Cell-free DNA as a diagnostic analyte for molecular diagnosis of vascular malformations

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Objectives

- Determine the feasibility of cell-free DNA (cfDNA) as an analyte for molecular diagnosis of vascular malformations (VM).
- Describe a multiplex droplet digital PCR (ddPCR) assay used to detect *PIK3CA* mutations.
- Report the use of cyst fluid cfDNA as an analyte for genetic testing.

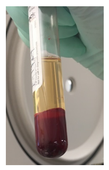
Introduction

- Sporadic VM are associated with activating somatic mutations.
  - Lymphatic malformations (LM): *PIK3CA*[1,2]
  - Venous malformations (VeM): *TEK, PIK3CA*[3,4]
  - Arteriovenous malformations (AVM): *MAP2K1, BRAF, KRAS*[5,6]
- New targeted therapies may be an option for patients with documented mutations.[7,8]
- Mutations are only detected in lesion tissue at very low levels.[1-6]
- Obtaining lesion tissue is invasive and carries significant risks.
- cfDNA has gained popularity in the cancer field for noninvasive and minimally invasive diagnostics.[9]

Methods

- Plasma and cyst fluid samples obtained from frozen biorepository or prospectively collected.
  - Prospective samples collected in Streck tubes: designed for cfDNA, decrease genomic DNA (gDNA) contamination.
- Plasma separated from whole blood or cyst fluid supernatant from pellet by centrifugation.
- cfDNA and gDNA isolated from plasma/cyst fluid supernatant and cyst fluid pellet, respectively.
- Droplet digital PCR (ddPCR) used to determine mutation status.
  - Samples with known mutations from prior testing: ddPCR singleplex screening
  - Samples with unknown mutation status: ddPCR multiplex screening

Results



Mutations detected in plasma cfDNA from patients with AVM, VeM, and LM

Plasma samples obtained for 38 individuals: 8 with AVM (15 samples), 3 with VeM (4 samples), 27 with LM (36 samples). All were screened for known mutations with singleplex ddPCR (Fig. 1). Only 4/38 (10.5%) of the individuals had a detectable mutation on ddPCR (Table 1, only patients with detectable mutations shown).

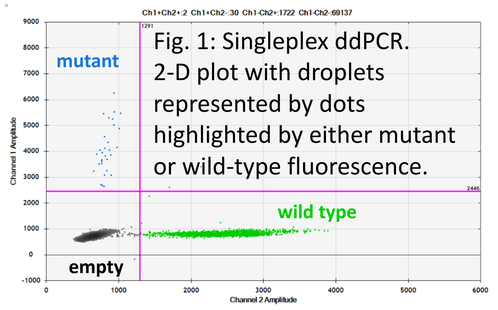


Fig. 1: Singleplex ddPCR. 2-D plot with droplets represented by dots highlighted by either mutant or wild-type fluorescence.

Patient	Age (y)	VM Type	Location	Mutation	Tissue VAF (%)	Volume (mL)	Plasma cfDNA VAF (%)
LR16-173	15	AVM	Ear	*MAP2K1* p.K57N	8.2	3.3 0.9 0.5	0.4 NEG NEG
LR17-049	18	AVM	Face, temple	*MAP2K1* p.Q5a6P	5.9	0.9 0.5	1.6 2.1
LR18-542	4	VeM	Face, buccal	*TEK* p.L914F	3.3	0.8	1.6
LR14-285	20	LM + CLOVES	Abdomen	*PIK3CA* p.E545K	1.8	8.0*	0.6

Table 1. Mosaic mutations detected in plasma cfDNA. * indicates collection in Streck tube. VAF – variant allele fraction; CLOVES – congenital lipomatosis, overgrowth, vascular malformations, epidermal nevi, and skeletal abnormalities.

Average DNA recovery was 21.8 ng. DNA recovery by volume was significantly lower from Streck tubes (n = 9) compared to frozen plasma from EDTA tubes (n = 46), (mean difference: -11.1 ng DNA/mL plasma). This is likely secondary to a larger amount of "contaminating" gDNA from white blood cells in the frozen plasma.

Mutations detected in cyst fluid cfDNA from patients with LM and known mutations

Seven individuals with known mutations from prior testing had cyst fluid available. Mutations were detected in cyst fluid cfDNA in 7/7 individuals (100%) (Table 2). 5/7 had higher variant allele fractions (VAF) in cyst fluid cfDNA compared to tissue. Only 3/7 pellet gDNA samples tested positive.

Patient	Age (y)	Location	*PIK3CA* Mutation	Tissue VAF (%)	Volume (mL)	Cyst fluid cfDNA VAF (%)	Cyst fluid pellet gDNA VAF (%)
LR16-145	1.0	Mediastinum	p.E542K	0.1	6.0	0.1	NEG
LR16-263	2 mo	Tongue/FOM	p.Q546K	9.5	9.0	0.9	NEG
LR16-266	1.4	Neck	p.H1047R	0.5	3.0	1.4	NEG
LR19-427	14	Parotid	p.E542K	1.1	3.3*	3.7	0.1
LR19-454	2.9	FOM/neck	p.E542K	3.6	6.5*	0.7	0.1
LR19-474	2.2	Neck	p.E545K	5.9	2.0*	7.9	11.9
LR19-545	14	Lower arm/hand	p.H1047R	2.2	10.2*	3.2	NEG

Table 2. Mosaic mutations detected in cyst fluid cfDNA. * indicates collection in Streck tube. VAF – variant allele fraction.

Multiplex ddPCR used for prospective mutation detection from cyst fluid cfDNA

A multiplex ddPCR previously developed for *PIK3CA* mutation detection in cancer[10] (Fig. 2) was used to screen cyst fluid cfDNA in individuals who had not had surgery previously. 4/5 cyst fluid cfDNA samples had mutations detected on multiplex ddPCR and confirmed on singleplex ddPCR (Table 3).

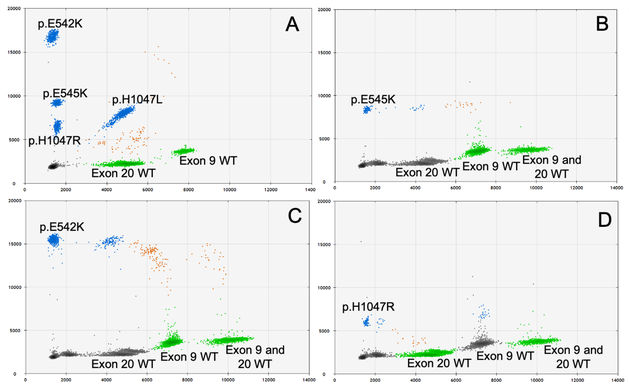


Fig 2. *PIK3CA* multiplex ddPCR. Positive controls for each of the four variants detected by multiplex ddPCR superimposed onto a single 2D fluorescence plot shows distinct separation of the four variant clusters and two corresponding wild-type (WT) clusters (A). Representative 2D-plots demonstrating multiplex results from cyst fluid cfDNA (B-D).

Patient	Age	Location	Volume (mL)	*PIK3CA* Mutation	Multiplex VAF (%)	Singleplex VAF (%)
LR16-265	11 yr	Axilla	6.0	p.H1047R	1.4	1.4
LR19-442	6 yr	Retro-peritoneum	9.0	p.E545K	0.2	0.2
LR19-443	1 mo	Axilla	2.0	NEG	NEG	-
LR19-446	9 mo	Chest wall	5.0	p.E542K	6.5	6.7
LR19-481	9 mo	Neck, lower face	6.5*	p.E545K	1.6	1.5

Table 3. Prospectively collected cyst fluid cfDNA has mutations detected by multiplex ddPCR and confirmed on singleplex ddPCR. * indicates collection in Streck tube. VAF – variant allele fraction.

Discussion

We detected somatic activating variants in cfDNA from plasma and cyst fluid in individuals with AVM, VeM, and LM. Multiplex ddPCR allowed for prospective molecular diagnosis from LM cyst fluid cfDNA in individuals without LM tissue resection. This represents an opportunity to initiate targeted medical therapy in VM patients prior to surgery. Furthermore, our results support future exploration of the use of cfDNA in diagnosis of non-neoplastic congenital disorders due to post-zygotic activating variants.