Overall, the above findings demonstrate the potential importance of the new field of EV-DNA fragmentomics.

3.4 | EV-ADD data scoring system

There has been a great effort from the ISEV community to provide sample preparation and EV isolation standards through the development of the MISEV guidelines. Using this as a reference, we aimed to include a compliance metric based on the

(a)

(b)

Diseases	Number of patients	Number of healthy subjects	EV-ADD metrics %	Types of DNA	EV DNA fragment size
Melanoma, Non-small-cell lung cancer, ...	43	0	77	dsDNA	Not reported

EV isolation methods	Ultracentrifugation 150,000 x g for 240 minutes
EV characterization	NTA, SEM, WB
EV subtypes studied	Exosomes
DNA isolation kits	ExoLution Plus platform Exosome Diagnositc
DNA quantification methods	Not reported
Volume of biofluids	0.5ml, 1ml, 2ml
Source	Human-plasma
Genes	BRAF, EGFR, KRAS
Mutations	V600E, E19del, L858R, G12, G13D
MtDNA	Not tested
EV markers	FLOT1, TSG101
Enzyme and Detergent	Not reported
Method of detection	Next generation sequencing
Sequencing Details	Platform: Exo1000, Reagent: Not available Sequencing depth: Not available, Sequencing: Not available, Human reference genome: Not available, Total DNA concentration used: Not available
Authors	Möhrmann et al 2018 Clinical Cancer Research
Year of publications	2018
Results	NGS exosome nucleic acids have high sensitivity to detect BRAF, KRAS, EGFR mutations compared to clinical testing of archival tumor and plasma cfDNA.
Applications	Biomarker and prognostic factor
PubMed ID	29051321
EVTrack ID	Not reported
EVTrack Score	Not available

FIGURE 2 **Search results on EV-ADD.** (**A**) An example of a query for "*KRAS* gene" as a search criterion in EV-ADD. (**B**) The database retrieves data on type of diseases, number of patients, EV-ADD data score system (% score), type of EV-DNA detected, source of biofluids, EV-DNA fragment size, methods of EV isolation, EV purification and characterization, subtypes of EVs, EV-DNA isolation techniques, EV-DNA quantification methods, volume of biofluids, enzymatic treatments, reference (Möhrmann et al., 2018), method of DNA detections, results, application, PubMed ID, EV-TRACK ID and score (if any). NTA = nanoparticle tracking analysis, SEM = scanning electron microscopy, WB = western blot

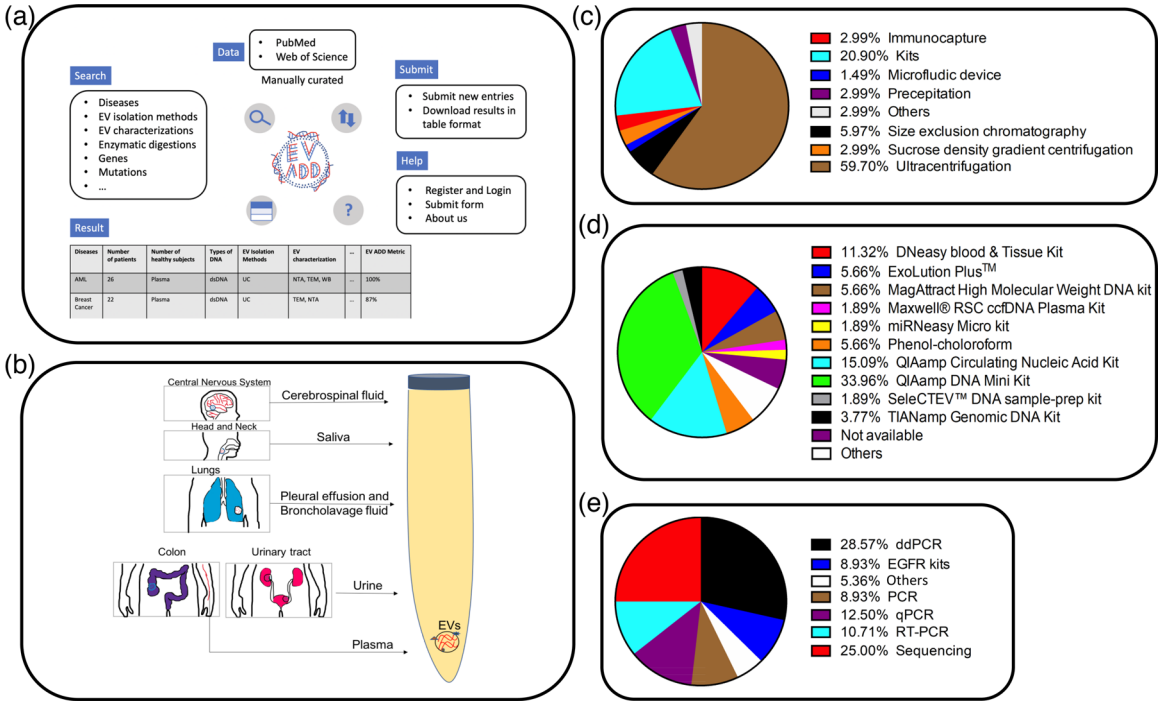

FIGURE 3 (**A**) An overview of the main functions in the EV-ADD. Published data on EV-DNA isolated from human biofluids is manually curated and annotated in a web-based application in the EV-ADD which can be searched and sorted based on various categories. (**B**) EVs can be isolated from human biofluids using (**C**) various EV purification techniques and (**D**) EV-DNA can then be isolated using commercial kits or in-house protocols. (**E**) Lastly, EV-DNA mutations, SNPs and CNVs can be detected using various PCR and sequencing techniques. UC = ultracentrifugation, ddPCR = Droplet Digital PCR, qPCR = Quantitative PCR, RT-PCR = Reverse Transcription PCR

reporting of sample preparation, EV isolation and characterization (Supplementary Table 2). This scoring system indicated that the majority of studies (60%) scored > 50%. In terms of EV isolation techniques, more than 50% of the studies reported the use of ultracentrifugation, while only 5% used size exclusion chromatography, despite its many advantages such as reproducibility, scalability, and low cost (Sidhom et al., 2020). Moreover, despite using ultracentrifugation for EV isolation, 83% of reported studies failed to report non-EV markers, indicating a lack of EV purity. It is noteworthy that relatively pure EVs can be obtained with the sequential use of three isolation techniques (size exclusion chromatography, sucrose density gradient centrifugation and ultracentrifugation) (Brennan et al., 2020). However, EV yield is significantly reduced, and various combinations of EV isolation techniques may lower the likelihood of detecting EV-DNA. Thus, starting human biofluid volume and isolation techniques will greatly influence the downstream applications. Finally, EV-ADD includes all the published EV-associated DNA studies without any restrictions based on the EV-ADD score.

4 | DISCUSSION

EV-DNA holds tremendous promise as both a biomarker and in understanding fundamental processes underlying cell-cell communication via EV cargo. As this field continues to grow, standardization of isolation, purification and analysis tools are needed. In this paper, we introduce EV-ADD, a dedicated knowledgebase for the research community to access data and methodologies on EV-DNA.

One of the goals of our database is to include all data that impacts EV isolation yield, quality and downstream analysis of EV-DNA. During the building of EV-ADD, we identified variations regarding many of these parameters. Differential ultracentrifugation was the conventional EV isolation method used, with approximately 50% of all reported studies using this technique. Surprisingly, the majority of these publications report different *g*-forces, rotor types and durations of ultracentrifugation (Allenson et al., 2017; Bart et al., 2021; Lazaro-Ibanez et al., 2014; San Lucas et al., 2016), all factors that significantly influence EV yield and cargo, in particular protein, DNA and RNA quantity and purity (Cvjetkovic et al., 2014; Théry et al., 2018). Consistency is needed in terms of isolation protocols when pelleting objects with similar sedimentation coefficients such as EVs. Differences were also noted in the types of EVs derived from human biofluids. For instance, studies reported the presence of cfDNA within the lumen of exosomes (Fernando et al., 2017; Kahlert et al., 2014). Another study demonstrated that the majority of large fragments of DNA, including tumor DNA are enriched in large-EVs compared to small-EVs (exosomes) (Vagner et al., 2018). Interestingly,

only low to negligible amounts of DNA were reported within EVs under normal physiological conditions and after acute physical exercise, with the emission of cfDNA being shown to be independent of EVs (Helmig et al., 2015; Lazaro-Ibanez et al., 2014; Neuberger et al., 2021).

However, it should be noted that the above-mentioned studies used ultracentrifugation and precipitation methods (Invitrogen) to isolate EVs. These crude and traditional ultracentrifugation methods are known to co-isolate impurities of non-vesicular aggregates, albumin, as well as heterogenous EV populations (Ludwig et al., 2019; Patel et al., 2019). Recent studies have shown a formation of complex biomolecule corona around the surface of EVs. For example, low density lipoprotein (LDL) corona was spontaneously formed as soon as LDL particles were mixed with the EVs (Sódar et al., 2016) and under genotoxic stress condition, mtDNA is observed on the surface of small EVs (Németh et al., 2017). Protein corona was also formed spontaneously on the surface of EVs derived from blood (Tóth et al., 2021). Moreover, various studies have shown that the Invitrogen precipitation method leads to polyethylene glycol (PEG) contamination, microvesicles as well as additional protein aggregates, which may give the false impression of isolating exosomes (Abramowicz et al., 2016; Patel et al., 2019). Finally, a lack of standardized terminology of EVs throughout the literature has resulted in inconsistent nomenclature in the cited literature. Therefore, the International Society of Extracellular Vesicle (ISEV) endorses the term 'extracellular vesicle' because currently there are no specific protein markers that have been established to identify exosomes and ectosomes (microvesicles/microparticles) (Théry et al., 2018; Witwer & Théry, 2019). DNA fragment sizes and quantity may also vary depending on the EV population isolated, emphasizing the importance of these considerations (Chang et al., 2020; Vagner et al., 2018). Variations may also reflect different sources of EVs (Jeppesen et al., 2019; Németh et al., 2017; Yokoi et al., 2019), EV isolation and DNA detection methods (Neuberger et al., 2021), and EV heterogeneity (Théry et al., 2018). Currently, EV-DNA isolation methods are not standardized, and are based on either commercially available kits (i.e. silica filtration and magnetic beads-based approach) or phenol chloroform method which can affect isolation efficiency and DNA fragment size (Sorber et al., 2017; Tagliaferro et al., 2021). Downstream analysis methods were also reported, such as Qubit fluorometer, nanodrop, Agilent bioanalyzer and qPCR, which present different levels of sensitivity and specificity.

Approximately 72% of EV-DNA studies reported in EV-ADD were performed on plasma, which contains a complex mixture of biomolecules (Leeman et al., 2018; Psychogios et al., 2011). Therefore, extensive pre-analytical steps are generally required before the EV isolation and purification steps. However, these pre-treatment aspects such as choice of blood collection tubes (Berckmans et al., 2019; Palviainen et al., 2020), centrifugation conditions (Vila-Liante et al., 2016), filtration, extraction method and blood processing time likely contribute to divergent results within the EV field (Bæk et al., 2016; Heatlie et al., 2020). We observed that ethylenediaminetetraacetic acid (EDTA)-containing plastic tubes were widely used for EV-DNA isolated from plasma. In addition, different centrifugation speeds and time have been applied to obtain platelet-poor plasma (PPP) and platelet-free plasma (PFP). Centrifugation of whole blood at 4°C may activate platelet and release platelet-activated particles (Arraud et al., 2014; Coumans et al., 2017; Witwer et al., 2013). Only five studies have reported filtration steps after the centrifugation procedure. One report suggested that 0.8 μm filter reduces the platelet contamination (Baranyai et al., 2015). Thus, measurement of residual platelets using CD41 and CD31 markers was recommended in the EV preparation (Aatonen et al., 2014; Cappellano et al., 2021; Venturella et al., 2019). To address these issues, an automated exosome isolation approach from undiluted whole-blood sample called 'acoustofluidic platform' has been developed, eliminating biofluid preprocessing steps and increasing exosome purity, yield and reproducibility with a shorter experimental time (Wu et al., 2017). The ISEV taskforce has recognized the importance of preprocessing of biofluids and provided a roadmap for blood preprocessing procedure for EV analysis (Clayton et al., 2019; Witwer et al., 2013). Importantly, ISEV blood workshops and symposiums (uEV 2022) are also being held to bring together EV researchers to provide updates, bringing us closer to the standardization of protocols. Extensive reviews on the standardization of blood collection and processing have been reported elsewhere (Coumans et al., 2017; Venturella et al., 2019). Taken together, these variations among studies introduce variabilities in analysis outputs, limiting inter-study comparisons of EV-DNA (EV-TRACK Consortium, 2017; Théry et al., 2018; Cvjetkovic et al., 2014). EV-ADD provides a repository of these experimental variables, aiding researchers to determine appropriate EV-DNA methodologies that are relevant to their research context (Supplementary Table 3A and B).

While liquid biopsy-based EV-DNA analysis may offer important advantages over cfDNA isolation, such as DNA protection from degradation, the clinical biomarker utility of EVs remains limited due to the complexity of isolating pure EV subtypes from biological fluids (Théry et al., 2006). Large volumes of plasma, ranging from 10 to 20 ml, are required for nucleic acid isolation from tumor-derived EVs, rendering it impractical for clinical use (Bernard et al., 2019; Cai et al., 2015; Castillo et al., 2018; San Lucas et al., 2016). Thus, ultrasensitive, efficient and state-of-the-art on-chip-based EV isolation assays are being tested in these settings (Chiriacò et al., 2018; Liang et al., 2017). These assays require very low amounts of plasma and can capture specific populations of EVs (tumor-specific EVs) based on surface markers, thus improving the sensitivity and specificity of detecting mutant molecules. In addition, single-EV-based liquid biopsy using a high-throughput Nano-bio Chip Integrated System for Liquid Biopsy (HNCIB) has shown promising results in detecting tumor-derived EV surface proteins (PDL1+) and internal cargo (mRNA/miRNA). A proof-of-concept study demonstrated that Glypican-1 (GPC1) is specifically enriched on cancer-derived exosome surface and only GPC1+ exosomes carry mutant KRAS transcript (G12D) (Melo et al., 2015). Simultaneous analysis of

multiple cargos in a single EV will improve the accuracy and sensitivity of disease markers that single parametric approaches may miss (Zhou et al., 2020).

While studies have reported the presence of EV-associated DNA in cell culture (Lee et al., 2014; Thakur et al., 2014) and biological fluids (Allenson et al., 2017; Fernando et al., 2018; Garcia-Silva et al., 2019; Jin et al., 2016; San Lucas et al., 2016), these observations have been challenged. Researchers have demonstrated that classical exosomes and small EVs do not carry double-stranded DNA and DNA-binding histones in specific cancer cell lines, and that active DNA emission is independent of exosome emission pathways, rather relying on the amphisome pathway (Jeppesen et al., 2019). However, simultaneous work demonstrated a mechanism whereby genomic DNA is loaded into exosomes via micronuclei. In the latter, authors demonstrated dsDNA spanning the entire human genome, thus reflecting the patient's genomic signature (Yokoi et al., 2019). A more recent study demonstrated that dsDNA recruitment into tumor-derived vesicles (TMV) occurs by activation of ADP ribosylation factor 6 (ARF6) with the cytosolic DNA sensor, cGAS and independent of amphisome pathway and micronuclei (Clancy et al., 2022). Taken together, data suggest that the loading of DNA into EVs may be context specific. The growing number of studies on EV-DNA will help to clarify the mechanisms of loading, biological role and research utility of EV-DNA.

While the EV research community is thriving, little consensus exists on optimal isolation and purification techniques for EVs and their cargo (EV-TRACK Consortium, 2017; Cvjetkovic et al., 2014; Lotvall et al., 2014). MISEV guidelines and other publications have reported the importance of pre-analytic parameters for human biofluids and EV isolation standardization (Lacroix et al., 2012; Muller et al., 2014; Mullier et al., 2013; Théry et al., 2018; Witwer et al., 2013; Yuana et al., 2015). Over the past decade, comprehensive and pure EV isolation techniques have been a major challenge in the EV field (Kalluri & Lebleu, 2020; Keerthikumar et al., 2016). Slight variations in isolation methods or within protocols lead to enrichment of certain EV subtypes and cargo, hindering reproducibility and large meta-analyses (EV-TRACK Consortium, 2017; Théry et al., 2018). A crowdsourcing knowledgebase named EV-TRACK, screens and compiles EV-focused publications to encourage standardization and advance the EV community (EV-TRACK Consortium, 2017). EV-ADD seeks to reach that same goal, providing a platform for users to find methodologies that have been successful in identifying EV-DNA. EV-ADD and EV-TRACK reveal a wide range of different protocols and reagents for EV and EV-DNA isolation within EV publications (EV-TRACK Consortium, 2017) (Table 1), highlighting the need to consider processing and analytical variables when interpreting data. Therefore, we have integrated an EV-ADD data metric system to validate experimental parameters and to ensure that relevant captured data in each article deposited in our database is reliable (EV-TRACK Consortium, 2017; Keerthikumar, S. 2016). Altogether, the EV-ADD metric highlighted a lack of standardization and data reporting in the EV-DNA field. Future studies following MISEV recommendations are critical for developing EV-DNA biomarker discovery and validation.

5 | CONCLUSION

Taken together, the field of EV-DNA is in its early stages, and as it grows, limitations persist, especially in the standardization of validated protocols, consistent data and interpretation of clinical correlations in disease. With exponential growth in EV-related publications, especially with the introduction of next-generation sequencing for EV-DNA-based liquid biopsy research (Castillo et al., 2018; Kahlert et al., 2014; San Lucas et al., 2016), a massive accumulation of experimental data will be generated and will require efforts to organize and continuously update databases. EV-ADD provides a knowledgebase of manually curated published EV-DNA data. Publicly and freely available, it begins to address many of the challenges in the EV field, providing a one-stop repository of experimental EV-DNA data across the literature. The current version of EV-ADD includes data from 76 published articles based on EVs isolated from human biofluids covering varying diseases. In the future, it will include EV-DNA data derived from *in-vitro* and *in-vivo* animal model systems. Additionally, EV researchers can register for free and deposit published EV-DNA findings directly into EV-ADD, thus centralizing experimental procedures, findings and interpretation into one platform. With its simplicity and easy accessibility, we hope that EV-ADD propels EV-DNA research forward and becomes an important resource for researchers in the EV field.

AUTHOR CONTRIBUTIONS

Mingyang Li, Thupten Tsering, Yunxi Chen, Prisca Bustamante and Julia V. Burnier participated in designing the EV-ADD website. Thupten Tsering, Amélie Nadeau, Alexander Laskaris, Mohamed Abdouh and Julia V. Burnier involved in drafting and reviewing the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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