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Mutational Profiling in suspected Triple-Negative Essential Thrombocythaemia using Targeted Next-Generation Sequencing in a Real World Cohort.

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Essential Thrombocythaemia (ET) is driven by somatic mutations involving the *JAK2*, *CALR* and *MPL* genes. Approximately 10% of patients lack driver mutations and are referred as 'triple-negative' (TN-ET). The diagnosis of TN-ET, however, relies on bone marrow examination which is not always performed in routine practice, and thus in the real world setting there are a group of cases with suspected TN-MPN.

In this real world cohort, suspected TN-ET patients were initially re-screened for *JAK2*, *CALR* and *MPL* and then targeted next-generation sequencing (NGS) was applied.

The thirty-five suspected TN-ET patients had a median age at diagnosis of 43 years (range 16-79) and follow-up 10 years (range 2-28). Median platelet count was $758 \times 10^9/L$ (range 479-2903). Thrombosis prior to and following diagnosis was noted in 20% and 17% of patients. Six patients were *JAK2V617F* and 2 patients were *CALR* positive on repeat screening. NGS results showed that 24 of 27 patients harboured no mutations. Four mutations were noted in 3 patients.

There was no evidence of clonality for the majority of suspected TN-ET patients with targeted NGS analysis. Detection of driver mutations in those who were previously screened suggests that regular rescreening is required. This study also questions the diagnosis of TN-ET without the existence of a clonal marker.

Introduction

Essential Thrombocythaemia (ET) is a *BCR-ABL1*-negative myeloproliferative neoplasm (MPN), characterized by clonal proliferation of the megakaryocytic lineage leading to thrombocytosis. The disease is complicated by thrombotic and haemorrhagic events, variable progression to myelofibrosis and rarely transformation to acute myeloid leukaemia.¹ ET is driven by generally mutually-exclusive somatic driver mutations involving the *JAK2*, *CALR* and *MPL* genes with reported frequencies at 50-60%, 20-30% and 1-5% respectively.² These mutations all induce constitutive activation of the JAK-STAT signalling pathway and its downstream effectors in a cytokine-independent manner leading to a myeloproliferative phenotype.

A small proportion of ET patients do not harbour any of the three common driver mutations and are referred to as 'triple-negative' (TN-ET). This cohort of patients accounts for 10-15% of ET diagnoses and appears to be a heterogeneous entity comprising of clonal and polyclonal diseases.³ Recent high-throughput sequencing studies, using targeted gene panels and whole-exome sequencing, have interrogated the genetic basis of TN-ET. These have identified mutations in genes involved in epigenetic regulation (*TET2*, *DNMT3A*, *ASXL1*, *EZH2*, *IDH1* and *IDH2*), RNA splicing (*SF3B1*, *SRSF2*, *U2AF1*) and regulation of cytokine signalling (*CBL*). Although mutations in these genes are not restricted to MPNs, their presence in TN-ET cohort can be of diagnostic value, providing evidence of clonality.³

This study aimed to determine the molecular profile of suspected TN-ET patients using next-generation sequencing (NGS) and to identify potential clonal biomarkers. Clinical outcomes including thrombo-haemorrhagic complications, myelofibrosis-free and leukaemia-free survival were also documented.

Methods

A real world cohort of 35 patients were included with a diagnosis of ET between the years of 1989-2018 according to the WHO classification. A retrospective audit of diagnosis and outcomes was undertaken.

Data collection

Demographic characteristics (age and gender) and clinical information (year of diagnosis, cardiovascular risk factors, blood parameters, bone marrow morphology, treatment and clinical outcomes) were determined. Bone marrow biopsy material was only available in 25 of the 35 cases and was reviewed blinded by an independent haematologist. The remaining 10 cases did not have a bone marrow but were considered likely to have a diagnosis of ET because in the past in the real world setting patients with a persistent elevated platelet count and no other cause have had a diagnosis of ET.⁴ Suspected TN-ET patients were included after excluding *JAK2V617F*, *CALR* exon 9 and *MPL* exon 10 mutations by conventional techniques (allelic specific PCR, DNA fragment analysis and Sanger sequencing, respectively).^{5,6}

Next Generation Sequencing

Genomic DNA was extracted from whole peripheral blood and retested for *JAK2V617F*, *CALR* exon 9 and *MPL* exon 10 mutations by conventional techniques. NGS was performed using the Illumina MiSeq™ platform and the Trusight Myeloid gene panel. Sequence variants were called using Somatic Variant Caller (Illumina), with variant filtering using a custom Classification Tree within the Cartagenia BENCH® software. Reporting required total read depth >100X for known variants and >300X for novel variants and allelic frequencies >5%.

RESULTS

Clinical characteristics and haematological values

Thirty-five suspected TN-ET patients, diagnosed between the years of 1989-2018, were included in the study. The median age at diagnosis was 43 years (range 16-79) and 83% were female (table 1). The median duration of clinical follow-up was 10 years (range 2-28). Full blood count parameters at presentation (prior to initiating treatment) are shown in table 1. Median platelet count at presentation was $758 \times 10^9/l$ (range 479-2903). Cardiovascular risk factors and thrombo-haemorrhagic complications prior to and following diagnosis are depicted in table 1. Thrombosis prior to and following ET diagnosis was noted in 20% and 17% of patients respectively. No patients had any significant haemorrhage. In this retrospective selected group of patients, no patients progressed to myelofibrosis or acute myeloid leukaemia. One patient died from metastatic disease of unknown origin, unrelated to the

haematological condition, however the diagnosis of ET was made in this particular patient in 1989 with a BM consistent with ET. Twenty-one patients (60%) were on cytoreductive therapy and fourteen (40%) on antiplatelet therapy alone.

Molecular Results

As the median duration of clinical follow-up was 10 years all cases were rescreened for the canonical driver mutations by conventional approaches.^{5,6} Of interest, six patients were *JAK2V617F* positive and two patients were *CALR* positive on repeat screening. (table 2). Twenty-seven patients were subsequently sequenced with the Trusight Myeloid gene panel. Four mutations were detected in three patients (table 3) with the remaining 24 patients not harbouring any identifiable mutations.

Of the 24 patients without an identifiable mutation, sixteen had available bone marrow biopsies. When reviewed by an independent haematologist twelve cases failed to meet the morphological criteria for MPN. However, 4 patients remained with a diagnosis of suspected TN-ET without the presence of a clonal marker (figure 1, table 4).

DISCUSSION

In this real world cohort of cases, we investigated the mutational profiles and reviewed the bone marrow biopsies of 35 cases of suspected TN-ET with a median clinical follow up of 10 years. The detection of *JAK2V617F* and *CALR* mutations in 23% patients when rescreened implies that the use of sensitive screening tools for driver mutations should be encouraged in the diagnostic workup of these cases. The rescreening of *JAK2V617F* used a sensitive real-time quantitative PCR assay that likely contributed to the enhanced identification of *JAK2* positive cases. Low allele burden *JAK2* mutants have been well described in other series of TN-MPN's but may also indicate clonal haematopoiesis of indeterminate potential.^{3,7,8} Sensitivity can be improved when using isolated granulocytes or platelets in comparison to whole blood.⁹ The use of NGS is further supported by the observation that atypical *JAK2* and *MPL* mutations have been identified in series of TN-MPN.¹⁰⁻¹² Our series prompted the histological review in cases with available material. Of 13 cases where no identifiable clonal marker was identified 4 patients showed morphological features of ET (figure 1, table 4).

Again these results imply that either the 4 cases had a polyclonal haematopoiesis and may be hereditary MPN disorders or the technical limitations of the assay prevented the discovery of a clonal marker. In this particular study we used the Illumina Trusight Myeloid gene panel which is limited to 54 genes and only exon 10 of the MPL gene and exon 12/14 of JAK2 are included with the potential of missing rarer mutations.

We recognise certain limitations of this study, including the fact that all patients did not have BM trephines as required for a WHO diagnosis of ET. As this is a retrospective series of cases with a median follow up of 10 years all available cases (n=25) were assessed by an independent Haematologist. The difference of opinion between the original diagnosis and that on review highlights the difficulty of producing completely reproducible morphological assessments in the real world setting. In this setting, patients with a persistent elevated platelet count and no other cause have in the past, been labelled as ET without BM.⁴ Now that clonal abnormalities are detected a raised platelet and the detection of a clone may be sufficient to have a diagnosis of ET.

This real world series demonstrates that a sizable proportion of patients do not have a clonal disease which raises issues concerning the management where cytoreductive therapy may not be warranted.

CONCLUSION

We have found no genetic evidence for clonality in the majority of patients diagnosed with suspected “triple-negative” ET using targeted myeloid gene panel NGS. We propose rrescreening for canonical driver mutations is indicated, in light of their detection upon repeat genotyping in this group of patients.

This study should encourage others to reconsider the diagnosis in a similar set of real world cases that exist within MPN clinics throughout the country.

Statements:

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References:

1. Cazzola M, Kralovics R. From Janus kinase 2 to calreticulin: the clinically relevant genomic landscape of myeloproliferative neoplasms. *Blood*. 2014;123:3714-9.
2. Tefferi A, Pardanani A. Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol*. 2015;1:97-105.
3. Cabagnols X, Favale F, Pasquier F *et al*. Presence of atypical thrombopoietin receptor (MPL) mutations in triple-negative essential thrombocythemia patients. *Blood*. 2016;127:333-42.
4. Harrison CN, Butt N, Campbell P, *et al*. Modification of British Committee for Standards in Haematology diagnostic criteria for essential thrombocythaemia. *Br J Haematol*. 2014;167:418-38
5. Catherwood MA, McAllister R, McCallion P *et al*. A molecular diagnostic algorithm for JAK2 V617F investigations in suspected myeloproliferative neoplasms. *Ir J Med Sci*. 2020;160:621-626.
6. Catherwood MA, Graham A, Cuthbert RJG *et al*. Absence of CALR Mutations in Idiopathic Erythrocytosis Patients with Low Serum Erythropoietin Levels *Acta Haematol*. 2018;139:217-19.
7. Milosevic Feenstra JD, Nivarthi H, Gisslinger H, *et al*. Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. *Blood*. 2016;127:325-32.
8. Steensma DP, Bejar R, Jaiswal S *et al*. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126:9-16.
9. Angona A, Fernández-Rodríguez C, Alvarez-Larrán A *et al*. Molecular characterisation of triple negative essential thrombocythaemia patients by platelet analysis and targeted sequencing. *Blood Cancer J*. 2016;6:e463.
10. Chang YC, Lin HC, Chiang YH, *et al*. Targeted next-generation sequencing identified novel mutations in triple-negative myeloproliferative neoplasms. *Med Oncol*. 2017;34:83-9.
11. Acha P, Xandri M, Fuster-Tormo F, *et al*. Diagnostic and prognostic contribution of targeted NGS in patients with triple-negative myeloproliferative neoplasms. *Am J Hematol*. 2019;94:E264-67.

12. Ju M, Fu R, Li H, et al. Mutation profiling by targeted sequencing of "triple-negative" essential thrombocythaemia patients. *Br J Haematol*. 2018;181:857-60.

Figure legends:

Figure 1a.

Patient number 25.

BM trephine biopsy (*haematoxylin & eosin*)

Hypercellular bone marrow with multiple pleomorphic and hyperlobulated megakaryocytes

Fig. 1b Patient number 33.

BM trephine biopsy (*haematoxylin & eosin*)

Hypercellular marrow with a large hyperlobulated megakaryocyte and a second megakaryocyte abutting it.

Table legends:

Table 1. Clinical and biological characteristics in 35 patients with suspected triple-negative essential thrombocythaemia.

Haematological and clinical information was collected at diagnosis; information concerning thrombosis and haemorrhage included events after diagnosis. ^aMedian (range).

Table 2.

Clinical and biological characteristics at most recent follow in 9 patients with suspected triple-negative essential thrombocythaemia in which driver mutations were detected on rescreening.

Haematological and clinical information was collected at retesting; ^aMedian (range), F/U median follow up, Hb-Haemoglobin, Plt-Platelets, Hct-Haematocrit %. * Patient 10 was diagnosed in 2003 and received hydroxyurea but was stopped due to the formation of a leg ulcer.

Table 3. Clinical and biological characteristics in 3 patients with suspected triple-negative essential thrombocythaemia found to have mutations with next generation sequencing.

Haematological and clinical information was collected at retesting; ^aMedian (range), F/U median follow up, Hb-Haemoglobin, Plt-Platelets, Hct-Haematocrit %

Table 4. Clinical and biological characteristics in 4 patients with suspected triple-negative essential thrombocythaemia without evidence of a clonal marker and histological suggestive of essential thrombocythaemia .

*Patient 27 was diagnosed as ET with a BM 3 years post presentation and by this stage progression seems to have occurred

Haematological and clinical information was collected at retesting; ^aMedian (range), F/U median follow up, Hb-Haemoglobin, Plt-Platelets, Hct-Haematocrit %. *

Age at diagnosis (years) ^a	43 (16-79)
Gender (M/F)	8/29
Haemoglobin (g/l) ^a	139 (96-164)
Haematocrit % ^a	40 (28-48)
Platelet count (x10 ⁹ /l) ^a	758 (479-2903)
Leucocyte count (x10 ⁹ /l) ^a	8.5 (4.2-17.2)
Neutrophil count (x10 ⁹ /l) ^a	5.4 (2.2-10.7)
Cardiovascular Risk Factors	
Hypertension, n (%)	14 (40%)
Hyperlipidaemia, n (%)	16 (46%)
Smoking, n (%)	6 (17%)
Diabetes Mellitus, n (%)	1 (2.8%)
Thrombo-haemorrhagic Complications	
Thrombosis prior to diagnosis, n (%)	7 (20%)
Thrombosis following diagnosis, n (%)	6 (17%)
Major Bleeding prior to diagnosis, n (%)	0 (0)
Major Bleeding following diagnosis, n (%)	0 (0)
Thrombosis prior to diagnosis, n (%)	7 (20%)

Table 1. Clinical and biological characteristics in 35 patients with suspected triple-negative essential thrombocythaemia

Patient	Age/Sex ^a at diagnosis	Hb (g/l)	Hct %	Plt x10 ⁹ /l	F/U	Cytoreductive Treatment	Trephine Review	Mutation
1	M,60	15	0.43	391	13	Y	P	<i>JAK2V617F</i> -9%
5	M,71	9.2	0.25	263	15	Y	P	<i>JAK2V617F</i> -13%
6	F,69	13.9	0.42	620	12	Y	P	<i>JAK2V617F</i> -8%
10	F,56	13	0.4	379	15	Y*	P	<i>JAK2V617F</i> -4%
18	F,25	11.3	0.34	453	13	Y	P	<i>CALR pos</i>
19	F,57	13.8	0.4	463	7	Y	P	<i>JAK2V617F</i> -15%
26	F,33	15	11.7	700	8	N	N/A	<i>JAK2V617F</i> -17%
34	59,F	14	0.4	514	2	N	N	<i>CALR pos</i>

Table 2. Clinical and biological characteristics at most recent follow in 9 patients with suspected triple-negative essential thrombocythaemia in which driver mutations were detected on rescreening.

*Patient 10 was diagnosed in 2003 and received hydroxyurea but was stopped due to the formation of a leg ulcer

Patient	Age/Sex ^a	Hb (g/l)	Hct %	Plt x10 ⁹ /l	F/U	Trephine Review	Mutation/%VAF
11	79,F	14	0.41	520	3	P	<i>MPL</i> c.1544G>T, p. (Trp515Leu)-8% <i>ASXL1</i> c.2196_2223del, p.(Gln733Glyfs*2)-30%
12	69,F	11.8	0.36	287	14	P	<i>TET2</i> c.5650A>G, p.(Thr1884Ala)-12%
31	42,F	12	0.36	352	12	P	<i>CBL</i> c.1259G>A, p.(Arg420Gln)-24%

Table 3. Clinical and biological characteristics in 3 patients with suspected triple-negative essential thrombocythaemia found to have mutations with next generation sequencing.

Patient ID	Age/Sex ^a	Hb (g/l)	Hct %	Plt x10 ⁹ /l	F/U	Cytoreductive Treatment	Trephine at diagnosis
25	75,F	12	0.37	615	2	Y	Increase in number of megakaryocytes with clustering and nuclear lobulation
27*	43,F	12	0.36	581	8	Y	Increased numbers of megakaryocytes. Pleomorphic in size with some large hyperlobated forms. Grade 2 Reticulin
30	70,M	11.8	0.33	517	3	Y	Normal to increased megakaryocytes. Predominance of large mature forms some with deeply lobulated nuclei
33	40,F	12.7	0.38	1018	3	N	Increased loosely, clustered and large megakaryocytes. Some megakaryocytes have abnormal nuclei. No fibrosis

Table 4. Clinical and biological characteristics in 4 patients with suspected triple-negative essential thrombocythaemia without evidence of a clonal marker and histological suggestive of essential thrombocythaemia.

*Patient 27 was diagnosed as ET with a BM 3 years post presentation and by this stage progression seems to have occurred.