The frequency of *JAK2* exon 12 mutations in idiopathic erythrocytosis patients with low serum erythropoietin levels

Melanie J. Percy, Linda M. Scott, Wendy N. Erber, Claire N. Harrison, John T. Reilly, Frank G.C. Jones, Anthony R. Green, Mary Frances McMullin

**MJP and LMS contributed equally to the manuscript and should be considered joint first authors*

From the Department of Haematology, Belfast City Hospital, Belfast BT9 7AB, Northern Ireland, UK (MJP, FGCJ, MFMM); Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 2XY, UK (LMS, ARG); Department of Haematology, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK (WNE, ARG); Department of Haematology, St Thomas' Hospital, London SE1 7EH, UK (CLH); Department of Haematology, Royal Hallamshire Hospital, Sheffield S10 2JF, UK (JTR); Department of Haematology, Queen's University, Belfast BT9 7AB, Northern Ireland, UK (MFMM).

Acknowledgments: we would like to thank the clinicians and scientists who supplied samples and clinical data, and to the erythrocytosis patients who took part in this study.

Funding: work in the ARG laboratory is funded by the UK Leukaemia Research Fund, the Leukemia and Lymphoma Society, and by the Wellcome Trust.

Manuscript received April 20, 2007. Accepted August 16, 2007.

Correspondence:
Melanie J. Percy PhD, Department of Haematology, Floor C, Tower Block, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, N. Ireland.
E-mail: melanie.percy@bll.n-i.nhs.uk

ABSTRACT

Background and Objectives

Idiopathic erythrocytosis (IE) is characterized by erythrocytosis in the absence of megakaryocytic or granulocytic hyperplasia, and is associated with variable serum erythropoietin (Epo) levels. Most patients with IE lack the JAK2 V617F mutation that occurs in the majority of polycythemia vera patients. Four novel *JAK2* mutant alleles have recently been described in patients with V617F-negative myeloproliferative disorders presenting with erythrocytosis. The aims of this study were to assess the prevalence of *JAK2* exon 12 mutations in IE patients, and to determine the associated clinicopathological features.

Design and Methods

A cohort of 58 IE patients with low to normal serum Epo levels and no known causative mutation were identified from 181 individuals diagnosed with IE. Patients' DNA samples were screened for the presence of a *JAK2* exon 12 mutation by allele-specific polymerase chain reaction and sequencing. Bone marrow trephines were examined for morphological abnormalities and the erythroid activity assessed immunohistochemically.

Results

Eight mutation-positive cases were identified, including one with a previously undescribed mutant *JAK2* exon 12 allele and another with biallelic involvement. The hematologic features of mutation-positive and mutation-negative patients were similar, although Epo-hypersensitive erythroid progenitors occurred exclusively in patients with an exon 12 mutation (p=0.0002; n=15). Patients' bone marrows were moderately hypercellular, as the result of erythroid hyperplasia, and several had mild megakaryocyte atypia.

Interpretation and Conclusions

JAK2 exon 12 mutations were detected in 27% of patients with low serum Epo levels, all of whom had Epo-independent erythroid progenitors. Consequently, IE patients presenting with either of these features should be tested for the presence of a *JAK2* mutation.

Key words: idiopathic erythrocytosis, *JAK2* exon 12 mutation.

Haematologica 2007; 92:1607-1614. DOI:10.3324/haematol.11643

Idiopathic erythrocytosis (IE) is a heterogeneous collection of rare hematologic disorders that can be either sporadic or familial in origin.[1] It is characterized by a raised red cell mass (greater then 125% of the predicated value) and an elevated hematocrit. IE is distinct from the myeloproliferative disorder, polycythemia vera (PV), as the red cell hyperplasia present in IE is not accompanied by elevations in the megakaryocytic or granulocytic lineages. Furthermore, PV is characterized by low serum erythropoietin (Epo) levels, whereas IE is associated with a wide range of Epo levels. This spectrum of Epo levels reflects the diverse molecular defects so far identified in IE, and can be used to divide patients into two broad groups. The first group is defined by having Epo levels that are below the range found in healthy individuals, suggesting the underlying molecular defect(s) in these cases may affect components of the Epo signal transduction pathway, such as the erythropoietin receptor (EpoR)[2,3] or the cytoplasmic tyrosine kinase, JAK2. However, the gain-of-function V617F JAK2 mutation, which is found in most PV patients as well as in approximately 50% of patients with essential thrombocythemia or primary myelofibrosis,[4-10] is either absent or present at a low frequency in patients with IE.[11-13] The second group is defined by having Epo levels that are raised or inappropriately normal given the erythroid hyperplasia in these individuals. Patients within this group may have aberrations in the oxygen sensing pathway,[1] which regulates the rate of Epo synthesized via the hypoxia inducible factor (HIF) transcription complex. The von Hippel Lindau (VHL) and prolyl hydroxylase domain 2 (PHD2) proteins are both involved in the proteasomal degradation of HIF, preventing transcription of HIF target genes such as *EPO*. Loss-of-function mutations have been described in the VHL[14-16] and PHD2[17] proteins in individuals with erythrocytosis and dysregulated Epo production.

Although the V617F JAK2 mutation occurs in the vast majority of patients diagnosed with PV, approximately 5% of individuals with well-defined PV are V617F-negative, even when sensitive detection methods are used to determine the *JAK2* genotype.[4] These cases warranted further investigation and, as a consequence, a novel myeloproliferative disorder was recently described.[18] This syndrome is associated with gain-of-function mutations in JAK2 which result from the deletion or substitution of nucleotides within *JAK2* exon 12. Four different mutant alleles have been described to date, and these all affect JAK2 residues located between positions 537 (F537) and 543 (E543), approximately 80 residues prior to the valine (V617) mutated in the majority of patients with a myeloproliferative disorder. Affected individuals present with erythrocytosis, but exhibit several clinical and laboratory features distinct from PV. In common with the V617F mutation, the exon 12 mutations confer Epo-independent growth both *in vitro* and *in vivo*, and in a murine bone marrow transplant model also give rise to a myeloproliferative phenotype characterized by a very pronounced erythrocytosis.[18]

Retrospective analysis of the patients' hematologic features at diagnosis revealed that a proportion of those with an exon 12 mutation failed to fulfill the diagnostic criteria for PV proposed by the Polycythemia Vera Study Group, but instead fulfilled sufficient criteria for a diagnosis of IE.[18] To determine the true prevalence of *JAK2* exon 12 mutations in patients with IE, we therefore assessed the *JAK2* status of 58 IE patients for whom a causative mutation had not been identified. Twenty-nine individuals had low serum Epo levels and the remainder exhibited inappropriately normal serum Epo levels for a raised hemoglobin.

Design and Methods

Patients

Patients with IE were referred from hospitals throughout the United Kingdom and Ireland.[16] Cases entered into the registry were those with a raised red cell mass in the absence of hyperplasia of other cell lineages which was not the result of various identifiable secondary causes, and which did not fulfill the diagnostic criteria for PV proposed by the British Committee for Standards in Haematology (BCSH).[19] A comparison between these criteria with those proposed by the World Health Organization[20] is provided in Table 1. Upon entering the study, all patients gave informed written consent approved by the Queen's University, Belfast Research Ethics Committee, according to the Helsinki protocol.

Mutation screening

Patients' total peripheral blood or purified granulocyte DNA was initially screened for the *JAK2* exon 12 F537-K539delinsL, K539L, H538QK539L and N542-E543del mutations using four separate allele-specific polymerase chain reaction (PCR) assays, as described previously.[18] Whenever a mutation was identified by allele-specific PCR, it was verified by sequencing the *JAK2* exon 12 PCR products. The nucleotide sequence of the E543-D544 mutant allele detected in patient 5 was confirmed by ligating *JAK2* exon 12 PCR products into pGEM-T (Promega, Southampton, UK), and sequencing individual bacterial colonies produced by the transformation of DH5α competent cells (InVitrogen, Paisley, UK).

Bone marrow histology

Bone marrow trephine biopsies, formalin-fixed and decalcified (n=5) or resin-embedded (n=2), were available for seven of the eight patients with exon 12 mutations. These were assessed for morphology using hematoxylin and eosin stained sections, and for reticulin. Immunoperoxidase staining was performed on the for-

malin-fixed trephine sections using an indirect immunoperoxidase method and diaminobenzidine substrate to assess erythroid (glycophorin A), granulocytic (myeloperoxidase) and megakaryocytic (CD61) activity. After hematoxylin counterstaining, staining was visualized by light microscopy.

Erythroid colony assays

Peripheral blood mononuclear cells were cultured in methylcellulose (H4531; StemCell Technologies, London, UK) in the presence or absence of 2 IU/mL Epo (Eprex, Janssen-Cilag, High Wycombe, UK) at a density of $6x10^5$ cells per 35 mm plate (in a final volume of 2 mL). At least 30×10^6 mononuclear cells were plated in the absence of exogenous Epo to ensure the detection of any Epo-independent erythroid colonies (EEC) in samples in which the mutant allele burden was low. All erythroid colonies were genotyped by direct sequencing.

Statistical methods

Frequencies of Epo-independent erythroid colonies were compared in IE patients with and without a *JAK2* exon 12 mutation using Fisher's exact test. The age and hematologic indices at presentation of exon 12 mutation-positive and mutation-negative patients were compared using an unpaired Student's t-test. *p* values less than 0.05 were considered statistically significant.

Results

We used a previously developed registry of IE cases[12,16,17] to assess the frequency of *JAK2* exon 12 mutations, and to determine the clinicopathologic features associated with these mutations. Of the 181 cases currently included in the IE registry, 14 had identified defects in the *EPOR*, *VHL*, or *PHD2* genes (Figure 1); none of the patients was positive for the V617F JAK2 mutation (*data not shown*). *JAK2* exon 12 mutation analysis was limited to those patients who lacked *EPOR*, *VHL* or *PHD2* mutations, and who had serum Epo levels in the low to normal range. This subgroup of 58 cases was selected because *JAK2* exon 12 mutations have previously been detected in patients with serum Epo levels in this range,[18] and because elevated Epo levels are suggestive of a dysregulated oxygen-sensing pathway. Twenty-nine of these selected cases had low serum Epo levels, and the remainder exhibited inappropriately normal serum Epo levels for a raised hemoglobin (Figure 1).

Screening granulocyte or total peripheral blood DNA samples from these 58 patients, using both allele-specific PCR and PCR sequencing, identified eight individuals (three of whom have been previously reported)[18] who were positive for a *JAK2* exon 12 mutation (Figures 2A and 2B). Four of the newly identified cases had the N542-E543del JAK2 mutation, often present at low levels in granulocyte DNA. The fifth case, in contrast, had a novel JAK2 mutation, E543-D544del, the nature of which was confirmed by sequencing cloned granulocyte PCR products (Figure 2C). This mutation was fortuitously identified using the allele-specific PCR

Table 1. Comparison of BCSH[19] and WHO[20] criteria for the diagnosis of PV.

	Criteria	*BCSH*	*WHO*
A1	Red cell nass	+	+
	Hematocrit	+	
	Hemoglobin		+
A2	No secondary cause	+	+
A3	Splenomegaly	+	+
A4	Abnormal karyotype	+	+
A5	EEC formation		+
B1	Platelets (>400×10⁹/L)	+	+
B2	Neutrophils (>10×10⁹/L)	+	
	WBC (>12×10⁹/L)		+
B3	Radiological splenomegaly	+	
	BM panmyelosis		+
	Low serum Epo	+	+
B4	Characteristic EEC growth	+	
	Criteria for PV	A1+A2+A3/A4 or A1+A2+Bx2	A1+A2+any A or A1+A2+Bx2

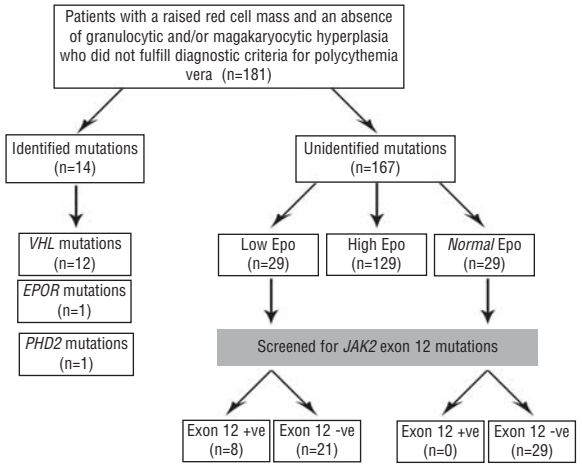

Figure 1. Mutation status of the patients in the idiopathic erythrocytosis registry. The molecular status of the *VHL*, *PHD2* and *EPOR* genes, and the serum Epo levels at diagnosis of patients included in the idiopathic erythrocytosis registry were used to identify a cohort of patients suitable for screening for a *JAK2* exon 12 mutation.

primer that had been designed to detect the N542-E543del mutation. The single base mismatch between the mutant *JAK2* allele and the PCR primer at position –4 (Figure 2C) did not appear to have compromised primer binding. The clinical data at diagnosis for all eight mutation-positive patients are listed in Table 2. The five newly identified patients each presented with a raised hematocrit (ranging from 0.54 to 0.68), and white cell and platelet counts that were not elevated. The phenotype of these individuals is, therefore, consistent with the recently identified myeloproliferative syndrome associated with *JAK2* exon 12 mutations.[18] All eight patients found to have a *JAK2* exon 12 mutation were from within the subgroup of IE patients with low serum Epo levels at diagnosis (n=29).

Comparison of the mutation-positive and mutation-negative patients with low serum Epo levels revealed that these two groups had similar hematologic indices (Table 3), although mutation-positive patients presented with significantly higher platelet counts ($p<0.005$) and lower Epo levels ($p<0.01$), and were also predominantly female. Mutation-positive patients tended to present at an older age, although this did not reach statistical significance ($p=0.09$). Due to an insufficient number of cases, we were unable to determine whether there are differences in the response to treatment between patients with and without an exon 12 mutation. We have, however, listed the patients' treatment details in Table 2. An additional difference between the mutation-positive and mutation-negative groups was noted when peripheral blood mononuclear cells from 15 IE patients were cultured in the presence and absence of exogenous Epo to evaluate the presence of EEC. EEC were found to be present exclusively in the eight mutation-positive patients studied ($p=0.0002$).

Patient 6 had a recent (November, 2006) granulocyte sequence trace that was suggestive of a biallelic *JAK2* mutation, with the peaks of the wildtype *JAK2* sequence lower than those of the mutant allele (Figure 3A). To confirm that this patient had a population of mutation-homozygous cells, erythroid colony assays were performed using the patient's peripheral blood mononuclear cells. Genotype analysis of 20 individual erythroid colonies grown in the presence of Epo identified that nine of these were mutation-heterozygous, and one colony was homozygous for the F537-K539delinsL JAK2 mutation (Figure 3A), despite the patient having apparently had only wildtype and mutation-heterozygous colonies a year earlier.[18] Half of the 62 EEC genotyped were homozygous mutant, suggesting that progenitors with biallelic *JAK2* exon 12 mutations may be better able to proliferate in the absence of Epo than mutation-heterozygous cells. These EEC also had a distinctive appearance, with their size and extent of hemoglobinization comparable to those of colonies grown in the presence of Epo (Figure 3B). In contrast, EEC from most patients, whether positive for an exon 12 or V617F JAK2 mutation, are generally smaller and paler than colonies grown in the presence of Epo (*LMS,*

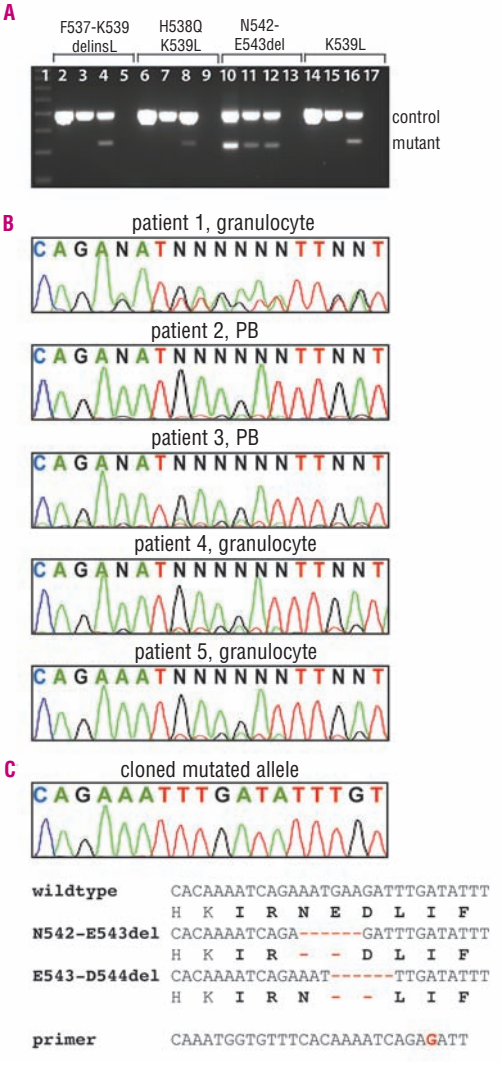

Figure 2. ***JAK2*** **exon 12 mutations occur in patients with idiopathic erythrocytosis. A. PCR was performed on total peripheral blood DNA using exon 12 control primers with primers specific for the F537-K539delinsL (lanes 2-5), H538QK539L (lanes 6-9), N542-E543del (lanes 10-13) or K539L (lanes 14-17) alleles. Assay examples for Patients 1 and 2 are shown (lanes 10-11); both were positive for the N542-E543del mutation. Patients previously described by Scott *et al.*[18] were used as positive controls in lanes 4, 8, 12 and 16. PCR negative controls for each allele-specific PCR reaction are lanes 5, 9, 13 and 17. Lane 1 contains a 100bp DNA size marker. B. All candidate mutations identified by allele-specific PCR were confirmed by PCR sequencing of purified granulocyte DNA (patients 1, 4 and 5) or total peripheral blood DNA (patients 2 and 3). The DNA sequence traces of the five new patients identified are shown. Sequencing of *JAK2* exon 12 in granulocyte DNA from patient 5 revealed a previously unidentified mutation in a minority of peripheral blood granulocytes. C. Sequencing of *JAK2* exon 12 cloned PCR products from this patient demonstrated that this mutation was a 6bp in-frame deletion that results in the deletion of JAK2 residues E543 and D544. The sequence of the allele-specific PCR primer, designed to detect the N542-E543del mutation, is shown; there is a one base mismatch (highlighted in red) that does not prevent the primer from detecting the E543-D44del mutation.**

unpublished data). We have now analyzed a total of 580 erythroid colonies cultured from seven patients with an exon 12 mutation (*reference 18 and this study*), and homozygous-mutant erythroid colonies have only been detected in the case reported here. Bone marrow trephines of seven of the eight mutation-positive patients were available for full assessment. In all cases, the bone marrow was hypercellular (60-80%), with the increase in cellularity primarily due to erythroid hyperplasia (Figure 4A). Morphological assessment and immunoperoxidase staining for glycophorin A (erythroid-specific) and myeloperoxide (myeloid-specific) revealed that the ratio of myeloid to erythroid cells ranged from 1:1 to 1:6, a significant reversal from the ratios observed in healthy controls (normal range, 2:1 to 5:1). Erythroid islands were prominent in all instances (highlighted by the glycophorin A staining), and showed normal differentiation from pronormoblasts to late normoblasts (Figure 4A). Granulocytopoiesis appeared normal in five patients, but was mild-

Table 2. Hematologic and clinical data for IE patients with a *JAK2* exon 12 mutation.

No.	Sex	JAK2 allele	Features at diagnosis							BM trephine summary	Retic.	M:E	EEC	Treatment
			Age	Hb	Hct	WCC	Plt	Epo	spleen					
1	F	N542-E543del	66	205	0.67	10.6*	206	3.0	N	mild erythroid hyperplasia	ND	1:1	Y	V, A
2	F	N542-E543del	32	N/A	0.60	5.2	297	<2.5	N	erythroid hyperplasia with prominent lymphoid aggregates	1-2	1:2	Y	V, A
3	M	N542-E543del	37	218	0.68	5.1	470	1.3	N	marked erythroid hyperplasia mild megakaryocyte atypia	2	1:1	Y	V, A
4	M	N542-E543del	67	194	0.61	7.7	293	3.7	N	erythroid hyperplasia mild megakaryocyte atypia lymphoid aggregates present	2∝	1:1	Y	V, A
5§	F	E543-D544del	73	172	0.54	5.3	314	2.5	N	N/A	ND	ND	Y	HU
6#	F	F537-K539delinsL	53	201	0.61	5.1	308	2.5	N	erythroid hyperplasia mildly increased granulocytopoiesis	0	1:1	Y	V, A
7#	F	N542-E543del	29	198	N/A	5.8	285	2.0	N	isolated erythroid hyperplasia	1	1:6	Y	V, A
8#	F	K539L	62	181	0.54	7.5	301	<2.5	N	erythroid hyperplasia with prominent lymphoid aggregates	2	1:1	Y	V

*Hb: hemoglobin (g/L); Hct, hematocrit; WCC: white cell count (×10⁹/L); Plt: platelet count (×10⁹/L); Epo: serum erythropoietin level (IU/mL) with normal range (5-25 IU/mL); Retic.: reticulin grade; M:E: ratio of myeloid to erythroid cells, assessed by glycophorin A and myeloperoxidase immunohistochemical staining; EEC: erythropoietin-independent erythroid colony growth (assayed 2006-2007); Y: yes; N: normal spleen size; V: venesection; HU: hydoxycarbamide; A: aspirin 75mg/day; N/A: not available; ND: not determined. *patient is a smoker. §the only patient to have experienced a thrombotic event (presenting with pulmonary embolism and cerebrovascular accident); #patients 1-5 are newly identified cases, patients 6-8 have been reported previously as patients 3, 5 and 8, respectively, in the study by Scott et al.[18] ∝: foci of increased reticulin deposition (grade 3) apparent.*

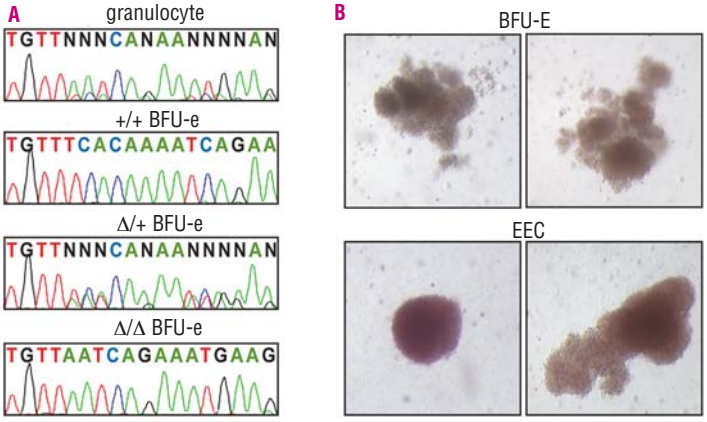

Figure 3. Bialleleic exon 12 mutations can occur, and enhance erythropoiesis *in vitro*. A. The *JAK2* exon 12 sequence trace obtained from the granulocyte DNA of patient 6 (top panel) was suggestive of biallelic involvement. Individual burst-forming units-erythroid (BFU-e) colonies were cultured from the patient's peripheral blood and genotyped by sequencing; typical examples of BFU-e with a wildtype (+/+; second panel), heterozygous F537-K539delinsL (Δ/+; third panel) or homozygous F537-K539delinsL genotype (Δ/Δ; bottom panel) are shown. B. Mutation-homozygous erythropoietin-independent erythroid colonies (EEC; bottom left and bottom right panels) are frequently as large as burst-forming units-erythroid (BFU-e) grown in saturating amounts (2 IU/mL) of erythropoietin. Both *JAK2* wildtype BFU-e (top left panel) and mutation-heterozygous BFU-e (top right panel) are shown. Original magnification, x63.

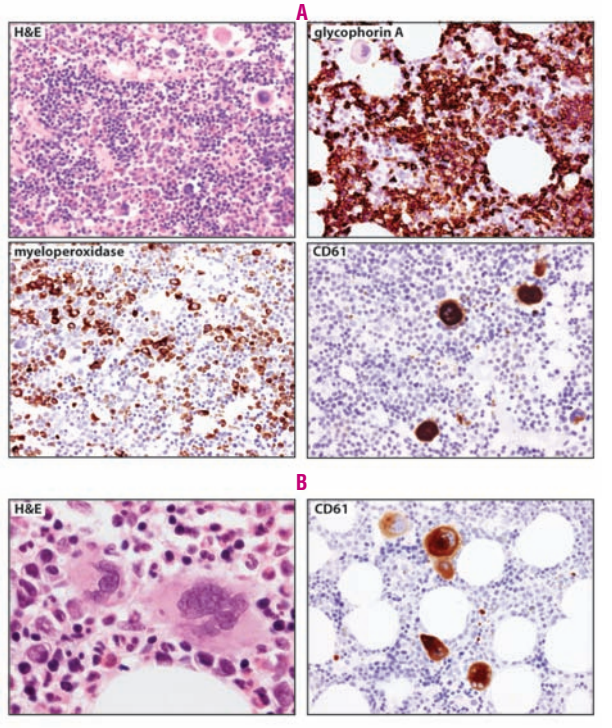

Figure 4. Isolated erythroid hyperplasia is typical in exon 12 mutation-positive cases. A. Hematoxylin and eosin-stained bone marrow trephine of patient 2 (H&E, upper left panel) indicated erythroid hyperplasia. Trephine sections stained for glycophorin A (upper right panel), myeloperoxidase (lower left panel) and CD61 (lower right panel) confirm the erythroid hyperplasia, in the presence of normal granulocytic and megakaryocyte activity. The staining was visualized by light microscopy (×400). B. High power magnification (×1000) image from a hematoxylin and eosin stained bone marrow trephine from Patient 4 demonstrated the presence of an occasional atypical large megakaryocyte with a hypolobated nucleus (left panel). A second trephine section, stained for CD61 (right panel), shows that most megakaryocytes in this patient had a normal morphology and that there was no significant megakaryocytic hyperplasia. Magnification, ×400.

Table 3. Comparison of hematologic and clinical data at diagnosis for IE patients with or without a *JAK2* exon 12 mutation.

JAK2 exon 12 status	*Mutant*	*Wild type*	
		Low Epo	*Normal Epo*
Demographics			
Number	8	21	29
Female, no. (%)	6 (75%)	9 (43%)	8[a] (31%)
Median age, years (range)	57.8 (29-73)	36.3 (10-65)	37 (15-70)
Hematological features			
Hemoglobin (g/L)			
mean±SD	196±15	197±16	186±12
median (range)	198 (172-218)	197 (163-232)	182 (170-222)
Hematocrit			
mean±SD	0.60±0.06	0.58±0.06	0.54±0.04[b]
median (range)	0.61 (0.54-0.68)	0.59 (0.47-0.71)	0.54 (0.49-0.64)
White cells (×10^9/L)			
mean±SD	6.5±2.0	7.2±2.6	8.1±1.81
median (range)	5.6 (5.1-10.6*)	6.6 (2.4-12.3)	8.0 (5.0-12.9)
Platelets (×10^9/L)			
mean ± SD	309±74	210±64[b]	227±70[c]
median (range)	299 (206-470)	198 (133-400)	214 (94-445)
Serum Epo (IU/mL)			
mean±SD	2.5±0.7	4.1±2.3[c]	10.2±4.7[d]
median (range)	2.5 (1.3-3.7)	4.2 (0.0- 9.0†)	9.5 (4.9†-25)

*Epo: erythropoietin level; SD: standard deviation; IU: international units. * patient smoked; † patients were stratified into low or normal Epo groups based upon the normal range of the assay used to test the sample. In most cases, the normal range was 5-25 IU/mL, but in several situations, it was 10-21 IU/mL and 4.2-24.2 IU/mL; [a] $p<0.05$ when compared to mutation-positive patients; [b] $p<0.005$ when compared to mutation-positive patients; [c] $p<0.01$ when compared to mutation-positive patients; [d] $p<0.001$ when compared to mutation-positive patients.*

ly increased in one (patient 6). In five cases, megakaryocytes were present in normal numbers and locations, and had normal morphology. In the other two cases, there was mild megakaryocytic hyperplasia and atypia: specifically, there were occasional large megakaryocytes with nuclear hypolobation (Figure 4B). Although the majority of megakaryocytes were in an interstitial location, a small number were paratrabecular. However, megakaryocyte clusters were not observed in any of the cases evaluated (Figures 4A and 4B). One or more interstitial lymphoid aggregates were seen in three cases. Reticulin was normal in three patients (grades 0-1), mildly increased (grade 2) in another two cases, and mildly to moderately increased (grade 2-3) in another one (Table 2).

Discussion

In spite of extensive investigations, the molecular basis of the erythrocytosis in the vast majority of IE cases remains elusive, although studies have identified several different molecular defects belonging to the Epo signalling and the oxygen sensing pathways.[1] Thus far, mutations in the *VHL* gene are the most frequent, but still constitute around only 10% of all IE cases,[21] whereas only two cases with a *PHD2* mutation have been reported to date.[17,22] EpoR mutations are also rare and account for the erythrocytosis in only a minority of cases. Studies that have included cases of familial erythrocytosis have suggested a prevalence of 12-15%,[23-25] whereas another study found only one EpoR mutation in 100 IE cases.[21] The description of the V617F JAK2 mutation in most cases of PV provided another candidate mutation, but several studies have shown this mutation is present very infrequently in IE patients.[11-13] The recent publication of four additional gain-of-function JAK2 mutations that are associated with a distinct myeloproliferative disorder characterized by predominant erythrocytosis[18] has raised the question of whether these *JAK2* mutations may be important in the pathogenesis of IE. Indeed, several of the patients in the study by Scott and colleagues were retrospectively found to have IE rather than PV. However, the incidence of *JAK2* exon 12 mutations in cases of IE and the clinicopathological features of the affected patients

have remained unclear. In this study, we demonstrated, by screening patients with defined IE and low serum Epo, that *JAK2* exon 12 mutations occur at a relatively high frequency in this group; that is, in eight of the 29 individuals tested (27%). Novel findings relating to the nature of the mutation in two cases are reported: one patient possessed a mutant allele, E543-D44del JAK2, which had not been identified previously. In another patient, a population of erythroid progenitors homozygous for F537-K539delinsL *JAK2* allele was present. Mutation homozygosity has not been observed in the other patients positive for an exon 12 mutation, and therefore appears to be a relatively infrequent phenomenon. In contrast, biallelic *JAK2* mutations have been detected in the majority of V617F-positive PV patients.[26,27]

Comparison of the clinical data of the mutation-positive and mutation-negative IE patients with low serum Epo levels revealed that these were very similar: hematocrits, hemoglobin concentrations and white cell counts did not differ appreciably. Mutation-positive individuals did present with significantly higher platelet counts, although these were within the range present in healthy controls. However, *in vitro* culturing of peripheral blood mononuclear cells revealed that EEC growth occurred in all those patients with an exon 12 mutation, but not in any of the cases with a wild-type *JAK2* genotype. Although considered a feature characteristic of myeloproliferative disorders, EEC have been documented in a minority of IE cases.[28,29] The observation that EEC are a frequent feature in IE patients with a *JAK2* exon 12 mutation is in striking contrast to the fact that most, but not all, patients with EpoR truncations fail to have EEC.[30] Consequently, patients presenting with an isolated erythrocytosis and low serum Epo level and/or EEC should be investigated for the presence of a mutation within *JAK2* exon 12.

It is important to stress that the patients initially diagnosed with IE and subsequently found to carry an exon 12 mutation do, in fact, have a myeloproliferative disorder. As such, these individuals may experience myelofibrotic transformation[18] and have an increased risk of hemorrhage or thrombotic complications (the latter occurred in Patient 5), thereby justifying the use of a *JAK2* exon 12 mutation screening approach that involves four separate allele-specific PCR assays to identify these cases. Furthermore, there is the potential for leukemic transformation, although none of the patients with an exon 12 mutation reported in the literature to date has developed leukemia. Further investigation should focus on the incidence of adverse events in patients with a *JAK2* exon 12 mutation.

In summary, *JAK2* exon 12 mutations were detected in 27% of IE patients with low Epo levels, making these the most common molecular defects identified within this subgroup to date. All patients positive for a *JAK2* exon 12 mutation had isolated erythroid hyperplasia, supporting the original description of a separate myeloproliferative syndrome associated predominantly with erythrocytosis.

Authors' Contributions

MJP designed and performed the research, analyzed the data and wrote the paper. LMS designed and performed the research, analyzed the data, and wrote the paper. ARG designed the research, analyzed the data, clinically assessed the patients, and wrote the paper. MFMM designed the research, analyzed the data, clinically assessed the patients, and wrote the paper. WNE performed research, analyzed data, wrote the paper. CNH clinically assessed the patients. JTR clinically assessed the patients. FGCJ set up the patient data base, and analyzed the data.

Conflict of Interest

The authors reported no potential conflicts of interest.

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