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Technical Note

A quantitative method for detection of circulating fms related tyrosine kinase 3 (FLT-3) in acute myeloid leukemia (AML) patients

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ABSTRACT

FMS related tyrosine kinase 3 (FLT-3) is a tyrosine kinase expressed in early hematopoietic precursor cells and has roles in survival, proliferation, and differentiation. Bone marrow expression and mutagenic analysis of FLT-3 in Acute Myeloid Leukemia (AML) patients is well-characterized. However, the levels of circulating FLT-3 in serum have not been previously described. In this study we describe a quantitative electrochemiluminescent immunoassay that detects FLT-3 in human serum. Using this method we find that AML patients have elevated levels of circulating FLT-3 and these levels correlated to the percent blast counts in the bone marrow (BM).

Technical note

1. Introduction

FMS related tyrosine kinase 3 (FLT-3) is a type III receptor tyrosine kinase expressed in hematopoietic stem/progenitor cells that has roles in survival, proliferation and differentiation (Takahashi, 2011). The major signaling pathways downstream of FLT-3 include PI-3-kinase, Ras/MAPK, and STAT5 (Takahashi, 2011). Increased FLT-3 expression in bone marrow leukemic blast cells has been observed in a majority of Acute Myeloid Leukemia (AML) patients (Gary Gilliland and Griffin, 2002). In addition, FLT-3 internal tandem duplication and tyrosine kinase domain mutations have been described in about 30% of primary AML specimen and correlate with disease progression (Ting Loke et al., 2012). Since FLT-3 can be up-regulated on the surface of AML blast cells, as well as in leukemic stem cells, it has generated interest as a target for large molecule therapies. Efforts to treat AML with FLT-3 targeted small molecule kinase inhibitors have been on-going for more than two decades and there is currently one FDA approved kinase inhibitor (midostaurin) and several more therapies in development (Levis, 2017; https://clinicaltrials.gov, n.d.). Despite the increased expression and known roles of FLT-3, to our knowledge no study documenting the presence of circulating FLT-3 in the serum has been described in AML. Herein, we describe a quantitative electrochemiluminescent immunoassay that measures FLT-3 in human serum and report on the concentrations of circulating FLT-3 in healthy donors and AML patients.

2. Materials and methods

2.1. Sample information

All human specimens were collected under Institutional Review Board approval with appropriate informed consent. In all diseased cases, materials obtained were surplus to standard clinical practice. Patient identity and PHI/identifying information were redacted from tissues and clinical data. Human tissue specimens were obtained from the following institutions: AML patient serum samples were obtained from Proteogenex (Culver City, CA). Healthy Donor serum samples were procured from the following sources: Amgen Tissue Bank (Thousand Oaks, CA), Proteogenex (Culver City, CA), Bio-Options (Brea, CA) and Lake Arrowhead Laboratory Consultants (Lake Arrowhead, CA).

2.2. Depletion of FLT-3 from pooled human serum

SpeedBead Magnetic Streptavidin Coated Particles (GE Healthcare Cat # 66152104010350) were washed and then incubated with 100-fold molar excess of biotinylated goat anti-FLT-3 (R&D systems, Cat # BAF812). The beads were incubated with the antibody at 4 °C for 6 h on a rotator. After a wash step, the antibody-coated beads were incubated with commercially sourced pooled human serum (Bioreclamation, Inc.) overnight with rotation at 4 °C. Magnetic force was then used to separate the now-depleted human serum from the bead-antibody-FLT-3 complex.

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2.3. FLT-3 electrochemiluminescent immunoassay

Quantitation of circulating FLT-3 in human serum was performed using an electrochemiluminescent immunoassay. MSD GOLD 96-well Streptavidin SECTOR plates (Meso Scale Discovery, Cat # L15SA-1) were coated with a biotinylated goat anti-FLT-3 (R&D systems, Cat # BAF812) as a capture reagent and incubated for 1 h at ambient room temperature with light shaking. Following a wash step, standards, controls, and samples were diluted 4-fold in buffer (Blocker BLOTTO in TBS - Thermo Fisher, Cat # 37530), added to the plate, and incubated for 2 h at ambient room temperature with light shaking. After another wash, a SULFO-TAG conjugated mouse anti-FLT-3 (R&D Systems, Cat # MAB8121) was added as a detection reagent and incubated for 1 h at ambient room temperature with light shaking. After a final wash step, 1× Read Buffer T (Meso Scale Discovery, Cat # R92TC) was added to the plates and the signal was detected by a Sector S 600 plate reader. Analyte serum concentrations were interpolated from a standard curve using human FLT-3 ECD-FLAG (produced by Biologics Group, Amgen, Inc.) in pooled human serum depleted of FLT-3. The bioanalytical data analyses were performed in GraphPad Prism Software 7.02.

2.4. Statistical analysis

Two-tailed unpaired *t*-tests (GraphPad Prism Software 7.02, La Jolla, CA) were used to assess statistical significance of differences. The *p* value of 0.05 was used to determine significant differences between any two groups. For precision and accuracy assessment, the %CV was calculated as (SD/mean concentration) × 100 and the %Bias was calculated as the (concentration measured-original/original) × 100.

Blast percentage (% Blast) in bone marrow was determined by counting cells in bone marrow using an optical microscope. This information was generated by Proteogenex (Culver City, CA) at the same time point at which the blood was drawn from the patients for the serum specimen. The correlation between the % Blasts in the BM and serum FLT-3 values were graphed, and Pearson correlation analysis was performed to generate both an "r square" value as well as a "*p* value."

3. Results and discussion

3.1. FLT-3 immunoassay development and performance

Monoclonal and polyclonal antibodies specific for FLT-3 were purchased from multiple vendors and tested as antibody pairs for detection of recombinant human FLT-3 ECD protein (data not shown). The antibody pair selected for further development included a polyclonal goat anti-FLT-3 as the capture reagent and a monoclonal mouse anti-FLT-3 (both from R&D Systems) as the detection reagent. This antibody pair was chosen due to its high signal to noise ratio, broad dynamic range, and cross-reactivity with non-human primate FLT-3 (data not shown).

Accurate quantitation of circulating proteins in serum is dependent upon the matrix in which standard and quality control (QC) samples are prepared. The highest accuracy is observed when the standards are prepared in the matrix that best resembles the background observed in the unknown samples (serum of the same species). However, assay sensitivity and accuracy can be impacted by endogenous target protein present in the serum used to prepare the standard curve samples. Additionally, non-specific matrix interference can produce false-positive signals even in the absence of the protein of interest. We compared standard curves of FLT-3 prepared in buffer (Blocker BLOTTO in TBS) or human serum pooled from multiple donors. In this experiment there were clear differences at the lower FLT-3 standard curve concentrations, as higher assay signals were observed in the serum samples relative to the buffer control (Fig. 1). To determine if these differences were due to non-specific matrix interference or the specific presence of FLT-3 in the pooled human serum, we depleted FLT-3 from this serum. The standard curve prepared in FLT-3-depleted serum was overlapping

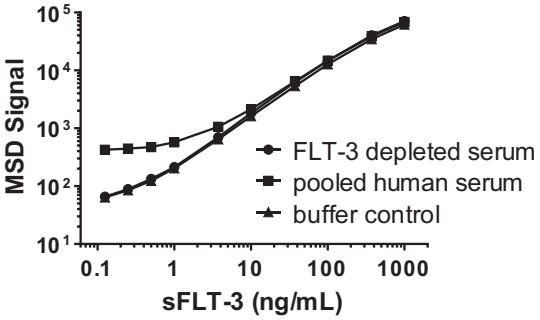


Fig. 1. Comparison of FLT-3 standard curves prepared in various matrices. Recombinant FLT-3-ECD protein standard curves were prepared in the indicated matrices prior to 4-fold dilution in buffer. The raw signal values for each sample in the FLT-3 electrochemiluminescent immunoassay are depicted. The data are displayed as mean +/− SD from 4 experiments, all run in duplicate.

with the buffer control curve (Fig. 1). This indicated that the higher signal observed at the low end of the pooled human serum standard curve was due to endogenous levels of FLT-3. These data support the use of either buffer or FLT-3 depleted serum as the matrix for standard and QC sample preparation to accurately quantitate FLT-3 from human serum samples.

Assay precision and accuracy was monitored across 4 independent measurements using standards and QCs prepared in FLT-3-depleted human serum. The assay performed well with a 4-log dynamic range of quantitation between the upper and lower limits of 1000 and 0.1 ng/mL, respectively. The bias and coefficient of variation were < 5% for all standards and < 15% for all QC samples (Table 1). These values were all within the established acceptance criteria of 20%.

Due to a rise in consumption of high-dose biotin supplements, there is a concern that assays that rely on a biotin-streptavidin reaction can be impacted by high serum levels of biotin. The FDA issued a guidance stating that potential interference of at least 1200 ng/mL biotin should be investigated in clinical assays (https://www.fda.gov/medicaldevices/safety/alertsandnotices/ucm586505.htm, 2017). At biotin levels ranging from 44 to 3600 ng/mL we detected no interference in detection of FLT-3 (data not shown), indicating that the impact of high-dose biotin is negligible in our assay.

3.2. Circulating FLT-3 in healthy donor and AML patient serum

Serum samples from healthy donor, newly diagnosed AML, and relapsed/refractory AML were obtained through commercial sources (Table 2) and assessed for FLT-3 concentrations using the assay described above (Table 1). The healthy donor serum samples had the lowest levels of circulating FLT3, ranging from 0.68 to 5.41 ng/mL with a mean of 2.18 ng/mL (Fig. 2A). Both subsets of AML patients had significantly higher levels of circulating FLT-3 than the healthy subjects (Fig. 2A). The newly diagnosed patients had a higher mean (42.4 ng/mL) than the relapsed/refractory (35.3 ng/mL) patients, but the difference between the subgroups of AML patients was not significant (Fig. 2A). These results indicate that serum FLT-3 levels could be used as a diagnostic biomarker for AML. An orthogonal approach was also performed on a subset of samples to confirm our findings. Using a peptide-based LC-MS approach we were able to show increased FLT-3 levels in AML patients, consistent with the immunoassay method described here (data not shown).

A) Serum samples from healthy donors, newly diagnosed or relapsed/refractory AML patients were assessed for FLT-3 concentrations by an electrochemiluminescent immunoassay. On the left each data point represents an individual donor sample, $n = 19–21$ per group, while the horizontal lines indicate the mean for each group. On the

Table 1
Inter assay precision and accuracy from standard (STD) and quality control (QC) samples for electrochemiluminescent FLT-3 immunoassay in FLT-3-depleted serum.

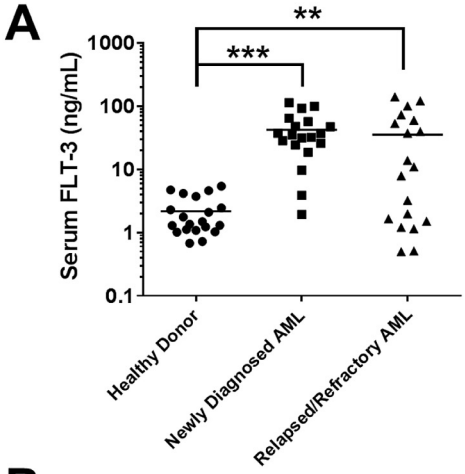
Batch (ng/mL)	STD 1000	STD 375	STD 100	STD 37.5	STD 10	STD 3.75	STD 1	STD 0.5	STD 0.25	STD 0.1	QC 750	QC 50	QC 2	QC 0.2
1	1022	372	95.2	39.3	10.2	3.71	0.999	0.499	0.242	0.127	672	47.1	1.98	0.231
2	1039	359	101.8	36.6	10.6	3.74	0.959	0.500	0.264	0.119	678	50.5	1.99	0.185
3	1089	354	95.2	38.4	10.4	3.93	0.968	0.495	0.243	0.116	649	46.8	1.97	0.178
4	1052	358	98.0	38.2	10.5	3.75	0.917	0.524	0.253	0.130	716	51.4	2.13	0.193
Mean	1051	361	97.6	38.1	10.4	3.78	0.961	0.505	0.251	0.123	679	49.0	2.02	0.197
S.D.	28.5	7.80	3.13	1.12	0.171	0.100	0.034	0.013	0.010	0.007	27.8	2.34	0.075	0.024
%CV	2.71	2.16	3.20	2.95	1.64	2.64	3.52	2.61	4.10	5.35	4.10	4.78	3.74	12.02
%Bias	5.10	−3.80	−2.45	1.67	4.25	0.87	−3.93	0.90	0.20	−1.60	−9.50	−2.10	0.88	−1.62

right the range of values observed and the mean for each group is depicted in the table. Two-tailed unpaired *t*-tests were used to assess statistical significance: *** indicates $p < .0001$, ** indicates $p = .0021$. B) % Blast in the bone marrow of each AML patient (Y-axis), along with the corresponding circulating FLT-3 concentrations (X-axis), are graphed for newly diagnosed (left) and relapsed / refractory (right). Pearson's correlation was used to assess the correlation between the two variables. The R squared values and statistical significance for each analysis are displayed within the graphs.

While it is clear that FLT-3 protein was increased in serum from AML patients, there was significant heterogeneity in circulating FLT-3 levels, particularly in the relapsed/refractory subset of patients. The heterogeneity could be due to many reasons, including disease status, variable bone marrow FLT-3 expression, and the impact of prior treatments. To address these points, we examined the correlation between the % blast in the bone marrow for each AML patient tested and the circulating FLT-3 values obtained (Fig. 2B). This analysis demonstrated a strong correlation between the percent blast count in the bone marrow and circulating FLT-3 levels for the relapsed/refractory subgroup ($p < .0001$), while the newly diagnosed subgroup was also statistically significant ($p = .0403$). These results suggest that the serum levels of FLT-3 accurately reflect disease status in the bone marrow, particularly for relapsed/refractory patients, supporting the use of this minimally invasive method to assess AML blasts. Further research is needed to determine what effect prior treatments have on serum levels of FLT-3. Additionally, the mechanism responsible for release of FLT-3 from cells into serum is unclear, as it could be due to proteolytic cleavage from the membrane or an alternative transcript that lacks the

Table 2
AML patient characteristics.

Patient number	Sex	Age	Disease status	Treatment
1	F	56	At diagnosis	No
2	M	72	At diagnosis	No
3	F	68	At diagnosis	No
4	M	53	At diagnosis	No
5	M	68	At diagnosis	No
6	M	65	At diagnosis	No
7	F	59	At diagnosis	No
8	M	43	At diagnosis	No
9	M	76	At diagnosis	No
10	F	63	At diagnosis	No
11	F	27	At diagnosis	No
12	F	51	At diagnosis	No
13	M	39	At diagnosis	No
14	F	24	At diagnosis	No
15	F	56	At diagnosis	No
16	M	47	At diagnosis	No
17	F	76	At diagnosis	No
18	F	65	At diagnosis	No
19	F	59	At diagnosis	No
20	M	55	Refractory	2 courses dacogen, 1 course cytosar
21	F	85	Refractory	Cytosar, mercaptopurine
22	M	62	Refractory	7 + 3 (cytosar, idarubicin)
23	F	51	Refractory	7 + 3 (cytosar, idarubicin)
24	F	65	Relapse	4 courses cytosar, mercaptopurine, vindaza
25	M	62	Relapse	2 courses 7 + 3 (cytosar, idarubicin), 1 course cytosar
26	F	53	Relapse	2 courses 7 + 3 (cytosar, idarubicin), remission consolidation 2 courses 7 + 3 (cytosar, idarubicin), HAM (cytosar, mitoxantrone), 4 courses 5 + 5 (cytosar, mercaptopurine).
27	F	34	Relapse	2 courses 7 + 3 (cytosar, idarubicin), 1 course - HAM (cytosar, mitoxantrone)
28	F	34	Relapse	Low dose cytosar, 7 + 3 (cytosar, idarubicin)
29	F	63	Relapse	7 + 3 (cytosar, idarubicin), 2 courses low dose cytosar
30	F	62	Relapse	7 + 3 (cytosar, idarubicin)
31	F	34	Relapse	7 + 3 (cytosar, idarubicin), mercaptopurine
32	M	55	Relapse	2 courses dacogen, cytosar
33	F	24	Relapse	7 + 3 (cytosar, idarubicin), HAM (cytosar, mitoxantrone)
34	F	53	Relapse	7 + 3 (cytosar, idarubicin)
35	F	44	Relapse	7 + 3 (cytosar, idarubicin)
36	F	68	Relapse	2 courses 5 + 2 (cytosar, idarubicin)
37	F	76	Relapse	cytosar
38	M	55	Relapse first early	2 courses dacogen
39	M	78	Relapse first early	7 + 3 (cytosar, idarubicin), low dose cytosar
40	F	66	Relapse first late	7 + 3 (cytosar, idarubicin), 5 + 2 (cytosar, idarubicin)



	Range (ng/mL)	Mean (ng/mL)
Healthy Donor	0.677-5.41	2.18
Newly Diagnosed AML	1.93-113	42.4
Relapsed/ Refractory AML	0.497-141	35.3

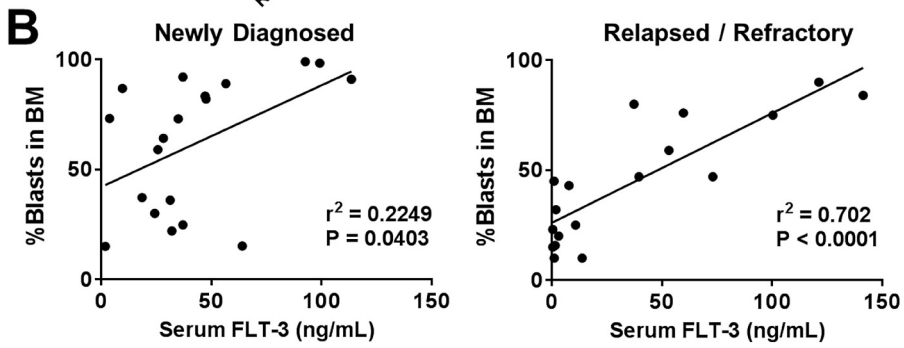


Fig. 2. AML patients have higher levels of circulating FLT-3 than healthy donors.

transmembrane domain, though it is worth noting that related tyrosine kinases KIT and CSF-1R are known to be proteolytically cleaved (Broudy et al., 2001; Rovida et al., 2001). Overall, this assay is useful for determining protein levels of FLT-3 in patient serum, and could be a minimally invasive and complementary addition to methods already in place to examine FLT-3 mutations via PCR.

4. Conclusion

Herein we have described an electrochemiluminescent immunoassay for the detection of FLT-3 in human serum samples. The assay performance met all acceptance criteria, with a 4-log quantitative dynamic range from 0.1 to 1000 ng/mL. This assay was implemented to detect circulating FLT-3 in healthy donor and AML patient serum samples. We discovered that both newly diagnosed and relapsed/refractory AML patients had significantly higher levels of FLT-3 in their serum than healthy donors. We further showed that the circulating FLT-3 levels correlated with bone marrow blast percentage in the relapsed/refractory AML subset. These results support the use of this standard immunoassay to determine serum FLT-3 levels as a minimally invasive biomarker test to assess AML disease status.

Declaration of interest

All authors were employed by Amgen, Inc. at the time the work was conducted.

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