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COPY NUMBER ALTERATIONS AND COPY-NEUTRAL LOSS OF HETEROZYGOSITY IN UKRAINIAN PATIENTS WITH PRIMARY MYELOFIBROSIS

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***Aim*: To examine frequencies and spectrum of genomic alterations in Ukrainian patients diagnosed with primary myelofibrosis (PMF). *Materials and Methods:* We enrolled 30 Ukrainian patients diagnosed with PMF who were previously tested for usual mutations in myeloproliferative neoplasms driver genes (*JAK2, MPL* and *CALR*). Genomic DNA samples were obtained from peripheral blood leukocytes of these patients. Copy number alterations and copy-neutral loss of heterozygosity (cnLOH) were assessed using a high-density CytoScan HD microarray platform. Statistical significance was evaluated by the Fisher exact test. *Results*: We identified frequent genomic alterations, but no significant difference in the rates of copy-number loss, copy-number gain, cnLOH, or multiple genomic alterations were found in the groups of PMF patients that were positive for one of the usual mutations in driver genes or negative for such mutations (33.3% and 55.6%, $p = 0.4181$, 19.0% and 11.1%, $p = 1.0000$, 61.9% and 44.4%, $p = 0.4434$, 33.3% and 55.6%, $p = 0.4181$, respectively). The most frequent alterations were cnLOH at 1p36-1p22, 9p24.3-9p13.3 and 11q12.3-11q25; copy number loss at 7q21-7q36.3 and 13q12.3-13q14.3. Copy number alterations and cnLOH commonly affected the *EZH2, LAMB4, CBL, CUX1, ATM, RB1* and *TP53* genes, in addition to *JAK2, MPL* and *CALR*. *Conclusion*: We demonstrated the spectrum of genomic alterations in the groups of the Ukrainian PMF patients with or without the usual mutations in the specific driver genes. We identified several potential genes, which may be involved in the myeloproliferative neoplasms development and their phenotype modification *(EZH2, LAMB4, CBL, CUX1, ATM, RB1* and *TP53)*.**

***Key Words*: primary myelofibrosis, copy number alterations, copy-neutral loss of heterozygosity, driver mutation.**

Primary myelofibrosis (PMF) is a clonal disorder of early hematopoietic stem cells manifesting as bone marrow fibrosis and pancytopenia and classified as BCR-ABL-negative myeloproliferative neoplasm (MPN) [1]. PMF patients are characterized by a severe disease course and worse prognosis, compared with patients of other MPN subtypes: polycythemia vera (PV) and essential thrombocythemia (ET). MPNs are driven by acquired somatic mutations of *JAK2*, *CALR* and *MPL* genes in more than 85% of cases [2].

Studies of chromosomal alterations in MPN patients revealed that they occurred more frequently in PMF patients (50%) compared with PV (15%) and ET (5%) patients. Some alterations were detected recurrently: deletions of 20q, 18q, 13q and 12p; trisomy of chromosomes 8 and 9; copy number gain at 1p, 9p, 17q; and various translocations [3, 4]. Chromosomal alterations in PMF patients, such as trisomy of chromosome 8, deletions of 7, 7q, 5, 5q, 12p, inversion of chromosome 3 are considered as an unfavorable karyotype and used for the risk stratification for these patients [5].

Improved methods for genomic alterations studies, from standard karyotyping with limited sensitivity to the high-resolution single-nucleotide polymorphism (SNP) arrays, allowed detection of short regions of copy number alterations and copy-neutral loss of heterozygosity (cnLOH). Thus, oncogenic microalterations can be studied, in particular, in MPN patients. This increases the search power for identification of potentially involved genes in MPN development (as 10–15% of MPN patients are negative for known mutations of the specific driver genes) [2, 5–7].

The aim of the study was to examine frequencies and spectrum of genomic alterations (copy number alterations and cnLOH) in Ukrainian PMF patients.

MATERIALS AND METHODS

30 Ukrainian PMF patients, previously tested for mutations in MPN driver genes (*JAK2, MPL* and *CALR*), were enrolled in the study. There were 13 *JAK2* V617F-positive, three *MPL* W515-positive and five positive for *CALR* gene mutations PMF patients. Nine PMF patients were negative for these mutations. Each patient signed an informed consent in accordance with the Declaration of Helsinki. The study was approved by the local Ethical Committee at the National Research Center for Radiation Medicine (Kyiv, Ukraine). DNA samples were obtained from peripheral blood leukocytes of PMF patients, using a Quiamp DNA extraction kit (Qiagen, Hilgen, Germany). Copy number alterations and cnLOH were assessed, using a High-density Affymetrix CytoScan HD oligo-SNP microarray platform (Affymetrix, Santa Clara, CA, US). Digested and labeled genomic DNA was hybridized to 2.67 million probes according to manufacturer's recommendations. The Chromosome Analysis Suite (ChAS) software version 3.1 (Affymetrix) was used to analyze the data. The genome assembly version GRCh37/hg19 was used as a reference. All genomic alterations were visually inspected and confirmed, and regions with poor quality

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Abbreviations used: cnLOH – copy-neutral loss of heterozygosity; ET – essential thrombocythemia; MPN – myeloproliferative neoplasm; PMF – primary myelofibrosis; PV – polycythemia vera; SNP – single-nucleotide polymorphism.

were excluded. The regions with at least 50 markers (over 200 kb (50 markers over 100 kb for leukemia regions) were considered for gains, 30 markers over 50 kb (15 markers over 20 kb for leukemia regions) — for losses, and a minimum length of 5 Mb (3 Mb for leukemia regions) — for cnLOH. An online catalog of human genes and genetic disorders Online Mendelian Inheritance in Man (OMIM) was used to identify leukemia-related genes within the altered regions. Statistical significance was evaluated by the Fishers exact test.

RESULTS AND DISCUSSION

Copy-number alterations and cnLOH were frequently found in Ukrainian PMF patients (Table, Fig. 1). There were 71.4% (15/21) of cases with genomic alterations among PMF patients positive for one of known driver gene *(JAK2, MPL* or *CALR*) mutations and 55.6% (5/9) of cases — among PMF patients negative for these mutations (p = 0.4311). However, here was no significant difference in the rates of copy-number loss, copy-number gain, cnLOH, or multiple genomic alterations, respectively, in these groups of PMF patients (33.3% and 55.6%, p = 0.4181; 19.0% and 11.1%, p = 1.0000; 61.9% and 44.4%, p = 0.4434; 33.3% and 55.6%, p = 0.4181). The most frequently altered regions were cnLOH at 1p36-1p22 (N = 6), 9p24.3-9p13.3 (N = 3) and 11q12.3-11q25 (N = 3); copy number loss at 7q21-7q36.3 (N = 5) and 13q12.3-13q14.3 (N = 2). Our findings are consistent with published data [3, 4, 7]. CnLOH at 9p, 1p and 19p duplicated the usual driver gene (*JAK2, MPL* or *CALR,* respectively) mutations in 33.3% (7/21) of cases among the subset of PMF patients that were positive for one of these mutations. The length of these altered regions varied from 13.7 to 93.6 Mb (Table, Fig. 2). A shorter genomic fragment (1 Mb) of cnLOH with duplicated *MPL* gene mutation was visually detected in a PMF patient (ID 842) and considered as leukemogenic.

In PMF patients, the most frequently affected genes due to copy number alterations and cnLOH in addition to *JAK2, MPL* and *CALR* were *EZH2, LAMB4, CBL, CUX1, ATM, RB1* and *TP53* genes (Fig. 3). Copy number losses of *EZH2* at 7q36.1 were detected in three PMF patients negative for usual driver gene mutations and in one *JAK2* V617F-positive PMF patient. Epigenetic regulator *EZH2* is a member of Polycomb Repressive Complex 2 which is involved in H3K27 trimethylation. Loss of function mutations and cytogenetic alterations of *EZH2* are frequently observed in MPN patients. Recent studies showed that *EZH2* alterations may be early events in leukemogenesis [8, 9].

Other studies demonstrated that *EZH2* loss can dramatically modify the myeloproliferative phenotype reducing survival in the presence of *JAK2* V617F muta-

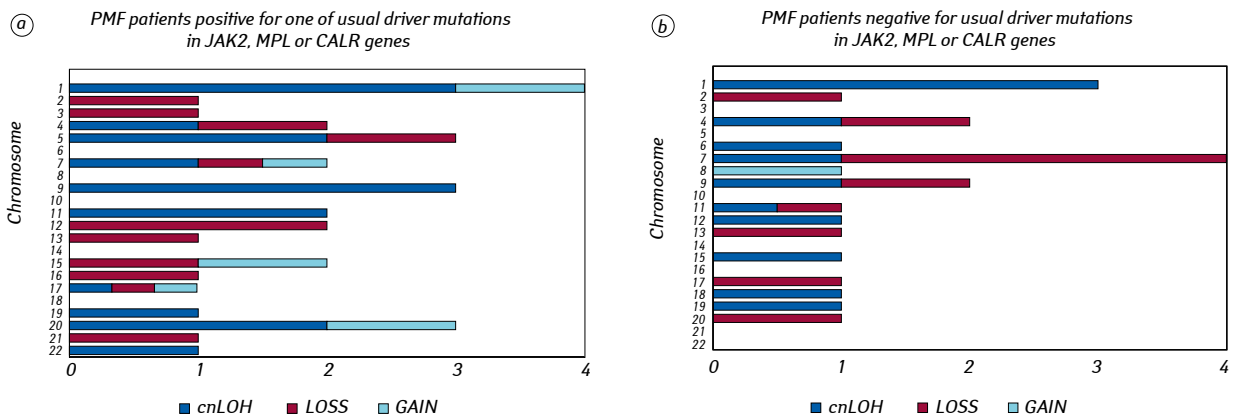

Fig. 1. Genomic alterations in Ukrainian PMF patients positive (*a*) and negative (*b*) for usual mutations in MPN driver genes (*JAK2, MPL* and *CALR)*. LOSS — copy number loss; GAIN — copy number gain

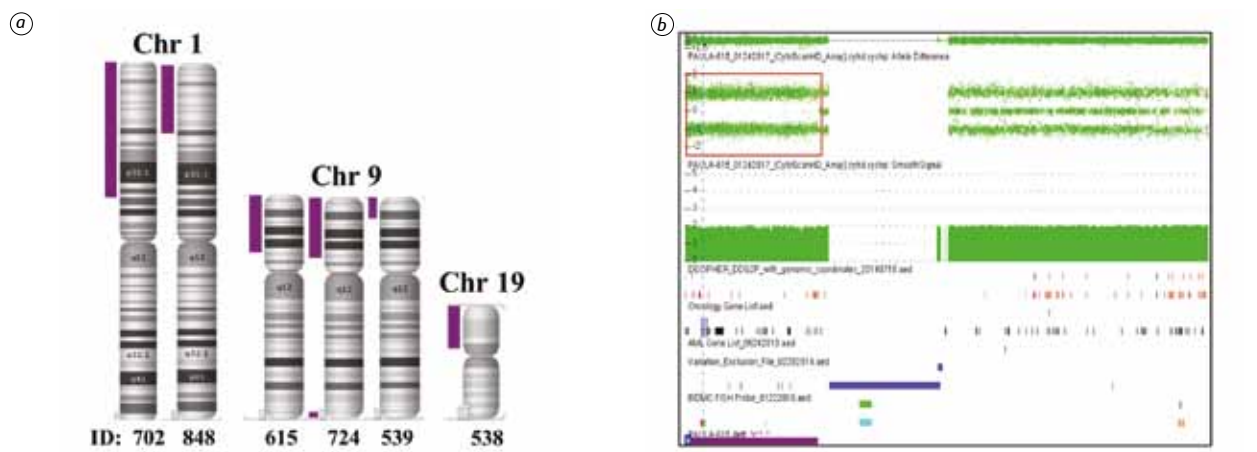

Fig. 2. The length of altered regions: *a* — cnLOH at chromosomes carrying usual mutations of known MPN driver genes; *b* — the region with cnLOH at 9p24.3p13.1 (35.9 Mb) in *JAK2* V617F-positive PMF patient (patient's ID 615)

Table. Copy-number alterations and cnLOH in PMF patients

Chr	Patients's ID	Driver gene mutated	Region altered	Type of alteration	Size, Mb	Genes within the regions	Altered DNA burden, %*
1	740	TN	1p36.32p36.22, 1q32.3q41	cnLOH	12.8	*RBP7*	100
	818	TN	1p36.33p34.3	cnLOH	34.8	*RAP1GAP, RBP7*	26
	615	*JAK2*	1q21.1q44	GAIN	103.8	*RBBP5, RBM34, TP53BP2*	85
	842	*MPL*	1p34	cnLOH	1	*MPL*	100
	848	*MPL*	1p36.33p33	cnLOH	48.2	*CSF3R, MPL, RAP1GAP, RBP7*	100
	983	*JAK2*	1p33p32.3	cnLOH	3		100
	702	TN	1p36.36p22.1	cnLOH	93.6	*CSF3R, JAK1, MPL, RAP1GAP, RBMXL1, RBP7, RPL5*	100
2	638	*JAK2*	2p22.2, 2p23.3, 2p25.3, 2q35	LOSS	3.3	*DNMT3A, TP53I3*	55–59
	702	TN	2q22.3q23.3	LOSS	3.9		54
3	852	*JAK2*	3p14.2	LOSS	0.3		100
4	740	TN	4q31.23q31.3	cnLOH	4.9	*FBXW7*	100
	1131	*JAK2*	4p16.3	LOSS	1.8		61
	926	TN	4q31.3	LOSS	0.364		100
	724	*JAK2*	4q22.3q23	cnLOH	3.5	*RAP1GDS1*	100
5	724	*JAK2*	5p13.2q11.2	cnLOH	9.7		100
	638	*JAK2*	5p, 5q multiple alterations	LOSS	134.4	*IRF1, RBM22, NPM1, DDX41*	24–88
	852	*JAK2*	5p15.2p14.3	cnLOH	6.1		100
6	740	TN	6q21q22.31	cnLOH	15.2		100
7	740	TN	7q21.3	cnLOH	4.3		100
	818	TN	7q22.3q36.2	LOSS	47.9	*BRAF, EZH2, LAMB4, POT1*	21
	846	TN	7q35q36.2	LOSS	6.8	*EZH2*	92
	638	*JAK2*	7q11.23, 7q11.23q21.11, 7q21.2q21.3	GAIN	12.9	*RBM48*	41–65
	638	*JAK2*	7q21.11q21.2, 7q21.3q36.3	LOSS	71.7	*BRAF, CUX1, EZH2, LAMB4, POT1, RBM33*	56, 57
	702	TN	7q21.3q31.31	LOSS	25.1	*CUX1, EZH2, LAMB4*	55
	842	*MPL*	7q36.1q36.2	cnLOH	3.3		100
8	846	TN	8p23.3q24.3	GAIN	146.4	*CSMD1, RAD21-AS1, RBM12B, RUNX1T1, TP53INP1*	76
9	615	*JAK2*	9p24.3p13.3	cnLOH	35.9	*FANCG, JAK2*	100
	818	TN	9q32q33.1	LOSS	5.5		20
	724	*JAK2*	9p24.3p13.1, 9q34.2q34.3	cnLOH	42.4	*FANCG, JAK2*	30, 100
	539	*JAK2*	9p24.3p23	cnLOH	13.7	*JAK2*	41
	702	TN	9q21.11q21.13	cnLOH	6.5		100
11	740	TN	11p15.5p15.4	LOSS	1.7		71
	740	TN	11q13.2q25	cnLOH	67.7	*ATM, CBL,*	100
	1131	*JAK2*	11q12.3q13.2, 11q13.3q25	cnLOH	72.1	*ATM, CBL, RBM7, TP53AIP1, RBM14, RBM4B*	26, 100
	1008	*JAK2*	11q23.3q24.1	cnLOH	6.4	*CBL*	100
12	615	*JAK2*	12p13.33p11.1	LOSS	34.7	*AEBP2, GPRC5A, KRAS*	93
	638	*JAK2*	12q multiple alterations	LOSS	12.5	*SH2B3, NCOR2*	47–61
	926	TN	12q21.2q21.31	cnLOH	4.1		100
13	702	TN	13q12.3q14.3	LOSS	19.8	*BRCA2, RB1*	80
	638	*JAK2*	13q14.13q14.3	LOSS	4.8	*RB1*	35
15	740	TN	15q23q24.2	cnLOH	7.7		100
	743	*CALR*	15q13.3	GAIN	0.433		91
	1014	*JAK2*	15q24.1q24.2	LOSS	1.4		46
16	743	*CALR*	16q23.1	LOSS	0.174		81
17	818	TN	17p13.3q21.2	LOSS	39.6	*PRPF8, RAP1GAP2, SUZ12, TP53*	19
	638	*JAK2*	17p13.1p11.2	cnLOH	9.1		100
	638	*JAK2*	17p13.3p13.1, 17q21.31q21.32, 17q21.33	LOSS	13.3	*PRPF8, RAP1GAP2, TP53*	50–55
	638	*JAK2*	17q23.2q25.3	GAIN	22.5	*RBFOX3, SRSF2*	54
18	702	TN	18q12.2q21.1	cnLOH	12.4	*SETBP1*	100
19	538	*CALR*	19p13.3p12	cnLOH	22.9	*CALR, CALR3, ELANE, JAK3, ZSWIM4*	100
	740	TN	19q12q13.12	cnLOH	3.5	*CEBPA*	100
20	904	*CALR*	20p13	GAIN	0.226		81
	842	*MPL*	20p13p12.3	cnLOH	7	*RAD21L1, RBCK1*	100
	702	TN	20q11.21q13.13	LOSS	18.8	*ASXL1, RBL1, RBM12, RBM39, RBPJL, TP53INP2, TP53RK*	80
	743	*CALR*	20q13.13q13.33	cnLOH	15.7	*CBLN4, CTCFL, DIDO1, GNAS, RTEL1, TP53RK*	100
21	724	*JAK2*	21q11.2	LOSS	0.335		53
22	703	*JAK2*	22q12.1q12.3	cnLOH	4.4		100

Note: Chr – chromosome; GAIN – copy-number gain; LOSS – copy-number loss; Mb – megabase.
*Percentage of DNA with identified cnLOH, GAIN or LOSS among studied DNA sample.

tion. During disease initiation stage, the cooperation between *EZH2* alterations and *JAK2* V617F mutations increases the ability of *JAK2* V617F-positive stem cells to self-renewal [10]. Interestingly, the mentioned *JAK2* V617-positive PMF patient (ID 638) with coexisting *EZH2* loss at 7q36.1 had multiple chromosome alterations indicating genomic instability potentially caused by this harmful initial combination (Fig. 4). This patient had additional copy number losses at 2p (involving epigenetic regulator *DNMT3A* and *TP53I3* gene which cooperates with p53 in cell death control); 5p and 5q; 12q (involving *SH2B3* gene which assists in JAK2-signaling regulation); 13q and 17q (involving well-studied across different malignancies *RB1* and *TP53* genes, respectively). Altered DNA burden for these regions with copy number losses ranged from 24 to 88%, but most of them were closed to 40–50%, suggesting their relation to the same leukemogenic cell clone. Proto-oncogene *CBL* encodes E3 ubiquitin ligase which negatively regulated JAK2-signaling due to JAK2 molecules ubiquitination and degradation. Even though, there is no evidence confirming ability of *CBL* gene mutations to drive disease, it was shown that they increase cell proliferation due to hypersensitivity to cytokines [11]. In the study we observed two *JAK2* V617-positive PMF cases with *CBL* homozygous loss, indicating that impaired ubiquitination of signaling molecules might

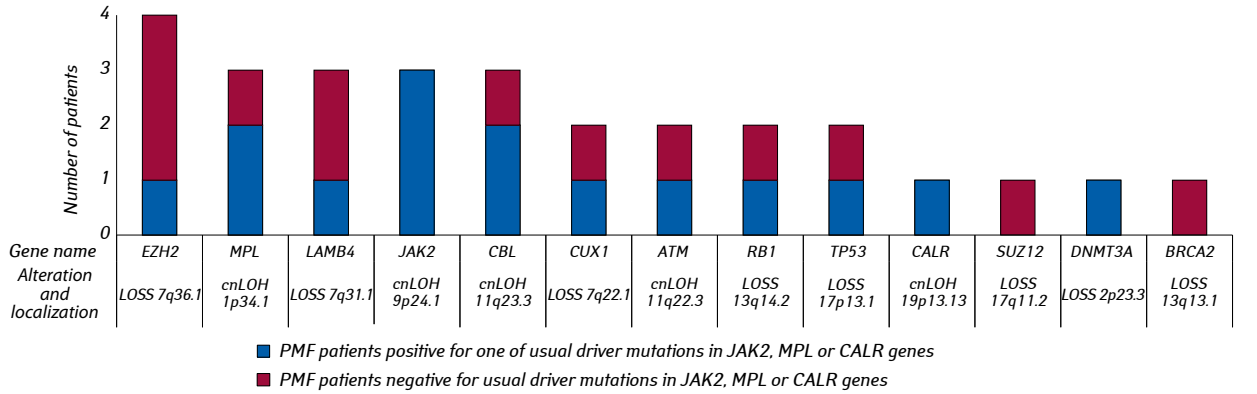

Fig. 3. Frequencies of affected genes with cnLOH and LOSS in Ukrainian PMF patients positive and negative for usual mutations in MPN driver genes (*JAK2, MPL* and *CALR)*. LOSS — copy number loss; GAIN — copy number gain

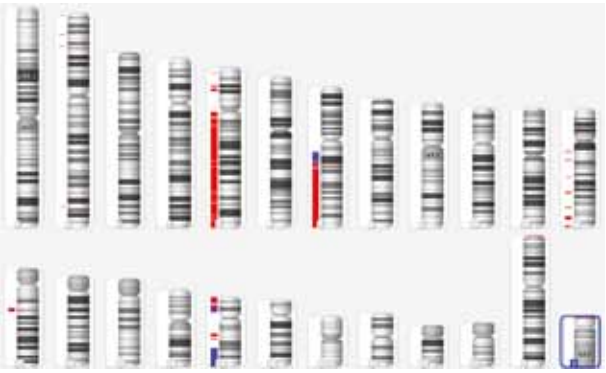

Fig. 4. Multiple genomic alterations (patient's ID 638)

give advantages to myeloproliferation. The *LAMB4* gene variants were reported in studies on myeloid neoplasms previously, but their biological function remains unknown in MPN [12]. cnLOH of DNA-damage response *ATM* gene and copy number loss of *TP53* suggest their contribution to the disease evolution due to loss of DNA repair function.

Another interesting gene was *POT1* which was deleted in two cases of PMF patients who harbored multiple genomic alterations (Table 1). This gene encodes a nuclear protein involved in the telomere maintenance, regulating its lengths, protecting chromosome ends from illegitimate recombination and abnormal chromosome segregation. Significantly shortened telomeres, activation of telomerase, and altered expression of telomere-associated proteins are common features of various hematologic malignancies [13].

Recent study reported the use of an oligonucleotide that targets the RNA template of a human telomerase reverse transcriptase and inhibits its activity in some PMF patients [14]. Thus, the role of impaired POT1 protein is most likely implemented in cooperation with other damaging genomic alterations.

CONCLUSIONS

The study demonstrates the spectrum of genomic alterations in Ukrainian PMF patients that are positive and negative for usual mutations in MPN driver genes *(JAK2, MPL* or *CALR)*. We have identified several genes potentially involved in the disease development and phenotype modification *(EZH2, LAMB4, CBL, CUX1, ATM, RB1* and *TP53)*.

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