A novel liquid biopsy method for detection of breast cancer

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Introduction

Improved technologies capable of characterizing system-wide changes associated with complex diseases will be required to be able to detect millions of proteins and their isoforms as well as multi-molecular complexes. We present a method for developing aptamer libraries using blood plasma exosomes that provides unprecedented system-wide coverage of native exosomal complexes.

ssODN library enrichment on individual patients plasma exosomes

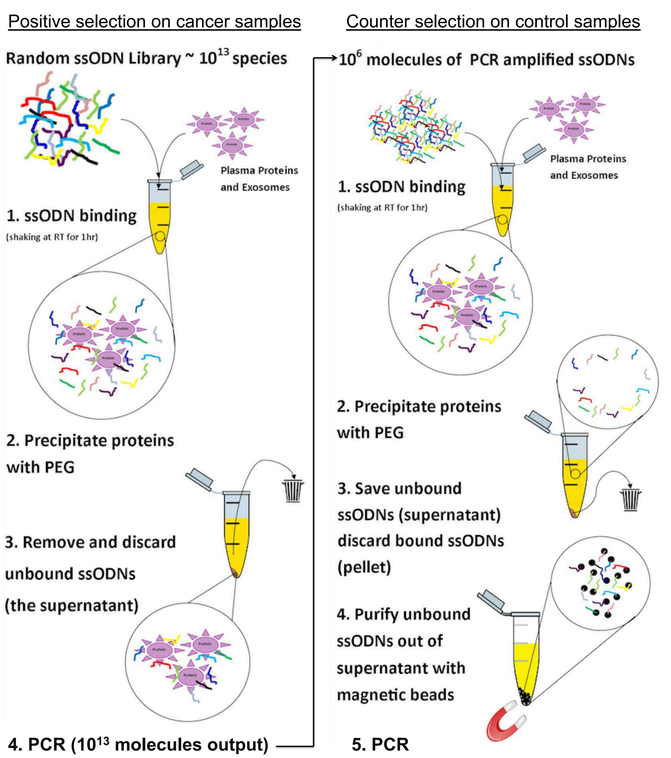


Fig. 1 Enrichment workflow: Random ssODN library (~10^{13} biotinylated ssODN species) was trained towards cancer specificity (positive selection) by incubating with plasma from cancer patients. Aptamer bound to exosomes were recovered using polyethylene glycol (PEG) followed by PCR. To remove binders to common targets, library was incubated with plasma from healthy and biopsy negative donors and unbound aptamers from the supernatant were collected (counter selection). Positive and counter selection iterations were performed for 12 rounds. Total12 libraries were trained toward individual breast cancer patients.

Exosomes isolated from blood plasma

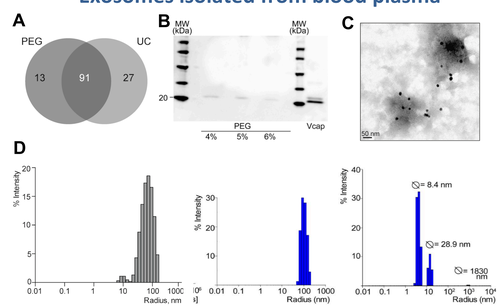


Figure 2. A) Overlap between exosome-associated proteins identified in PEG- or ultracentrifugation (UC). **B**) Western blot, CD9 Ab detected target protein in plasma exosomes, precipitated with PEG. Positive control is UC isolated exosomes from VCaP cell line. **C**) TEM images of PEG precipitated exosomes (EV) visualized by anti-CD9 antibody coupled gold-nanoparticles (black spheres). **D**) Dynamic light scattering (DLS) of plasma EV' size distribution isolated by PEG precipitation (left), UC (middle) and exosome-free HSA solution (right).

Enriched libraries binding exosomal proteins

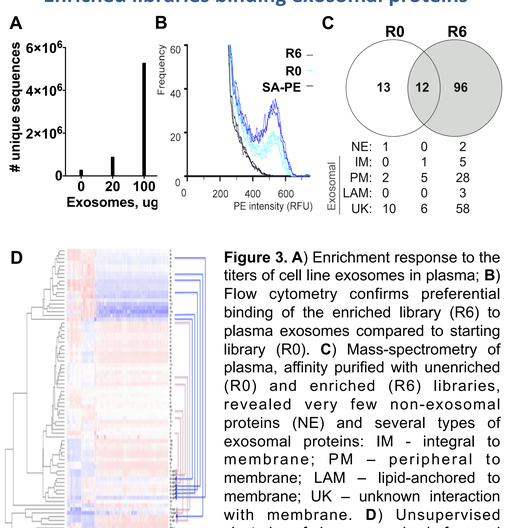


Figure 3. A) Enrichment response to the titers of cell line exosomes in plasma; **B**) Flow cytometry confirms preferential binding of the enriched library (R6) to plasma exosomes compared to starting library (R0). **C**) Mass-spectrometry of plasma, affinity purified with unenriched (R0) and enriched (R6) libraries, revealed very few non-exosomal proteins (NE) and several types of exosomal proteins: IM - integral to membrane; PM – peripheral to membrane; LAM – lipid-anchored to membrane; UK – unknown interaction with membrane. **D**) Unsupervised clustering of plasma samples before and after exosome depletion.

Validation of enriched libraries on corresponding cases

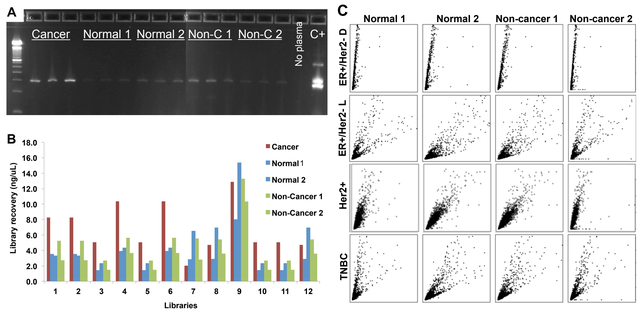


Figure 4. Recovery of enriched libraries from probing corresponding cases shown as PCR product (**A**) and quantified by QuBit (**B**). **C**) NGS raw data after volume normalized input per flow cells. ER positive and Her negative ductal breast carcinoma case (ER+/Her2-D) was used for enrichment of library C4 and can be detected with this library based on NGS data (top row) or directly from the total recovery in (**A**) and (**B**). Other enrichment cases, ER+/Her2-Lobular breast carcinoma, Her2 positive and triple negative (TNBC) can be detected as well, but might require NGS readout as well.

Training enriched libraries on unrelated cancer cases

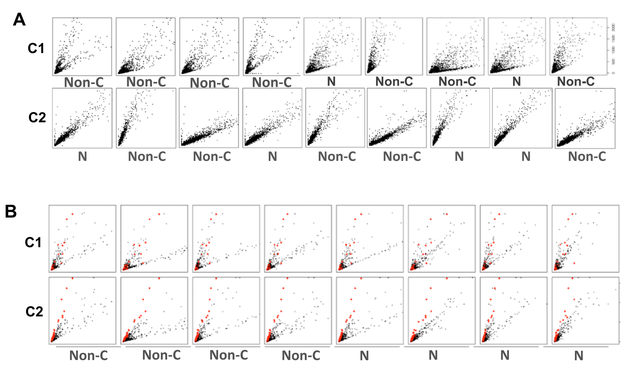


Figure 5. A) Post-enrichment test of library C7-R12, enriched on ER+/Her2- lobular breast cancer carcinoma case C1 for 12 rounds, shows bifurcated profile of case C1, while unrelated case C2 has uniform distribution of sequences. C7 library was recovered from case C2 and served as round 13 of enrichment on unrelated case. **B**) Post-enrichment test of library C7-R13 shows that profiles of the cases C1 and C2 became more similar and informative sequences, selected for C2 are mostly specific for C1 as well. This suggests that one additional enrichment round on unrelated case allowed to expand library specificity and detect unrelated case.

Validation of enriched libraries on unrelated cases

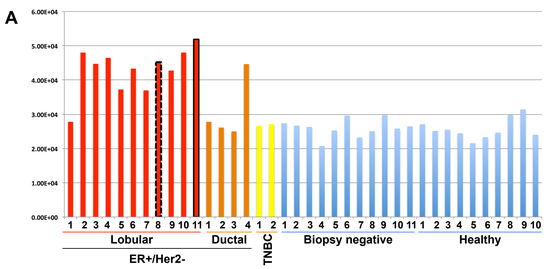
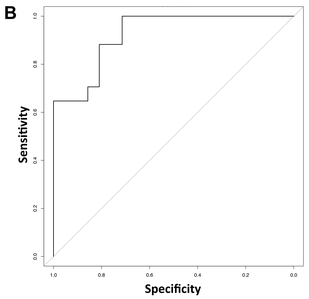


Figure 6. C7-R13 library test on unrelated cases. **A**) Lobular carcinoma vs controls. Based on T test p values < 0.0001 and fold change >1.5, 41 sequences were selected. Chart shows that lobular cases are separated from controls, according to the enrichment logic (y axes: normalized counts sum). Misclassified lobular case 1 is progesteron negative, while all others are positive. Detected ductal carcinoma case 4 is micropapillary carcinoma, while all others are regular ductal carcinoma. Controls: solid frame lobular case 11 is enrichment cases in rounds 1-12; dashed frame case 8 – round13.



B) All cancer types vs controls. Based on either wilcoxon ranksum test, KS test or T test p values < 0.0005, and fold change > 1.2, 497 aptamers were selected. ROC curve for all cancer cases versus control cases (AUC =0.924).

Summary

- ✓ Serial enrichment on individual plasma exosome samples promotes evolution of aptamer libraries to dramatically improve identification of PR+/ER+/HER2- patients
- ✓ 100% of independent PR+/ER+/HER2- patients were correctly identified using the evolved library
- ✓ The evolved library also maintained the ability to detect other breast cancer subtypes (ROC AUC = 0.924)
- ✓ This technology is uniquely suited for profiling complex systems and phenotypes because it is based on physical evolution at the population level, NOT algorithm training
- ✓ More libraries for other subtypes are currently being built
- ✓ Basic research on feasibility of aptamer library enrichment directly on blood plasma and specificity of enriched aptamer library binding exosomal proteins was shown in *Domenyuk et al. Plasma Exosome Profiling of Cancer Patients by a Next Generation Systems Biology Approach. Scientific reports 7, 2017*, doi:10.1038/srep42741.