Pathology (April 2019) **51(3)**, pp. 301–307

HAEMATOLOGY

Calreticulin mutation specific CAL2 immunohistochemistry accurately identifies rare calreticulin mutations in myeloproliferative neoplasms

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Summary

Mutations of the multifunctional protein calreticulin (CALR) are recognised as one of the main driver alterations involved in the pathogenesis of Philadelphia negative myeloproliferative neoplasms (Ph$^-$ MPN) and also represent a major diagnostic criterion in the most recent World Health Organization classification of myeloid neoplasms. Nowadays, quantitative assessment of the driver mutations is gaining importance, as recent studies demonstrated the clinical relevance of the mutation load reflecting the size of the mutant clone. Here, we performed for the first time a manual and automated quantitative assessment of the CALR mutation load at protein level using CAL2, a recently developed CALR mutation specific monoclonal antibody, on a cohort of 117 patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF) and compared the CALR protein mutation loads with the *CALR* mutation load values established by a molecular assay. Eighteen different *CALR* mutations were detected in the cohort of the 91 CALR mutant cases. Mutation loads of the *CALR* mutations were between 13% and 94% with mean value in PMF cases significantly higher than ET cases (49.94 vs 41.09; t-test, p=0.004). Cases without CALR mutation (n=26) showed no or only minimal labelling with the CAL2 antibody, while all 18 different types of CALR mutations were associated with CAL2 labelling. The *CALR* mutation load showed a significant correlation (p=0.03) with the occurrence of major thrombotic events, with higher mutation load in patients presenting with these complications. We report a 100% concordance between the mutation status determined by immunohistochemistry and the CALR molecular assay, and we extend the applicability of this approach to 16 rare CALR mutations previously not analysed at protein level.

Key words: Myeloproliferative neoplasms; calreticulin mutation; mutation load; CAL2 immunohistochemistry.

Received 21 August, revised 31 October, accepted 8 November 2018
Available online 31 December 2018

INTRODUCTION

Essential thrombocythemia (ET) and primary myelofibrosis (PMF) are classical Philadelphia-negative (Ph$^-$) myeloproliferative neoplasms (MPNs) characterised by a progressive nature due to exuberant production of fully differentiated cells in chronic phase.[1] Since the discovery of the clonal Janus-kinase 2 (JAK2) and myeloproliferative leukaemia protein (MPL) mutations in Ph$^-$ MPNs in 2005 and 2006, respectively, our understanding of the genetic and cellular basis of these diseases has increased dramatically.[2–6] In 2013, mutations of a multifunctional protein, calreticulin (CALR), were described by two independent studies in the majority of *JAK2* and *MPL* negative MPNs, with *CALR* mutations detected in 25–35% of the entire ET and PMF patient cohorts.[7,8] Since 2013, more than 50 different types of frameshift mutations in exon 9 of *CALR* have been described, with type 1, a 52 bp deletion (c.1092_1143del), and type 2, a 5 bp insertion (c.1154_1155insTTGTC) representing approximately 80–90% of all *CALR* mutations.[7,8] The vast majority of the non-type 1/2 mutations can be categorised as type 1-like, type 2-like variants based on the predicted helix propensity of the mutant amino acid sequence.[9,10] Clinically, *CALR* mutations are associated with favourable prognosis in both ET and PMF; however in PMF, this effect appears to be restricted to type 1 and type 1-like mutations.[9,11–13] In contrast to PMF, the presence of *CALR* mutations does not appear to affect the long-term survival of patients with ET.[14,15]

Testing of *CALR* mutations has become an integral part of the routine diagnostic algorithm of MPNs as a major

Print ISSN 0031-3025/Online ISSN 1465-3931 © 2018 Royal College of Pathologists of Australasia. Published by Elsevier B.V. All rights reserved.
DOI: https://doi.org/10.1016/j.pathol.2018.11.007

diagnostic criterion, and is usually performed at a molecular level together with analysing *JAK2* V617F and *MPL* mutations using qualitative as well as quantitative methods. Since the mutation load of these driver mutations appears to be associated with various clinical features of the patients,[16–19] the importance of quantitative assessment of mutations in ET and PMF is being increasingly recognised.[20,21]

More recently, antibodies against mutated CALR emerged, and based on the novel C-terminus of the mutant protein, these enable detection of the *CALR* mutations at protein level, using immunohistochemistry (IHC).[22] CAL2, a commercially available antibody specific for mutated CALR has demonstrated high sensitivity and specificity in detecting various CALR mutations in three smaller Ph$^-$ MPN cohorts, with the staining almost exclusively localised to the megakaryocytes.[23–25] However, these studies included only a very few cases with rare *CALR* mutations without precise annotations.

Here, we performed a quantitative expression analysis of CALR mutations using the CAL2 antibody on a cohort of 117 patients with ET or PMF, including 18 different CALR mutation types, and we assessed the correlation between the abundance of CALR mutations at protein level and *CALR* mutation load detected in the corresponding peripheral blood or bone marrow aspirate samples using a molecular method.

MATERIAL AND METHODS

Study population

Our cohort included 117 patients diagnosed with Ph$^-$ MPNs at the Semmelweis University and University of Pécs, Hungary, between 2002 and 2016. Before sample collection, written informed consent was received from all patients. According to the World Health Organization (WHO) 2008 criteria, 69 of 117 patients had ET (28 males, 41 females; median age 58.9 years, range 4–84 years), and 48 patients had PMF (24 males, 24 females; median age 64.5 years, range 31–90 years). Ninety-one of these patients carried a *CALR* mutation, while the remaining 26 cases with *JAK2* V617F or *MPL* mutation, or triple-negative were used as controls. The study was conducted in accordance with the Declaration of Helsinki.

Quantitative assessment of *CALR* mutations using fragment analysis

Genomic DNA was extracted from peripheral blood (n=101) or bone marrow aspirate (n=16) specimens obtained from the 117 Ph$^-$ MPN patients using the High Pure PCR Template Preparation Kit (Roche, USA) according to the manufacturer's recommendations. *CALR* exon 9 mutations were screened by high-resolution fragment size profiling of fluorescence-labelled polymerase chain reaction (PCR) products using capillary electrophoresis on an ABI3500 genetic analyser (Life Technologies, USA) with the following primers: *CALR* forward primer 5′-6-FAM-AGTTTGGCAACGAGACGTG-3′ and *CALR* reverse primer 5′-GAGTCTCACAGAGACATTATTTGG-3′. *CALR* mutational load ($CALR^{mut}$) was calculated as the relative ratio of peak heights: $CALR^{mut}/(CALR^{mut} + CALR^{wild\text{-}type})$ as described before.[17] Bidirectional Sanger sequencing was performed to determine the exact type of the *CALR* mutations.

Detection of *CALR* mutations using immunohistochemistry

Formalin fixed, paraffin embedded (FFPE) bone marrow samples from 91 MPN patients with different *CALR* mutations were selected for IHC based CALR mutation analysis using CAL2, an antibody specific for the mutant CALR protein.[22] The types and distribution of the *CALR* mutations included in this study are summarised in Table 1. Bone marrow samples from 26 patients with known *JAK2* V617F and *MPL* mutation status or triple-negative genotype were included as negative controls.

Four-µm sections were cut from decalcinated, FFPE bone marrow biopsy samples. Immunohistochemical reaction using a commercially available monoclonal antibody recognising mutated calreticulin (CAL2 clone, DIA-CAL-250; Dianova, Germany) was performed on dewaxed sections following endogenous peroxidase blocking with hydrogen peroxide pre-treatment for 20 min and antigen retrieval (at 100°C for 40 min in an electric cooker using a buffer of Tris-EDTA (pH 9.0). The antibody dilution was 1:100. Sections were stained using a biotin-free anti-mouse IgG polymer-peroxidase conjugate system (Dako Cytomation, Denmark). Immunoreactions were revealed using a diaminobenzidine (DAB) chromogen-hydrogen peroxide substrate for 5 min. Immunostained slides were digitalised using a Pannoramic 250 Flash III scan instrument (3DHISTECH, Hungary).

Table 1 Types and distribution of CALR mutations and negative controls included in the study

CALR mutation	Description	Type 1-like vs Type 2-like	Total *n*	ET *n*	PMF *n*	CAL2 IHC+
Type 1	c.1092_1143del	Type 1-like	47	26	21	47/47
Type 2	c.1154_1155insTTGTC	Type 2-like	23	19	4	23/23
Type 7	c.1102_1153del	Type 1-like	2	2	0	2/2
Type 8	c.1104_1137del	Type 1-like	1	1	0	1/1
Type 24	c.1120_1138del	Other	1	1	0	1/1
Type 33	c.1154_1155insATGTC	Type 2-like	1	1	0	1/1
'Other'	c.1099_1150del52	Type 1-like	2	0	2	2/2
'Other'	c.1100_1145del46	Type 1-like	1	1	0	1/1
'Other'	[c.1102A>G, c.1103_1136del34]	Type 1-like	1	1	0	1/1
'Other'	c.1103_1136del34	Type 1-like	1	1	0	1/1
'Other'	[c.1103A>T, c.1104_1137del34]	Type 1-like	1	0	1	1/1
'Other'	c.1105_1156del52	Type 1-like	1	1	0	1/1
'Other'	c.1110_1141del32	Type 1-like	1	0	1	1/1
'Other'	c.1116_1146del31	Type 1-like	1	0	1	1/1
'Other'	c.1125_1146del22	Other	1	0	1	1/1
'Other'	c.1135_1138del4	Other	1	0	1	1/1
'Other'	c.1155_1158GGAG>TTGTCAGAA	Type 2-like	1	0	1	1/1
'Other'	c.1158_1159insAGGACAAGGAG	Type 2-like	1	0	1	1/1
Combined mutations						
Type 1 + Type 2	c.1092_1143del + c.1154_1155insTTGTC	NA	1	0	1	1/1
Type 1 + rare mutation	c.1092_1143del + c.1149_1152GGAC>TTGTCA	NA	1	0	1	1/1
JAK2 V617F + Type 1	c.1092_1143del	NA	1	1	0	1/1
Negative controls						
Genotype						
Triple-negative			14	8	6	0/14
JAK2 V617F mutant			7	4	3	0/7
MPL W515K/L mutant			4	2	2	0/4
MPL S505N mutant			1	0	1	0/1

ET, essential thrombocythemia; IHC, immunohistochemistry; PMF, primary myelofibrosis.

Quantification of the mutant CALR protein expression

The expression of the mutated calreticulin protein as detected by immunohistochemistry was assessed both manually and using automated image analysis. The intensity of calreticulin expression was scored manually as 0, 1, 2 or 3 when no, mild, moderate or strong expression was detected in individual cells, respectively. One hundred megakaryocytes in each slide were analysed by two investigators independently. The individual scores of cells were added together to formulate the manual H-score (H_{man} score) ranging from 0 to 300. The ratio of non-megakaryocytic cells showing mutated calreticulin expression was also determined.

An automated image analysis application (DensitoQuant module; 3DHISTECH) was used to assess CAL2 labelling. The cytoplasms of one hundred megakaryocytes were manually highlighted as region of interest. Intensity of immune labelling was assessed for each pixel within the region of interest. A value from 0 to 3 was assigned to each pixel in a similar fashion that was used manually. The H_{auto} score was determined using the following formula: (ratio of weak positive pixels) + (ratio of moderate positive pixels) × 2 + (ratio of strong positive pixels) × 3 = H_{auto} score. The H_{auto} score ranged from 0 to 300 and was comparable to the H_{man} score.

RESULTS

Fragment analysis

The 91 cases with *CALR* mutation included 55 patients with ET and 36 patients with PMF. Eighteen different *CALR* mutations were seen, with type 1 and type 2 being the most frequent, amounting to 80% of all cases. Two cases showed two distinct *CALR* mutations within the same sample, while one case showed coexistence of a *JAK2* V617F and a type 1 CALR mutation (Table 1). Mutation loads of the *CALR* mutations were between 13% and 94% with mean value in PMF cases significantly higher than ET cases (49.94 vs 41.09; t-test, p=0.004), with this difference being more pronounced in the case of type 1 and type 1-like mutations. Indeed, cases with type 1 and type 1-like mutations showed significantly higher mean mutation load values compared to cases with type 2 and type 2-like mutations (47.44 vs 37.38; t-test, p=0.003) regardless of the diagnosis. The mutation load values obtained from bone marrow samples were significantly higher (p=0.0075) compared to the mutation loads measured in the peripheral blood samples; however, it is important to note that these samples do not represent paired specimens from the same patients and this comparison is also limited by the small number of bone marrow samples (Fig. 1).

Thirty-two cases showed >50% mutation loads, indicating that at least a subpopulation of the cells carried a homozygous mutation. This finding was associated with the diagnosis of PMF (15/70 ET cases vs 17/47 PMF cases), as well as with type 1 or type 1-like mutations (27/62 type 1 or type 1-like mutations vs 5/26 type 2 or type 2-like mutations); however, significance was not reached (p=0.0932 and p=0.051, respectively).

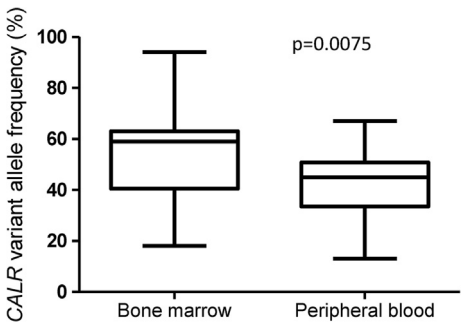

Fig. 1 Comparison of mutation loads determined in peripheral blood versus bone marrow specimens. Mutation load values obtained from bone marrow samples (n=16) were significantly higher (p=0.0075) compared to the mutation load values measured in the peripheral blood samples (n=75), however it is important to note that these samples do not represent paired specimens from the same patients and this comparison is also limited by the small number of bone marrow samples.

Immunohistochemistry

Cases without CALR mutation (n=26) showed no or only minimal labelling with the CAL2 antibody. H-scores evaluated manually (H_{man} score) were between 0 and 54 (8.6±3.0 mean value), the cases only showed megakaryocytes with dim expression. Automated image analysis yielded H_{auto} scores between 0 and 91 (10.0±5.0 mean value). All these cases were scored CALR wild-type by the two independent observers as well. CALR mutation positive cases (n=91) showed H_{man} scores between 62 and 276 (144.5±4.9 mean value), with an average of 45.7 (±2.6) % megakaryocytes demonstrating moderate to strong CALR expression. Automated image analysis resulted in H_{auto} scores between 98 and 287 with mean values of 186.5 (±2.9) and 68.5 (±1.28) % for the H_{auto} scores and CALR expressing megakaryocytes, respectively. Using cut-off values of 58 and 95 for H_{man} and H_{auto} scores, respectively, resulted in accurate discrimination of CALR mutation negative and positive cases (Supplementary Fig. 1).

Immunohistochemistry revealed no significant difference in CALR staining pattern between ET and PMF cases or different types of *CALR* mutations. All 18 different types of CALR mutations analysed in our study were associated with CAL2 labelling. Sixteen of these mutations had not been analysed using the CAL2 antibody before, demonstrating the applicability of this IHC approach to identify the rare CALR variants (Fig. 2).

Megakaryocytes with CALR expression showed no distinctive morphological features when compared to CALR negative cells within a sample, although immature cells with high nucleus to cytoplasmic ratio tended to have more intensive labelling. Forty-six percent (42/91) of the cases (27 ET and 15 PMF) with CALR mutation showed labelling of non-megakaryocytic cells as well. The range of positive cells was between 0 and 65%, with these cells appearing as granulocytic precursors (Fig. 3).

Next, we tested whether the CALR mutation load as determined by immunohistochemistry showed any correlation with the clinical and laboratory parameters of the patients. The H_{man} scores did not show a correlation with age of the patients, lactate dehydrogenase levels, platelet and white blood cell count, haemoglobin and haematocrit values (data not shown). However, patients with major thrombotic events presented with significantly higher CALR mutation load (p=0.03) compared to the patients without thrombotic events (Fig. 4).

Correlation of molecular and immunohistochemical studies

Manual and automated evaluation of CALR staining showed significant correlation (p<0.001). Although the percentage of megakaryocytes with moderate to strong labelling (Fig. 5) as well as the proportion of CAL2 positive non-megakaryocytic cells tended to be higher in cases with higher CALR mutation loads, statistical significance was not reached, and neither the

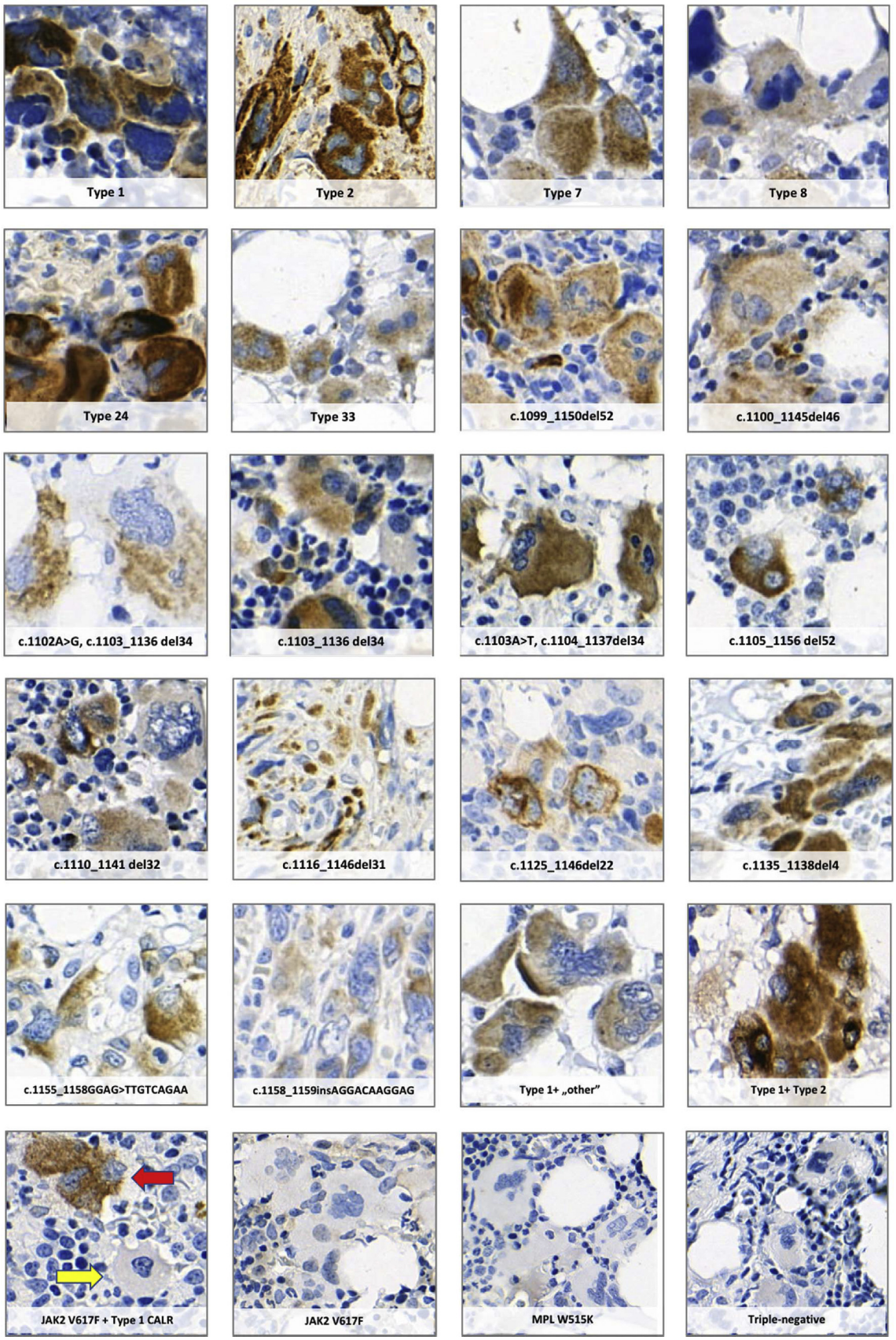

Fig. 2 Positive staining of megakaryocytes in MPN cases with 18 different *CALR* mutations using the CAL2 monoclonal antibody. *JAK2* V617F or *MPL* mutation positive cases and samples from patients with triple-negative genotype were used as negative controls. The red and yellow arrows indicate a CALR mutation positive and a CALR mutation negative megakaryocyte in a case with a concomitant *CALR* and *JAK2* V617F mutation, respectively.

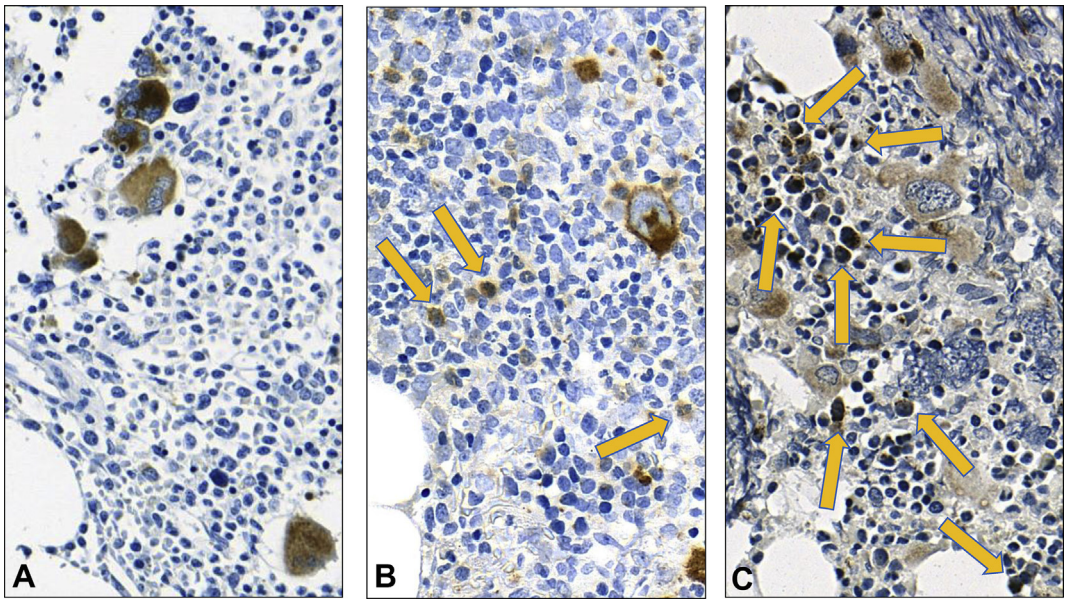

Fig. 3 Demonstration of the heterogeneity observed in positively staining non-megakaryocytic cells in MPN cases with *CALR* mutation using the CAL2 monoclonal antibody. (A) No or minimal CAL2 positivity in non-megakaryocytic cells. (B) Faint CAL2 positivity in a few non-megakaryocytic cells indicated by arrows, representing mostly granulopoietic cells based on morphology, however, no further immunohistochemistry was performed to specify the lineage. (C) Strong cytoplasmic positivity in a higher proportion of non-megakaryocytic cells (indicated by arrows).

H_{man} nor the H_{auto} score demonstrated any significant association with mutation loads determined by molecular studies.

DISCUSSION

JAK2, *CALR* and *MPL* mutations are well recognised as driver mutations in myeloproliferative neoplasms. The allele burden of *JAK2* V617F, and more recently of *CALR* mutations, is known to associate with clinical parameters including risk of thrombosis, fibrosis of the bone marrow as well as disease progression.[17,26–28] Therefore quantitative evaluation of these mutations is gaining importance nowadays.

Recently, detection of somatic mutations on protein level using mutation specific antibodies and immunohistochemistry, e.g., *BRAF* V600E mutations in hairy cell leukaemia, has emerged as an attractive approach, representing a viable (rapid and cost-effective) alternative in laboratories with limited access to standardised molecular assays. The CALR mutation specific monoclonal antibody CAL2 was generated by Stein *et al.* and they reported sensitive and highly specific detection of CALR mutations in a cohort of 52 patients including five rare mutations.[25] Suitability of the CAL2 antibody to detect CALR mutations using FFPE material was confirmed by two subsequent studies on a limited number of cases including three additional rare variants.[23,24]

Our study represents the largest cohort of CALR mutant MPN patients (n=91) analysed using the CAL2 antibody to date and we demonstrate, for the first time, the applicability of this approach to reliably detect 16 different rare CALR mutations. We have performed quantitative manual and digital image-based analysis of CALR mutations to assess the tumour burden at protein level. All CALR mutation positive cases showed CAL2 staining, while the CALR negative cases showed no or minimal labelling. We observed a 100% concordance between the mutation status determined using the CAL2 antibody versus the molecular assay.

CALR staining was predominantly localised to the megakaryocytes, as reported before;[22–25] however, none of these studies quantified the CALR tumour burden determined by immunohistochemistry and the proportion of non-megakaryocytic cells with CAL2 labelling. In our study, 46% of the cases demonstrated CAL2 staining of non-megakaryocytic cells mostly appearing as myeloid precursors, with up to 65% CAL2 positivity of the non-megakaryocytic cells. This is in line with the emergence of CALR mutation in early multipotent progenitors capable of erythroid as well as myeloid differentiation.[8] The predominant expression of mutant CALR in megakaryocytes is explained by the relative overexpression of the wild-type CALR protein in megakaryocytes in comparison to other cell types.[22,24,29]

We and others previously reported correlation of the *CALR* mutation load determined by molecular assays with various clinical and laboratory parameters, including risk of thrombosis, risk of myelofibrotic transformation, presence of fibrosis

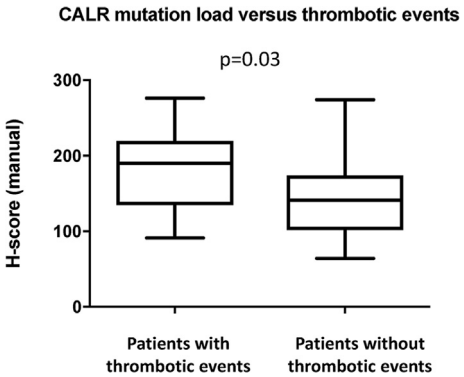

Fig. 4 Patients with major thrombotic events (n=9), including arterial thromboses (ischaemic stroke, acute myocardial infarction, popliteal artery occlusion, transient ischaemic attack and angina pectoris) and venous thromboses (pulmonary embolisation, femoral vein thrombosis, portal vein thrombosis and hepatic vein thrombosis) characterised with significantly higher CALR mutation load as defined by H-scores derived from quantitative analysis of CAL2 immunohistochemistry staining when compared to patients without thrombotic events (n=43), (p=0.03, t-test).

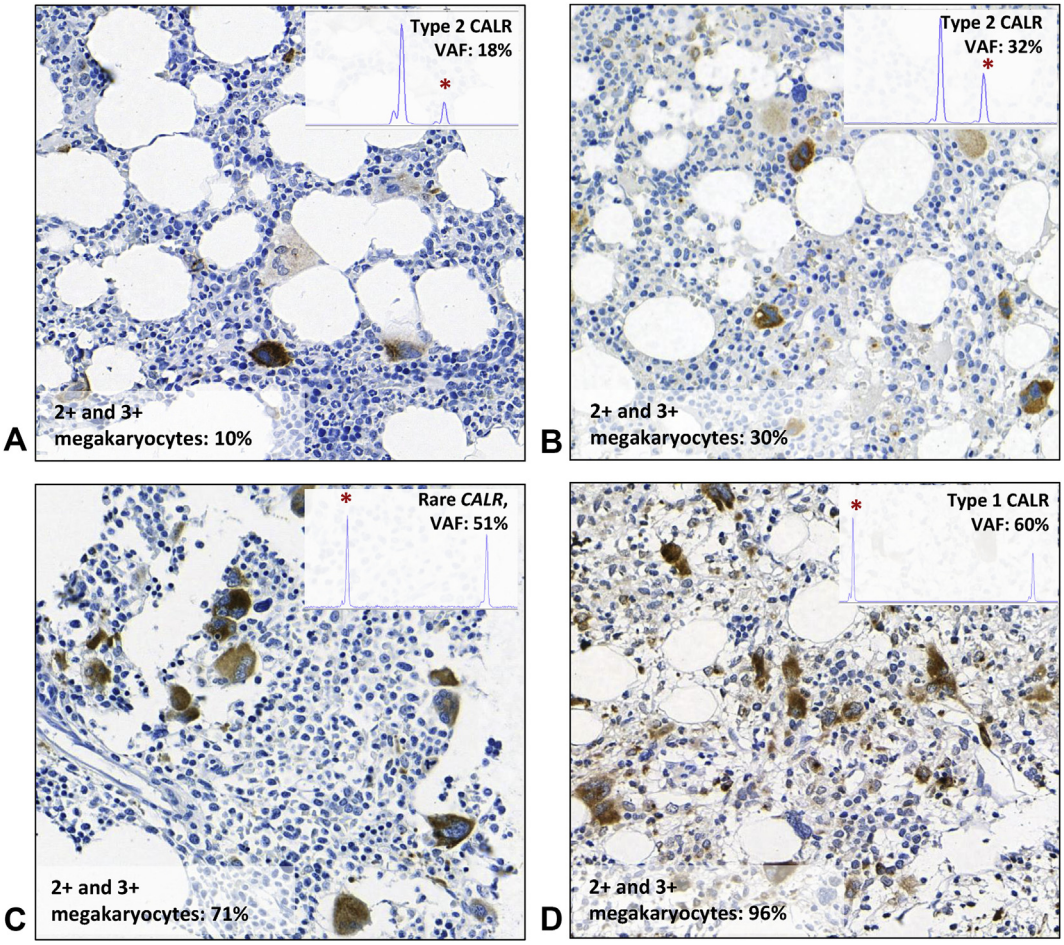

Fig. 5 Demonstration of CAL2 staining in the context of megakaryocyte proportion and *CALR* mutation loads. (A–D) Representative images with a trend observed between the proportion of megakaryocytes with moderate (2+) and strong (3+) CAL2 staining and mutation loads determined by molecular assay. (Red asterisks denote mutant CALR peak in electropherogram obtained from molecular assay.)

in bone marrow, splenomegaly, lactate dehydrogenase levels and platelet counts.[17,30,31] The CALR mutation load as determined by immunohistochemistry in this study, however, showed a significant correlation only in the context of thrombotic events, with patients presenting with major thrombotic events characterised by higher CALR mutation load. With thrombotic events being one of the most frequent complications in patients with MPNs, this observation may be of interest as we have previously reported higher incidence of thrombotic events in patients with higher *CALR* mutation load values.[17]

In an attempt to correlate the tumour burden readouts defined by immunohistochemistry and fragment analysis, we compared the CALR H_{man} scores with the *CALR* mutation load values. Only a trend was observed between percentage of megakaryocytes with moderate to strong labelling, and the proportion of CAL2 positive non-megakaryocytic cells with the *CALR* mutation load values. However, this analysis may have been hampered by the fact that the mutation load values reflect the percentage of peripheral granulocytes and monocytes with *CALR* mutation, while the CALR H-scores mainly represent the ratio of megakaryocytes with expression of mutated CALR protein, and by the homozygous state of *CALR* mutations as also demonstrated in approximately one-third of the cases analysed in this study.

In summary, we performed a quantitative CALR mutation analysis using a CALR mutation specific antibody on a cohort of 91 MPN patients with CALR mutation. We demonstrate 100% concordance with molecular assays and confirm the applicability of this approach to identify CALR mutations in FFPE tissue material. Further, we extend these findings to 16 rare mutations previously not analysed by this approach, and demonstrate a correlation between the occurrence of thrombotic events and CALR protein mutation load.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2018.11.007.

Conflicts of interest and sources of funding: This work was funded by the Momentum grant (LP- 95021) of the Hungarian Academy of Sciences, the NVKP_16-1-2016-0004 grant of the Hungarian National Research, Development and Innovation Office (NKFIH) and the by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the molecular biology thematic programme of the Semmelweis University. The authors state that there are no conflicts of interest to disclose.

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