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MOLECULAR PROFILING OF Bcr-Abl NEGATIVE MYELOPROLIFERATIVE NEOPLASMS, GOING BEYOND DRIVER MUTATIONS, SINGLE CENTER EXPERIENCE

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Abstract

The classical Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) comprise essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV). Major complications responsible for disease-related mortality are thromboembolic and hemorrhagic events, and in a subset of MPN patients, disease transformation to secondary acute myeloid leukaemia or PMF can occur.

Besides well-established driver mutations, further molecular studies, revealed novel somatic mutations i.e. non-driver mutations, whose occurrence may precede or follow the acquisition of driver mutations and can contribute in phenotypic variability, and disease progression.

In order to justify these observations, we examined the mutational profile of 78 patients with MPNs using next-generation sequencing for the detection of non-driver mutations in correlation with clinical presentation.

Somatic driver mutations were detected in 64 (78%) while 14 (17, 9%) were triple negative MPN cases. Most prevalent driver mutation was JAK2V617F mutation 54 (69, 2%), followed by CALR (10, 2%) and MPL in two (2, 5%). Fourteen (17, 9%) were triple negative cases. Mutations in ASXL1 gene were detected in one patient with ET and one with PMF, while mutations in TET2 gene were detected in three ET, two PV and two PMF patients, all of them JAK2V617F positive.

TP53 mutation was present in three patients, one with PV and two with ET. More than two non-driver mutations were seen in one patient with prefibrotic i.e hypercellular phase of myelofibrosis.

Larger studies are needed to conclude whether the landscape of non-driver mutations differs among cohorts and how their presence affects clinical presentation.

Keywords: myeloproliferative neoplasms, JAK2V617F mutation, non-driver mutations

Introduction

The classical Philadelphia chromosome (Ph) negative myeloproliferative neoplasms (MPN) comprise essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV). They are a group of mutually related clonal hematologic disorders characterized by excess accumulation of terminally differentiated myeloid cells (white blood cells, erythrocytes and platelets).

Epidemiologically, the MPNs are designated as rare diseases, with incidence of around 1-2 per 100,000 for PV and ET, and 0.3 per 100,000 for PMF, with median age at diagnosis at 60 years old, although they can also develop in young adults and children.

They are generally more common in males with the exception of ET which is more prevalent among females. Clinical presentation may vary from asymptomatic cases in whom the diagnosis is established through routine blood count, to clinically symptomatic cases that usually present with nonspecific symptoms such as headache, weakness, fatigue, night sweats, blurred vision, pruritus, thrombotic events, and early satiety with pain or discomfort in the upper left side of abdomen due to splenomegaly [1].

Major complications responsible for disease related mortality are thromboembolic and hemorrhagic events, and in a subset of MPN patients, disease transformation to secondary acute myeloid leukemia (AML) or PMF (for ET and PV patients) can occur.

Highest risk of blast transformation have patients with PMF, with an incidence rate of 20% in the first decade after diagnosis, and lowest risk have ET patients with <1% incidence rate in the first decade of diagnosis, while PV patients are in the middle with 2.3% incidence [2].

In the evaluation of patients with suspected MPN, other causes must be excluded. For polycythemia it is hypoxemia as a result of smoking, pulmonary or cardiac disease, erythropoietin secreting tumors (e.g. renal cell carcinoma), drug induced because of corticosteroid, androgen or erythropoiesis-stimulating agent therapy, while for thrombocytosis it is acute inflammatory conditions (infection, surgery, or malignancy), and are usually transient and resolve after the resolution of the precipitating process [3,4].

Widely used and accepted classification and diagnostic criteria for MPN is the 2016 World Health Organization (WHO) diagnostic criteria that encompasses the combination of clinical, morphological, and molecular genetic features as the most suitable attempt to define these entities that frequently overlap [5].

Treatment decision can be challenging and needs careful identification of risk factors that can further enhance the risk for thrombosis or bleeding in these patients, so treatment is directed towards prevention of thromboembolic and hemorrhagic incidents together with good quality of life preservation.

Genomic studies carried out in 2005 have identified several somatic mutations in the majority of patients with MPNs, of which the most common was the sense mutation at exon 14 of the JAK2 gene leading to abnormal functioning of the Janus kinase 2 (JAK2) protein and finally dysregulation with constitutive activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway responsible for normal hematopoiesis. It is a somatic mutation that involves substitution of valin to phenylalanine at codon 617 (JAK2V617F) and it is present in 95% of PV, and in approximately 55-60% of ET and PMF patients, and was soon adopted as a molecular diagnostic criteria [6].

In 5% of PV patients that are negative for JAK2V617F mutation, JAK2 exon 12 mutation can be identified [4].

Moreover, other two mutations have been identified and were found responsible for JAK-STAT pathway dysregulation, such as mutations in the MPL gene (present in <5% of ET patients, and 8% of PMF cases), and CALR gene (present in 30% of ET and PMF patients). These three mutations are designated as “driver” mutations, restricted only in MPNs, and have correlation with the clinical phenotype of the disease and response to treatment. Around 10-15% of ET and PMF patients lack all three driver mutations, known as triple negative MPNs and seem to have worse prognosis [7].

Further studies on molecular profiling of MPN patients using Next Generation Sequencing (NGS), revealed novel somatic mutations i.e non-driver mutations, whose occurrence may precede or follow the acquisition of driver mutations and can contribute in phenotypic variability, disease progression or shortened survival [7, 8].

It is interesting to note that these somatic mutations are not limited to MPNs only, but can also occur in a broad range of myeloid neoplasms, and involves genes in the spliceosome machinery (SF3B1,

U2AF1, and SRSF2), as well as genes encoding for several epigenetic modifiers (TET2, DNMT3A, IDH1/2, EZH2, and ASXL1) [8, 9].

Moreover, non-driver mutations are incorporated in the Mutation Enhanced International Prognostic Scoring System (MIPSS70) for PMF patient's ≤70 years old, eligible for bone marrow transplantation, the only curative option for this specific group. Continuing researches on these non-driver mutations can finally fully uncover and clarify the complex molecular pathogenesis of BCR-ABL1 negative MPN's [8].

In order to justify these observations, we examined the mutational profile of 78 patients with MPNs, in whom bone marrow biopsies for fibrosis tracking, and next generation sequencing (NGS) for detection of non-driver mutations were performed and were correlated with clinical presentation`.

Material and methods

A total of 78 patients with MPN of which 14 with PV, 54 with ET and 10 with PMF, who were diagnosed and followed at the University Clinic of Hematology, Skopje, RN Macedonia, were included in this study. The diagnosis was made according to 2016 WHO criteria and the median follow up period was 42 months (12-72). Median age of the patients at diagnosis was 53 (18-76).

Genomic DNA from Peripheral blood and bone marrow mononuclear cells was extracted with the Mag Core HF 16 Plus automated nucleic acid extractor (RBC Bioscience, Taiwan) using the Mag Core® Genomic DNA Whole Blood Kit according to the manufacturer protocol.

Detection of the most common driver mutations (c.1849G>T (p.Val617Phe) in the JAK2 gene, in the exon 9 of the CALR gene and c.1543_1544delinsAA (p.Trp515Lys), c.1544G>T (p.Trp515Leu), c.1542_1544delinsAGC and c.1514G>A (p.Ser505Asn) in the MPL gene) was performed by multiplex fluorescent allele-specific PCR using custom designed primers [9].

Targeted next generation sequencing analysis (NGS) was performed on the Mini sequencing system (Illumina, San Diego, CA, USA) using the Archer® VariantPlex® Core Myeloid panel that targets specific molecular markers (DNA copy number variation, DNA SNP In/del and DNA structural variants) in 37 genes (ANKRD26, BRAF, ABL1, IDH1, IDH2, JAK2, KIT, KRAS, PHF6, PTPN11, RUNX1, SETBP1, SF3B1, ASXL1, CALR, CBL, CEBPA, DNMT3A, GATA1, GATA2, ETNK1, ETV6, EZH2, MPL, NPM1, NRAS, STAG2, TET2, TP53, U2AF1, SRSF2, BCOR, CSF3R, FLT3, WT1, ZRSR2 and DDX41) frequently mutated in myeloid malignancies. For each reaction a total of 200ng genomic DNA (PBMC's) was used for library preparation. DNA libraries were prepared and multiplexed according to the Archer® VariantPlex® HS-HGS protocol, as recommended. Subsequently, the obtained DNA libraries were Quantified and normalized by quantitative PCR (qPCR) using the KAPA Universal Library Quantification kit (F. Hoffmann-La Roche Ltd) to an amount permitting a theoretical coverage of at least 250 reads per targeted sequence/per patient. The sequencing reactions were performed using MiniSeq Mid Output Kit (300-cycles) and the NGS data was aligned and annotated using the Archer Analysis software v5.1.

Bone marrow biopsy and aspiration was performed using local anesthesia, and detection of stromal structural fibers was made with reticulin and trichrome staining. Grading of fibrosis was made according the European Consensus on grading of bone marrow fibrosis (grade 0-3).

The correlation of the mutations with various laboratory and clinical parameters was done by using Mann-Whitney and Kruskall Wallis student's tests using the Stat graphics 4.3 software.

Clinical and laboratory data at diagnosis were obtained from the patients' charts according to the protocol approved by the Institutional Review Board of the University Clinic of Hematology, Skopje, RN Macedonia, and were collected at diagnosis and during follow up until start of treatment.

Results

In our study, among 78 MPN patients, somatic driver mutations were detected in 64 (78%) while 14 (17, 9%) were triple negative MPN cases. Of the three driver mutation most prevalent was

JAK2V617F mutation 54 (69, 2%), followed by CALR (10, 2%), MPL mutation in two (2, 5%), and 14 (17, 9%) were triple negative cases.

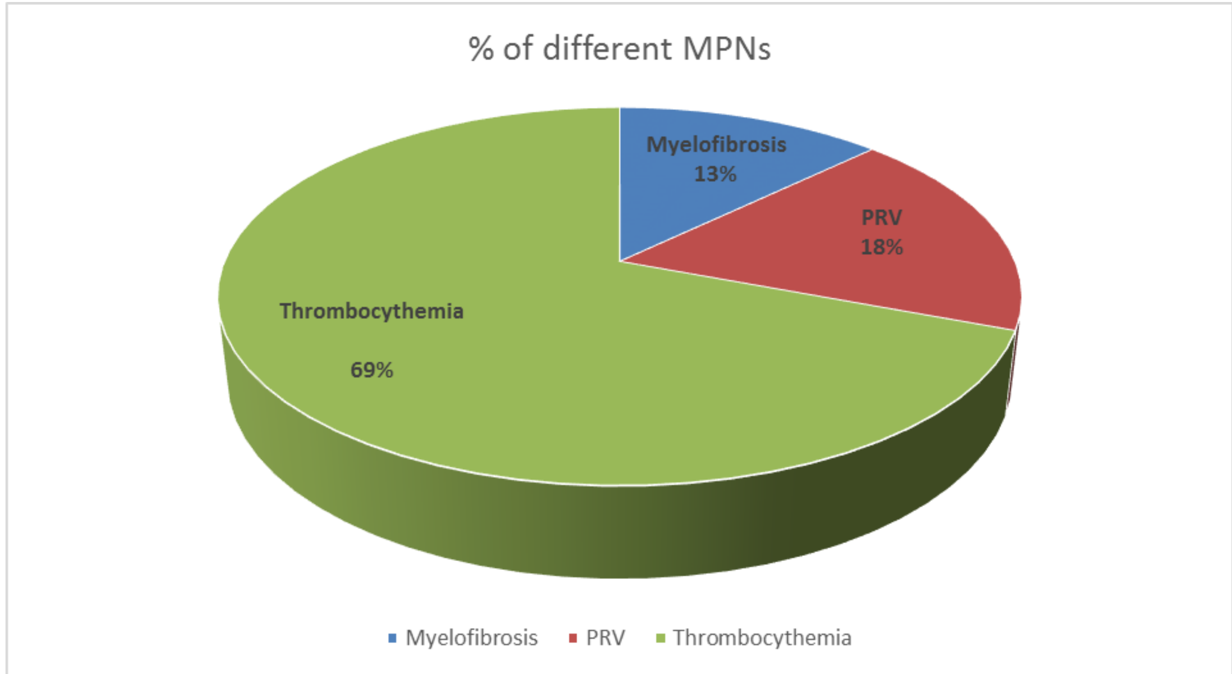

Chart 1. Distribution of different MPNs

Table 1. Demographic, laboratory and clinical characteristics of 78 MPN patients according to driver mutations

Diagnosis	Total patients		Gender	No of patients	Mutations			Age	Initial blood counts				Follow up blood counts			
	Number	%			Type	Number	%		Hb g/L	Le x 10^9/L	Tr x 10^9/L	Hct %	Hb g/L	Le x 10^9/L	Tr x 10^9/L	Hct %
Myelofibrosis	10	12.82	M	4	JAK2+	7	70 %	63.8 (44-72)	162.2 (123-191)	26 (10-51)	509 (363-638)	49.6 (44-60)	155.2 (118-189)	14.5 (7.9-34)	418.6 (128-698)	46 (33-56)
			F	6	triple neg	3	30 %		107.6 (84-120)	14.6 (5-25)	1017 (771-1200)	34.3 (26-39)	107 (94-115)	5.8 (5.3-5.9)	981.5 (831-1238)	33.3 (33-34)
PV	14	17.95	M	9	JAK2+	13	92.85 %	57.8 (27-74)	168.2 (133-196)	14.1 (6-25)	480.25 (173-770)	54.4 (49-65)	160.5 (141-200)	10.2 (7-14)	506.2 (161-932)	49 (42-63)
			F	5	triple neg	1	7.14 %		156 (133-189)	8.8 (5-15)	349.6 (161-539)	52.2 (44-58)	152.7 (135-175)	10.8 (6-15.9)	392.3 (195-660)	44.8 (39-50)
Thrombocythemia	54	69.23	M	25	CALR+	8	14.8 %	49.6 (18-76)	130 (117-147)	5.9 (8.4-12.6)	945.6 (681-1264)	39.8 (38-42)	133.2 (117-150)	8.5 (6-12.9)	1001.6 (407-1279)	39 (40.7-43)
					JAK2+	34	62.9 %		156 (94-204)	10.7 (5.5-22)	830.5 (413-1952)	47 (35-62)	149.8 (113-181)	9.95 (3.6-24)	704.35 (265-1400)	45.5 (39-50)
			F	29	MPL	2	3.7 %		131	7.5	816	40	140	8.8	777	42
					triple neg	10	18.5 %		134 (128-143)	10.3 (7.4-14)	1077.5 (819-1251)	42.3 (39-46)	146.7 (136-171)	10 (6.2-12.2)	1077.5 (382-1715)	43.8 (39-49)
P values (Kruskall Wallis)								0.03	0.01	0.01	0.00003	0.002	0.01	0.5	0.00001	0.003
P values (Mann Whitney)								0.04	ET/PV 0.001	ET/PMF 0.003	ET/PMF 0.01	PMF/PV 0.01	ET/PV 0.0004	/	ET/PV 0.00001 ET/PMF 0.0003	ET/PV 0.003 ET/PMF 0.0003

Of 54 (69, 2%) ET patients, the JAK2V617F mutation was detected in 34 (62, 9%), 8 patients (14, 8%) were CALR mutation positive, 2 (3, 7%) were MPL mutation positive, and 10 pts. (18,5%) were negative for all of the three driver mutations. Median age at diagnosis in the ET group was 49.6 (18-76) with a slight female predomination.

Of 14 (25.9%) PV patients, the JAK2V617F mutation was present in 13 (92, 9%) of them, and one patient was triple negative (7, 1%). Comparison of the age at diagnosis was lowest in the ET group 49.6 (18-76), followed by the PV group 57, 8 (27-74) and PMF group with 63.8 (44-72) (p 0.03).

There was no statistically significant difference in the ET group regarding platelet count at diagnosis between JAK2V617F, CALR, or MPL positive and triple negative ET patients (p 0.05). Comparison of hemoglobin (Hb) level between the three groups, showed highest Hb levels in PV patients, with statistically significant difference comparing with ET patients (p 0.001). Highest leucocyte count was seen in PMF patients, with statistically significant difference with leucocyte count in ET patients (p 0.003). Platelet count as expected, was highest in ET patients (ET/PMF p 0.01).

High platelet count in PMF patients was due to the fact that most of them had early phase myelofiborsis. Hematocrit level was highest in the PV group (ET/PV p 0.01).

Major thrombotic events were recorded in 22 (28%) of the patients. These patients had higher platelet counts than the 56 patients who did not experienced major thrombotic events (953x10^9/L compared with 642 x10^9/L (p 0.004). Bone marrow examination confirmed the diagnosis in all patients and helped distinguishing prefibrotic PMF from MPN with panmyeloic proliferation.

Average spleen size was 12,5cm (11-17), with largest diameter recorded in PMF patients in comparison to ET and PV patients but with no statistically significant difference (p 0.1).

NGS of 16 MPN patients from our cohort revealed presence of several non-driver mutations including ASXL1, TET2, DNMT3A, TP53, CBL, ANKRD26 and SRSF2 mutation. From 16 patients, 11

were initially JAK2V617F mutation positive, 4 of them had CALR mutation and one patient was triple negative. Two patients didn't show presence of non-driver mutations.

The main objective was the observation whether disease burden has correlation with possible presence of non-driver mutations.

Table 2. Patient's characteristics and non-driver mutations

	Patients	Hb g/L	Le x 10^9/L	Tr x 10^9/L	Hct %
Myelofibrosis	**2**	**136**	**33.5**	**624.5**	**42.5**
F	1	**123**	**51**	**638**	**38**
ASXL1,TET2,KRAS,SRSF2					
M	1	**149**	**16**	**611**	**47**
TET2					
PRV	**4**	**167.25**	**11.35**	**528**	**53.75**
F	1	**133**	**14**	**761**	**49**
ANKRD26, TP53					
M	3	**179**	**10**	**450**	**55**
DNMT3A,TET2		178	14	321	58
FLT3,DNMT3		170	11	770	50
WT1		188	6.4	260	58
Thrombocythemia ess.	**8**	**149.75**	**9.075**	**751**	**44.5**
F	5	**151**	**9**	**763**	**47**
DNMT3A,ASXL1		140	8	469	42
TET2		204	10.2	670	62
TET2, CEBPA		138	12	1274	48
TP53		142	5.5	481	43
ANKRD26,TP53		133	7.4	921	38
M	3	**147**	**10**	**731**	**41**
DDX41,TET2		135	8.5	629	40
PHF6		163	11	745	43
ANKRD26,CBL		143	10	819	40

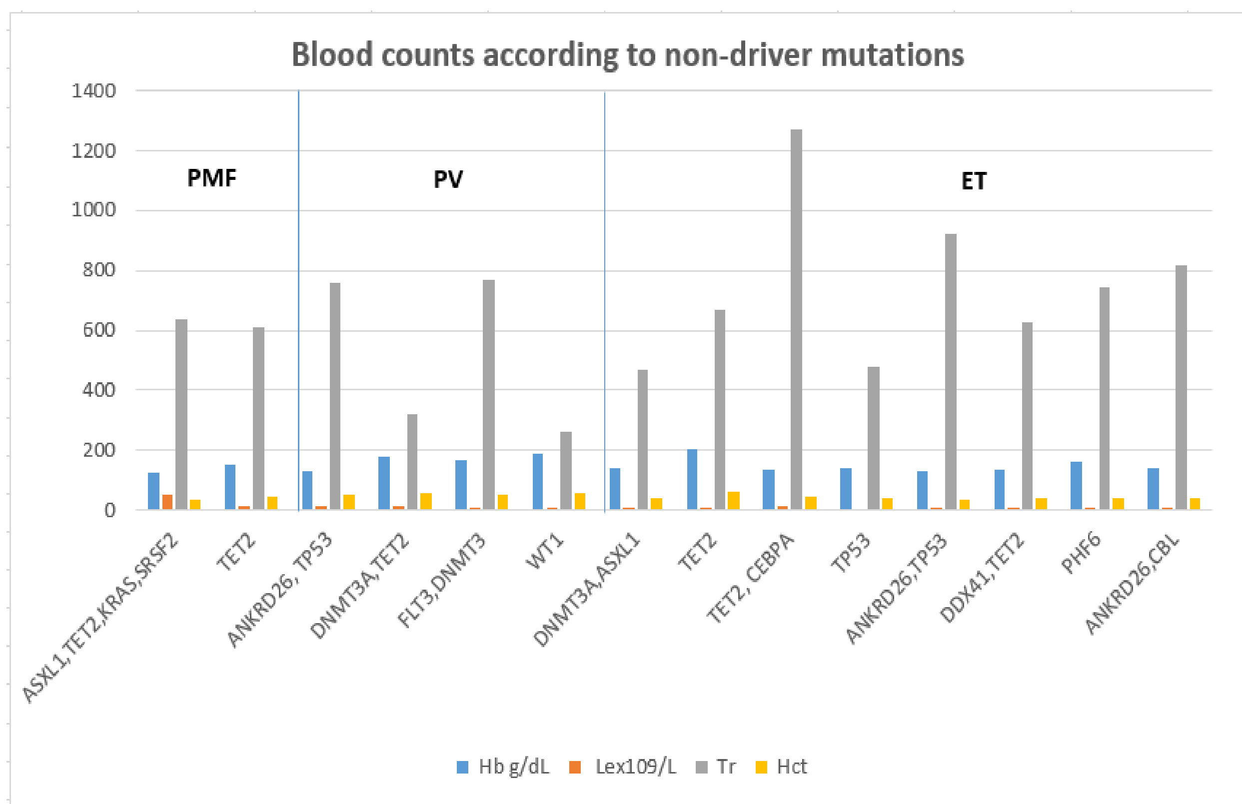

Chart 2. Blood counts according to non-driver mutations

Mutations in ASXL1 gene were detected in two patients, one with ET, and one with PMF, while mutations in TET2 gene were detected in three ET, two PV and two PMF patients all of whom were JAK2V617F mutation positive.

Three patients, one with PV and two with ET, revealed presence of mutation in TP53 gene. More than two non-driver mutations were seen in one patient with prefibrotic i.e hypercellular phase of myelofibrosis.

Discussion

Studies have showed that molecular status, including presence of one of the driver mutations (JAK2, CALR, or MPL) and other non-driver mutations (ASXL1, SRSF2, CBL, and IDH1/2 among others) affects diagnosis and prognosis of patients with BCR-ABL1 negative MPNs. Management of patients with these heterogeneous group of diseases begins with estimating the prognosis, disease burden, and prevention of vascular events [10,11].

Genetic and genomic testing are of increasing importance in the diagnosis, and risk assessment in order to appropriate management of patients with Bcr-Abl negative MPN's. Studies worldwide showed that ASXL1 mutations correlated with constitutional symptoms, leukocytosis, and presence ≥1% circulating blasts, in our study it was detected in two patients, one with PMF (hypercelular phase due to grade 1 bone marrow fibrosis) and one with ET, correlating with progressive deterioration of the blood count, and increasing platelet count, respectively.

Mutation in TET2 was detected in three ET, two PV and two PMF patients all of whom were JAK2V617F mutation positive, thus supporting already published data regarding the role of TET2 mutations in molecular biology of different myeloid malignancies [12].

Three patients, one with PV and two with ET, revealed presence of mutation in TP53 gene, but its association with progressive disease was not seen in our study, since our patients are showing indolent disease course, with stable blood counts without any treatment so far, except acetylsalicylic acid.

More than two non-driver mutations were detected in one patient with prefibrotic i.e hypercellular phase of myelofibrosis and supported the concept that the number of mutations also matters in being predictive for more aggressive disease and worse outcome since blood count deterioration in the follow up period was seen [12].

Conclusion

Contemporary molecular technics are essential for diagnosis and identification of prognostic markers in MPN patients, especially in so-called triple negative MPN cases where there is possibility for a reactive cause rather than a neoplasm, so more comprehensive analyses are needed in order to confirm disease clonality.

Novel studies suggest that identifying the key driver mutations will become only a part of the diagnostic work-up of MPN patients especially in triple negative cases. However, the appropriate use of these new diagnostic tools in a clinical setting can be challenging, due to diversity of available genetic targets but still limited evidence base [13].

The main objective of our study was to analyze whether the landscape of non-driver mutations differs among cohorts, but in order to make more specific correlation between their presence and clinical presentation, larger group of patients is needed.

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