Routine Screening for *KIT* M541L is Not Warranted in the Diagnostic Work-up of Patients with Hypereosinophilia

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Hypereosinophilia (HE), defined as peripheral blood eosinophil counts persistently >1.5 $x10^9$/L, [1] is seen in a wide range of reactive and clonal disorders. In hypereosinophilic syndrome (HES), HE results in potentially life-threatening organ damage as a consequence of eosinophilic tissue infiltration and/or release of toxic granule proteins [2]. The underlying causes of HE are complex, heterogeneous and incompletely understood, but an important subset is characterized by clonal HE driven by an underlying acute or chronic myeloid or lymphoid neoplasms [2] [3].

Clonal HE is frequently associated with somatic mutations that result in the constitutive activation of specific tyrosine kinases (TK), in particular PDGFRα, PDGFRβ, FGFR1, ABL, JAK2 or FLT3 [2]. Most commonly, translocations or other genomic rearrangements generate TK fusion genes, e.g. *FIP1L1-PDGFRA*, *ETV6-PDGFRB, ZMYM2-FGFR1*, *PCM1-JAK2* and many others [2], but some cases test positive for activating TK point mutations such as *KIT* D816V or *JAK2* V617F [4]. Identifying driver fusion genes is critical for clinical management, for example patients with *PDGFRA* or *PDGFRB* rearrangements show excellent responses to imatinib, with most cases achieving sustained haematological and molecular remissions [5,6] [7]. Clinical benefit has also been achieved with TK inhibitors (TKI) for other abnormalities, for example ruxolitinib and sorafinib for cases with *JAK2* or *FLT3* fusions, respectively [8,9].

Some cases of HE show clinical responses to imatinib despite no evidence for a recognized underlying abnormality in an imatinib-sensitive fusion gene [10]. For many of these cases responses are transient [5] but occasional longer term responders have been reported [7]. Recently it has been suggested that a somatically-acquired *KIT* variant c.1621 A>C; p.Met541Leu (hereafter referred to as *KIT* M541L) is associated with imatinib response in HE patients negative for *PDGFRA*/*B* rearrangements [11]. Whilst this finding is supported by the fact that M541L has been reported to increase the sensitivity of the KIT receptor to stem cell factor [12], it remains controversial because this variant is a recognised inherited single nucleotide polymorphism (rs3822214) with a minor allele frequency of 0.08 in the ExAC database (http://exac.broadinstitute.org/) and is classified as benign/likely benign on ClinVar (www.ncbi.nlm.nih.gov/clinvar/). Nevertheless, it has been suggested that HES patients should be screened for *KIT* M541L, as positive cases may benefit from imatinib treatment [11].

To evaluate the significance of *KIT* M514L in HE, we aimed to (i) compare the *KIT* M541L allele frequency between patients referred for investigation of HE and normal healthy controls (ii) investigate the variant allele frequency (VAF) in positive HE cases to determine if *KIT* M541L mutations may be acquired somatically and (iii) investigate the *KIT* M514L status in cases negative for *PDGFRA/B* rearrangements who responded to imatinib.

We screened healthy controls (n=214) and patients referred for investigation of *FIP1L1-PDGFRA* negative HE (n=220) for *KIT* M541L using an amplification refractory mutation system (ARMS) PCR designed to amplify allele specific products of different sizes, and able to detect *KIT* M541L down to 5% VAF (Figure 1a). Forty two (19%) of HE cases tested positive for *KIT* M541L compared to 38 (18%) of healthy controls. The allele frequency between the control and HE groups was indistinguishable (0.095 versus 0.098; P=0.91; Fishers exact two tailed test). Digital droplet PCR (ddPCR) was used for patients heterozygous for *KIT* M541L by the ARMS assay to determine whether the variant burden was close to 50% (consistent with a constitutional polymorphism) or significantly <50% (suggestive of a somatic mutation). Of the 42 *KIT* M541L heterozygous HE cases, 40 had sufficient DNA for analysis. The median allele burden was 50.2% (range 47.9%-56.0%), indistinguishable from that need in healthy controls (n=28) and thus consistent with all instance of *KIT* M514L in HE being constitutional (Figure 2).

Finally, we studied pre-treatment DNA from 3 patients with HES negative for *PDGFRA/B* rearrangements who were treated with imatinib (400 mg/day) and showed normalization of eosinophil counts at a median of 0.8 months (range, 0.4-5.0) after treatment for a duration of 13.6 months (range, 3.7-44.8). None of the three imatinib responders tested positive for KIT M541L prior to treatment (Figure 1b).

In conclusion, (i) we found no increased prevalence of KIT M541L in HE patients compared to controls, (ii) no case where *KIT* M514L was acquired somatically, and (iii) this variant was not present in three HES patients who responded to imatinib. Whilst we cannot exclude the possibility that *KIT* M541L may be acquired somatically in very rare cases, we conclude that

there is no clinical value in screening for this variant on a routine basis in patients with HE or HES.

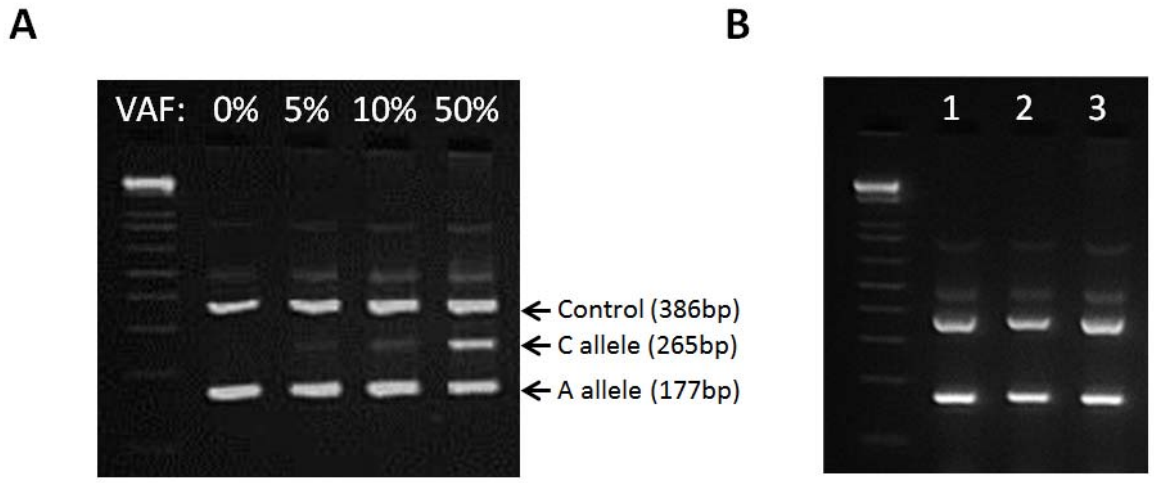

Figure 1: ARMS PCR to detect KIT M541L. (A) Concentration gradient demonstrating detection of KIT M541L at different %VAF. (B) Absence of *KIT* M541L ARMS in three *PDGFRA/B* negative HES patients who responded to imatinib. A tetra-primer ARMS assay was designed using http://primer1.soton.ac.uk/primer1.html with inner primers designed to specifically amplify the normal and mutant *KIT* M541L sequences and outer primers to produce a positive control band for each reaction. Inner primers included mismatches to maximise allele discrimination (shown in lower case) and wild type/mutant specific bases (underlined). PCR primers were: forward outer (FO), 5'-GTACAATGTAACCAAGGTGAAGCTCTGA-3'; reverse outer (RO), 5'-ACAACCTTCCACTGTACTTCATACATGG-3'; forward wild-type-specific (Fwt), 5'-TAGCTGGCATGATGTGCATTATTGcG<u>A</u>-3'; reverse mutant specific (Rmt), 5'-ACCTGTAAATATTTGTAGGTCAGAAT<u>t</u>A<u>G</u>-3'. FO, RO and Rwt primers were all used at a final concentration of 0.5µM and Rmt was used at 1µM in PCR reactions using AmpliTaq Gold DNA polymerase, 25ng genomic DNA an annealing temperature of 60°C, and 35 cycles. Each product was a different size that could be clearly resolved on a 3% agarose gel stained with ethidium bromide.

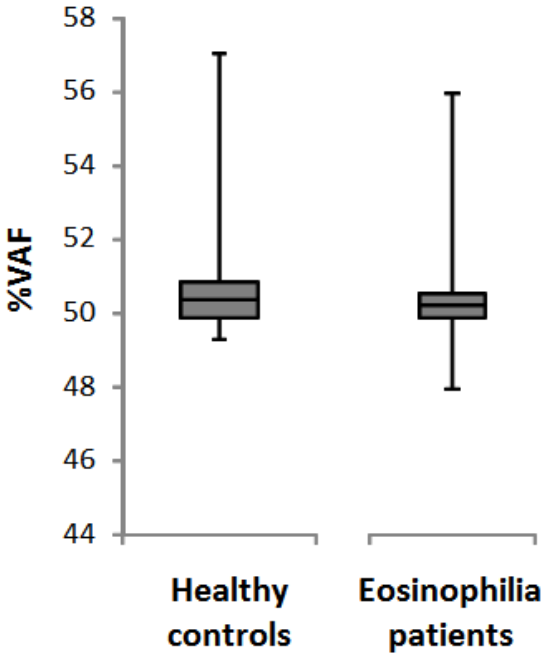

Figure 2: VAF for *KIT* M541L positive samples (n=40) and heterozygous healthy controls (n=28) tested by ddPCR. Mutant and wild-type specific probes were designed with 5' 6FAM and HEK reporters respectively and 3' BHQ1 (black hole quencher) and were used in the same reaction as forward and reverse primers. Probes and primers were: wild-type probe, 5'-[HEX]TGCATTATTGTGATGATTCTGACCTAC[BHQ1]-3'; mutant probe, 5'-[HEX] TGCATTATTGTGCTGATTCTGACCTAC[BHQ1]-3'; forward primer, 5'-TGCTGATTGGTTTCGTAA-3'; reverse primer, 5'-CCTTCCACTGTACTTCATA-3'. Reactions were prepared with 117ng sonicated genomic DNA, 0.25µM of each probe, 0.9µM of each primer and 'no dUTP' Supermix (BioRad, Watford, UK). Droplets were prepared using QX200 Droplet Generator (BioRad). Amplification was performed with an annealing temperature of 58°C for 40 cycles and droplets were measured using QX200 Droplet Reader (Biorad). Each sample was run in triplicate, with *KIT* M541L positive, negative and no template controls for each run. %VAF was calculated by dividing the concentration of *KIT* M541L mutant droplets by the total concentration and multiplying by 100. Only samples with an accepted droplet number of over 10000 were included as lower droplet numbers may produce less reliable results. Plots show the median, range and interquartile range.

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