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14. ABSTRACT
The overarching goal of my laboratory is to investigate whether circulating LO, a novel class of atypically large (1-10μm diameter), bioactive extracellular vesicles (EVs), which are released by highly invasive and metastatic amoeboid tumor cells in the plasma, and contain abundant RNA, miRNA, DNA, and protein cargo, report clinically relevant information and tumor-specific genomic alterations, thus representing a valuable alternative and/or complement to other technologies proposed as a means of liquid biopsy. Collectively our findings indicate that DNA analyses of LO in blood (plasma) may provide a faithful representation of the genome of the tumor cells of origin. Because we have performed also comparative analysis with other EVs and shown that *LO are a source of high quality and abundant DNA and they contain the whole genome of donor tumor cells*, our overall objective is to **test whether enumeration and genomic profiling of LO circulating in patient blood allow early detection of metastatic PC and identification of clinically significant PC-specific genomic aberrations**, thereby overcoming the current limitations of the "liquid biopsy"

15. SUBJECT TERMS
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Table of Contents

Page

Accomplishments

In the second year of the DoD-funded project, we performed the very first large-scale DNA analysis of two distinct extracellular vesicle (EV) populations, large (L-) and small (S-)EVs, isolated from 2ml plasma specimens obtained from two patients with metastatic castration resistant prostate cancer (mCRPC). For each patient, sequencing data/genetic profile of matching metastatic tissue biopsy was available to us through Dr. Edwin Posadas at Cedars-Sinai Medical Center. L- and S-EVs were isolated by differential centrifugation. Total amount of dsDNA in the L-EV fractions was 7.32 ng and 228.3 ng, in the S-EV fractions the amount of dsDNA was 9.33 ng and 3.45 ng, respectively. After standard quality control of the EV DNA samples, whole exome sequencing at 1500-2000x was performed at the Genomics Resources Core Facility at Weill Cornell Medical College, New York, USA. Germline DNA (buffy coat) from each patient was used as control. For data analysis of this highly reach sequencing, we established a collaboration with Dr. Francesca Demichelis, University of Trento, Italy. Dr. Demichelis has established a pipeline for analysis of whole sequencing data on cell free (cf)DNA (PMID: 25232177, PMID: 26537258). Somatic copy number aberrations and mutations specific for mCRPC were identified.

In the L-EV DNA from one of the two patients, a tissue-quality tumor signal showing 65% tumor purity and 2.86 ploidy was detected. Moreover, this tumor signal was represented by typical mCRPC genomic alterations, such as amplifications of *AR* and *MYC*, and deletions in *BRCA2* and *RB1* (Figure 1). When we compared these results with the results of the FoundationOne test, which detects all classes of genomic alterations in more than 300 cancer-related genes, on the matched tumor tissue from this patient, we identified the same alterations (e.g. *AR* amplification, *BRCA2* deletion). Importantly, plasma L-EV DNA reported additional mCRPC-specific genomic aberrations that had not been detected in the tissue biopsy (e.g. *MYC* amplification, *RB1* deletion). This suggests that the DNA associated with circulating L-EVs might be more informative in comparison with the DNA extracted from tissue biopsy, because it contains genetic information from different metastases throughout the body. The S-EV DNA from the same patient exhibited a similar profile (not shown).

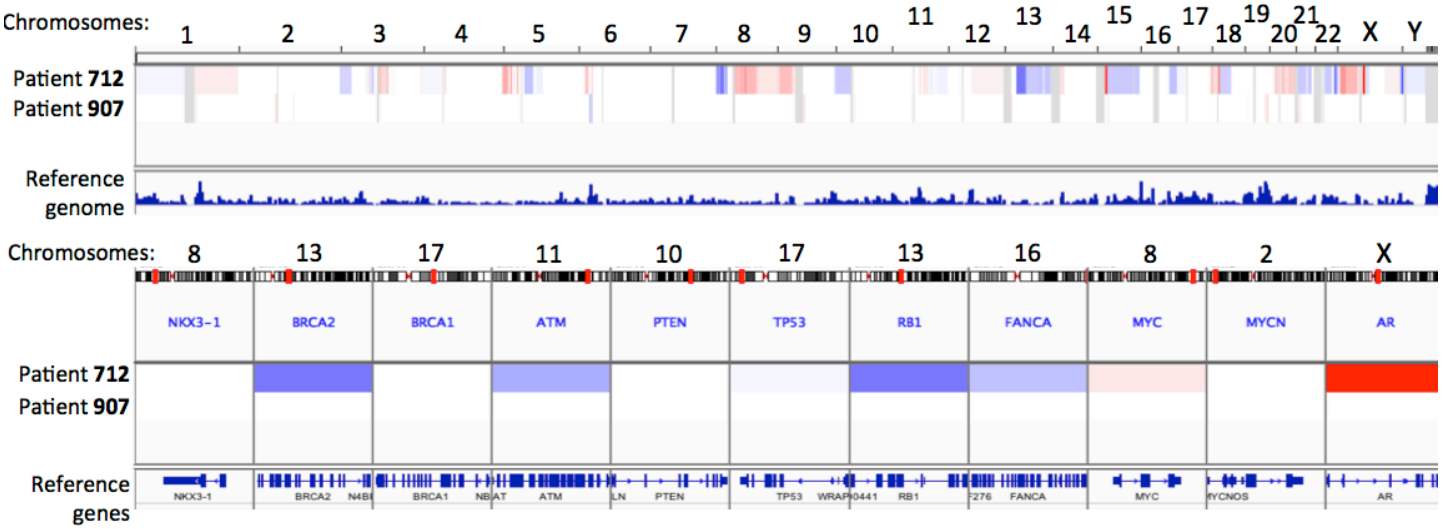

Figure 1. **EV-associated DNA reflects typical for mCRPC genomic alterations**. Upper panel shows genetic landscape across all chromosomes; amplifications are marked in red, deletions are marked in blue. Lower panel shows genomic aberrations in the genes commonly altered in mCRPC.

We have demonstrated that most of the tumor DNA circulating in prostate cancer patient plasma is associated with L-EVs (*Vagner, Spinelli, et al. Journal of Extracellular Vesicles 2018*). However, the tumor signal detected in the L-EV fraction might also arise from the circulating cfDNA, which might co-precipitate with L-EVs or/and be associated with the surface membrane of vesicles. To find out if the tumor signal detected in the L-EV fraction originates from the DNA content of these vesicles (and, thus, may provide different from the circulating cell-free tumor DNA information), we (i) developed a nuclease treatment protocol for EVs, to get rid of extravesicular DNA, and (ii) established a system that allows us to distinguish between the extravesicular DNA and the DNA contained inside the EVs.

(i) First, we treated the samples with nucleases, DNaseI and ExoIII, to quantitatively assess the amount of extravesicular and intravesicular EV-associated DNA. Briefly, EVs from PC3 cell line were spiked into 1 ml of plasma obtained from healthy donors. L- and S-EV fractions were isolated from plasma specimens and DNA was extracted either from EVs directly, without any pre-treatment, or following pre-treatment with nucleases (Figure 2, upper panel). We observed that the amount of DNA recovered from either L- or S-EV fractions was substantially reduced when EVs had been pre-treated with nucleases (Figure 2). This result suggested that a large portion of EV DNA is extravesicular and accessible to nucleases. However, more than 1/3 of the DNA in L-EVs and 1/10 of the DNA in S-EVs was clearly intravesicular leading us to the conclusion that a portion of the DNA is enclosed into EVs, and the relative portion of DNA in L-EVs is higher than in S-EVs – possibly due to the fact that the L-EV volume is ~1,000 times larger than the S-EV volume.

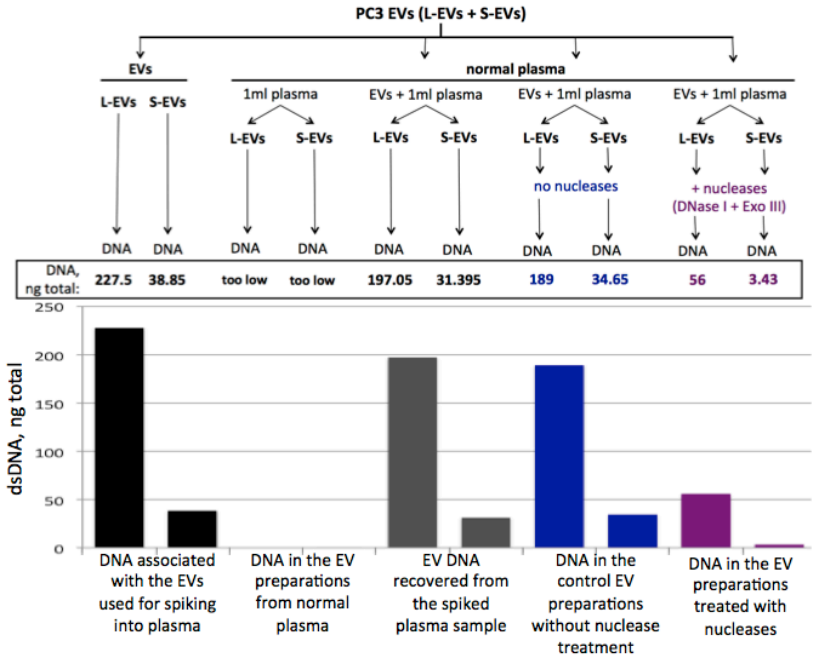

Figure 2. Large portion of EV-associated DNA is extravesicular. Experimental design (upper panel) and quantification of the results (lower panel) of the spike-in experiment. PC3-derived EVs were spiked into 1 ml of plasma from healthy donors. Following EV isolation by ultracentrifugation, the EV DNA was extracted with or without pre-treatment with nucleases. The DNA yield was quantified by High Sensitivity dsDNA Qubit Assay.

(ii) We next sought to confirm that the DNA that resists nuclease treatment (upon pre-treatement with nucleases), is indeed intravesicular. In order to do so, PC3-derived EVs were spiked into 1 ml of plasma from healthy donors together with genomic (g)DNA extracted from non-cancerous cells (primary lung fibroblasts IMR90, ATCC® Number: CCL-186™). PC3 cells exhibit a highly aberrant genome and have a homozygous deletion of *PTEN* among other gains and losses that manifest as somatic copy number variations (CNV). Therefore, we reasoned that we would be able to distinguish between the intravesicular PC3 DNA and extravesicuar IMR90 DNA based on the detectability of the *PTEN*, which would be undetectable in the intravesicular PC3 DNA but detectable in the extravesicular IMR90 DNA by digital (d)PCR. Thus, the presence of the *PTEN* dPCR product would indicate the presence of extravesicular DNA in the sample, and the absence of the *PTEN* dPCR product would indicate that only intravesicular DNA is present in the sample. We used dPCR with specific *PTEN* primers to test this hypothesis.

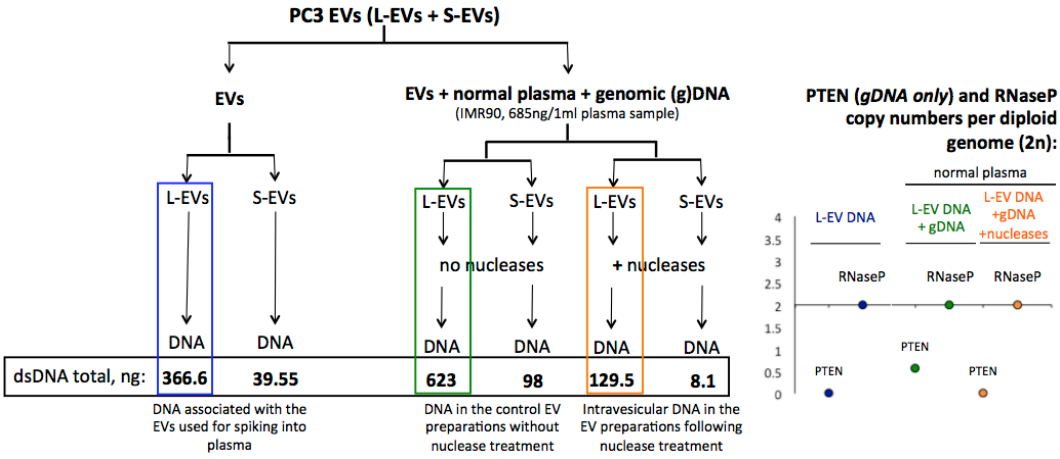

Figure 3. Nuclease treatment digests DNA outside of the EVs leaving the intravesicular DNA intact. The scheme on the left shows experimental design and quantification of the results. PC3-derived EVs and IMR90 gDNA were spiked into 1 ml of plasma from healthy donors. Following EV isolation by ultracentrifugation, the EV DNA was extracted with or without pre-treatment with nucleases. The DNA yield was quantified by High Sensitivity dsDNA Qubit Assay and PTEN copy number per diploid genome was quantified by dPCR (plot on the right).

After spiking PC3-derived EVs and IMR90 gDNA into plasma, L- and S-EVs were isolated from the plasma specimens, and DNA was extracted from the EVs directly or following nuclease treatment (Figure 3) – like in the previous experiment.

In agreement with the previous result, there was a substantial decrease in the amount of DNA obtained from the nuclease-treated EVs (Figures 2 and 3). To find out the intra- or extravesicular localization of the EV-associated DNA, dPCR was performed on the L-EV DNA samples and *PTEN* CNV per diploid genome was quantified using RNaseP as a reference gene. We found that *PTEN* transcripts were present in the DNA from the untreated L-EVs, which meant that extravesicular gDNA had co-precipitated with the vesicles and was present in the L-EV DNA sample (Figure 3, green). However, *PTEN* transcripts were undetectable in the DNA from the nuclease-treated L-EVs, suggesting that the extravesicular gDNA had been digested by nucleases and only intravesicular DNA was present in the sample (Figure 3, yellow and blue).

Future Directions:
The EVs used for the whole exome sequencing experiment described above were not pre-treated with nucleases. Therefore, it cannot be concluded if the tumor signal detected in the EV DNA originated from the intravesicular DNA, or the mixture of intra- and extravesicular circulating tumor DNA. As a next step, we are planning on interrogating the origin of the tumor signal in the plasma EV fractions by comparing, side-by-side, the tumor signal in the DNA extracted from (i) plasma (circulating cfDNA), (ii) EVs without nuclease treatment (like in the original experiment), and (iii) EVs treated with nucleases. For this experiment, 6 ml of plasma obtained from mCRPC patients will be provided by our collaborator Dr. Posadas, and will be split in 3 x 2 ml aliquots. One aliquot will be used for the direct extraction of cfDNA (i) and the remaining two aliquots will be used for EV isolation followed by the DNA extraction with or without nuclease treatment ((ii) and (iii)). After standard quality control of the EV DNA samples, whole exome sequencing will be performed and data analyzed as described above.

In parallel experiments, during the first year of the DoD funding, we identified molecules whose alterations are responsible for nuclear membrane instability and provided evidence that this results in the shedding of EVs that contain nuclear material. During the second year of the DoD funding, these results were published (*Reis-Sobreiro et al., Cancer Res. 2018*). We have now also determined the size of the intact vesicular DNA, which supports our hypothesis that EVs can contain nuclear material. To accomplish this, we lysed EVs directly in agarose plugs and resolved EV DNA by pulse-field gel electrophoresis (PFGE). Resolution of high molecular weight DNA, which was possible with this method, revealed that L-EVs contain DNA fragments up to 2 Mbp (Figure 4A). In addition, DNA fragments in the size range of 100 Kbp–2 Mbp were enriched in L-EVs compared to whole cells and were undetectable in S-EVs (Figure 4A). This is in agreement with our previous results suggesting a distinct process of DNA packaging in L-EVs. We have also analysed the high molecular weight DNA contained in plasma-derived EVs. Even though the amount of EV DNA obtained from 1 ml of plasma was significantly lower than the amount of DNA in our *in vitro* system, the size of the intact DNA in plasma-derived L-EVs was in the size range of 100 kbp–2 Mbp (Figure 4B), replicating our *in vitro* findings. The amount of DNA in S-EVs was, again, undetectable.

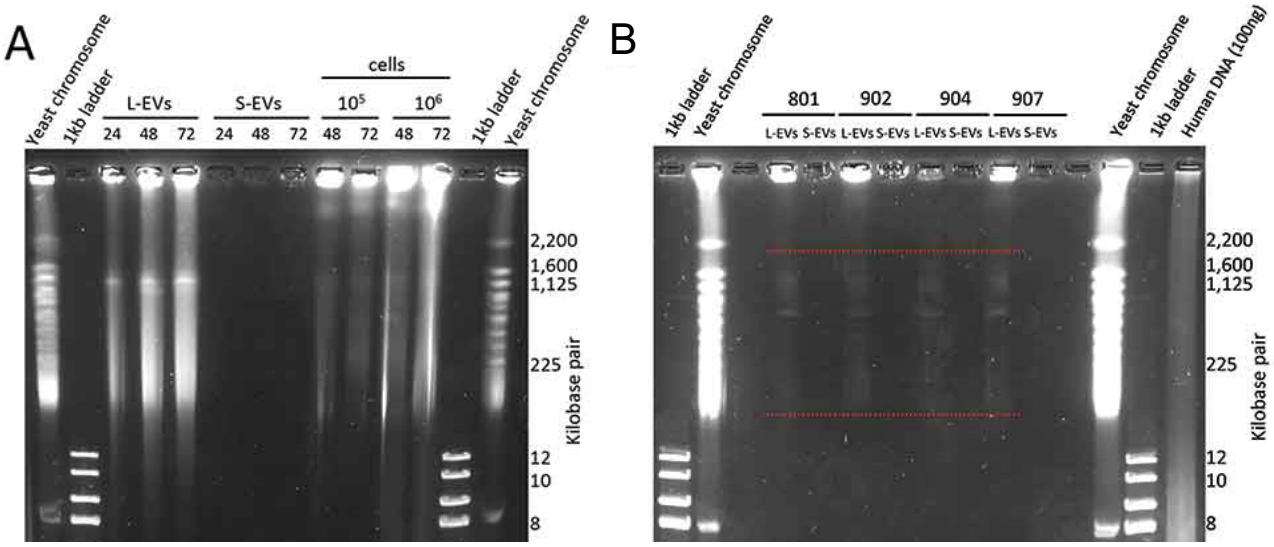

Figure 4. EVs contain high molecular weight DNA. A. PC3 L-EV and S-EV DNA was extracted in agarose plugs by incubation in lysis buffer for 24, 48 or 72 h, and high molecular weight DNA was resolved by PFGE, which revealed that L-EVs contain large DNA fragments (100 kbp–2 Mbp). **B**. EVs were isolated from 1 ml of plasma obtained from mCRPC patients, EV DNA was extracted in agarose plugs by incubation in lysis buffer for 48 h, and high molecular weight DNA was resolved by PFGE. Similar to L-EVs *in vitro*, patient plasma-derived L-EVs contain high molecular weight DNA (100 kbp–2 Mbp) (indicated by red dashed lines).

Products:

Eight manuscripts were published under this second year of funding, one more is under review in Proteomics, and one more is in preparation.

1. Zijlstra A, **Di Vizio D**. Size matters in nanoscale communication. Nat Cell Biol. 2018 Mar;20(3):228-230.
2. Vagner T, Spinelli C, Minciacchi VR, Balaj L, Zandian M, Conley A, Zijlstra A, Freeman MR, Demichelis F, De S, Posadas EM, Tanaka H, **Di Vizio D**. Large Extracellular Vesicles carry most of the tumor DNA circulating in prostate cancer patient plasma. J Extracell Vesicles. 2018 Aug 7;7(1):1505403
3. Reis-Sobreiro M, Chen JF, Novitskaya T, You S, Morley S, Steadman K, Gill NK, Eskaros A, Rotinen M, Chu CY, Chung LWK, Tanaka H, Yang W, Knudsen BS, Tseng HR, Rowat AC, Posadas EM, Zijlstra A, **Di Vizio D**, Freeman MR. Emerin Deregulation Links Nuclear Shape Instability to Metastatic Potential. Cancer Res. 2018 Nov 1;78(21):6086-6097.
4. Lee J, Yoon YJ, Kim JH, Dinh NTH, Go G, Tae S, Park KS, Park HT, Lee C, Roh TY, **Di Vizio D**, Gho YS. Outer Membrane Vesicles Derived From Escherichia Coli Regulate Neutrofil Migration by Induction of Endothelial IL-8. Front Microbiol. 2018 Oct 11;9:2268.
5. Soekmadji C, Hill AF, Wauben MH, Buzás EI, **Di Vizio D**, Gardiner C, Lötvall J, Sahoo S, Witwer KW. Toward mechanisms and standardization in extracellular vesicle and extracellular RNA studies: results of a worldwide survey. J Extracell Vesicles. 2018 Oct 24;7(1):1535745.
6. Hillis AL, Lau AN, Devoe CX, Dayton TL, Danai LV, **Di Vizio D**, Vander Heiden MG. PKM2 is not required for pancreatic ductal adenocarcinoma. Cancer Metab. 2018 Oct 23;6:17.
7. Rotinen M, You S, Yang J, Coetzee SG, Reis-Sobreiro M, Huang WC, Huang F, Pan X, Yáñez A, Hazelett DJ, Chu CY, Steadman K, Morrissey CM, Nelson PS, Corey E, Chung LWK, Freedland SJ, **Di Vizio D**, Garraway IP, Murali R, Knudsen BS, Freeman MR. ONECUT2 is a targetable master regulator of lethal prostate cancer that suppresses the androgen axis. Nat Med. 2018 Dec;24(12):1887-1898
8. Théry C, Witwer KW, Di Vizio and several others. Minimal information for studies of extracellular vesicles 2018 (MISEV2019): a position statement of the international society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018 Nov 23;7(1):1535750.

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