EVALUATING THE PROGNOSTIC SIGNIFICANCE OF CIRCULATING mRNAs IN CHILDREN WITH REFRACTORY OR RELAPSED NEUROBLASTOMA (RR-NBL); a BEACON-Neuroblastoma biomarker study

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Background

- Children with high-risk neuroblastoma have poor outcomes. Early identification of children at greatest risk of relapse could mean timelier modifications of treatment to improve outcomes.
- We have previously established that high levels of the adrenergic (ADR) neuroblastoma mRNAs paired-like homeobox 2B (PHOX2B) and tyrosine hydroxylase (TH) in blood of children with newly diagnosed stage M neuroblastoma predicts outcome (PMID: 24590653), a finding that is now being exploited clinically.
- In the current study we have extended these investigations to children with relapsed or refractory neuroblastoma (RR-NBL) treated in the BEACON-neuroblastoma trial (NCT0238527).
- As neuroblastoma harbours cells with an ADR and a more mesenchymal (MES) phenotype, which may contribute to progression and relapse (PMID: 37142597/PMID: 37142597) we have investigated the expression of candidate MES mRNAs in blood from children with RR-NBL.

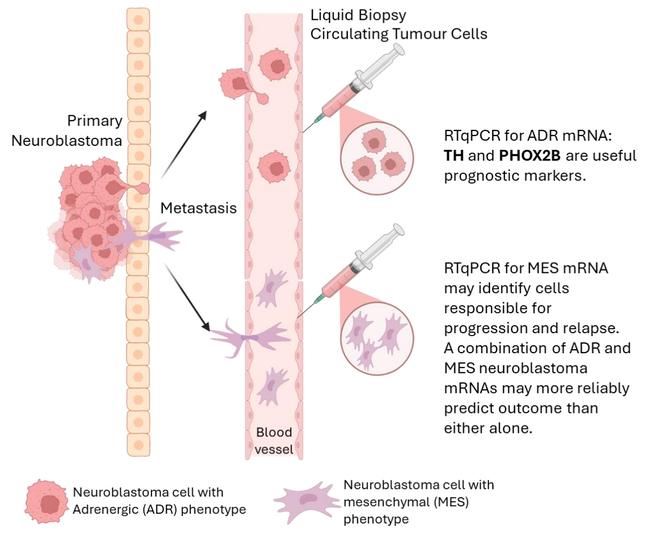


Figure 1. Schematic of the hypothesis and aims of this project.
Neuroblastomas harbour cells that express ADR and MES gene products. The detection of these gene products in blood may a useful marker of circulatory tumour cells, predicting progression and relapse.

Methods

- RNA was extracted from blood samples (n=2ml) collected in PAXgene™ blood RNA tubes at baseline (n=74), after cycle 2 (n=42), cycle 4 (n=31), cycle 6 (n=24) and end (n=25) of treatment as previously described (PMID: 17023157).
- The level of ADR and MES mRNAs in all samples was quantified by RTqPCR using standardised protocols or TaqMan Low Density Arrays (TLDA). In addition to RR-NBL clinical samples, TLDA-RTqPCR was also performed on RNA derived from normal blood (pools of adult blood) or a patient with low risk NB disease.
- The prognostic power of TH and PHOX2B mRNAs was evaluated using Kaplan-Meier survival curves and Cox proportional hazards regression.
- Euclidean clustering of normalised TLDA data was used to generate heat maps identifying both ADR and MES gene signatures.

Workflow

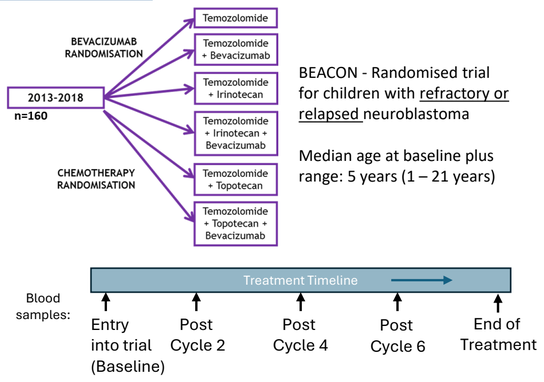


Fig 2a. Sequential blood samples were collected from neuroblastoma patients treated in the BEACON-Trial for relapsed /refractory high-risk patients.

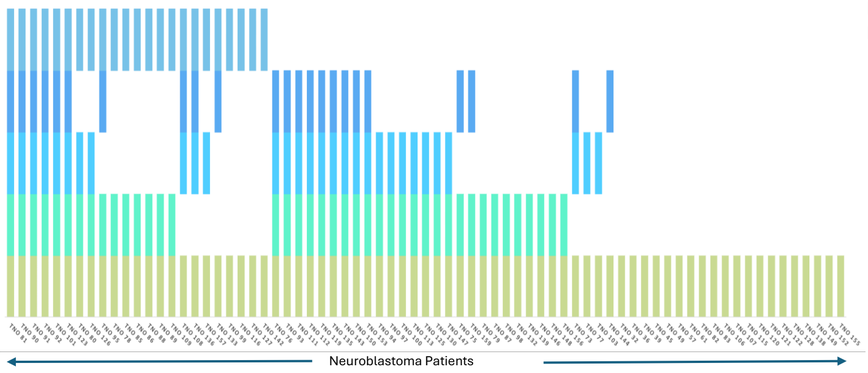


Fig 2b. Distribution of sequential samples throughout the cohort

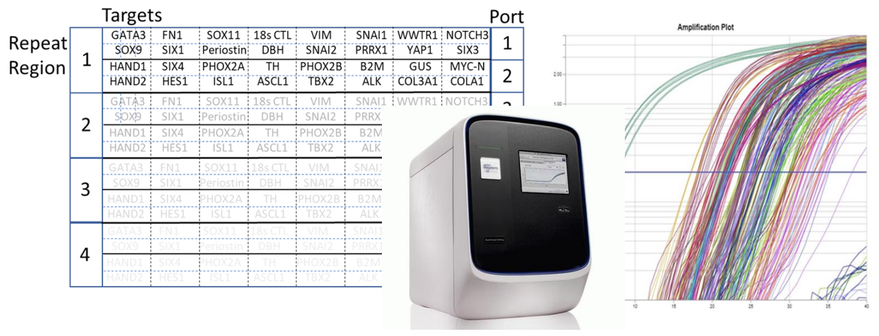


Fig 2c. Initial studies that established detection of TH and PHOX2B in peripheral blood as prognostic markers used standard RTqPCR techniques. To investigate the potential of MES biomarkers as prognostic indicators we undertook a systematic literature review and populated a TaqMan Low Density Array (TLDA) with the most promising candidates. This allowed the screening of large number of samples for multiple potential MES biomarkers as well as established ADR mRNAs and controls.

Results

Detection of TH and PHOX2B mRNAs (ADR markers) by RTqPCR are prognostic of progression-free and overall survival

The detection of PHOX2B or TH mRNAs at baseline (at entry on BEACON trial) correlated with progression-free survival (PFS) and overall survival (OS) in RR-NBL. As shown in the table below, the correlation was stronger when both PHOX2B and TH were detected.

	PFS			OS		
	TH	**PHOX2B**	**TH and PHOX2B**	**TH**	**PHOX2B**	**TH and PHOX2B**
Number PCR positive/Total (% positive)	48/88 (55)	52/88 (59)	40/88 (46)	48/88 (55)	52/88 (59)	40/88 (46)
Hazard Ratio	1.45	1.46	2.68	1.47	1.47	2.84
95% confidence interval of Hazard Ratio	1.25-1.69	1.25-1.70	1.65-4.35	1.22-1.77	1.22-1.77	1.71-4.72
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 1. Cox proportional hazards regression was run to assess prognostic ability of mRNA for PFS and OS

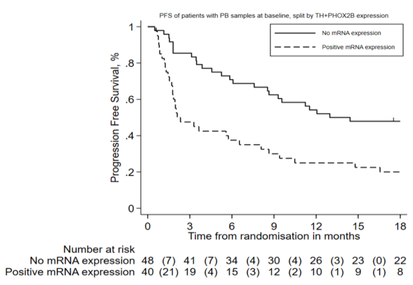


Fig 3. Kaplan-Meier curve comparing PFS for patients with no detectable TH and PHOX2B mRNAs at baseline compared to those with detectable TH and PHOX2B mRNAs.

TaqMan Low density Array (TLDA) validation

To ensure that TLDAs are an appropriate tool to systematically assay clinical samples for multiple mRNA targets we employed several layers of controls. This included negative controls, genes of known expression levels and house-keeping genes. In addition, we generated a positive control sample (1:1 mix of IMR-32 and MG63 RNA) for analysis on the TLDAs to evaluate their reproducibility and maintain QC alongside clinical samples through the course of this project. (Fig 4)

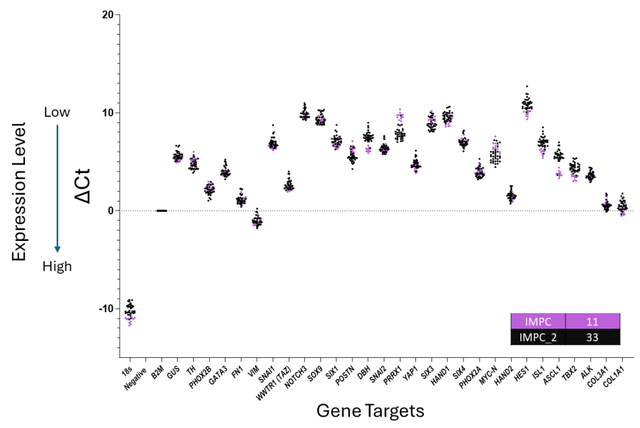


Fig 4. ΔCt (normalised to B2M) of positive control samples for each target on the TLDA. Two batches of control RNA were used, indicated by black and purple dots

TLDA data of 196 blood samples taken from 74 patients throughout the BEACON trial.

For every blood sample a ΔCt was generated for each target mRNA. The results for all time points are plotted in Fig.5, along with data derived from a multi-donor adult blood (red dots) and a single low risk patient (blue dot).

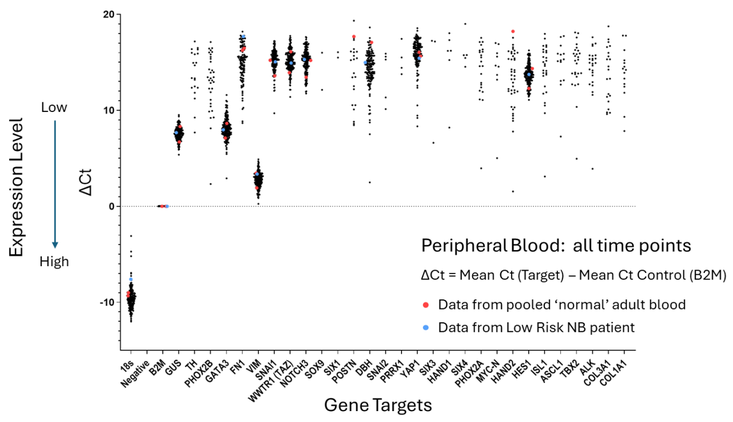


Fig 5. ΔCt (Mean Ct Target – Mean Ct B2M) for all samples assayed with the TLDA. All signals with a >35 Ct Mean Ct were removed.

The mRNA levels for TH and PHOX2B correlated well between standard RTqPCR and TLDA (R^2 = 0.95 and 0.93, when a >35 Ct cut-off was applied). TLDA detection was highest in baseline samples (TH: 41%, PHOX2B: 56%), aligning with the results from standard RTqPCR (Table 1). Detection was rare in other samples, with a slight increase at EoT (Fig. 6).

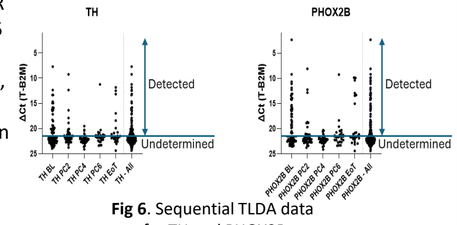


Fig 6. Sequential TLDA data for TH and PHOX2B

Euclidian clustering of TLDA data

Heat maps generated by hierarchal Euclidian clustering of ΔCt values generated from analysing all samples, segregates the patient cohort into two distinct groups. (labelled A and B on Fig. 6). Some MES-mRNAs are detected in the blood of nearly all patients (e.g. WWTR1 (TAZ), SNA1L, NOTCH3, YAP1, HES1 and DBH) whilst others (e.g. ALK, PHOX2A, HAND2, ISL1 and TBX2 – Cluster B in Fig 6) are only detected in minority of samples.

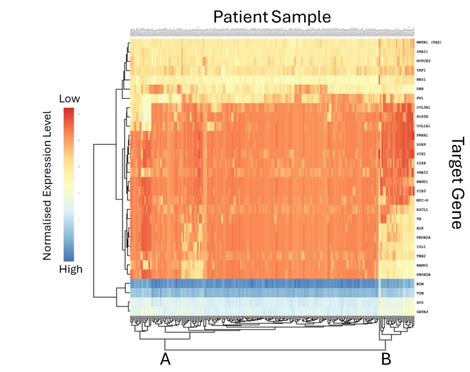
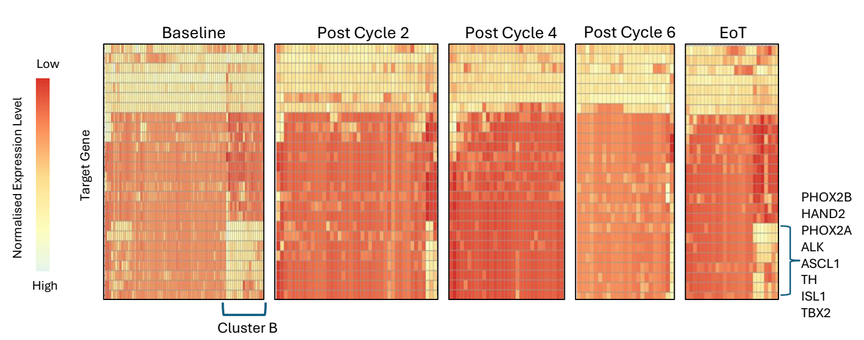


Fig 7 (left) . Hierarchal clustering of ΔCt (Mean Ct Target – GeoMean) of all 74 patients at all clinical time-points.

Fig 8 (below). Hierarchal clustering of ΔCt (Mean Ct Target – GeoMean) of all 74 patients segregated by clinical time point.



Segregating the cohort by time course (Fig. 7) suggests that cluster B is present at baseline, recedes during treatment but is detectable again at end of treatment. This clinical significance of this has yet to be determined.

Conclusions

The neuroblastoma mRNAs TH and PHOX2B can be detected in the blood of relapsed and refractory patients on the BEACON trial and are prognostic of both progression-free survival and overall survival.

TaqMan Low Density Arrays are a useful to evaluate the level of a panel of mRNAs in blood.

Clustering analysis demonstrates there is a panel of neuroblastoma associated MES mRNAs which segregates the patients treated in the BEACON trial into distinct groups. The clinical significance of these signatures is currently being evaluated.

References

1) Viprey VF *et al,* J Clin Oncol. 2014 Apr 1;32(10):1074-83. doi: 10.1200/JCO.2013.53.3604. Epub 2014 Mar 3. PMID: 24590653. **2)** van Groningen T *et al,* Nat Genet. 2017 Aug;49(8):1261-1266. doi: 10.1038/ng.3899. Epub 2017 Jun 26. PMID: 28650485. **3)** Thirant C *et al* 2023 May 4;14(1):2575. doi: 10.1038/s41467-023-38239-5. PMID: 37142597; PMCID: PMC10160107. **4)** Viprey Vf *et al* . Eur J Cancer. 2007 Jan;43(2):341-50. doi: 10.1016/j.ejca.2006.08.007. Epub 2006 Oct 4. PMID: 17023157.

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