Extracellular vesicle-associated repetitive element DNAs as candidate osteosarcoma biomarkers

Linda Cambier1, Kevin Stachelek1,2, Martin Triska1, Rima Jubran3, Manyu Huang4, Wuyin Li4, Jianying Zhang5, Jitian Li4,5 & David Cobrinik1,2,6,7*

Osteosarcoma (OS) is the most common malignant bone tumor in children and young adults. Despite that high-risk factors have been identified, no test for early detection is available. This study aimed to identify circulating nucleic acid sequences associated with serum extracellular vesicle (EV) preparations at the time of OS diagnosis, as a step towards an OS early detection assay. Sequencing of small nucleic acids extracted from serum EV preparations revealed increased representation of diverse repetitive element sequences in OS patient versus control sera. Analysis of a validation cohort using qPCR of PEG-precipitated EV preparations revealed the over-representation of HSATI, HSATII, LINE1-P1, and Charlie 3 at the DNA but not RNA level, with receiver operating characteristic (ROC) area under the curve (AUC) ≥ 0.90. HSATI and HSATII DNAs co-purified with EVs prepared by precipitation and size exclusion chromatography but not by exosome immunocapture, indicative of packaging in a non-exosomal complex. The consistent over-representation of EV-associated repetitive element DNA sequences suggests their potential utility as biomarkers for OS and perhaps other cancers.

Osteosarcoma (OS) is the most common malignant bone tumor of children, adolescents, and young adults, representing approximately 1% of newly diagnosed cancers in adults, and 3–5% in children1,2. With current treatment regimens, patients with non-metastatic OS have 5-year survival rates above 65% whereas the ~25% of patients presenting with metastases have a 5-year survival of less than 20%3,4. As such, early detection of OS prior to metastasis could significantly improve outcomes.

Early detection is especially needed in individuals who are predisposed to OS either genetically or through iatrogenic exposures. OS occurs at increased rates in several monogenic hereditary cancer syndromes such as retinoblastoma (RB1)5, Li-Fraumeni syndrome (TP53), Bloom syndrome (RECQL2), Werner syndrome (RECQL3), and Rothmund-Thomson syndrome (RECQL4). OS also occurs with increased frequency in children exposed to radiation or alkylating agents, in Diamond–Blackfan anaemia patients and in adults with bone disorders such as Paget’s Disease. Combined genetic predisposition and exposure to DNA damaging agents confers particularly high risk; for example, relative risk for children with hereditary retinoblastoma increased from ~69 without such treatments to ~302 for radiotherapy and ~539 for radiotherapy plus chemotherapy in the largest treatment-stratified analysis5.

The need for OS biomarkers is reflected in a large yet inconclusive literature. Early studies focusing on bone markers such as alkaline phosphatase showed highly variable increases in OS patients6. Later proteomic studies revealed two as-yet uncharacterized OS-associated proteins7 whereas studies of miRNAs showed variable

1The Vision Center and The Saban Research Institute, Children’s Hospital Los Angeles, 4650 Sunset Blvd, MS163, Los Angeles, CA 90027, USA. 2Cancer Biology and Genomics Program, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA. 3Division of Hematology/Oncology, Children’s Hospital Los Angeles, Los Angeles, CA 90027, USA. 4Henan Luoyang Orthopedic Hospital (Henan Provincial Orthopedic Hospital)/Henan Provincial Orthopedic Institute, 82 Qiming Road, Luoyang 471002, Henan, China. 5Department of Biological Science, The University of Texas at El Paso, El Paso, TX 79968, USA. 6Department of Ophthalmology and Roski Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA. 7Department of Biochemistry and Molecular Medicine and Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA. 8email: jitianlee@hotmail.com; dcobrinik@chla.usc.edu
A recent study identified 56 miRs that were upregulated in pre-treatment OS patient plasma11; however, among the top candidates (miR-21, miR-221, and miR-106a), levels increased by only ~ 2.4–8-fold and sensitivity was at best ~ 85%. An alternative approach is to detect aneuploidy via cell-free DNA (cfDNA) whole genome sequencing, yet at present this has limited sensitivity12 due to the dilution of tumor with non-tumor cfDNA. Currently no biomarkers have been shown to reliably detect naïve pre-symptomatic OS in predisposed individuals13.

In this study, we aimed to identify circulating biomarkers that distinguish OS patients from healthy controls, as a step towards a liquid biopsy for early OS detection. Liquid biopsies may detect circulating tumor components including cfDNA, tumor cells, and extracellular vesicles (EVs)14–19, a category that includes exosomes, shedding vesicles, microparticles, retroviral-like particles, ectosomes, microvesicles, oncosomes, and apoptotic bodies20,21. EVs are released by most if not all cells22 and carry components of their cell of origin such as proteins, lipids, metabolites, and various types of RNA23,24. Among the different types of EVs, exosomes and oncosomes are more highly produced by cancer cells than by normal cells, are often present at increased levels at cancer diagnosis, may further increase during tumor progression15, and carry cargo that reflects metastatic progression and treatment response25,26. Moreover, EV preparations may contain exosomal as well as non-exosomal tumor components. Thus, we aimed to identify OS biomarkers in serum-derived EV preparations.

To identify EV-associated OS biomarkers we compared the abundance of nucleic acid sequences in OS patient versus control serum EV preparations. Specifically, we sequenced small nucleic acids extracted from EV preparations and examined differential representation of unique as well as repetitive element sequences which are often produced and may be released by cancer cells27, including by OS cells28. We then evaluated whether the same sequences were differentially represented in different patient cohorts and by different EV isolation and analytic methods. Through these approaches we identified circulating EV-associated repetitive element DNA sequences that were more abundant in OS sera compared to healthy sera in two patient cohorts.

Results

Over-representation of repetitive element sequences in OS EV preparations. To identify OS biomarkers, we compared nucleic acid sequences associated with EV preparations from sera of OS patients and healthy controls. Initial analyses were performed on a discovery cohort of treatment-naïve OS patients from Children’s Hospital Los Angeles (CHLA) and Henan Luoyang Orthopedic Hospital (HLOH), comprised of males and females between 5 and 29 years old and presenting with different OS types. Control cohorts were comprised of healthy siblings of hereditary retinoblastoma patients who had not developed retinoblastoma (hereditary retinoblastoma controls; HRCs) and unrelated approximately age-matched healthy individuals (healthy controls; HC) (Table 1a). EV preparations were made with the commercial ExoQuick kit based on polyethylene glycol (PEG) precipitation, with recognition that EV as well as non-EV components are isolated29,30. Nanoparticle tracking analysis of each sample revealed similar size distributions of control and OS EVs between 50 and 150 nm, which is characteristic of exosomes (Fig. 1a,b). EV concentrations were not significantly higher in sera from OS patients compared to controls (Fig. 1c). Likewise, EV concentrations were similar in OS patient serum from USA (CHLA) and China (HLOH) and for different patient ages, genders, and OS types (Fig. 1c–e).

To detect differentially represented EV-associated RNA and DNA sequences, nucleic acids were extracted from OS and control EV preparations using SeraMir small RNA enrichment kit (SBI) without DNase treatment and a sequencing library was built by addition of a 5′-RNA adapter and a 3′-DNA adapter followed by PCR amplification and sequencing. Comparison of uniquely mapped sequences using DESeq21 identified 107 significantly over-represented genes and 587 significantly under-represented genes (≥ twofold change, p.adj < 0.05) in OS samples (Supplementary Fig. 1). However, the over-represented sequences had poor OS sensitivity and specificity (not shown). As a far greater proportion of genes were under-represented in OS samples, we considered whether our analysis of uniquely mapped sequences ignored potentially relevant over-represented non-uniquely mapped sequences. Thus, we evaluated the differential representation of the major repetitive element categories after aligning reads to RepeatMasker32. This indicated that OS serum EV preparations had greater representation of almost all repetitive element categories including the most abundant LINE1, LTR/ERV, α-satellite and SINE/Alu categories but not the low complexity ribosomal RNA and scRNA sequences (Supplementary Fig. 2A, B). The most highly represented sequence was the LINE1 family member L1P1 (Supplementary Fig. 2C).

While these analyses revealed consistent over-representation of repetitive element sequences in OS serum EV preparations, the identities of the most over-represented elements were uncertain since programs that are not specifically designed for repetitive element detection may erroneously map repetitive element reads33. To more accurately define the differential repetitive element representation, sequences were evaluated with TEtranscripts, which maps repetitive element sequences more accurately and quantitatively than non-dedicated programs34. Using default settings with reads mapped to the GRCh38 genome, TEtranscripts confirmed that OS samples had far more significantly under-represented than over-represented sequences in comparison to control samples (Fig. 2a) and showed that most of the under-represented sequences were single-copy genes (Fig. 2b). In contrast, a vast proportion of repeat element sequences were over-represented in OS EV preparations (Fig. 2c) albeit with only 19 significantly over-represented versus four significantly under-represented (Fig. 2d). Among significantly over-represented repeat elements, Human Satellite I (HSATI) had the highest fold change (log2 (6.14), p.adj = 0.007) (Fig. 2d and Table 2, Analysis 1).

Because GRCh38 contains numerous alternative assemblies that are enriched for repetitive elements that might siphon repetitive element reads, adds synthetic centromeric repeat sequences, and hard-masks certain centromeric and genomic repeat arrays35, we considered whether these features might affect the ability to detect differential representation of unique or repetitive element sequences. To address this possibility, we re-performed TEtranscripts analysis with reads aligned to hg19, which lacks the GRCh38 alternative assemblies. This identified
| Source   | Sample | Gender | Age (yrs) | OS type                        |
|----------|--------|--------|-----------|--------------------------------|
| a. Discovery cohort (nucleic acid sequencing) |        |        |           |                                |
| **Osteosarcomas** |        |        |           |                                |
| CHLA     | OS-C1  | M      | 7         | High grade, extensive necrosis |
|          | OS-C2  | F      | 13        | Chondroblastic, necrosis      |
|          | OS-C3  | M      | 12        | Conventional                  |
|          | OS-C4  | M      | 5         | High grade, small cell variant|
|          | OS-C7  | M      | 17        | Conventional secondary to RB   |
|          | OS-C8  | M      | 14        | Conventional secondary to RB   |
| HLOH     | OS-H6  | M      | 16        | Osteoblastic                  |
|          | OS-H7  | M      | 17        | Osteoblastic                  |
|          | OS-H11 | F      | 25        | Osteoblastic                  |
|          | OS-H19 | M      | 15        | Fibroblastic                  |
|          | OS-H20 | M      | 17        | Osteoblastic                  |
|          | OS-H27 | M      | 29        | Osteoblastic                  |
| **Controls** |        |        |           |                                |
| CHLA     | HC1    | F      | 17        |                                |
|          | HRC1   | M      | 5         |                                |
|          | HRC2   | F      | 14        |                                |
|          | HRC3   | F      | 11        |                                |
|          | HRC4   | F      | 9         |                                |
| HLOH     | HH12   | M      | 10        |                                |
|          | HH17   | M      | 14        |                                |
|          | HH21   | M      | 21        |                                |
|          | HH23   | M      | 16        |                                |
|          | HH25   | M      | 27        |                                |
|          | HH28   | F      | 21        |                                |
|          | HH29   | F      | 11        |                                |
| b. Validation cohort (qPCR) |        |        |           |                                |
| **Osteosarcomas** |        |        |           |                                |
| CHLA     | OS-C1  | M      | 7         | High grade, extensive necrosis|
|          | OS-C3  | F      | 12        | Conventional                  |
|          | OS-C5  | M      | 13        | Conventional                  |
|          | OS-C9  | M      | 19        | Conventional                  |
| HLOH     | OS-H21 | M      | 43        | High grade, extensive necrosis|
|          | OS-H24 | M      | 46        | Osteoblastic                  |
|          | OS-H25 | F      | 16        | Osteoblastic                  |
|          | OS-H34 | M      | 14        | Osteoblastic                  |
| **Controls** |        |        |           |                                |
| CHLA     | HC2    | F      | 38        |                                |
|          | HC3    | M      | 38        |                                |
| IR       | HI1    | M      | 20        |                                |
|          | HI2    | M      | 19        |                                |
|          | HI3    | F      | 20        |                                |
|          | HI4    | F      | 18        |                                |
|          | HI5    | M      | 20        |                                |
|          | HI6    | M      | 19        |                                |
|          | HI7    | M      | 20        |                                |
|          | HI8    | F      | 18        |                                |
|          | HI9    | F      | 19        |                                |
|          | HI10   | F      | 20        |                                |

Table 1. Osteosarcoma patient and control donor characteristics. OS-C, osteosarcoma from CHLA; OS-H, osteosarcoma from HLOH; HC, healthy control from CHLA; HH, Healthy control from HLOH; HI, healthy control from Innovative Research Inc.; HRC, hereditary retinoblastoma sibling control.
15 significantly over-represented repeat elements, of which Human Satellite II (HSATII) had highest fold change (log$_2$ (2.73), $p$.adj = 0.002), and one significantly under-represented element in OS versus control sequences (Table 2, Analysis 2 and Supplementary Fig. 3). The significantly differentially represented repetitive elements identified using hg19 had little overlap with those identified when mapping to GRCh38.

Validation of over-representation of repeat elements in OS EV preparations. We next examined whether the increased representation of repetitive elements was evident in a validation set of mostly distinct samples. The validation cohort consisted of treatment-naïve OS patients from CHLA and HLOH including males and females between 7 and 46 years old and presenting with various OS types as well as approximately age-matched healthy individuals (Table 1b). The validation cohort was independent of the discovery cohort except for re-analysis of OS1 and OS3, which were the only samples with a sufficient quantity to re-test. To assess the repetitive element over-representation, EV-associated nucleic acids were isolated and evaluated using methods that differed from the discovery cohort analyses: EVs were isolated by PEG6000 precipitation instead of ExoQuick, nucleic acids were extracted using the miRNeasy micro RNA extraction kit (Qiagen) instead of SeraMir, and repetitive elements were examined by reverse transcription and quantitative PCR (RT-qPCR) instead of sequencing. Similar to the discovery cohort, EV concentrations were not significantly higher in sera from OS patients compared to controls (data not shown).

RT-qPCR was used to analyze four representative repetitive element categories including the HSATI and HSATII satellite sequences that were most differentially over-represented in TETranscripts Analyses 1 and 2 (Table 2), the LINE1 P1 family member (L1P1) that showed the highest fold change in the RepeatMasker analysis (Supplementary Fig. 2), and Charlie 3, another over-represented repetitive element with a significant log$_2$ fold change of 4.36 (20.5-fold increase) in TETranscripts Analysis 1 (Table 2, Fig. 2d). RT-qPCR reactions yielded the predicted product sizes for HSATI (406 bp), L1P1 (83 bp), and Charlie 3 (104 bp). RT-qPCR of HSATII yielded prominent products of 85, 134, 183 and 281 bp, in agreement with HSATII genomic structure (Supplementary
The HSATII and Charlie 3 products were confirmed to represent the predicted sequences by Sanger sequencing. For each sample, RT-qPCR was performed on the same proportion of total EV nucleic acid extracted from the same serum volume and was normalized against a spike-in RNA. The analyses confirmed the over-representation of HSATI, HSATII, L1P1, and Charlie 3 sequences in OS EV preparations (12.42-fold, \( p = 0.0040 \); 3.33-fold, \( p = 0.062 \); 3.56-fold, \( p = 0.016 \); 12.6-fold, \( p = 0.0007 \); respectively) (Fig. 3a). In contrast, the single-copy gene HECDT2, chosen on the basis of a 4.67 log2 fold change in TETranscripts analysis, did not show a significant difference (Supplementary Fig. 5A and B), and was deemed to have been spuriously identified, possibly due to biases in the library construction method. The over-representation of HSATI, HSATII, L1P1, and Charlie 3 sequences was similar after removal of the OS1 and OS3 samples that were also used in the discovery cohort (Supplementary Fig. 6A). Thus, the over-representation of repetitive element sequences initially detected by nucleic acid sequencing was confirmed in a validation cohort using RT-qPCR.

**Over-representation of repetitive element DNA but not RNA in OS EV preparations.** As our nucleic acid isolation and analysis methods could detect RNA as well as DNA sequences, we examined the nucleic acid origin of the over-represented repetitive element sequences by performing qPCR without reverse transcription. With this approach, the HSATI, HSATII, L1P1 and Charlie 3 sequences were significantly over-represented in OS versus control EVs (22.18-fold, \( p = 0.0015 \); 3.7-fold, \( p < 0.0001 \); 2.86-fold, \( p = 0.0015 \); 10.29-fold, \( p = 0.011 \); respectively) (Fig. 3b), indicative of differential representation of repetitive element DNA, rather than RNA. Evaluation of the sensitivity and specificity by Receiver Operating Characteristic (ROC) curves yielded area under the curves (AUCs) of 0.86 or greater for each repetitive element (Fig. 3c). As for the RT-qPCR analyses, results were similar after removal of OS1 and OS3 (Supplementary Fig. 6B and C).
of the nucleic acids (Fig. 4b), with the majority of the remaining nucleic acid likely representing protected EV. DNase treatment slightly decreased the amount of nucleic acid whereas RNase A eliminated most but not all samples, whereas RNase A treatments had no significant effect (Fig. 4a). Bioanalyzer assessments revealed that and double-stranded RNA and RNA strands in RNA-DNA hybrids39. After these treatments, nucleic acids were NaCl in order to cleave single-stranded RNA as well as in the absence of NaCl in order to cleave single-stranded treatment with DNase I or RNase A prior to nucleic acid extraction. RNase A treatments were performed in 1 M tion of RT-independent products, PEG-precipitated EV preparations from four OS and four control sera were Exosome-associated repetitive element DNAs (HSATI, HSATII, L1P1 and Charlie 3 DNAs) are associated with EVs, we examined whether they co-purified with EVs prepared by size exclusion chromatography but not exosome immunoaffinity capture. To further evaluate if repetitive element DNAs control for possible artifactual generation of RT-independent products, PEG-precipitated EV preparations from four OS and four control sera were treated with DNase I or RNase A prior to nucleic acid extraction. RNase A treatments were performed in 1 M NaCl in order to cleave single-stranded RNA as well as in the absence of NaCl in order to cleave single-stranded and double-stranded RNA and RNA strands in RNA-DNA hybrids39. After these treatments, nucleic acids were extracted with the miRNeasy Micro kit and HSATI and HSATII abundance were assessed by qPCR. In these analyses, DNase I treatment eliminated 97~99% of HSATI and 80~99% of HSATII signals in both OS and control samples, whereas RNase A treatments had no significant effect (Fig. 4a). Bioanalyzer assessments revealed that DNase treatment slightly decreased the amount of nucleic acid whereas RNase A eliminated most but not all of the nucleic acids (Fig. 4b), with the majority of the remaining nucleic acid likely representing protected EV RNA. The repetitive elements’ sensitivity to DNase I prior to nucleic acid extraction implied that the repetitive element DNAs were over-represented, whereas single copy genes were not over-represented, in OS EV preparations. Thus, major repetitive elements DNAs (HSATI, HSATII, L1P1, Charlie 3) were over-represented, whereas single copy genes were not over-represented, in OS EV preparations. HSATI, HSATII, L1P1 and Charlie 3 DNAs were similarly increased in OS samples from USA (CHLA) and China (HLOH) and in high-grade as well as non-high-grade OS samples (Supplementary Fig. 7). In contrast, qPCR of IL17A and ZNF3 sequences, which are within 1 Mb of HSATI on chromosome 22 (chr22q11.21) and within 40 Mb of HSATII on chromosome 7 (chr7q22.1), respectively, did not show a significant difference between OS and control EVs (Supplementary Fig. 8A and B). Thus, major repetitive elements DNAs (HSATI, HSATII, L1P1, Charlie 3) were over-represented, whereas single copy genes were not over-represented, in OS EV preparations. To further evaluate the abundance of repetitive element DNAs and control for possible artifactual generation of repetitive element DNAs were over-abundant in OS compared to control EV preparations whereas their RNAs were either undetectable or present in similar quantities. To assess whether OS serum EV preparations might also have an increased abundance of repetitive element RNAs, PEG-precipitated EVs were prepared, treated with DNase I, and the remaining nucleic acids extracted and examined by RT-qPCR. In these samples, no amplification signal was detected for HSATI or Charlie 3, while HSATII and L1P1 products were reduced ~32~64-fold compared to non-DNase I treated samples and showed no significant difference in control and OS samples (Fig. 4c and data not shown). Thus, HSATI, HSATII, L1P1, and Charlie 3 DNAs were over-abundant in OS compared to control EV preparations whereas their RNAs were not sequestered inside of EVs.

| Analysis 1 (mapped to GRCh38) | log2FC | p.adj | Analysis 2 (mapped to hg19) | log2FC | p.adj |
|-------------------------------|--------|------|-------------------------------|--------|------|
| HSATI:Satellite:Satellite     | 6.14   | 0.007| HSATII:Satellite:Satellite    | 2.73   | 0.002|
| LTR685a:Gypsy:LTR            | 5.77   | 0.003| L1MA6:L1:LINE                | 2.65   | 0.041|
| LTR75:ERV-LTR                | 5.07   | 0.042| MSTR:ERV:MaLR:LTR            | 2.52   | 0.001|
| LTR16:E2:ERV-LTR             | 5.07   | 0.001| (GAATG)n:Satellite:Satellite  | 2.51   | 0.035|
| LTR16:A1:ERV-LTR             | 5.01   | 0.014| Charlie4:chAT-Charlie:DNA    | 2.37   | 0.011|
| Tigger8:Char-Mar-Tigger:DNA  | 4.60   | 0.025| Harlequin-int:ERV1:LTR       | 2.28   | 0.032|
| MLT1H2:ERV1:MaLR:LTR         | 4.54   | 0.016| L1M4:L1:LINE                 | 2.05   | 0.038|
| BLACKJACK:chAT-Blackjack:DNA | 4.43   | 0.029| L1MB8:L1:LINE                | 2.03   | 0.005|
| Charlie4:chAT-Charlie:DNA   | 4.36   | 0.030| L1PB4:L1:LINE                | 2.00   | 0.021|
| MER113:chAT-Charlie:DNA      | 4.11   | 0.019| L1MC3:L1:LINE                | 1.90   | 0.046|
| MLT1A1:ERV1:MaLR:LTR         | 4.05   | 0.015| L1PB2:L1:LINE                | 1.89   | 0.026|
| L1MCb:L1:LINE                | 3.98   | 0.037| L1MEc:L1:LINE                | 1.88   | 0.047|
| MLT2B2:ERV-LTR               | 3.75   | 0.018| MLT1A0:ERV1:MaLR:LTR         | 1.81   | 0.016|
| HSATII:cent:Satellite        | 3.63   | 0.025| L1MB7:L1:LINE                | 1.80   | 0.028|
| LTR32:ERV-LTR                | 3.25   | 0.042| L1MDa:L1:LINE                | 1.45   | 0.041|
| Charlie4:chAT-Charlie:DNA   | 3.20   | 0.034| L1PA12:L1:LINE               | -2.27  | 0.002|
| CER:Satellite:Satellite      | 3.18   | 0.021|                               |        |      |
| MLT1E2:ERV-LTR               | 2.92   | 0.029|                               |        |      |
| L1MC4a:L1:LINE               | 2.71   | 0.040|                               |        |      |
| MER74A:ERV-LTR               | -2.61  | 0.041|                               |        |      |
| L1PA16:L1:LINE               | -2.96  | 0.029|                               |        |      |
| FAM:Ahu:SINE                 | -3.05  | 0.015|                               |        |      |
| FordPref:chAT-Tip100:DNA     | -5.34  | <0.001|                               |        |      |

Table 2. Repeat elements significantly differentially represented in OS versus control EV preparations (p. adj < 0.05; elements examined in the validation cohort in bold italics).

Co-purification of OS-associated repetitive element DNAs with EVs in size exclusion chromatography but not exosome immunoaffinity capture. To further evaluate if repetitive element DNAs that were more abundant in OS patient PEG-precipitations (here termed ‘OS-associated’ repetitive element DNAs) are associated with EVs, we examined whether they co-purified with EVs prepared by size exclusion chromatography (SEC) and exosome immunoaffinity capture. SEC yields more pure EV populations40,41 with lower protein contamination compared to PEG precipitation42,43, whereas exosome immunoaffinity capture uses well-characterized surface markers CD9 or CD81 to highly purify intact exosomes41,44.
We first examined if repetitive element DNAs co-purify with SEC-isolated EVs from two control and two OS samples. Nanoparticle tracking analyses revealed that the control and OS EVs both eluted from size exclusion columns solely in fractions 6 and 7 (Fig. 5a,b) and had similar size profiles (Fig. 5c,d). qPCR analyses revealed that the OS EV fractions had more abundant HSATI and HSATII DNA (Fig. 5e), as observed with the PEG-isolated EVs of the same samples (Fig. 5f). Similarly, HSATI and HSATII levels were higher in the same OS versus control sera when normalized to EV concentration (Supplementary Fig. 9A and B). Furthermore, nucleic acids obtained from arbitrarily selected non-EV fractions from one control and one OS fractionation (Fig. 5g,h) revealed no detectable HSATI and only minimal HSATII, which was not higher in OS samples (Fig. 5i,j). Thus, HSATI and HSATII DNAs co-purified with EVs in SEC with a greater abundance in OS compared to control sera, similar to that of PEG preparations.

We next assessed whether OS-associated repetitive element DNAs co-purified with EVs in exosome CD9 or CD81 immunoaffinity capture. In pilot studies, we confirmed that our immunocapture approach enriched for exosomes by single particle interferometric reflectance imaging sensing (SP-IRIS) using an ExoView instrument45. SP-IRIS analyses showed that a similar number of EV particles eluted from CD9 immunoaffinity capture from

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Over-representation of repetitive elements in OS compared to control EV preparations in a validation cohort. (a) Violin plots representing relative abundance of HSATI, HSATII, L1P1 and Charlie 3 by RT-qPCR of control (n = 6–11) and OS (n = 7–8) serum EV preparations. RT-qPCR was normalized against C. elegans external spike-in miR-39–3p RNA. White lines represent median. (b) Violin plots representing relative abundance of HSATI, HSATII, L1P1 and Charlie 3 DNA by qPCR, in the absence of reverse transcription, in control (n = 12) and OS (n = 8) serum EV preparations. qPCR was performed on equal proportions of nucleic acid extracted from 200 ul of OS and control serum. White lines represent median. (c) Diagnostic value of HSATI, HSATII, L1P1 and Charlie 3 DNA sequences in serum OS preparations. ROC curves were generated using data in (b). Groups were compared using two-tailed, unpaired, Mann Whitney U test; *p < 0.05; **p < 0.01; ***p < 0.001.
control (21,006 particles, n = 1) and OS sera (21,264 ± 374 (SEM) particles, n = 2), that the eluted particles could be re-immunocaptured on the microarray-based solid phase chip coated with antibodies to exosomal surface markers (Fig. 5k,l), and that the re-captured particles were from 50 to 80 nm diameter (characteristic of exosomes) (Fig. 5m,n) and expressed various combinations of exosomal markers CD81, CD63, and CD9, similar to the PEG precipitated EV preparations of the same samples (Supplementary Fig. 10). Nanoparticle tracking analyses of the CD9 and CD81 immunoaffinity capture eluates showed particle size distributions similar to that of PEG precipitations but larger than reported by SP-IRIS (Fig. 5o,p, Supplementary Fig. 11A and B), as expected46. However, in contrast to PEG- and SEC-isolated EVs, CD9 and CD81 immunoaffinity captured EV preparations showed no significant difference in HSATI and HSATII DNA abundance between control and OS samples (Fig. 5q,r and Supplementary Fig. 11C). Thus, the OS-associated HSATI and HSATII DNAs failed to co-purify with EVs during immunocapture in contrast to their co-purification with EVs isolated via PEG precipitation or

Figure 4. Repetitive element sensitivity to DNase in EV preparations. (a) Abundance of HSATI and HSATII sequences determined by qPCR in control (n = 4) and OS (n = 4) serum EV preparations isolated with PEG and either untreated (UT) or pretreated with DNase I or RNase A with or without NaCl prior to nucleic acid extraction. Treated groups were compared to untreated groups using unpaired Kruskal–Wallis test with uncorrected Dunn’s test where each comparison stands alone; *, p < 0.05. Error bars represent standard deviation of biological replicates. (b) Bioanalyzer electropherograms of equal proportions of nucleic acids prepared from 200 ul of representative control and OS EV preparations that were untreated or treated with DNase I or RNase A prior to nucleic acid extraction. (c) Violin plots representing relative abundance of HSATII, L1P1 and Charlie 3 by RT-qPCR in control (n = 4) and OS (n = 4) serum EV preparations pre-treated with DNase I. qPCR was normalized against C. elegans external spike-in miR-39–3p RNA. White lines represent median.
were sensitive to DNase I, indicating that they were not a protected EV cargo (Fig. 4a), in contrast to DNAs in non-OS sera that were not tightly bound to CD9+ or CD81+ exosomes. In a validation cohort and found to reflect repetitive element DNAs that co-purified with circulating EVs but were not tightly bound to CD9+ or CD81+ exosomes. HSATI and HSATII were the most significantly increased upon mapping to the GRCh38 and hg19 genome builds. The over-represented repetitive element sequences were confirmed in a validation cohort and found to reflect repetitive element DNAs that co-purified with circulating EVs but were not tightly bound to CD9+ or CD81+ exosomes. HSATI and HSATII were distinguished from other repetitive elements in that they were enriched in serum EV preparations but not in total cfDNA, implying that they segregated into distinct complexes in the circulation of OS patients.

Our detection of increased repetitive element DNAs in OS patient sera was enabled by a novel screening approach. First, in the discovery cohort, we prepared serum EVs using a precipitation method that concentrates exosomes as well as other EVs and non-vesicular constituents, which enlarged the population of biomarker candidates. Second, we did not treat EVs or nucleic acid preparations with DNase, which enabled isolation of DNAs as well as RNAs and further diversified the potential biomarker pool. Third, we isolated nucleic acids using small RNA preparation kits that also captured repetitive element DNAs, and we built our sequencing library by direct ligation of adapters to extracted DNAs as well as RNAs, using an activity with properties similar to T4 RNA ligase, which allowed the discovery of differentially represented DNA as well as RNA species. Finally, we evaluated repetitive element sequences that include diverse satellite and non-satellite categories that may comprise more than two-thirds of the human genome. Repetitive elements are often ignored in human sequencing studies because of the complexity involved in properly aligning short sequencing reads to highly repetitive regions as well as poor understanding of their functional relevance. However, the paucity of over-represented single copy sequences in OS patient EV preparations prompted us to consider whether repetitive element sequences might be over-represented.

To examine differential repetitive element sequence representation, we initially used RepeatMasker to align sequence reads against the Repbase library of known repeats. This revealed an over-representation of all repetitive element categories in OS serum EV preparations, with the LINE1 family member L1P1 as the most significantly over-represented species (Supplementary Fig. 2). To more accurately identify differentially represented repetitive elements, we used T1Transcripts, which assigns both uniquely and ambiguously mapped reads to all possible gene and transposable element-derived transcripts in order to statistically infer the correct gene or transposable element abundances. T1Transcripts analyses confirmed that repetitive elements were over-represented in OS serum EV preparations and identified HSATI, HSATII and Charlie 3, among others, as significantly over-represented (Table 2). Different elements were identified when reads were aligned to GRCh38 or to hg19, likely due to the presence of alternative repetitive-element-enriched sequence assemblies in GRCh38.

We validated the increased abundance of selected repetitive element sequences in a second patient cohort. In the validation set, we observed their increased abundance via qPCR, without reverse-transcription and in a DNase-sensitive manner, implying differential representation of repetitive element DNAs. Repetitive element DNA sequences were similarly increased in OS samples from USA (CHLA) and China (HLOH) and did not correlate with the OS grade, suggesting that these elements are produced independently of OS type. EV-associated repetitive element DNAs showed a high sensitivity and specificity for sera of patients with an OS diagnosis, with a significant AUC > 0.9 for HSATI, HSATII and L1P1. However, the sensitivity was diminished by omitting the EV preparation step, particularly for HSATI and HSATII (Fig. 6), suggesting that these OS-associated repetitive element DNAs are segregated from bulk cfDNA.

At present the physical state of the repetitive element DNAs that enables their enrichment from OS patient sera is unknown. Prior studies detected repetitive element DNAs in growth medium of human OS cells, yet their physical state was not characterized. In serum EV preparations, the OS-associated repetitive element DNAs were sensitive to DNase I, indicating that they were not a protected EV cargo (Fig. 4a), in contrast to DNAs in cultured cancer cell-derived microvesicles. As repetitive element DNAs co-purified with EVs in SEC precipitation and SEC but not in CD9 or CD81 immunoaffinity capture, they might associate with exosomes too weakly to survive immunocapture, associate with CD9- and CD81-negative exosomes, associate with non-exosome EVs,
Figure 5. Co-purification of OS-associated repetitive element DNAs with EVs in size exclusion chromatography but not exosome immunoaffinity capture. (a,b) Representative protein concentration (blue line) and EV concentration (black bars) elution profiles from control (a) and OS (b) serum separated by size exclusion chromatography (SEC). (c,d) Representative size distribution of control (c) and OS (d) serum EV particles in pooled fractions 6 and 7 analyzed by nanoparticle-tracking. (e,f) Relative abundance of HSATI and HSATII DNA in two control and two OS SEC (e) and PEG (f) EV fractions as defined by qPCR. (g,h) Representative protein concentration (blue line) and nucleic acid concentration (black bars) elution profiles from control (g) and OS (h) SEC separations as in (a) and (b). (i,j) Abundance of HSATI (i) and HSATII (j) in pooled EV fractions 6–7 and non-EV fractions on one control and one OS SEC analysis as evaluated by qPCR. (k–n) SP-IRIS analyses by ExoView of EVs isolated by CD9-immunoaffinity capture. (k,l) Representative concentration of control (k) and OS (l) fluorescent EV particles immunocaptured on the CD9, CD81 and CD63 antibody spots. Results depict the mean of the measurement of triplicate spots ± SEM, subtracted for IgG spot values and adjusted by dilution factor. (m,n) Representative size distribution of control (m) and OS (n) label-free EV particles immunocaptured on the CD9, CD81 and CD63 antibody spots. Results depict the mean of the measurement of triplicate spots ± SEM, subtracted for IgG spot values. (o,p) Size distribution and particle number of control (o) and OS (p) EVs isolated by CD9 immunoaffinity capture and analyzed by nanoparticle-tracking. (q,r) Violin plots representing abundance of HSATI (m) and HSATII (n) of control (n = 6) and OS (n = 8) immunoaffinity capture of CD9-positive exosomes evaluated by qPCR. White lines represent median. Groups were compared using two-tailed, unpaired, Mann Whitney U test; *p < 0.05. Similar CD81 immunoaffinity capture results in Supplementary Fig. 11.
or participate in large non-vesicular complexes that co-purify with EVs in the PEG and SEC methods. Despite prior reports that DNA is present in cancer exosomes or small EVs27,50–52, a recent reassessment demonstrated that small vesicles are not vehicles of active DNA release and that double stranded DNA was associated with non-vesicular entities that are extruded from cancer cells53. Similar to our results, this reassessment did not observe DNA associated with immunoaffinity capture of CD81-positive exosomes, further supporting that exosomes do not contain DNA or tightly associate with other particles that contain DNA. Understanding the biogenesis of repetitive element DNAs that are enriched in OS patient EV preparations could provide insight into OS pathogenesis as well as a tool with which to detect asymptomatic OS or other conditions in which EV-associated repetitive element DNAs are elevated.

A limitation of this study is that it is based on two relatively small patient cohorts and examined a mixture of EV-associated DNAs and RNAs in the discovery cohort. Thus, the sensitivity of the repetitive element DNA analyses for OS and the specificity for OS versus other cancers may be refined by defining differential DNA representation in a larger series of OS as well as other cancers and non-cancerous conditions. Nevertheless, the results suggest that EV-associated repetitive element DNAs are among the most sensitive markers of newly diagnosed OS identified to date, with ROC curve AUCs of 0.90 for HSATI and 0.97 for HSATII (Fig. 3c). By comparison, in prior OS biomarker analyses, circulating miRNAs had at best ROC curve AUCs of 0.833–0.955, yet were significantly elevated in only a subset of many similar miRNA screens11,54–56. Likewise, a deep sequencing approach detected circulating tumor DNA aneuploidy in only 50% of treatment-naïve OS patients12. Still, in this study, the increased repetitive element DNAs were observed in sera of already-diagnosed OS patients, and further refinements are likely to be required in order to detect repetitive element DNAs produced by smaller, incipient tumors prior to their clinical appearance.

A final question raised by our findings is whether similar EV-associated repetitive element DNAs are increased in the circulation of patients with other cancers. Notably, centromeric and pericentric repetitive element RNA sequences, particularly alpha satellites and satellite II and III sequences, were reported to be overexpressed in testicular, liver, ovarian, and lung cancers compared to corresponding normal tissues57. The pericentric human satellite II (HSATII) RNA was reported to be the most differentially expressed satellite subfamily in pancreatic
cancer tissue and was also overexpressed in lung, kidney, ovarian, colon and prostate cancers\textsuperscript{58,59}. Moreover, \textit{HSATII} was one of the six most up-regulated satellite sequences in a study comparing fresh bone and OS samples by RNA-seq\textsuperscript{12}. \textit{LINE-1} was also overexpressed in pancreatic and prostate tumor samples\textsuperscript{60,61}. However, although \textit{LINE1} and other repetitive element RNAs were detected in cancer cell-derived EVs in culture\textsuperscript{27}, their up-regulation has not been reported for circulating cell-free RNA in cancer patients. Our detection of the enriched DNA templates of these repetitive element RNAs in the OS patient circulation raises the possibility that circulating repetitive element DNAs may also be enriched in additional cancer types.

Materials and methods

Patients and samples. This study was reviewed and approved by the institutional review board at Children’s Hospital Los Angeles (approval no. CCI-13-00223) and at Henan Luoyang Orthopedic Hospital (approval no. 2015-01). All participants gave a written informed consent. Parents/Legally authorized persons gave informed consent on behalf of the all minors and subjects above 14 years old gave assent. All analyses were conducted in accordance with relevant guidelines and regulations. Blood samples were collected during a clinically indicated venipuncture from previously untreated patients with primary diagnosis of OS and from volunteer subjects with no known medical conditions, i.e. healthy controls. Control sera for the validation cohort were obtained from local volunteer subjects and from Innovative Research Inc. (Novi, MI, USA). Blood was drawn in serum separator collection tubes (SST), clotting was allowed for 30 min at room temperature in vertical position and then tubes were centrifuged at 1000g for 10 min at 4 °C. Serum was collected, immediately aliquoted, and stored at −80 °C.

Discovery cohort: EV isolation, nucleic acid extraction, and sequencing. Serum EVs were isolated using ExoQuick (System Biosciences Inc. (SBI), Mountain View California, USA) and aliquots frozen. One aliquot was used for NTA analyses and on confirmation of high EV purity aliquots were thawed and nucleic acid extracted using SeraMir (SBI) without DNase treatment, according to manufacturer instructions. The sequencing library was constructed using TailorMix miRNA Sample Preparation (SeqMatic) with a selection of small nucleic acids from 140 to 300 bases. 5′-RNA adapters and 3′-DNA adapters (SeqMatic, personal communication) were directly ligated to nucleic acid substrates, followed by PCR amplification. Libraries were sequenced to generate single-end 50 bp reads on MiSeq 500 platform (Illumina).

Validation cohort: EV isolation and nucleic acid extraction. Serum was cleared by centrifugation at 3000 × g for 15 min at 4 °C. For polyethylene glycol (PEG) precipitation, 50–200 ul of cleared serum was combined with an equal volume of freshly prepared 16% PEG 6000 (Sigma-Aldrich) in 1 M NaCl, to give a final concentration of 8%, incubated for 30 min on ice, centrifuged in a tabletop microfuge at 16,000 × g for 2 min at room temperature (Eppendorf, model 5424 R using an FA-45-24-11 fixed angle rotor) and the pellet resuspended in a volume of PBS equal to that of the starting serum volume. For size exclusion chromatography (SEC), ~ 300 ul of cleared supernatant was centrifuged at 10,000 g for 30 min at 4 °C in a fixed angle rotor and loaded onto a glass Econo-column (Bio-Rad, 10 cm height, 1.5 cm diameter) packed with Sephacryl S-300 High Resolution Collector (Bio-Rad). For each fraction, the protein concentration and the presence of EVs was characterized by gravity flow and eluate collected in 20 fractions of 15 drops (~ 500 ul) on a Model 2110 Fraction Collector (Bio-Rad). For each fraction, the protein concentration and the presence of EVs was characterized by Bradford method (Bio-Rad) and nanoparticle tracking analysis (see below), respectively. EV fractions were concentrated on a 100 kDa Amicon ultra centrifugal filter (Millipore) from 2 × 500 µl to a final volume of ~ 100 ul. Immunoaffinity capture of CD81 + or CD9 + EVs was carried out using the Exo-Flow Exosomes Purification Kit (SBI, Mountain View California). Briefly, 200 ul of cleared serum was precipitated with 200 ul of 16% PEG 6000 as above, and the pellet re-suspended in 200 ul of PBS. 50 ul of this EV preparation were incubated in 20 µl of anti-CD81 or anti-CD9 pre-coated magnetic beads (9.1 µm) on a rotating rack at 4 °C overnight. CD81 + or CD9 + EVs were eluted from the beads in the Exosome Elution Buffer at 25 °C for 30 min.

Nucleic acids were extracted from 20 to 200 ul of EV preparations (or from 50 ul of serum) using miRNeasy Micro kit (Qiagen) and suspended in 14 ul of RNase/DNase-free H₂O (depending the initial volumes of serum) according to the manufacturer’s instruction. For samples intended for reverse-transcription, a spike-in control (\textit{C. elegans} miR-39-3p) miRNA mimic (Qiagen) was added (1.6 × 10⁸ copies) after the lysis step. Nucleic acid size and concentration were analyzed on an RNA Pico 6000 chip using an Agilent Bioanalyzer (Agilent, Palo Alto, CA, USA), equipped with Expert 2100 software, which generated an electrophoretic profile and the corresponding ‘pseudo’ gel of the sample. After separation, nucleic acid sizes were normalized to a 25 bp RNA marker. Samples showing nucleic acids of > 200 bp were eliminated from the study.

Particle size and concentration measurement by nanoparticle tracking analysis. EV preparations were analyzed by nanoparticle tracking using a NanoSight NS300 (Malvern, Worcestershire, UK.) configured with a high sensitivity sCMOS camera (OrcaFlash2.8, Hamamatsu C11440, NanoSight Ltd). In brief, each sample was mixed by vortexing, and subsequently diluted in particle-free PBS to obtain a concentration within the recommended measurement range (10⁸–10⁹ particles/mL), corresponding to dilutions from 1:100 to 1:500. After optimization, settings were kept constant between measurements. Ambient temperature was recorded manually and did not exceed 25 °C. Approximately 20–40 particles were in the field of view for each measurement. Three videos of 30 s duration were recorded for each sample. Experiment videos were analyzed using NTA 3.2 Dev Build 3.2.16 software (Malvern).
Single particle interferometric reflectance imaging sensing (SP-IRIS).

EVs from PEG and immunocapture preparations were analyzed on ExoView R100 platform (Nanoview Biosciences, MA). Briefly, EVs within these preparations were immunocaptured on a multiplexed microarray chip with CD9, CD81 CD63, and CD41a antibody spots, as well as negative control IgG antibody spots to determine the level of non-specific binding, and then probed for CD9, CD81, CD63, and CD41a surface markers with respective additional fluorescent antibodies. EVs from PEG preparation and eluted EVs from immunoinaffinity were diluted in solution A (Nanoview Biosciences, MA). The samples were incubated on the ExoView Tetraspanin Chip (EV-TC-TTS-01) placed in a sealed 24-well plate for 16 h at room temperature. The chips were then washed three times in 1 ml PBST for 3 min each on an orbital shaker. Then, chips were incubated with ExoView Tetraspanin Labeling A/Bs (EV-TC-AB-01) that consist of anti-CD81 Alexa-555, anti-CD63 Alexa-488, and anti-CD9 Alexa-647. The antibodies were diluted 1:5000 in PBST with 2% BSA. The chips were incubated with 250 µL of the labeling solution for 2 h. The chips were then washed once in PBST, three times in PBST followed by a rinse in filtered deionized water and dried. Immunocaptured EVs on the microarray chip were imaged on a single EV-basis with the ExoView R100 reader using the nScan2 2.9 acquisition software. The data were then analyzed using the NanoViewer tool (Nanoview Biosciences, MA) that counts and sizes fluorescent nanoparticles immunocaptured on the antibody spots. For exosome analysis the size window was selected to include particle sizes from 50 to 200 nm.

Reverse transcription (RT) and qPCR.

Equal volumes of nucleic acid prepared as above were reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) in 20 ul volume according to the manufacturer’s protocol. 0.5 ul of the samples produced with or without the RT step were analyzed in 10 ul qPCR reactions with iQ Green Supermix (Bio-Rad) on an ABI 7900 Fast Real-Time PCR System (Applied Biosystems) with the following cycling parameters: 94 °C, 30 s; 59 °C, 15 s; 68 °C, 25 s for 35 cycles. Relative sequence abundance was determined by the ΔΔCt method. In most PCR runs, a negative control with no nucleic acid template was added and never generated PCR product. PCR primers were designed manually to have a melting temperature of 58 °C and to generate amplicons of ~ 100 bp or as previously described for HSATI and HSATII (Supplementary Fig. 4) and obtained from Integrated DNA Technologies:

- **HSATI** F: 5′-TATGTTGCGCTTTGGAATT-3′, **HSATI** R: 5′-TGAGATGAAGAAATTACAGAGGCT-3′ (amplicon: 406 bp);
- **HSATII** F: 5′-ATTCCATTCTGATGATTCC-3′, **HSATII** R: 5′-GGAACCGAAGTAACTCTTAGAATG-3′ (prominent amplicons: 85, 134, 183 and 281 bp);
- **L1P1-orf2** F: 5′-ATCAGAGATTCTCAAAACACCTCTAC-3′; **L1P1-orf2** R: 5′-AGAGTGATGCTGCAGGAATG-3′ (amplicon: 83 bp);
- **Charle3** F: 5′-ACAAAAGGACGTGAACGAGCCTGC-3′, **Charle3** R: 5′-TCCAGTCTACTCGTAACTCTG-3′ (amplicon: 104 bp);
- **HECDT2** F: 5′-TGTGGAAGACCTTCCAGAAGATGATGAAA-3′, **HECDT2** R: 5′-GAGGGATGCGATTCTTCTTATAAGT-3′ (amplicon: 131 bp).

DNase I and RNase A treatment of EV preparations.

Intact EV preparations were treated with DNase I (Qiagen) in RDB buffer for 15 min at room temperature and then inactivated for 10 min at 70 °C. Intact EV preparations were treated with RNase A (Thermo Scientific) at final concentration 0.4 ug/ul with or without NaCl at final concentration 1 M for 10 min at 37 °C and inactivated by RNase inhibitor (Takara) at final concentration 1 M for 10 min at 37 °C.

RNA-Seq data processing, alignment and analysis.

Fastq files were aligned to GRCh38 for analysis 1) or hg19 (for analysis 2) using STAR using the parameters recommended for TEtranscripts (i.e. allowing for up to 100 alignments per read)24, and the resulting BAM files were processed using TEtranscripts to quantify both non-repetitive element and repetitive element abundance.

Statistical analysis.

Groups were compared using two-tailed, unpaired, Mann Whitney U test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). All analyses were performed using Prism 8 software (GraphPad).
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Author contributions
L.C. established hypotheses, designed studies, performed experiments, analyzed data, and wrote the paper. K.S. managed samples and performed RNA-seq and repetitive element analyses. M.T. performed RNA-seq analyses. R.J., J.L., M.Y., W.L., and J.Z. accrued, annotated, and provided samples. D.C. established hypotheses, designed studies, accrued samples, co-authored data, and co-wrote the paper.

Competing interests
D.C. and L.C. are inventors on a U.S. provisional patent, Children’s Hospital Los Angeles as applicant, relating to REPETITIVE ELEMENT DNAs.

Additional information
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Correspondence and requests for materials should be addressed to J.L. or D.C.

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