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Verification of co-existence of *BRAF V600E* and *NRAS Q61* mutations in congenital melanocytic naevi (CMN)

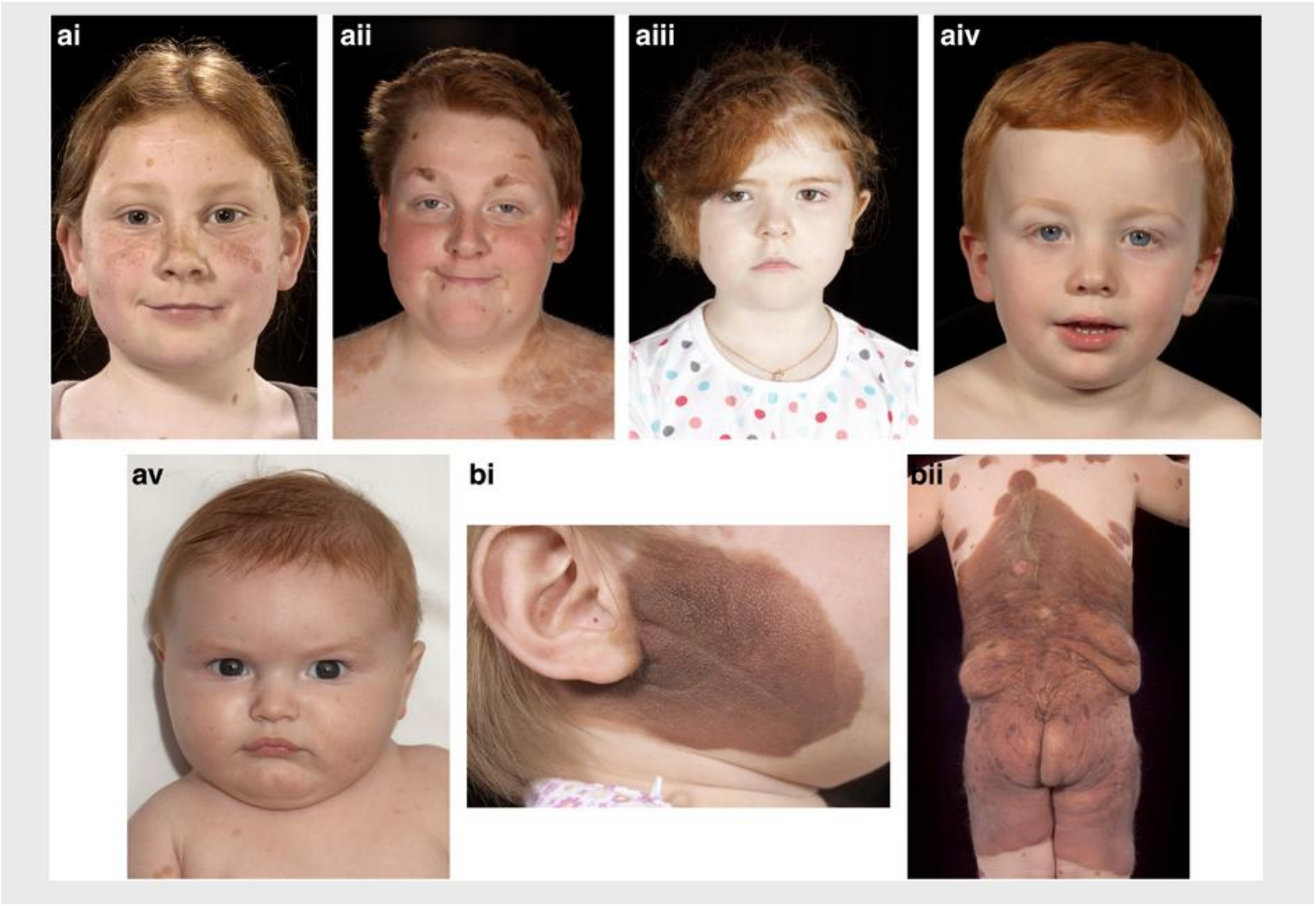

Figure 1: Clinical images of children with congenital melanocytic naevi (CMN) [1]

Introduction

Congenital Melanocytic Naevi (CMN) develop *in utero* and are therefore present at birth unlike acquired melanocytic naevi (AMN) which develop post-natally. Small single CMN are common, occurring in about 1 in 100 babies, but multiple or very large CMN are rare, affecting approximately 1 in 20,000 infants. Hundreds of well-defined pigmentary birth defects can be present in an individual with CMN, covering up to 80% of the body's surface.

CMN is a known risk factor for neurodevelopmental abnormalities and developing melanoma in postnatal life[(2)]. Increasing size of the naevi has been found to be associated with increased risk of both conditions[(3)].

CMN does not follow a Mendelian pattern of inheritance. Phenotypic abnormalities in this condition are considered to be due to a causative somatic mutation [(4, 5)].

AMNs frequently harbor oncogenic mutations. *BRAF* (V-raf murine sarcoma virus oncogene homolog B1) mutations are present in 59% of melanoma cell lines. It is located on chromosome 7, where the commonest mutation is glutamic acid substitution for valine at codon 600 (V600E). *NRAS* (neuroblastoma ras viral oncogene homolog) mutations are present in approximately 15% of cases with melanoma and the most frequent alterations are codon 61 mutations[(6-8)]. This is a RAS protein involved in the control of key cell signaling pathways. Transformation from guanosine diphosphate bound to the active guanosine triphosphate bound state allows RAS to act as a molecular switch, contributing to the activation of *BRAF*, one of three closely related Raf proteins, as well as phosphatidylinositol 3-kinase. This thereby activates the RAF/MEK/ERK pathway and Akt, respectively (see figure 2) [(9, 10)].

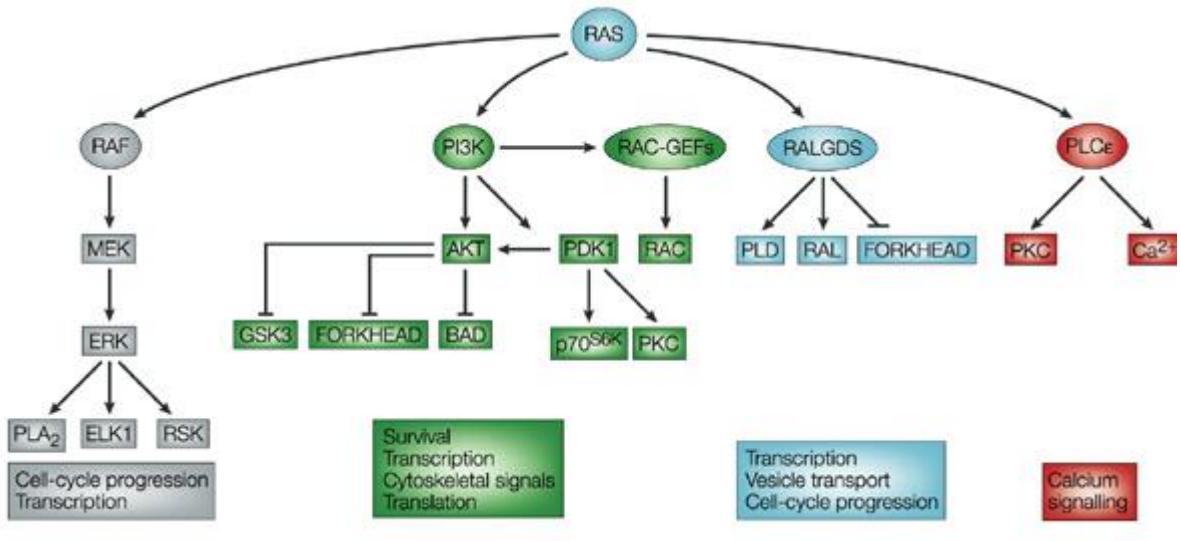

Figure 2: Diagram to show targeting RAS signaling pathways [(10)]

Previous studies have stated that *BRAF* and *NRAS* mutations found in melanocytic lesions are mutually exclusive [8, 11]. In recent unpublished data, however, a large cohort of 85 CMN patients had affected skin samples tested for *NRAS* and *BRAF* mutations, using standard Sanger sequencing and diagnostic laboratory high-resolution melt (HRM) techniques. These results revealed surprisingly that approximately 70% of the sample cohort carried both mutations (although one mutation often had a lower variant percentage).

Melanomas which harbor the *BRAF V600E* mutation are currently treated with *BRAF* inhibitors such as Vemurafenib[12]. However this mutation specific therapy is contraindicated in individuals with *NRAS* mutations due to paradoxical activation of MAPK pathway[13]. Therefore this unpublished data is highly relevant for future treatment, at least for patients with large or multiple CMN and further laboratory validation is required to identify potential changes that could be made to the current treatment regimen if *BRAF* and *NRAS* mutations coexist in an individual [14, 15].

Therefore, the aim of the study was to determine the concordance of the first and second BRAF results and verify the presence of *BRAF V600E* mutations in confirmed *NRAS* mutant CMN samples.

Materials and methods

59 anonymised DNA samples extracted from CMN formalin-fixed, paraffin-embedded (FFPE) tissue samples were genotyped using EntroGen's B-Raf codon 600 mutation analysis kit, a real time PCR-based assay that detects V600E in the codon 600 of B-Raf gene. The mutation analysis was carried out in accordance with the Analysis kit protocol (see below). Alterations (shown in Table 2) were made to reagent volumes as the protocol has been devised to perform an analysis on 5 different mutations; V600E, V600K, V600R, V600D and V600M.

4 DNA samples had a concentration of 100ng/πl and were diluted to give a final DNA concentrations of 20ng/πl.

Reagent Preparation

Table 1

Components	**Volume**
2X PCR reaction mix	15µl
Primer mix	6µl
DNA sample	20ng
Molecular grade water	Adjust to make total 30 µl

1. Master mix was prepared for each sample.

Table 2			
Reagents	**Final concentration**	**Volume per sample for 5 mutations (w/10% overage)**	**Volume per sample for 1 mutation**
2X Reaction Master Mix	1X	82.5 µl	15µl
DNA, Positive control mix or Water	20ng	5.5 µl	1µl
Nuclease free water		44 µl	8µl

2. Samples were vortexed and centrifuged for 10 seconds at 2,000rpm.
3. 24µl of master mix were dispensed in 61 wells. Sample well allocation recorded.
4. 6µl of V600E Mutation Detection Primer Mix added to each well.
5. Samples mixed by pipetting up and down several times.
6. The plate was sealed with optical sealing film (ABI plates).
7. The plate was centrifuged for 1,500 rpm for 1 minute.

Instrument setup

The assay was performed using an StepOne Plus™ Real-Time PCR system (Applied Biosystems®) using StepOne Software.

1. File>New Experiment>Advanced Setup selected
2. Experiment name created and 61 wells selected.
3. Type of experiment: comparative Ct. Reagents: Taqman. Plate setup in left navigation panel selected.
4. Two targets added: FAM(*BRAF*) and VIC(Control). NFQ-MGB selected for the Quencher for both targets.
5. Sample names for the experiment inserted on the right side of the screen.
6. Both targets assigned to all sample wells including PC and NTC.
7. Passive Reference Dye: None
8. 'Run method' on left panel selected.
9. Sample volume: 30µl.
10. Cycling parameters set up as shown:

Table 3			
Temperature	Time	Cycles	Data Collection
95°C	10 min	X1	Off
95°C	15sec	X40	Off
95°C	30 sec		Off

11. Prepared plate loaded and run started.
12. After one hour the analysis is complete
13. Input of data into SPSS database

Data analysis

1. Criteria used to analyse the data in the real-time PCR instrument software (Applied Biosystems 7500 Step 1 Plus):

- Manual threshold,

  FAM (*BRAF*) threshold: 10,000,

  VIC (Control) Threshold: 1,000,

- automatic baseline

2. Positive control wells and VIC Ct selected. The threshold was set and the amplification plots for each sample were checked to assess if each reaction had loaded properly.

The table below explains the interpretation of results using VIC threshold (reproduced from kit manual).

Table 4		
If VIC Ct is:	**Analysis description**	**Action**
Between 26 and 31	Ideal Ct range	Continue with step 3
Below 26	Overloaded reaction	Dilute DNA and re-test
Above 31	Insufficient DNA, fragmentation, or presence of PCR inhibitors in the DNA	Increase DNA input per reaction and re-test
Not present	Reaction components or DNA added incorrectly	Do not continue with analysis this sample has failed

3. Positive control wells and FAM Ct selected. The threshold was set and the amplification plots for each sample were checked to assess the presence of signal. Higher amounts of mutant variant produced a lower Ct value.
4. Ct values exported to a file using export function of Step One v.2.3 software. The table below shows the analysis steps used for the FAM channel (BRAF)

Table 5	
If FAM is:	**Mutation status**
37 or lower	Positive
Above 37	Re-test sample more DNA (5-10 times). If Ct value has decreased by 1 cycle, the sample is positive. If the Ct value stays the same or is absent, the sample is negative
Absent	Negative

Results

Results were consistent with previous findings obtained from sequencing and high resolution melt. 2 samples which have previously been identified as a low level variants and the experiment confirmed them as *BRAF* mutations. 1 sample was reconfirmed as a *BRAF* mutant. 1 sample was identified as a low level variant. *BRAF* Ct for this sample was 37.37 (above the threshold to be considered to have a positive mutation status but may be consistent with the low level variant status) and therefore requires re-testing.

24 out of the total 61 samples did not amplify. According to the protocol this may be due to one of the reaction components or DNA not being added correctly. These samples should therefore be re-tested.

Previous methods have displayed a higher number of positive samples. As several samples did not amplify, 14 results were not obtained for previously identified BRAF mutants**.**

The table below displays a comparison between previous methods used (HRM and sequencing) and this experiment:

Table 6					
BRAF Mutation Positive		**Second BRAF Method Results Cross tabulation**			
Count					
		Second BRAF Method Results			Total
		wild type	BRAF V600E mutation	failed to amplify	
BRAF Mutation Positive	.00	21	0	8	29
	1.00	12	4	13	29
Total		33	4	21	58

Conclusions and future work

From the results obtained, the co-existence of *BRAF V600E* and *NRAS Q61* mutations in congenital melanocytic naevi has been verified by a second diagnostic grade method, in all samples in which the second test worked.

The results were consistent with previous results obtained using sequencing and High Resolution Melt. 4 mutations identified as having the *NRAS* mutation also had the *BRAF* mutation. A higher number of mutants may have been identified if all samples had amplified therefore *BRAF* mutants that did not amplify could be re-tested in due course to identify other mutations and validate co-existence further.

These results will need to be replicated in a larger or second cohort of CMN patients, and will also be checked in a cohort of AMN samples. Functional work will need to be undertaken to check whether both mutations could be within the same cell, or whether this result implies two populations of cells within the same naevus.

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