Recurrent activating *STAT5B* N642H mutation in myeloid neoplasms with eosinophilia

Nicholas C. P. Cross,[1,2] Yvette Hoade,[1,2] William J. Tapper,[1] Gonzalo Carreno-Tarragona,[1,2] Tiziana Fanelli,[3] Mohamad Jawhar,[4] Nicole Naumann,[4] Iwo Pieniak,[1] Johannes Lübke,[4] Sahra Ali,[5] Kaljit Bhuller,[6] Sonja Burgstaller,[7] Catherine Cargo,[8] Jamie Cavenagh,[9] Andrew S. Duncombe,[10] Emma Das-Gupta,[11] Paul Evans,[8] Peter Forsyth,[12] Philip George,[11] Charlotte Grimley,[11] Fergus Jack,[13] Laura Munro,[14] Varun Mehra,[15] Kavita Patel,[16] Ali Rismani,[17] Gabriela Sciuccati,[18] Rowena Thomas-Dewing,[19] Patrick Thornton,[20] Andres Virchis,[21] Simon Watt,[22] Louise Wallis,[23] Alastair Whiteway,[24] Kris Zegocki,[25] Barbara J. Bain,[26] Andreas Reiter[4] & Andrew Chase[1,2]

[1] Faculty of Medicine, University of Southampton, Southampton, UK

[2] Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury, UK

[3] Center Research and Innovation of Myeloproliferative Neoplasms, AOU Careggi, University of Florence, Firenze, Italy

[4] University Hospital Mannheim, Heidelberg University, Mannheim, Germany

[5] Hull & East Yorkshire Hospitals NHS Trust, Hull, UK

[6] University Hospitals of Leicester NHS Trust, Leicester, UK

[7] Klinikum Wels-Grieskirchen, Wels, Austria

[8] HMDS, St. James's University Hospital, Leeds, UK

[9] St Bartholomew's Hospital, London, UK

[10] University Hospitals Southampton, Southampton, UK

[11] Nottingham University Hospitals NHS Trust, Nottingham, UK

[12] Raigmore Hospital, Inverness, UK

[13] Poole Hospital NHS Trust, Poole, UK

[14] York Teaching Hospital NHS Trust, York, UK

[15] King's College Hospital, London, UK

[16] Mid Yorkshire Hospitals NHS Trust, UK

[17] Whittington Health & University College London Hospitals, London, UK

[18] Hospital de Pediatria "Prof. Dr. Garrahan", Buenos Aires, Argentina

[19] Salford Royal Hospital, Salford, UK

[20] Beaumont and Connolly Hospitals, Dublin, Ireland

[21] Barnet & Chase Farm Hospitals, Wellhouse Lane, Barnet, UK

[22] Manchester University NHS FT, Manchester, UK

[23] Royal Bournemouth Hospital, Bournemouth, UK

[25] Southmead Hospital, Bristol, UK

[25] Whipps Cross University Hospital, London, UK

[26] St Mary's Hospital, London, UK.

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43   Correspondence to:

44

45   Professor N.C.P. Cross

46   Wessex Regional Genetics Laboratory

47   Salisbury NHS Foundation Trust

48   Salisbury SP2 8BJ, UK

49

50   Tel: +(44) 1722 429080

51   Fax: +(44) 1722 331531

52   email: ncpc@soton.ac.uk

53

54

Abstract

Determining the underlying cause of persistent eosinophilia is important for effective clinical management but remains a diagnostic challenge in many cases. We identified *STAT5B* N642H, an established oncogenic mutation, in 27/1715 (1.6%) cases referred for investigation of eosinophilia. Of the 27 mutated cases, a working diagnosis of hypereosinophilic syndrome (HES; n=7) or a myeloid neoplasm with eosinophilia (n=20) had been made prior to detection of *STAT5B* N642H. Myeloid panel analysis identified a median of 2 additional mutated genes (range 0-4) with 4 cases having *STAT5B* N642H as a sole abnormality. *STAT5B* N642H was absent in cultured T cells of 4/4 positive cases. Individuals with *SF3B1* mutations (9/27; 33%) or *STAT5B* N642H as a sole abnormality had a markedly better overall survival compared to cases with other additional mutations (median 65 months versus 14 months; hazard ratio = 8.1; P<0.001). The overall survival of *STAT5B* mutated HES cases was only 30 months, suggesting that these cases should be reclassified as chronic eosinophilic leukemia, not otherwise specified (CEL-NOS). The finding of *STAT5B* N642H as a recurrent mutation in myeloid neoplasia with eosinophilia provides a new diagnostic and prognostic marker as well as a potential target for therapy.

Running title: *STAT5B* N642H in eosinophilia

Keywords: STAT5B, eosinophilia, myeloid neoplasms

84   **Introduction**

85

86   Eosinophilia, defined as an elevation of the peripheral blood (PB) eosinophil count above

87   0.5×10^9/L, is conventionally divided into three main categories: primary, secondary (reactive)

88   and idiopathic. Primary eosinophilia is a clonal hematologic disorder in which the

89   eosinophils form part of the neoplastic clone. Secondary, non-clonal eosinophilia may be

90   driven by a wide range of underlying conditions including allergic disorders, autoimmunity,

91   infectious diseases, lymphoproliferative disorders, solid tumors, drug reactions and other

92   conditions. Idiopathic eosinophilia is a diagnosis of exclusion when no primary or secondary

93   cause can be identified[1].

94

95   Clonal eosinophilia is seen in the context of a myeloid neoplasm and particularly the World

96   Health Organisation (WHO)-defined entities 'chronic eosinophilic leukemia, not otherwise

97   specified' (CEL-NOS) and 'myeloid and lymphoid neoplasms with rearrangement of *PDGFRA*,

98   *PDGFRB* or *FGFR1* or with *PCM1-JAK2*, *ETV6-JAK2* or *BCR-JAK2*' (MLN-eo). Clonal

99   eosinophilia may also be associated with other WHO subtypes of myeloproliferative

100   neoplasm (MPN-eo) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN-eo)[1-3].

101

102   Identifying the underlying cause of eosinophilia is important for patient management but

103   can be challenging in the absence of an overt myeloid neoplasm or discernible secondary

104   cause. Primary eosinophilia is strongly associated with constitutively activated tyrosine

105   kinase (TK) signalling, and to date more than 70 TK fusion genes have been identified in

106   myeloid neoplasms as a consequence of reciprocal translocations or other genomic

107   rearrangements[3]. Identification of these fusions usually confirms a specific diagnosis and is

108   an indication for targeted therapy. Notably, imatinib induces rapid and durable complete

109   clinical, hematologic and molecular remissions in >90% of patients with a *PDGFRA* or

110   *PDGFRB* fusion gene, conferring excellent progression-free and overall survival (OS) [4, 5].

111   Fusions involving *JAK2*, *FGFR1* or *FLT3* are associated with a more aggressive clinical course

112   and may be responsive to other small molecule inhibitors.[6-8] Some TK-fusion negative

113   eosinophilia cases test positive for *KIT* D816V or *JAK2* V617F, whereas others have

114   mutations in a range of genes associated with myeloid neoplasms such as *TET2*, *ASXL1*, *EZH2*

115   or *SETBP1*.[9,10, 11] It has been suggested that a rapid and durable response to corticosteroids

116   is uncommon in cases with primary eosinophilia and instead points towards a diagnosis of

117   secondary eosinophilia, if that is not already apparent.[12]

118

119   In this study we have used genomic approaches to focus on the identification of novel

120   somatic abnormalities in patients with suspected primary eosinophilia. We have identified a

121   recurrent somatically acquired point mutation in *STAT5B* leading to an N642H substitution

122   in several cases. This mutation is known to activate STAT5B but was previously thought to

123   be restricted to lymphoproliferative disorders

124

125

126   **Methods**

127   *Patients and samples.* Our study included samples from individuals referred for routine

128   diagnostic analysis of persistent unexplained eosinophilia and/or patients diagnosed with

129   MPN or MDS/MPN with eosinophilia according to standard morphologic, hematologic and

130   laboratory criteria [2]. Cases that tested positive for *FIP1L1-PDGFRA* or other recognised TK

131   fusion genes were excluded. The study design adhered to the tenets of the Declaration of

132   Helsinki and was approved by the National Research Ethics Service (UK) Committee South

133   West and the institutional review board of the Medical Faculty of Mannheim, Heidelberg

134   University as part of the 'German Registry on Disorders of Eosinophils and Mast Cells'. DNA

135   or RNA was extracted from total PB or bone marrow (BM) leukocytes using standard

136   procedures. T-cells were selected using CD3 microbeads (Miltenyi Biotech, Cologne,

137   Germany) and stimulated to divide by co-cultivation with CD2/CD3/CD28 in the presence of

138   interleukin-2 (T-cell activation/expansion kit; Miltenyi Biotech).

139

140   *RNAseq analysis*. RNAseq data was analysed for point mutations using an in house pipeline.

141   Briefly, raw RNAseq data in fastq format were aligned to the reference genome

142   (human_g1k_v37) using STAR aligner and a two-step process. In the first step, splice

143   junctions were detected by an initial alignment of the fastq files. In the second step, final

144   alignments were determined using the splice junctions as a guide.[13] After alignment the

145   bam files were sorted, de-duplicated and read groups were added using Picard

146   (http://broadinstitute.github.io/picard). In preparation for variant calling, GATK was used to

147   hard-clip intronic sequences (SplitNCigarReads), reassign mapping qualities

148   (ReassignOneMappingQuality) and recalibrate base quality scores (BaseRecalibrator).

149   Variants were called using GATK HaplotypeCaller and ignoring soft clipped bases, which

150   minimises false positive and false negative calls. A final set of high quality variants in variant

151   call format (VCF) was produced by selecting variants with a phred-scaled confidence

152   threshold of 20 and excluding variants with low depth corrected quality scores (QD<2),

153   significant strand bias (FS>30) or SNP clusters of 3 or more SNPs within 35 base pairs.

154   ANNOVAR [14] was used to annotate variants with respect to genes, databases of known

155   polymorphisms (Exome Aggregation Consortium, 1000 genomes, exome sequencing project,

156   dbSNP), and pathogenicity estimates (avsift, polyphen etc). A shortlist of potentially relevant

157   variants was created by applying additional filters which excluded; non-coding variants,

158   synonymous SNVs and variants with an alternate allele frequency greater than 1% in public

159   databases of known variation: 1000 genomes (1000G;

160   www.ncbi.nlm.nih.gov/variation/tools/1000genomes/), exome sequencing project (ESP;

161   http://evs.gs.washington.edu/EVS/) and exome aggregation consortium (ExAC;

162   http://exac.broadinstitute.org/). Variants passing these filters were further highlighted if

163   they were; located in a manually curated list of candidate genes (n=581 genes), annotated

164   as pathogenic in Clinvar, overlapped with a somatic mutation in hematopoietic and

165   lymphoid tissue in COSMIC (https://cancer.sanger.ac.uk/cosmic) or were recurrently

166   mutated.

167

168   *Amplification-refractory mutation system (ARMS) test for STAT5B N642H*. A tetra-primer

169   ARMS assay[15] was designed using http://primer1.soton.ac.uk/primer1.html with inner

170   primers to specifically amplify the normal and mutant *STAT5B* alleles and outer primers to

171   produce a positive control *STAT5B* band for each reaction. PCR primers were: forward outer

172   (FO), 5'-CGATCAGGAAACACGTAGATAAGGTAATT-3; reverse outer (RO), 5'-

173   AAATGGAGATTTCTATTGGAGCCATTAT-3; forward wild-type specific (Fwt), 5'-

174   TCTCTGGTGGTAAAAGGCATCAGGTT-3; reverse mutant specific (Rmt), 5'-

175   TTATTGATCTAGAGGAAAGAATGTTTTAGC-3. The FO and RO were used at a final

176   concentration of 0.5μM whereas the inner primers Fwt and Rmt were used at 2.5μM.

177   Amplification reactions were performed using AmpliTaq Gold DNA polymerase, 25ng

178   genomic DNA at an annealing temperature of 60°C for 35 cycles. Cases showing mutant

179   bands after agarose gel electrophoresis were confirmed by independent amplification

180   followed by Sanger sequencing.

181

182   *Other mutational analysis*. Sanger sequencing was used to screen for other *STAT* gene

183   mutations using primers designed to amplify from cDNA: *STAT3* exons 20-22 (5'-

184   GGGCCATCTTGAGCACTAAG-3' and 5'-CACAGATAAACTTGGTCTTCAGG-3'); *STAT5A* exons 16-

185   17 (5'-GGACCTTCTTGTTGCGCTTT-3' and 5'-GGCGGTCAGGAAACACATAG-3') and *STAT5B*

186   exons 16-17 (5'- AGTGACTCAGAAATTGGCGG-3' and 5'-GGCCTGGTCCATGTACGT-3'). ARMS

187   assays were designed for JAK3 V722I and SOCS1 Q201H. For JAK3 V722I a standard tetra

188   primer assay was designed using PCR primers: forward outer (FO), 5'-

189   CAATAGACCCACCCCAATCTCCCCAGAC-3'; reverse outer (RO), 5'-

190   GCAAGGAAGTGGATCCCTGATCCCACTT-3'; reverse wild-type-specific (Rwt), 5'-

191   GCCACGGTCTGGGAAGTGTTTAGTGtCG-3'; forward mutant specific (Fmt), 5'-

192   ATCCAGGGCACTGATGGGCATGGTTAT-3'. FO, RO, Rwt and Fmt primers were all used at a

193   final concentration of 0.5μM in PCR reactions using AmpliTaq Gold DNA polymerase, 25ng

194   genomic DNA an annealing temperature of 68°C for 35 cycles. Design of a tetra primer assay

195   for SOCS1 Q201H proved to be difficult and therefore we used a simplified mutation-specific

196   assay with primers forward (F) 5'-CCAGGAGGGGGAGGACCCCCTCAAGAGG-3' and reverse

197   mutant specific (Rmt), 5'-CGCGACTACCTGAGCTCCTTCCCCTTCGAC-3' at an annealing

198   temperature of 70°C for 35 cycles. All ARMS assays were validated with control samples,

199   and positive/negative controls were included on each run. The Illumina Trusight Myeloid

200   Sequencing Panel (Illumina, San Diego, CA) was used to screen *STAT5B* mutated samples for

201   additional somatic mutations. Samples were processed according to the manufacturer's

202   protocol, run on an Illumina Miseq and results interpreted using Alissa Interpret (Agilent,

203   Cheadle, UK) using a variant allele frequency (vaf) cut off of ≥5%.

204

205   *Colony growth and sequencing.* Mononuclear cells from cryopreserved or fresh primary

206   cells were grown in methylcellulose with cytokines, without erythropoietin (Methocult

207   H4035 Optimum, no EPO, Stemcell Technologies (Vancouver, BC, Canada). DNA was

208   prepared for sequencing using the PicoPlex whole genome amplification kit (Rubicon

209   Genomics Inc., Ann Arbor, MI). Colonies were first plucked into 80 μl phosphate buffered

210   saline, spun down and resuspended in 2.5 μl of PicoPlex cell extraction buffer followed by

211   DNA extraction and amplification according to the manufacturer's instructions. The DNA

212   was then cleaned up using the QIAquick PCR purification kit (Qiagen, Hilden Germany) prior

213   to amplification and Sanger sequencing using primers designed to target specific mutations.

214

215   *Statistical analysis.* OS probabilities were estimated using the Kaplan-Meier method and

216   compared by the log-rank (Mantel-Cox) test using SPSS v25 (IBM Corporation, Armonk, NY,

217   USA). OS was defined as the time between diagnosis and the date of death or last contact.

218

219   **Results**

220

221   *STAT5B N642H mutation identified by RNAseq.* We previously described RNA-seq analysis to

222   search for cryptic fusion genes in 14 patients with MPN-eo or idiopathic hypereosinophilia

223   with a normal karyotype [6]. Reanalysis of these data for possible point mutations identified

224   10 candidate variants in 6 cases (Table 1). No variants were seen in 8 cases, including the 2

225   previously reported to have *DIAPH1-PDGFRB* or *ZMYM2-FLT3* fusions.[6] Two of the 6 cases

226   had known myeloid driver mutations: *JAK2* V617F in case E166 and *SF3B1* K666N in case

227   E11076. Of the other variants, *STAT5B* N642H (NM_012448: c.A1924C) was seen in two

228   cases (E11076 and E11493) and stood out as this is a known driver mutation in

229   lymphoproliferative disorders.[16] Furthermore this mutation was recently reported in the T-

230   cells and other cell lineages of two young children with eosinophilia.[17]

231

232   *Identification of additional cases with STAT5B mutations*. We used an ARMS assay to rapidly

233   screen additional retrospective cases for *STAT5B* N642H (Figure 1a). In an initial screen we

234   found that 2/30 cases with suspected MPN-eo tested positive compared to 0/74 cases with

235   other chronic myeloid neoplasms. Subsequent screening focused only on cases referred for

236   investigation of eosinophilia: of 1671 cases screened, 23 further cases tested positive.

237   Overall, therefore, we found that 27/1715 (1.6%) eosinophilia patients harbored *STAT5B*

238   N642H. For 25/26 cases confirmed by Sanger sequencing, the mutation specific peak was at

239   a similar height or somewhat lower than the wild type peak, suggesting that the majority of

240   leukocytes were heterozygous for *STAT5B* N642H. In one sample (E492) only the *STAT5B*

241   mutant peak was seen suggesting the dominance of a homozygous or hemizygous mutant

242   clone (Supplementary Figure 1). In 4/4 cases tested the *STAT5B* mutant clone was not

243   detected in cultured T-cells confirming that it was acquired somatically (Supplementary

244   Figure 2).

245

246   *Other STAT mutations.* Other somatic *STAT5B and STAT3* mutations have been identified in

247   lymphoproliferative disorders. To test if these mutations might also be associated with

248   persistent eosinophilia, we sequenced known mutation hotspot regions of *STAT3* and

249   *STAT5B* in 153 cases but no variants were seen apart from *STAT5B* N642H. No human

250   mutations have been reported in *STAT5A* but due to the high homology with *STAT5B* we

251   also sequenced part of this gene, but again no variants were found (149 cases).

252

253   *Additional mutations in STAT5B-mutated cases*. We screened all 27 *STAT5B* N642H cases for

254   mutations in other genes associated with myeloid neoplasms (Figure 1B). Overall, mutations

255   were seen in a median of two additional genes (range 0-4), with 4 cases showing no

256   additional variants (Group 1). Of note, 9 cases had *SF3B1* mutations (Group 2) of which 4

257   were detected as sole additional abnormalities, 3 were seen in combination with single

258   mutations in *TET2* or *DNMT3A* and 2 had additional mutations in genes encoding signalling

259   or splicing components. Fourteen cases did not have *SF3B1* mutations but instead had a

260   diverse range of epigenetic, signalling, transcription factor and other splicing mutations with

261   no clear patterns emerging in terms of co-mutated genes (Group 3). All additional mutations

262   are detailed in Supplementary Table 1.

263

264   *Clinical features associated with STAT5B N642H and other mutations*. The clinical features

265   for all cases are summarised on Table 2. There was a preponderance of males (19 males, 8

266   females) and the median age was 70 (range 7-89; n=27) with a median eosinophil count at

267   presentation of 6×10^9/L (range 0.5-27; n=26). In most cases eosinophilia was apparent at

268   diagnosis but in 3 cases this was acquired during the course of myelodysplastic syndrome

269   (MDS; cases E2594 and E11837) or MPN (case E13661). Basophilia was noted in several

270   cases. A working diagnosis of idiopathic hypereosinophilic syndrome (HES) had been made

271   in 7 cases prior to the finding of *STAT5B* N642H and CEL-NOS in 2 cases, supported by the

272   finding of an additional chromosome 8 by cytogenetic analysis in both cases. The Kaplan-

273   Meier estimate for OS of *STAT5B*-mutated HES cases was only 30 months, which is very

274   short compared to published series[11], and suggests that these cases should be reclassified as

275   CEL-NOS[10]. The remaining 18 cases had been diagnosed with another myeloid neoplasm,

276   most commonly a subtype of MDS/MPN (n=11). The PB and BM from a representative case

277   with CEL-NOS and *STAT5B* N642H as a sole abnormality is shown on Figure 2.

278

279   There was no obvious correlation between the clinical diagnosis and the molecular

280   classification described above. For example the 7 cases initially diagnosed with HES were

281   split between molecular group 1 (n=1), group 2 (n=2) and group 3 (n=4) and the 9 cases with

282   *SF3B1* mutations had been diagnosed with 7 different entities, including HES (n=2) and 6

283   WHO-defined subtypes of myeloid neoplasms. There was, however, a clear correlation

284   between molecular features and outcome. Focusing on patients with eosinophilia at

285   diagnosis, the OS for cases in group 3 was markedly inferior to cases in groups 1 and 2

286   [median 14 months versus 65 months; hazard ratio (HR) = 8.1 (95% CI: 1.9 - 23); P<0.0004,

287   Figure 3A]. By contrast age, gender and white cell count were not significantly associated

288   with OS in this relatively small group, whereas eosinophil count approached significance (≤

289    $6x10^9$/L, 49 months versus > $6x10^9$/L, 17 months; P=0.06). No significant effect of mutation

290   number was seen when all mutations were considered but, interestingly, when genes

291   strongly associated with age-related clonal hematopoiesis (i.e. *DNMT3A* and *TET2*) were

292   excluded from the analysis, the OS for cases with mutations in 2 or more additional genes

293   was significantly worse than that of cases with 0 or 1 additional mutations [median 18

294   months versus 50 months; HR = 6.5 (95% CI: 2.1 – 30) P=0.001, Figure 3B].

295

296   As for therapy, 5 patients were treated with imatinib for at least 2 months but none

297   responded. One patient with *KIT* D816V-positive systemic mastocytosis with associated

298   hematologic neoplasm (SM-AHN) responded to midostaurin but the response was lost after

299   9 months. Some patients showed clinical improvement and reduction in eosinophil counts

300   with corticosteroids but in most cases this was partial and/or transient.

301

302   *Clonal hierarchy*. To understand if acquisition of *STAT5B* N642H is an early or late event in

303   the development of myeloid neoplasia, we genotyped myeloid colonies grown from 4 cases

304   with multiple mutations. For case E10926, 6 mutations in 5 genes had been identified by

305   bulk analysis. By colony analysis we found a major clone with 5 mutations that included

306   *STAT5B* N642H and *KRAS* V14I but not *KIT* D816V, and a minor clone that included *KIT*

307   D816V but not *STAT5B* N642H or *KRAS* V14I. All colonies tested positive for *SF3B1* and two

308   independent *TET2* mutations and thus branching evolution can be inferred with *STAT5B*

309   N642H acquired in a later subclone. For 2 of the other cases linear evolution was apparent

310   with *STAT5B* and *ASXL1* mutations acquired as late events. The fourth case was

311   uninformative with all colonies positive for the 3 mutations detected on bulk analysis

312   (Figure 4A). For two cases with increasing eosinophil counts, the *STAT5B* N642H vaf, as

313   estimated by Sanger sequencing, increased over time (Figure 4B).

314

315   *Assessment of other variants identified by RNAseq*. Other recurrent mutations identified by

316   RNAseq were also considered. Both *PRF1* N252S and *SOCS1* Q210H were identified in two

317   individuals (Table 1). *PRF1* encodes perforin 1, a gene associated with familial

318   hemophagocytic lymphohistiocytosis. However the N252S variant has been shown to be a

319   non-functional rare single nucleotide polymorphism (SNP) and was therefore not considered

320   further[18]. *SOCS1* encodes suppressor of cytokine signalling 1, which takes part in a negative

321   feedback loop to attenuate cytokine signalling and therefore has obvious potential

322   relevance to myeloid neoplasia. The status of the Q210H variant is unclear: it is seen at a

323   frequency of 0.2-0.6% in the 1000G, ESP and ExAC control datasets, as are some known

324   somatic driver mutations such as *JAK2* V617F. For one case we tested buccal cells and found

325   the variant was constitutional (data not shown). To test the possibility that Q210H might be

326   more widespread in myeloid neoplasia we tested cases referred for investigation of

327   eosinophilia (n=151), mastocytosis (n=75), MDS/MPN (n=54) and healthy controls (n=88).

328   Just 3 (1%) cases tested positive (one with mastocytosis, 2 with MDS/MPN) and none of the

329   controls (P=0.99; Fisher's exact test) suggesting that this variant is an irrelevant, rare

330   polymorphism that happened to be present in 2 of the cases analysed by RNAseq. *JAK3*

331   V722I was seen in a single case and has been reported previously as an activating

332   mutation[19] but is also present at 0.4-1% in control datasets and its pathogenicity has been

333   disputed. We found that 4/307 cases with suspected myeloid neoplasia tested positive for

334   *JAK3* V722I (one of which also had the mutation in cultured T-cells) as well as 3/88 normal

335   controls (P=0.19), again suggesting it is an irrelevant polymorphism. Similarly, *RPS19* T55M

336   is also believed to be a polymorphism[20].

337

338

Discussion

STAT5 is a key component of cytokine-induced signal transduction cascades, and a critical downstream mediator of transformation by oncogenic tyrosine kinases such as BCR-ABL1, JAK2 V617F, FLT3-ITD and ZNF198-FGFR1[21-25]. STAT5 is encoded by two different genes, *STAT5A* and *STAT5B*, located closely together at chromosome 17q11.2 that encode proteins with >90% amino acid identity and largely redundant functions. Nevertheless, targeted disruption of *STAT5A* and *STAT5B* gives rise to distinct phenotypes in mice and only *STAT5B* has been reported to be a target of mutations in cancer[26].

STAT5A N642H was initially identified as a constitutively activating mutation in a random *ex vivo* mutagenesis screen[27]. The corresponding mutation in *STAT5B* was subsequently identified as a somatically acquired driver mutation in 1-37% of patients with various lymphoid neoplasms including large granular lymphocytic leukemia[16], paediatric T-cell acute lymphoblastic leukemia[28], T-cell prolymphocytic leukemia[29], γδ-T-cell lymphoma[30] and two cases of lymphocyte-driven early onset nonclonal eosinophilia with urticaria, dermatitis and other features[17]. The most frequent mutation seen in these T-cell disorders is *STAT5B* N642H, but other mutations are also seen, specifically *STAT5B* Y665F and *STAT3* Y640F, N647I and D661V/Y [31]. Isolated reports have identified single cases of myeloid neoplasms, specifically chronic neutrophilic leukemia[32] and MLN-eo[33], that tested positive for *STAT5B* N642H as well as 2 cases that developed clonal hematopoiesis following aplastic anemia[34]. Our findings are the first to identify *STAT5B* N642H as a recurrent mutation in myeloid neoplasms with eosinophilia.

We demonstrated the absence of *STAT5B* N642H in cultured T-cells from 4 cases, one of whom (case E12614) was in molecular group 1, i.e. had no additional mutations. All 4 group 1 cases, and indeed most cases in this study, had *STAT5B* vafs which suggested the great majority of leukocytes were heterozygously mutated (Supplementary Figure 1). Although we cannot exclude the possibility that some T-cell subsets might be part of the mutant clone, our data strongly suggest that *STAT5B* N642H drives primary eosinophilia irrespective of the presence or absence of additional mutations.

371   Cytokine stimulation results in phosphorylation of STAT5B by receptor or non-receptor

372   tyrosine kinases. Dimerization of tyrosine phosphorylated (pY) STAT5B is mediated by trans-

373   SH2 domain/phosphotyrosine binding, and the dimers then translocate to the nucleus and

374   activate transcription of target genes [26]. N642 is located within the SH2 domain of STAT5B,

375   close to the phosphotyrosine-binding loop. Rather than being constitutively active, the

376   N642H mutant shows prolonged pY-STAT5 levels upon cytokine stimulation due to greatly

377   enhanced stability of N642H homodimers[30]. Transgenic expression of *STAT5B* N642H under

378   the control of the *Vav1* promoter (which is believed to be active in all hematopoietic cell

379   types, including stem cells) resulted in transplantable CD8+ T-cell neoplasia. Although

380   eosinophilia was not noted, there was a mild expansion of mature and immature myeloid

381   lineage cells[35]. The apparent absence of myeloid neoplasia with eosinophilia in this mouse

382   model may be the result of a number of factors, including the precise expression of *STAT5B*,

383   the effect of other somatic mutations, the cell type in which *STAT5B* N642H arises and the

384   rapid onset of T-cell neoplasia.

385

386   We observed a striking influence of additional mutations on patients with *STAT5B* N642H

387   that mirrors established associations in related myeloid disorders, particularly the relatively

388   good prognostic influence of *SF3B1* mutations and the adverse influence of multiple

389   mutations, both of which have been described in MDS[36, 37]. In addition, only one case with

390   an *SF3B1* mutation tested positive for one or more mutations in *SRSF2*, *ASXL1* and *RUNX1*

391   (S/A/R), known to be an adverse prognostic factor in advanced SM[38], whereas 10/14 cases

392   in group 3 were S/A/R positive (P=0.009; Fisher's exact test).

393

394   The combination of *JAK2* V617F and *SF3B1* mutations is associated with MDS/MPN with ring

395   sideroblasts and thrombocytosis [39]. In our series, the working diagnosis of patients with

396   *STAT5B* N642H and *SF3B1* mutations ranged from HES to MDS/MPN (Table 2). Stored

397   material from most cases was not available for central morphological review but we

398   envisage that prospective analysis of new cases will help to define more accurately the

399   features associated with *STAT5B* N642H in the presence or absence of *SF3B1* mutations.

400

401   Previous studies have shown that STAT5A is required for eosinophil differentiation of cord

402   blood-derived CD34+ cells [40]. Our findings, however, suggest that *STAT5B* N642H may be a

13

403   driver of eosinophilia. First, in most of our cases eosinophilia was apparent at diagnosis and

404   the level of *STAT5B* N642H as assessed by Sanger sequencing indicated that the mutation

405   was present in the majority of cells. In 4 of these cases *STAT5B* N642H was detected as a

406   sole abnormality, although we cannot exclude the possibility of mutations in genes not

407   covered by the myeloid panel. Second, analysis of 2 cases with increasing eosinophil counts

408   showed an increase in the *STAT5B* N642H vaf. Three additional cases acquired eosinophilia

409   during the course of their disease but unfortunately samples were not available for analysis

410   from the pre-eosinophilia phase.

411

412   The finding that STAT5B N642H shows prolonged activation following cytokine signalling

413   suggests that targeting upstream tyrosine kinases may ameliorate the activity of mutant

414   STAT5B. Indeed, the T-cell neoplasms induced by transgenic *STAT5B* N642H was markedly

415   supressed by JAK1/2 inhibition[35]. Furthermore, a small molecule inhibitor of STAT5B

416   dimerization has been shown to inhibit the growth of *FLT3*-ITD positive AML cells[41]. Further

417   studies will be required to determine if myeloid disorders associated with *STAT5B* N642H

418   are targetable with small molecule inhibitors.

419

420

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426

427   **Conflicts of interest**

428   None of the authors have any relevant conflicts of interest or disclosures

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431

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588

589

Figure legends

592   Figure 1. (A). ARMS assay for *STAT5B* N642H. Heterozygotes have 3 bands - control band

593   (243bp), wild type (wt) specific band (166bp) and mutant allele specific band (124bp). Using

594   serial dilutions of DNA from a mutated case we estimated that the assay can detect *STAT5B*

595   N642H at a variant allele frequency of 10%; (B) summary of mutations for all 27 *STAT5B*

596   N642H mutated cases as determined by ARMS PCR, Sanger sequencing and myeloid panel

597   analysis. Group 1 are cases with no additional mutated genes, group 2 have additional

598   mutated genes that include *SF3B1* and group 3 have additional mutated genes that do not

599   include *SF3B1*. ARCH = genes strongly associated with age-related clonal hematopoiesis.

600   Full details of additional mutations are given in Supplementary Table 1.

601

602   Figure 2. Peripheral blood film (A), bone marrow smear (B) and bone marrow trephine at

603   x20 (C) and x100 (D). The blood film showed 40% neutrophils, 28% eosinophils, 4% basophils,

604   12% lymphocytes and 7% monocytes. The eosinophils were morphologically close to normal

605   with only a very minor degree of vacuolation and hypogranularity but there was an increase

606   in non-lobated forms. Bone marrow cellularity and megakaryocyte numbers are increased.

607   Increased cellularity is due to an increase in all three granulocyte lineages (neutrophils,

608   basophils and eosinophils). The trephine biopsy sections show hypercellularity,

609   disorganisation and an increase in cells of neutrophil and eosinophil lineages. Reticulin was

610   not increased.

611

612   Figure 3. Kaplan-Meier plots showing overall survival (OS) estimates for the *STAT5B* N642H

613   mutated cases who had eosinophilia at diagnosis and had follow up data available (n=23). (A)

614   Comparison of the 3 molecular groups shows that cases in group 3 (those with additional

615   mutated genes that do not include *SF3B1*) have an inferior OS compared to all other cases

616   (median 14 months versus 65 months; P<0.0004). (B) OS for cases with mutations in 2 or

617   more additional genes (excluding *DNMT3A* and *TET2*) was significantly worse than that for

618   cases with 0 or 1 additional mutations (median 18 months versus 50 months; P=0.001). Of

619   the 9 cases with mutations in ≥2 additional genes, 8 were in group 3.

620

621   Figure 4. (A) Clonal hierarchy for 4 patients. The numbers indicate the number of colonies

622   with that genotype, e.g. for case E11825, 5 colonies were mutant for *TET2* and *SF3B1* but

623   not *STAT5B* and 9 colonies were mutant for all 3 genes. Light grey indicates an inferred

624   ancestral clone. (B) Sequential analysis of two cases with increasing eosinophil counts

625   (values indicated are ×10^9/L). Case E11825 had an elevated an eosinophil count at diagnosis,

626   with eosinophilia increasing over time co-incident with an increase in *STAT5B* N642H vaf.

627   Case E13661 was diagnosed initially with PV in 1988 and *STAT5B* N642H was not detected in

628   a sample taken 6 years later. Slightly elevated eosinophil counts were first noted in 2014,

629   and a sample from this time was positive for *STAT5B* N642H. The mutant vaf and eosinophil

630   counts increased over the next 4 years.

631

632

633 Table 1. Candidate variants identified by RNAseq analysis

634

Case	Gene	Refseq	cDNA	protein	vaf
E166	*JAK2*	NM_004972	c.G1849T	p.V617F	1.00
E166	*SOCS1*	NM_003745	c.G630C	p.Q210H	0.48
E11072	*RPS19*	NM_001022	c.C164T	p.T55M	0.48
E11072	*SOCS1*	NM_003745	c.G630C	p.Q210H	0.86
E11075	*PRF1*	NM_001083116	c.A755G	p.N252S	0.39
E11076	*SF3B1*	NM_012433	c.G1998T	p.K666N	0.32
E11076	*PRF1*	NM_001083116	c.A755G	p.N252S	0.67
E11076	*STAT5B*	NM_012448	c.A1924C	p.N642H	0.43
E11493	*STAT5B*	NM_012448	c.A1924C	p.N642H	0.47
E11500	*JAK3*	NM_000215	c.G2164A	p.V722I	0.56

635

636

637   Table 2. Clinical summary of *STAT5B* N642H mutant cases

638

Case	Sex	Age (y)	Status (m)	wcc	plt	Hb	eos	Initial diagnosis	MG
E143	M	69	D (107)	16	280	100	1.5	aCML	2
E228	M	65	n/a	n/a	n/a	n/a	n/a	HES	2
E492	F	7	D (13)	30	343	147	11	Myeloid sarcoma	3
E1237	M	89	D (12)	15	488	148	6	MPN-U	3
E2323	M	70	D (12)	61	107	101	10	CMML	3
E2335	M	63	D (9)	28	113	158	0.7	MDS/MPN	3
E2594	M	58	D (14)	14	128	81	4	MDS-SLD; eosinophilia at 3 years	2
E2810	M	81	D (7)	18	409	94	10	MPN-U	2
E2935	F	10	D (48)	47	157	110	27	HES	1
E3065	F	82	D (28)	25	150	100	2	CMML + MM	1
E3321	M	86	D (45)	21	n/a	152	6	HES	2
E4349	M	61	D (12)	15	16	132	5	CMML	3
E5735	M	68	D (31)	12	106	111	5	HES	3
E10926	M	70	D (16)	142	111	73	1.4	SM-AHN	2
E11076	M	72	A (68)	21	556	149	6	MDS/MPN-U	2
E11493	M	76	D (23)	42	275	92	19	MDS/MPN-U	3
E11825	F	61	A (61)	9	438	108	1.2	MDS/MPN-RS-T	2*
E11837	M	72	D (14)	11	120	156	7	MDS-MLD; eosinophilia at 3 years	1
E12398	M	77	D (15)	24	34	140	7	HES	3*
E12433	F	51	A (35)	19	151	90	3	MDS/MPN-U	2
E12601	F	64	D (14)	28	267	116	10	HES	3
E12614	F	15	A (48)	28	655	133	4	CEL-NOS	1*
E12885	F	75	D (23)	24	238	110	3	MDS/MPN	3*
E13191	M	83	A (9)	29	401	148	13	HES	3
E13206	M	85	D (4)	39	229	142	20	CEL-NOS	3
E13493	M	70	D (9)	17	195	65	6	MDS/MPN-U	3
E13661	M	42	A (60)	12	267	125	0.5	PV; eosinophilia at 25 years	3

639
640   Sex: male (M) or female (F); Age in years (y) at presentation; Status: dead (D) or alive (A) at specified number
641   of months (m) after presentation or first detection of eosinophilia (E2594, E11837, E13661); white cell count
642   (wcc), platelets (plt) and eosinophils (eos) $\times 10^9$/L at diagnosis or first detection of eosinophilia; hemoglobin (Hb)
643   in g/L; diagnosis: atypical chronic myeloid leukemia (aCML), hypereosinophilic syndrome (HES),
644   myeloproliferative neoplasm, unclassifiable (MPN-U); myelodysplastic/myeloproliferative neoplasm
645   (MDS/MPN), myelodysplastic syndrome with single lineage dysplasia (MDS-SLD), chronic myelomonocytic
646   leukemia (CMML), multiple myeloma (MM), systemic mastocytosis with associated hematological neoplasm
647   (SM-AHN), MDS/MPN, unclassifiable (MDS/MPN-U), MDS/MPN with ring sideroblasts and thrombocytosis
648   (MDS/MPN-RS-T), MDS with multilineage dysplasia (MDS-MLD), chronic eosinophilic leukemia, not otherwise
649   specified (CEL-NOS), polycythemia vera (PV); molecular group (MG): 1 = no additional mutations; 2 = *SF3B1*
650   mutated; 3 = additional mutations but *SF3B1* unmutated; not available (n/a). * indicates the 4 cases for whom
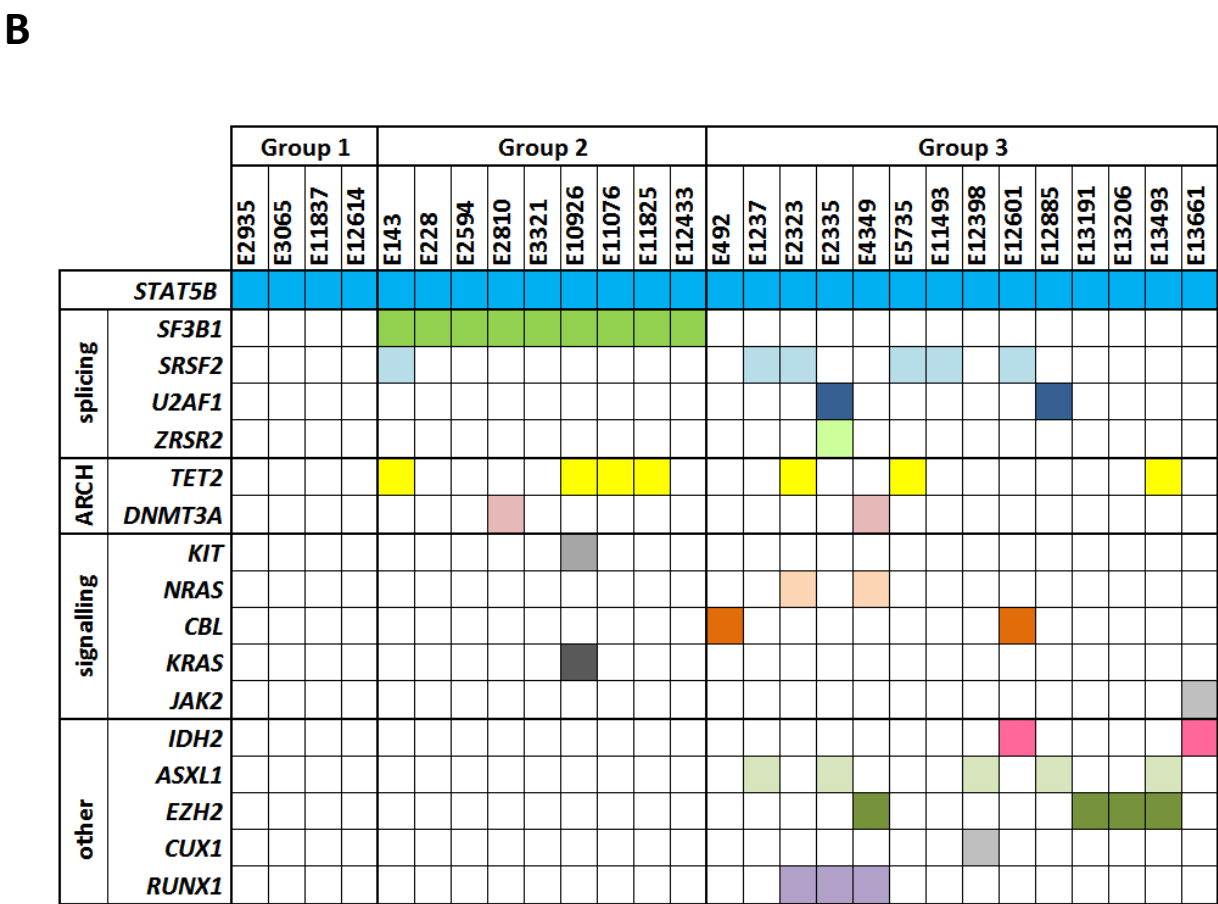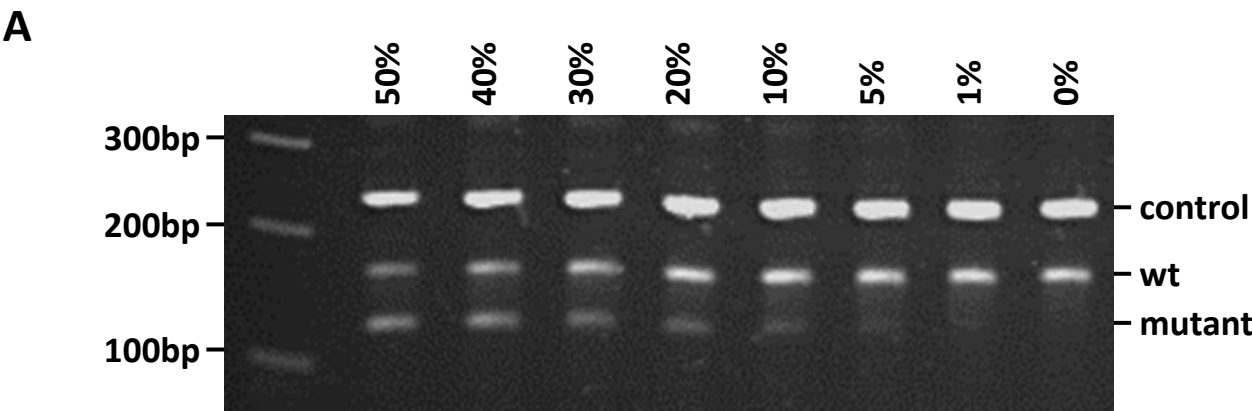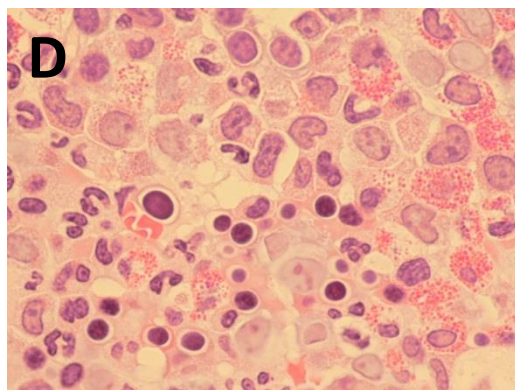651   *STAT5B* N642H was absent in cultured T-cells.

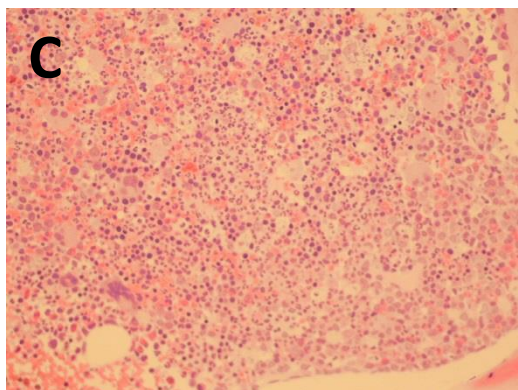652

653

654

655

656

Figure 1

Figure 3

Figure 3

A

E10926

SF3B1
TET2*
TET2del

→ SF3B1
TET2*
TET2del
STAT5B
KRAS
n=15

→ SF3B1
TET2*
TET2del
KIT
n=2

E11825

TET2
SF3B1
n=5

→ TET2
SF3B1
STAT5B
n=9

E12398

STAT5B
CUX1
n=14

→ STAT5B
CUX1
ASXL1
n=10

E12885

STAT5B
ASXL1
U2AF1
n=14

B

E11825

2014; eos = 1.2   2015; eos = 5.8

E13661

1994; PV   2014; eos = 0.4   2018; eos = 3

Figure 4