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SOMATIC GENE VARIANTS IN UNRESECTABLE CUTANEOUS MELANOMA CELLS AND DETECTION OF PHARMACOGENOMIC MARKERS: INFLUENCE ON STRATEGY OF EFFECTIVE CANCER TREATMENT

The study **aimed** to identify the clinically relevant gene variants in unresectable cutaneous melanoma samples from Ukrainian patients using NGS technology and to investigate some pharmacogenomic markers useful for the development of cancer treatment strategies. **Materials and Methods.** 30 samples of unresectable cutaneous melanomas of various localizations and differentiation grades were analyzed. The Ion Torrent NGS technology of targeted gene sequencing (Custom AmpliSeq™ Cancer Hotspot and Pharmacogenomic panels) was applied to identify the genetic alterations, which were classified using Franklin by Gennox database and custom pharmacogenomic Ion Reporter Software pipeline. **Results.** A total of 148 different gene alterations were identified in 40 genes (SNVs, MNVs, INDELs) by the Cancer Hotspot Panel, revealing the mutation patterns consistent with the international data. However, notable discrepancies exist, such as a high *KRAS* mutation rate (29.3%), predominantly in stage III tumors, suggesting their role in tumor aggressiveness and progression. We identified the frequent *TP53* and *BRAF* mutations, with *BRAF* V600E being the most common, and observed a higher prevalence of *BRAF* mutations in females. *TP53* mutations were prevalent (59.3%) and varied with age and sex, though their prognostic significance requires further validation. A second key novel finding was the detection of *FLT3* mutations in 22.2% of the samples, with a significantly higher prevalence in stage IV disease, suggesting a potential role of *FLT3* in melanoma progression and warranting further investigation as a prognostic biomarker and a potential target for the existing *FLT3* inhibitors. Ultimately, our pilot analysis of pharmacogenomic markers underscored their potential clinical utility in choosing personalized treatment decisions. For instance, the identification of a patient with the rs35599367 (G/A) risk allele for adverse drug reactions underscores the value of using pharmacogenetic data to make a correct selection between approved therapies. **Conclusions.** Our study presents the first comprehensive analysis of somatic mutations and pharmacogenomic markers in unresectable cutaneous melanoma tissue samples from Ukrainian patients. We found that while common somatic variants generally align with global trends, the mutational landscape in this cohort presents several unique features: a high *KRAS* mutation rate and its apparent stage-specific prevalence, and a high *FLT3* mutation rate, predominantly in stage IV tumors. Further validation on a larger number of samples, as well as more exhaustive analysis employing alternative methods, is necessary to substantiate these findings and to facilitate more effective melanoma treatment.

Keywords: NGS, clinical relevance, gene variants, melanoma, pharmacogenomic markers, effective cancer treatment.

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Melanomas are aggressive malignant neoplasms with high metastatic potential [1]. According to the National Cancer Registry of Ukraine, in 2022, skin melanoma was among the most prevalent oncological pathologies detected in both men and women aged 18—29 and 30—44 years [2]. The mortality rate due to this pathology was approximately 28% of the total number of cases detected during the specified period. Approximately three-quarters of melanoma patients received surgical treatment, while only a quarter of patients received combined and comprehensive treatment [2]. Radiation therapy and chemotherapy emerged as the predominant combined modality. In recent years, there has been significant progress in the field of oncology with the development of novel treatment methods, including targeted therapy and immunotherapy. However, these treatment modalities have limited efficacy due to the adverse effects and drug resistance [3].

The molecular heterogeneity of melanoma is a primary factor contributing to therapeutic ineffectiveness. It is well established that melanomas exhibit a broad spectrum of somatic mutations. The most prevalent mutations have been identified in genes such as *BRAF, NRAS, KIT, NF1,* and *TP53*, among others. This finding is corroborated by the database evidence, particularly that from the COSMIC (https://cancer.sanger.ac.uk/cosmic/). Identifying a specific spectrum of genetic alterations in melanoma cells allows for the categorization of biologically distinct subtypes that respond differently to treatment. The most well-known and common *BRAF* mutations in melanoma (especially V600E) are associated with sensitivity to BRAF/MEK inhibitors [4]. However, some patients with these mutations do not respond to therapy or develop resistance [5]. In such cases, pharmacogenomic markers, defined as inherent genetic variations in patients, may hold significant implications. These polymorphisms, which occur in genes associated with metabolism, transporters, immune regulation, and other processes, can influence the effectiveness of targeted and immunotherapy treatments, as well as the toxicity and tolerability of pharmaceuticals [6, 7].

Although identifying clinically significant genetic features is standard practice for diagnosing and predicting treatment efficacy in patients with cancer, particularly melanoma, pharmacogenetic factors are rarely considered in current treatment protocols [8]. The study aimed to identify clinically relevant gene variants in unresectable cutaneous melanoma samples from Ukrainian patients using NGS technology and to investigate some pharmacogenomic markers useful for the development of cancer treatment strategies.

Materials and Methods

Patient samples. The present study examined a total of 30 Ukrainian melanoma patients, age range from 28 to 76 years, males and females in approximately equal proportion (Table 1). The patients

Table 1. **Clinical and pathological characteristics of melanoma cases**

Sample No.	Age	Gender	Breslow (mm)	Stage (by Clark)
1	50	F	2.5	III
2	29	F	1.5	III
3	62	F	1.3	III
4	62	M	5.2	IV
5	33	M	1.1	IV
6	44	M	0.67	III
7	33	F	0.7	III
8	43	F	3	IV
9	32	M	6	III
10	57	F	2.2	III
11	66	F	0.3	III
12	42	F	6	III
14	43	M	0.7	III
16	49	M	3.75	IV
17	63	F	0.9	III
20	36	F	1.5	III
22	56	F	2.7	IV
24	64	M	4.5	IV
25	37	F	1	III
26	76	M	3	IV
27	41	F	1.3	III
28	55	M	0.6	III
30	50	M	2.3	III
32	26	F	2.25	IV
34	28	M	1.2	III
35	71	M	3.75	III
36	32	F	5	IV
41	49	M	6	IV
44	37	M	1	III
46	38	F	4.3	IV

presented with unresectable cutaneous melanomas of varying locations and differentiation grades, and stages III to IV of the disease by Clark.

The Helsinki Declaration was followed in conducting human sampling. The ethical approval was obtained from the Ethics Commission of the National Cancer Institute of Ukraine and the Ethics Committee of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (protocol number: 255/8, March 12, 2024).

Melanoma sample genotyping by targeted Ion Torrent NGS. Library preparation and sequencing. Genomic DNA (gDNA) from biopsy materials of cancer tissue samples was extracted using the GeneJET FFPE DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). 15 ng of gDNA were used for library preparation according to the manufacturer's protocol. DNA was amplified using Custom AmpliSeq™ Hotspot Panel Primer Pool and Custom AmpliSeq™ Pharmacogenomic Panel Primer Pools. PCR pools for each sample were combined and subjected to primer digestion with FuPa reagent (Thermo Fisher Scientific, Inc., USA). The libraries were indexed using the Ion Xpress Barcode Adapter Kit. The adapter-ligated library (amplicons) was purified using MagMAX Pure Bind beads (Thermo Fisher Scientific, Inc., USA). Quantification of the final libraries was performed using an Ion Library TaqMan™ Quantitation Kit on the QuantStudio™ 5 Real-Time PCR System Kit (Thermo Fisher Scientific, Inc., USA), following the manufacturer's protocols. The obtained libraries were diluted to ~70 pM and then pooled together for further processing. The clonal amplification of the barcoded DNA library on Ion Sphere Particles (ISP) was performed on the Ion Chef System using the Ion 540 Kit-Chef, according to the manufacturer's instructions (Thermo Fisher Scientific, Inc., USA). The template-positive ISPs were then loaded onto an Ion 540 Chip for sequencing using an Ion GeneStudio S5 Plus Sequencer, following the manufacturer's protocol (Thermo Fisher Scientific, Inc., USA).

Ion torrent bioinformatics analysis, variants filtering, assessment of pathogenicity (clinical significance), pharmacogenomics markers, and clinical treatment protocols. Primary sequence analysis was performed as described by us previously [9]. The pathogenicity (clinical significance) of genetic mutations was evaluated according to the ACMG-AMP and Oncogenic classifications [10—12]. Detection of pharmacogenomics markers was carried out using a custom-designed Ion Reporter Software pipeline (Thermo Fisher Scientific, Inc., USA). The data processed through the Ion Reporter pharmacogenomics pipeline were then classified and filtered using the ClinPGx knowledgebase (https://www.clinpgx.org/). The data were statistically processed using OpenEpi software and Fisher's test, as well as odds ratio (OR) calculation.

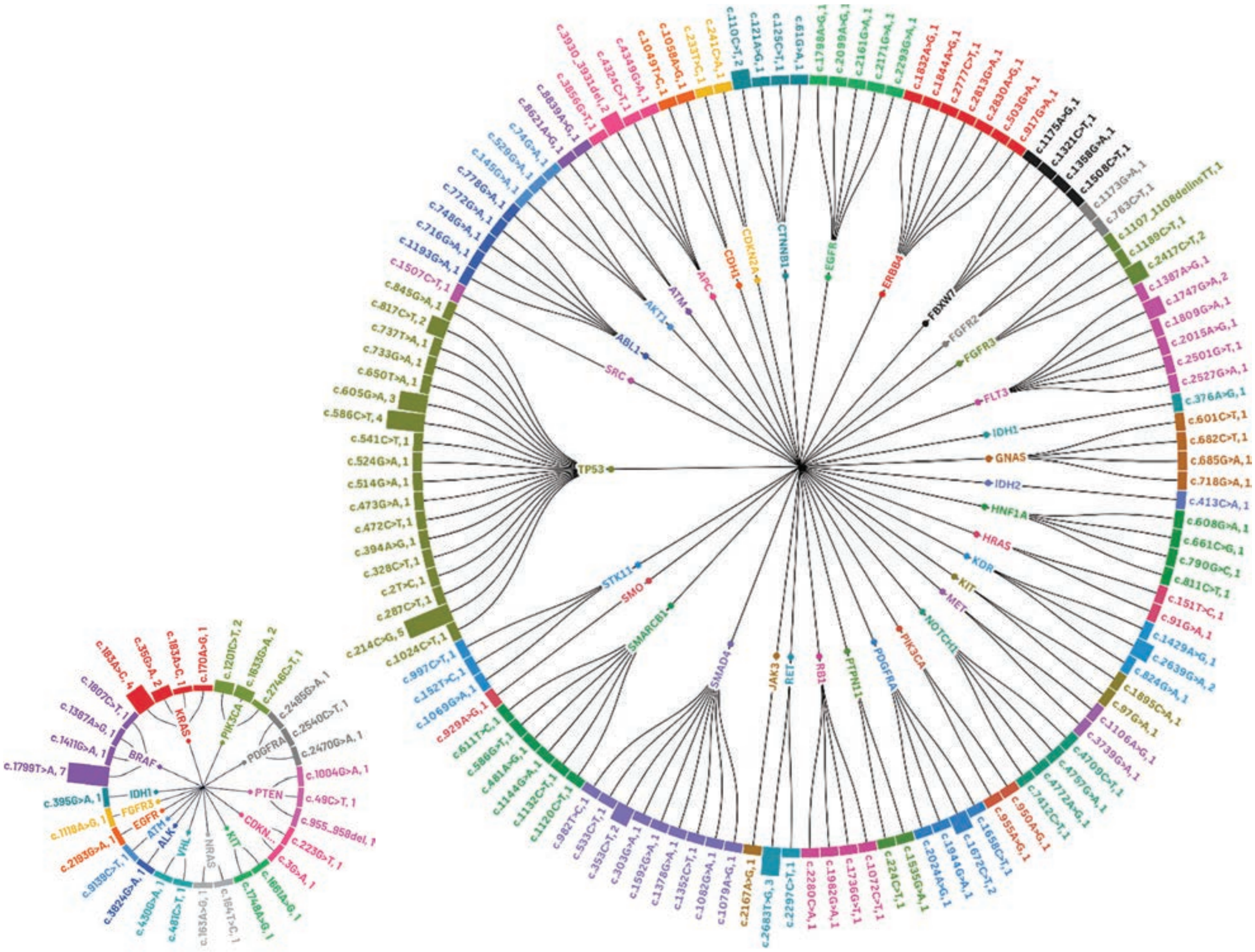
The analysis of melanoma treatment strategies was conducted taking into account the Ukrainian standards and approved treatment protocols by the State Expert Center of the Ministry of Health of Ukraine (GC 2023-1064, 09.06.2023, https://www.dec.gov.ua) and methods approved by the FDA and EMA.

Results

Clinically relevant gene variants in melanoma samples determined by the Custom Ion AmpliSeq™ Hotspot panel. A Custom Ion AmpliSeq™ Hotspot panel for the mutation screening of 51 cancer-related genes was designed using Ion AmpliSeq™ Designer (https://www.ampliseq.com). The design included hotspot regions of 51 oncogenes and tumor suppressor genes, all of which had been previously associated with cancer. Overall, it represented approximately 23 kb of the target DNA sequence and 224 amplicons in 1 pool of primers for multiplex PCR. Using targeted sequencing with the Custom AmpliSeq™ Hotspot Panel and subsequent processing using Franklin by Genoox of 30 melanoma samples of Ukrainian patients, 177 genetic variant alterations were classified by Franklin as Tier 1—3 variants in 27 cancer samples. The Franklin by Genoox platform was used primarily for variant filtering, analysis, and identification of variants for reporting. According to the AMP/ASCO/CAP Tier Classification for Somatic Variants, the detected variants fell into three tiers: Tier I (variants of strong clinical significance) — the largest group, Tier II (variants of potential clinical significance), and Tier III (variants of unknown clinical significance) (Fig. 1).

A total of 148 different unique gene variants were identified in 40 genes (Fig. 2). Two variants were classified as Tier 1: *BRAF* c.1799T>A (V600E), which was detected in 7 samples, and *NRAS* c.164T>C, detected in 1 sample (Fig. 1, *a*). The other 28 variants were classified as Tier 2 and 118 as Tier 3 (Fig. 1, *a, b*).

The largest number of mutations was found in the *TP53* gene — 18 different variants in 16 cases. 9, 7,



a *b*

Fig. 1. (*a*) Distribution of the number of identified Tier 1—2 altered genetic variants; (*b*) Tier 3 altered genetic variants by genes

and 6 different variants were detected in the *SMAD4, PDGFRA,* and *ERBB4* genes, respectively. Five variants were detected in each of the *ABL1, EGFR, FLT3, PIK3CA,* and *SMARCB1* genes. The other genes had between 1 and 4 detected variants (Fig. 1 and 2).

The average number of detected variants per patient was 6.56. The maximum number of variants identified in a single sample was 7. These detected Tier1-Tier3 genetic alterations belonged to single-nucleotide variants (SNVs) or insertions and deletions (INDELs).

Three tumor-associated genes (*BRAF, KRAS,* and *PIK3CA*) showed the presence of clinically associated mutations in several melanoma samples at a specific gene location (Fig. 1, *a*). Gene variants of gene *BRAF* c.1799T>A and *KRAS* c.183A>C were detected in 7 and 5 melanoma samples, respectively. The following 3 mutation variants, *KRAS* c.35G>A, *PIK3CA* c.1201C>T, and c.1633G>A, were detected twice each.

The most clinically significant mutations in Tier 1—2 groups were found in 22 melanoma samples. 39 cases of Tier 1—2 gene variants were detected. They were represented by 30 gene variants in 14 genes (Fig. 1, *a*). Among them, there were 10 Pathogenic and 11 Likely pathogenic mutations according to the ACMG classification. Oncogenic classification showed the presence of 3 most oncogenic mutations, *PTEN* c.49C>T, *PTEN* c.955_958del, and *ATM* c.9139C>T, with the highest Oncogenicity scores of 15, 13, and 10, respectively. In the ClinVar database, 6 of the identified mutations are listed as oncogenic, namely *BRAF* c.1799T>A, *IDH1* c.395G>A, *KRAS* c.183A>C, *KRAS* c.35G>A, *PIK3CA* c.1633G>A, and *PTEN* c.955_958del.

Comparison of clinicopathological characteristics of nonresectable cutaneous melanoma patients based on the presence of mutations in the altered genes. Given the variants of the *BRAF* gene identified in 10 unresectable cutaneous melanoma pa-

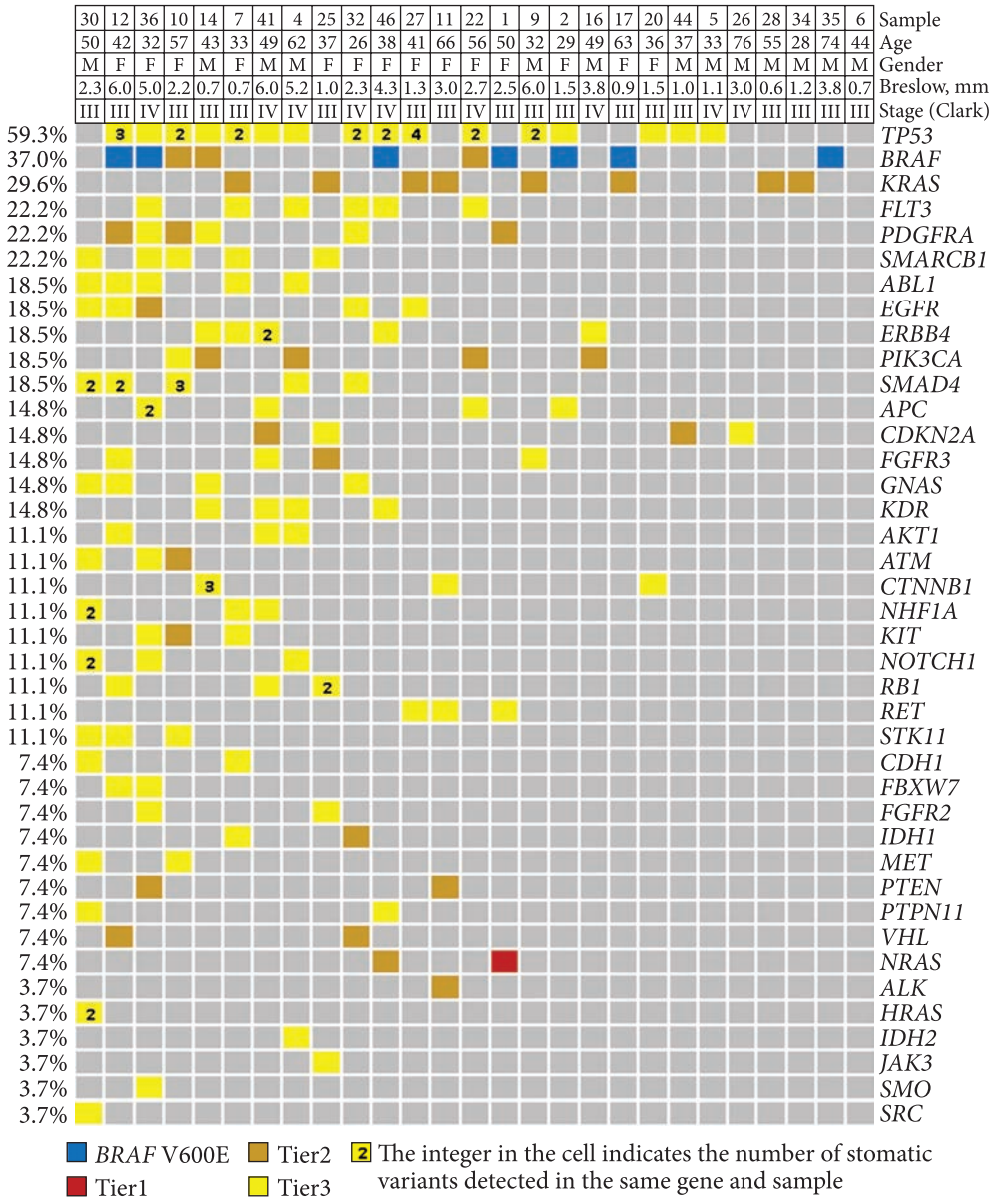


Fig. 2. Oncoplot based on targeted hotspot region sequencing showing the altered genes (rows) for 27 unresectable cutaneous melanomas (columns)

tients (a frequency of 37%), we were able to analyze their association with the clinicopathological features. A comparative analysis revealed that somatic mutations in the *BRAF* gene are statistically significantly more prevalent in unresectable cutaneous melanoma samples from women than from men ($p = 0.034$). In the present study, *BRAF* mutations were detected in the samples from 8 out of 14 females and 2 out of 13 males (Fig. 2). It is noteworthy that there was no statistically reliable difference ($p = 0.052$) in the prevalence of *BRAF* V600E mutation itself in the samples of females (6) and males (1).

In the presence of *TP53* variants, no statistically significant differences were observed between the genders (Fig. 2). However, it is noteworthy that the carriers of two or more *TP53* variants in unresectable cutaneous melanoma tissue were significantly ($p = 0.043$) more common among females (7 cases) than males (1 case). Furthermore, we found that somatic variants in the *TP53* gene are significantly ($p = 0.049$) more prevalent in the melanomas from patients under the age of 50 compared to the samples from older patients (Fig. 2).

We also found a significant association between the presence of the somatic variants in the *KRAS* ($p = 0.039$) and *FLT3* ($p = 0.016$) genes and the disease stage by Clark. A comparative analysis revealed that somatic mutations in the *FLT3* gene

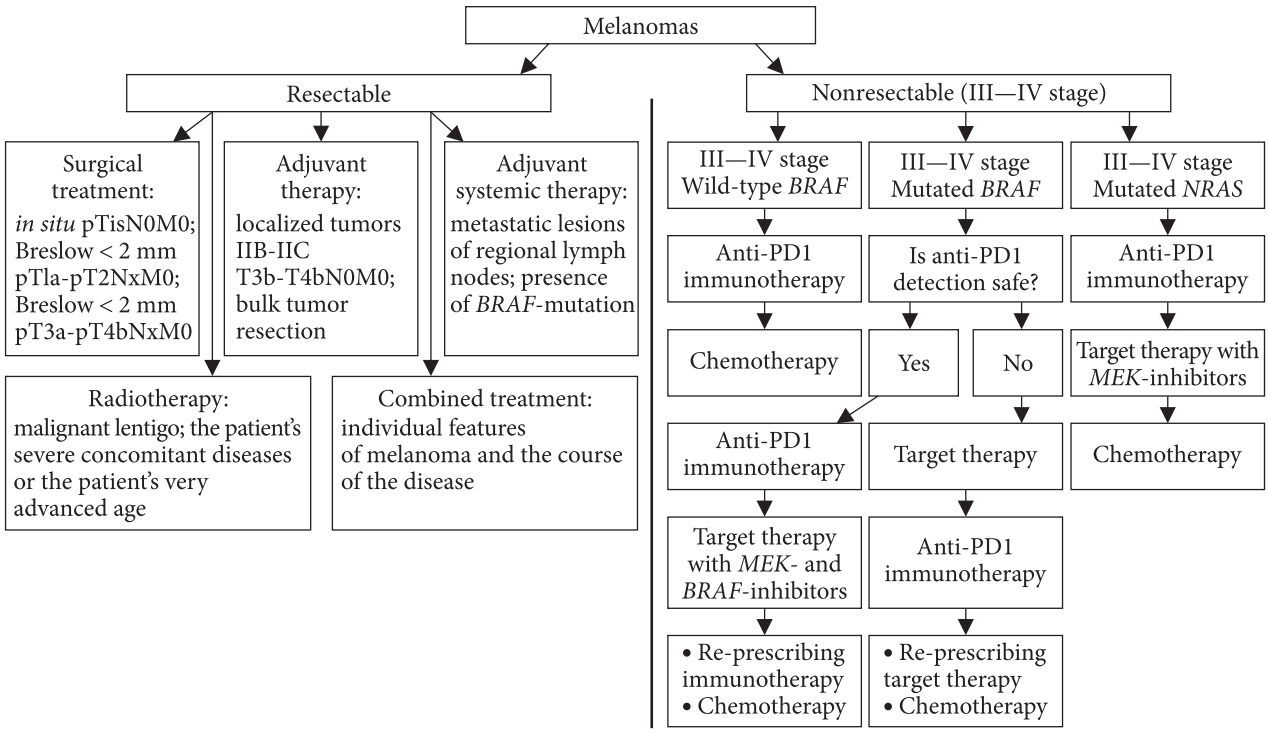


Fig. 3. A brief outline of the melanoma treatment protocol in Ukraine

were statistically significantly more prevalent in the stage IV melanoma samples compared to the stage III samples. All variants identified in this study within the *KRAS* gene were detected in 8 samples of stage III tumors, whereas the *KRAS* variants were not detected in any stage IV case (Fig. 2).

The most clinically relevant variants for melanoma are *BRAF* gene mutations, especially at the position 600 — *BRAF* V600E (c.1799T>A). It is a Tier 1 and Likely Oncogenic mutation. When this mutation is present in melanoma cells, the FDA approves certain targeted drugs for treatment. Another mutation belonging to Tier 1 was found in the studied tumor samples: this is *NRAS* c.164T>C. There are no FDA-approved melanoma drugs for this mutation, but there are C- and D-level drugs being investigated for *NRAS* gene alterations in melanomas and FDA-approved drugs for other cancer types. In addition, some genes, including *BRAF, NRAS, ATM, CDKN2A, KIT, KRAS*, and *PTEN*, have therapeutic significance for melanoma with the A-D approval levels. Only four of the identified gene variants have FDA-approved drugs for other types of cancer and are of therapeutic significance, namely *BRAF* c.1799T>A, *IDH1* c.395G>A, *KRAS* c.183A>C, and *PIK3CA* c.1633G>A.

Clinically approved Ukrainian protocol of melanoma treatment and types of treatment according to pathohistological characteristics and genetic features of melanoma samples. In Ukraine, the protocol for the treatment of melanoma approved by the Ministry of Health of Ukraine on June 9, 2023, number GS 2023-1064 is currently valid. A brief outline of the protocol is presented in Fig. 3.

This study examined samples of stage III and IV melanomas considered unresectable. Table 2 presents the selection of 1—3 lines of therapy according to the adopted Ukrainian protocol, clinicopathological characteristics of the samples, and their genetic features, in particular *BRAF* status. As seen, the samples belong to 3 treatment groups: stage III and IV wild-type *BRAF*, stage III mutated *BRAF*, and stage IV mutated *BRAF*. In the stage III and IV groups with mutated *BRAF*, there is a choice depending on the safety of first-line therapy. According to the protocol approved in Ukraine, PD-1 blockers are the standard first-line therapy, regardless of the *BRAF* or *NRAS* status. However, there are cases when immunotherapy in the first line is not safe and requires the appointment of targeted therapy: high levels of lactate dehydrogenase, high tumor burden, and aggressive course of the disease, which will leave insufficient time for the development of an effective antitumor immune response.

For tumors with wild-type *BRAF* stage 3 and 4, second-line therapy is chemotherapy with some drug options or their combination: Dacarbazine, Te-

Table 2. **Treatment types according to pathohistological characteristics and genetic features of melanoma samples**

BRAF status (WT/Mut)	Stage	First-line therapy	Second-line therapy	Third-line therapy	Sample number
WT	III	Pembrolizumab	Dacarbazine, Temozolomide, Carboplatin and Paclitaxel		3, 6, 7, 9, 11, 20, 25, 27, 28, 30, 34, 44
	IV				4, 5, 16, 26, 32, 41
Mut	III	Safe IT: Pembrolizumab / or TT: Dabrafenib and Trametinib	After IT: Dabrafenib and Trametinib/ After TT: Pembrolizumab	After TT: Re IT or ChT/ After IT: Re TT or ChT	1, 2, 10, 12, 14, 17, 35
Mut	IV	Safe IT: Pembrolizumab / or Dabrafenib and Trametinib or Vemurafenib and Cobimetinib	After IT: Dabrafenib and Trametinib or Vemurafenib and Cobimetinib / After TT: Pembrolizumab	After TT: Re IT or ChT/ After IT: Re TT or ChT	22, 36, 46

Notes: IT— immunotherapy (Pembrolizumab); TT — target therapy (Dabrafenib and Trametinib or Vemurafenib and Cobimetinib); ChT— chemotherapy (Dacarbazine, Temozolomide, Carboplatin, and Paclitaxel).

mozolomide, Carboplatin, and Paclitaxel, while for the tumors with mutated *BRAF*, second-line therapy depends on the choice for first-line therapy (immunotherapy or targeted therapy). Therefore, after immunotherapy, it is necessary to choose targeted therapy, that is, Dabrafenib and Trametinib or Vemurafenib and Cobimetinib. It depends on the stage of melanoma. If targeted therapy was chosen for first-line therapy, then the second-line therapy protocol recommends choosing immunotherapy (Pembrolizumab). Third-line therapy is intended only for tumors of stage 3—4 with mutated *BRAF* or *NRAS*.

Pharmacogenomic markers in patients with melanoma, determined by Custom* AmpliSeq™ *Pharmacogenomic panel. A Custom AmpliSeq™ Pharmacogenomic panel for the screening of 204 ClinPGx Level 1-2 of evidence cancer drug-related markers in 121 genes was designed using the Ion AmpliSeq™ Designer (https://www.ampliseq.com). The design included loci of 204 Level 1—2 of evidence pharmacogenomic markers, which have been previously associated with 30 most commonly used cancer drugs (https://www.clinpgx.org/page/clinAnnLevels). Overall, it represented approximately 39 kb of the target DNA sequence and 208 amplicons divided into 2 pools of primers for the multiplex PCR.

Level 1A summary annotations describe variant-drug combinations that have variant-specific prescribing guidance; Level 1B describes variant-drug combinations that have a high level of evidence supporting the association but no variant-specific prescribing guidance; Levels 2A and B describe variant-drug combinations with a moderate level of evidence supporting the association; Level 3 describes variant-drug combinations with a low level of evidence supporting the association, and Level 4 does not support an association between the variant and the drug phenotype [13]. ClinPGx annotates peer-reviewed published literature that reports associations between genetic variants and drug response. The associations between a variant and a drug phenotype from a single publication are recorded as Variant Annotations (VA) with no level of evidence.

As shown in Table 2, the Ukrainian protocol for unresectable melanoma treatment involves the use of 9 different drugs in various cases. However, it should be noted that the pharmacogenetic markers at the evidence Level 1 or Level 2 for these drugs have not yet been described. For the drugs Pembrolizumab, Dabrafenib, Trametinib, and Cobimetinib, the Level 3 and Level 4 markers are also missing. However, our panel included and let us analyze in this study Level 3 markers and ClinPGx Variant Annotations for Dacarbazine, Temozolomide, Carboplatin, Paclitaxel, and Vemurafenib.

Our panel includes only one marker CYP1A2*3, which is associated with Dacarbazine [14]. Furthermore, we did not identify CYP1A2*3, associated with decreased clearance of Dacarbazine in any of the studied samples. In addition, in this study, we were able to genotype samples for both markers, rs1045642 and rs35599367, associated with toxic side effects of the targeted therapy drug Vemurafenib [15]. As shown in Table 3, 1 of 3 patients who could have been prescribed therapy with Vemurafenib in combination with Cobimetinib turned out to be a carrier of rs35599367 (G/A), associated with increased risk for grade ≥3 nausea,

grade 1–4 hyperbilirubinemia, and cutaneous squamous cell carcinoma.

Furthermore, the present study enabled the genotyping of 17 patients with stage III and IV wild-type *BRAF* tumors who could have been prescribed chemotherapy with some drug options or their combination: Dacarbazine, Temozolomide, Carboplatin, and Paclitaxel (Table 4).

As shown in Table 4, we were able to genotype samples for 5 ClinPGx Variant Annotations markers and 28 evidence Level 3 pharmacogenetic markers associated with Paclitaxel, 7 ClinPGx Variant Annotations markers and 16 Level 3 markers associated with Carboplatin, as well as 1 ClinPGx Variant Annotations marker and 2 Level 3 markers associated with Temozolomide.

Discussion

To our knowledge, this is the first report describing the distribution of somatic mutations in unresectable melanomas of Ukrainian patients and interpreting them using the current international classification systems, as well as the first attempt to analyze the pharmacogenomic markers associated with the drugs that are approved by the Ukrainian protocol of melanoma treatment.

A thorough review of the scientific literature allowed us to conclude that our data on the most common somatic variants are generally consistent with those reported in other studies [16—19]. Notwithstanding, some discrepancies were observed. For instance, the detection of somatic *KRAS* mutations in 29.3% of unresectable cutaneous melanoma samples in our cohort is a striking finding, especially when contextualized with the existing literature. Previous studies have consistently reported the *KRAS* mutation rates in cutaneous melanoma to be low, typically around 5% [17, 18]. Higher *KRAS* mutation rates have been observed in mucosal and female genital tract melanomas (up to 32%), but not in the cutaneous subtypes [20—22]. The elevated frequency in the studied unresectable cases may reflect the selection for more aggressive or therapy-resistant tumor clones, since the *KRAS* mutations are associated with poor prognosis and resistance to targeted therapies in melanoma and other cancers [23, 24]. The most common *KRAS* mutation detected was c.183A>C (Q61H) in 5 samples, followed by c.35G>A (G12D) in 2 samples, and c.170A>G (D57G) in 1 sample. The predominance of Q61H in our cohort aligns with the findings that this allele is more frequent in melanoma compared to other cancers, where codon 12 mutations (like G12D) are more common [21]. D57G is less common but has been reported as an activating mutation [21].

Strikingly, the *KRAS* mutations were present in 8 of 18 stage III samples but absent in all 9 stage IV samples, suggesting a stage-specific pattern of *KRAS* involvement in melanoma progression. While *RAS* mutations (including *NRAS* and *KRAS*) have been associated with tumor progression and thicker, more advanced primary melanomas [24], our data suggest that the *KRAS*-mutant clones may be selected against or outcompeted as melanoma progresses to distant metastasis. This is consistent with findings that *KRAS* mutations are often mutually exclusive with other driver mutations and may define a distinct biological subset [25]. Additionally, studies of brain metastases have shown that the *KRAS* mutations are clonal and concordant with the extracranial disease, but their frequency remains low in widespread metastatic settings [26]. Alternatively, the microenvironmental or therapeutic pressures in stage IV disease may favor other oncogenic pathways.

As shown by our findings, 18 variants of the *TP53* gene were identified in the tumor tissue from 59.3% of patients, making it the most frequently mutated gene. However, as expected, the most common single nucleotide variant in melanoma samples in our cohort is V600E (c.1799T>A) in the *BRAF* gene, which was found in 7 cases (25.9%). The somatic

Table 3. **Genotyping results for patients with stage IV *BRAF*-mutated tumors**

Sample number	Age	Gender	Breslow	Genotype by ABCB1 rs1045642	Genotype by CYP3A4 rs35599367
22	56	F	2.7	A/G	G/A
36	32	F	5	G/G	G/G
46	38	F	4.3	A/G	G/G

Table 4. **Genotyping results for patients with stage III and IV wild-type *BRAF* tumors**

Level of evidence			4	5	6	7	9	11	16	20	25	26	27	28	30	32	34	41	44	← Sample	Gene	Phenotype Categories	Related publication PMID
Pacli-taxel	Carbo-platin	Temo-zolomide	62	33	44	33	32	66	49	36	37	76	41	55	50	26	28	49	37	←Age			
			M	M	M	F	M	F	M	F	F	M	F	M	M	F	M	M	M	← Gender			
			5.2	1.1	0.7	0.7	6.0	0.3	3.8	1.5	1.0	3.0	1.3	0.6	2.3	2.3	1.2	6.0	1.0	← Breslow			
			IV	IV	III	III	III	III	IV	III	III	IV	III	III	III	IV	III	IV	III	← Stage			
VA	VA		A/G	G/G	A/A	A/G	G/G	A/G	G/G	A/G	G/G	A/A	A/G	A/G	A/G	A/A	A/G	A/G	G/G	rs1045642	ABCB1	E/T	PMC4990373, 24599932, PMC11050576
3	VA		A/A	C/C	A/C	A/C	C/C	C/C	C/C	A/C	C/C	A/C	A/C	A/C	C/C	C/C	A/C	C/C	C/C	rs2032582	ABCB1	E/T	36988399, 24599932
3	VA	3	A/A	G/G	A/G	A/G	G/G	A/A	G/G	A/G	G/G	A/G	A/G	A/G	G/G	A/A	A/G	A/A	A/G	rs1128503	ABCB1	T/M	34280009, 28447211, 33589792
3		3	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/T	C/C	C/T	C/C	C/T	C/C	C/T	rs2229109	ABCB1	M	12065748
3			T/T	T/T	T/T	T/C	T/T	T/T	T/T	T/C	T/T	T/C	T/T	T/C	T/T	T/C	T/T	T/T	T/T	rs9282564	ABCB1	M	12065748
3	3		A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/T	A/A	A/A	A/A	rs17309872	ACSS2	E	21636554
3			C/C	C/C	C/A	C/A	C/A	C/A	C/A	C/A	A/A	C/A	A/A	C/A	A/A	C/C	C/A	C/C	C/A	rs12415607	CASP7	E	22441531
3			C/C	T/T	T/T	C/T	T/T	T/T	T/T	C/T	T/T	T/T	C/T	C/T	T/T	T/T	T/T	T/T	T/T	rs7921977	CASP7	E	22441531
3			T/T	T/T	T/C	T/T	T/T	T/T	T/C	T/C	T/T	T/T	T/T	T/C	T/T	T/T	T/T	T/C	T/T	rs4353229	CASP7	E	22441531
3			G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	rs1127687	CASP7	E	22441531
3			C/C	C/C	C/G	C/C	C/C	C/G	C/G	C/C	C/C	C/G	C/C	C/G	C/C	C/C	C/G	C/G	C/C	rs2227310	CASP7	E	22441531
3			A/A	A/A	C/C	A/A	A/A	A/A	A/A	A/A	A/A	A/C	A/C	A/A	A/C	A/A	A/C	A/A	A/A	rs2072671	CDA	T	33462346
3			G/G	C/G	C/C	C/G	C/G	C/G	C/C	C/G	C/G	C/C	C/G	C/C	C/C	C/G	C/G	C/G	C/G	rs1056836	CYP1B1	T	2459993
3			T/T	T/T	T/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/C	T/T	rs10509681	CYP2C8	T/M	11668219, PMC3660078
3			C/C	G/C	G/G	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/G	G/C	rs1113129	CYP2C9	T	20212519
VA			G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	rs35599367	CYP3A4	T	PMC3686845
3	3		C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	rs776746	CYP3A5	E/T	24704000, 26179145
3	3		T/T	T/C	T/C	T/T	T/C	T/C	T/C	T/T	T/T	T/C	T/C	C/C	T/T	T/T	T/C	T/C	T/C	rs9981861	DSCAM	E	21079520
3			C/C	C/C	C/A	C/C	A/A	C/C	C/A	C/A	C/C	C/A	C/A	C/A	C/A	C/C	C/A	A/A	C/A	rs3212986	ERCC1	T	25495407
3			G/A	G/A	G/G	G/A	G/A	G/A	G/G	G/A	G/A	G/G	G/A	G/A	G/G	G/A	G/A	G/A	G/A	rs10771973	FGD4	T	PMC3445665
3	3		G/C	G/C	G/G	C/C	C/C	G/C	G/C	G/G	C/C	G/C	G/C	G/C	C/C	C/C	G/C	G/G	G/C	rs11623866	FNTB	E	PMC4631186

End of Table 4

Level of evidence			4	5	6	7	9	11	16	20	25	26	27	28	30	32	34	41	44	← Sample	Gene	Phenotype Categories	Related publication PMID
Pacli-taxel	Carbo-platin	Temo-zolomide	62	33	44	33	32	66	49	36	37	76	41	55	50	26	28	49	37	←Age			
			M	M	M	F	M	F	M	F	F	M	F	M	M	F	M	M	M	← Gender			
			5.2	1.1	0.7	0.7	6.0	0.3	3.8	1.5	1.0	3.0	1.3	0.6	2.3	2.3	1.2	6.0	1.0	← Breslow			
			IV	IV	III	III	III	III	IV	III	III	IV	III	III	III	IV	III	IV	III	← Stage			
VA	VA	VA	G/G	A/G	A/G	A/A	A/A	A/G	A/G	A/G	A/A	A/G	A/A	A/A	A/A	A/G	A/A	A/G	A/A	rs1695	GSTP1	T	36988399
3	3		C/C	C/C	C/C	C/T	C/C	C/T	C/C	C/T	C/C	C/C	T/T	C/C	C/T	C/C	C/T	C/C	C/C	rs1801368	MAD1L1	Other	23407047
3	3		A/A	G/A	G/A	G/G	G/G	G/A	G/A	G/G	G/G	G/A	G/G	G/A	G/A	G/G	G/A	G/G	G/A	rs726501	MAP3K1	E	PMC3124814
3	3		T/T	T/C	T/C	T/T	T/T	T/T	T/C	T/C	T/T	T/T	T/T	T/C	T/C	T/C	T/C	T/T	T/C	rs16886403	MAP3K1	E	PMC3124814
VA	VA		G/G	G/A	G/G	G/G	G/G	G/A	G/A	G/A	G/G	G/A	G/A	G/G	G/G	A/A	G/A	G/A	G/G	rs1801133	MTHFR	E	21605004, 19307503
3	3		T/T	T/T	T/T	G/G	T/T	T/T	T/T	T/T	T/T	T/T	T/T	G/T	T/T	T/T	T/T	T/T	T/T	rs544093	OPRM1	T	PMC3798385
3			G/G	G/C	G/G	C/C	G/C	G/C	G/G	G/G	G/C	G/C	G/C	G/G	G/C	C/C	G/C	G/G	G/C	rs747199	SLC29A1	E	24361227
3	3		G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	T/G	G/G	G/G	G/G	rs4149117	SLCO1B3	T	26641474, 26267044
3	3		A/A	A/A	A/A	A/A	A/A	A/A	G/A	A/A	A/A	A/A	G/A	A/A	A/A	G/A	A/A	A/A	A/A	rs7311358	SLCO1B3	T	26641474
3			G/G	A/G	G/G	G/G	A/A	A/G	A/G	A/G	A/A	A/A	G/G	A/G	A/A	A/G	A/G	A/A	A/A	rs4880	SOD2	T	25495407
3	3		C/C	G/C	C/C	C/C	C/C	G/C	G/C	G/C	C/C	G/C	G/G	G/C	C/C	C/C	G/C	C/C	C/C	rs139887	SOX10	T	PMC3798385
VA	VA		Wt/Wt	Wt/Wt	Wt/del	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/Wt	Wt/del	Wt/Wt	rs11280056	TYMS	E/T	23263912, 21980041
	3		G/A	G/A	G/A	G/G	G/G	G/A	G/G	G/A	G/G	G/G	G/A	G/G	G/G	G/A	G/G	G/A	G/A	rs1800629	TNF	T	31616045
	3		G/G	T/T	G/G	T/G	T/T	T/G	G/G	T/G	T/T	T/G	T/G	T/T	T/G	G/G	T/G	G/G	T/T	rs13181	ERCC2	T	25069034
	3		A/G	A/A	G/G	A/G	A/G	A/G	A/G	A/G	A/G	A/A	A/G	A/G	A/G	A/G	A/A	A/G	A/G	rs11615	ERCC1	T	27498158
	3		A/G	G/G	A/G	A/A	A/A	A/A	A/A	A/G	G/G	A/G	A/G	A/G	A/A	A/G	A/G	A/G	A/A	rs12659	SLC19A1	E	16875718
	VA		Ref	Ref	Mut	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Mut	Ref	Mut	Ref	Ref	Ref	***DPYD*** Ref	DPYD	E	PMC10298094
PGx score			3.8	1.6	1.9	1.7	1.0	1.6	1.2	3.2	1.0	1.7	1.6	1.7	2.3	4.3	1.8	1.4	1.8				

Notes: Genotypes highlighted in red are associated with reduced efficacy (E), and/or increased toxicity (T), and/or impaired metabolism (M). Genotypes highlighted in green are associated with increased efficacy and/or reduced toxicity. PGx score — ratio of the number of "red" genotypes to the number of "green" genotypes; VA — ClinPGx Variant Annotations marker.

TP53 mutations are among the most common genetic alterations in cutaneous melanoma, though their reported frequency varies (typically 20%–30%, but higher in some cohorts) [16—19]. Our finding of the higher *TP53* variant prevalence in patients under 50 aligns with the evidence that the mutation rates and selection pressures can differ by age and tumor stage [26, 27]. However, most large-scale studies did not consistently report strong age-specific differences, suggesting they may be cohort-dependent or influenced by environmental/genetic factors. The observed higher frequency of the multiple *TP53* variants in females is consistent with some studies that note sex-based differences in gene expression and mutation patterns, but robust, consistent sex-specific associations for *TP53* in melanoma remain limited and require further validation [28, 29]. The *TP53* somatic mutations are frequent in melanoma, but their prognostic and predictive value is complex and context-dependent, with the strongest evidence for a negative impact on anti-CTLA-4 therapy outcomes [30]. Further research is needed to clarify their role in melanoma management.

Our data also indicate a significantly higher prevalence of the *BRAF* mutations in unresectable cutaneous melanoma samples from females compared to males (57% vs. 15%, $p = 0.034$). This aligns with some large-scale studies showing that the *BRAF* V600E mutation is more frequent in women, while the V600K mutation is more common in men [31]. However, other studies have not found significant sex-based differences in overall *BRAF* mutation rates [32].

In the design of the Cancer Hotspot panel, our objective was to incorporate as many known significant therapeutic, diagnostic, and prognostic markers as possible for the maximum number of solid and hematological tumor types. For these reasons, 6 variants of the *FLT3* gene were identified in the tumor tissue from 7 patients (22.2%), making it the fourth most frequently mutated gene in our study. *FLT3* mutations are well-characterized in hematological cancers, especially acute myeloid leukemia (AML) but not in melanoma. To our knowledge, only one study directly addresses *FLT3* mutations in the context of a solid tumor, specifically glioma, where the *FLT3* mutations were associated with significantly poorer overall survival, suggesting their potential pathogenic role in this tumor type [33].

The findings of this study indicate that the somatic *FLT3* mutations are significantly more prevalent in stage IV compared to stage III cutaneous melanomas. The increased prevalence of *FLT3* mutations in stage IV melanomas suggests that these mutations may be associated with disease progression or metastatic potential. It is possible that the *FLT3* mutations act as «passenger» mutations in early stages but acquire functional significance in the metastatic context, or that they represent a subset of patients with distinct biological features. This is consistent with the broader concept that certain genetic alterations accumulate as tumors advance, potentially conferring selective advantages such as an increased invasiveness or resistance to therapy [34]. Notably, lower *FLT3* expression was associated with more advanced pathological stages and higher T grade, and higher *FLT3* expression correlated with better prognosis [35, 36]. This suggests a complex role for *FLT3* in melanoma biology, potentially involving both gene expression and mutational status.

This finding is novel and suggests that, while being rare, the *FLT3* mutations may have clinical relevance in certain solid tumors. Additional studies are needed to clarify whether the *FLT3* mutations are true drivers of metastasis or simply correlate with advanced disease, and to determine their impact on patient outcomes. If the *FLT3* mutations are indeed more common in advanced melanoma, they could serve as biomarkers for disease progression or poor prognosis. Given the success of the *FLT3* inhibitors in hematologic malignancies, there may be a rationale to explore targeted therapies in *FLT3*-mutant metastatic melanoma, pending the functional validation.

Pharmacogenetics is increasingly recognized as a multifactorial polygenic trait. The polygenic risk scores offer a promising approach to predict drug response, but their clinical utility is currently limited by methodological and population challenges. In the next stage of the study, an attempt was made to evaluate the possibility of using the pharmacogenetic markers as part of a personalized melanoma treatment strategy and as a supplement to the analysis of therapeutic markers such as *BRAF* V600E.

According to the pathohistological characteristics, identified by Cancer Hotspot panel genetic features of melanoma samples and treatment protocol in Ukraine, the samples belong to 3 treatment groups: stage III and IV wild-type *BRAF*, stage III mutated

BRAF, and stage IV mutated *BRAF.* The analysis of pharmacogenetic markers was possible for 5 of 9 drugs that constituted first-line and second-line treatment for these three groups (Table 2). We did not identify *CYP1A2*3,* which is associated with decreased clearance of Dacarbazine in any of the samples we studied [13]. Therefore, in this study, no conclusions can be drawn regarding therapy with Dacarbazine. But 1 of 3 patients who could have been prescribed therapy with Vemurafenib in combination with Cobimetinib turned out to be a carrier of rs35599367 (G/A) associated with increased risk for grade ≥3 nausea, grade 1—4 hyperbilirubinemia, and cutaneous squamous cell carcinoma (Table 3) [14]. The treatment protocol in Ukraine entails the administration of combinations of Dabrafenib and Trametinib or Vemurafenib and Cobimetinib for the treatment of stage IV mutated *BRAF* melanoma. Medical professionals would be better equipped to select one of these two combinations if they were aware of the rs35599367 risk allele.

For the treatment group stage III and IV wild-type *BRAF,* we analyzed 38 pharmacogenetic markers associated with Temozolomide, Carboplatin, and Paclitaxel (Table 4). The group under discussion included 17 patients who, according to the second-line therapy protocol, could have been prescribed chemotherapy, with some drug options or their combination. Limited data prevent us from using the polygenic risk score (PRS-PGx) methods. Therefore, we calculated a primitive PGx-score to assess the generalized contribution of all 38 markers (Table 4) — ratio of risk genotypes ("highlighted in red") to protective genotypes ("highlighted in green") identified in the sample for each of 38 markers. The risk genotypes are associated with reduced efficacy, and/or increased toxicity, and/or impaired metabolism. The protective genotypes are associated with increased efficacy and/or reduced toxicity.

The most widely used Carboplatin and Paclitaxel combination chemotherapy provides a moderate disease control in melanoma, but the response rates and survival are low. As a combination, these drugs yield objective response rates (ORR) of 13—20%, disease control rates (DCR) of 34—47%, and 12-month OS rates of 36—43% in advanced melanoma; toxicities are mainly hematologic but manageable [37, 38]. As shown by our findings, in a cohort of 17 patients, 2 (12%) have a significantly lower PGx score of 1. It can be assumed that these patients should have demonstrated a complete and partial response to Carboplatin and Paclitaxel combination chemotherapy, as their proportion is similar to that indicated in the aforementioned studies. Nevertheless, we do not have data to reliably assess the possibility of predicting the effectiveness of melanoma therapy based on pharmacogenetic markers. Ongoing research and larger, more diverse studies are essential to realize the full potential of PRS in personalized medicine.

Our study presents the first comprehensive analysis of the somatic mutations and pharmacogenomic markers in unresectable cutaneous melanoma tissue samples from Ukrainian patients. We found that while common somatic variants generally align with global trends, the mutational landscape in this cohort presents several unique features. Most notably, the high *KRAS* mutation rate (29.3%) and its apparent stage-specific prevalence indicate potential selection against *KRAS*-mutant clones during metastasis. Additionally, a high *FLT3* mutation rate (22.2%) was observed, predominantly in stage IV tumors, suggesting a possible association with disease progression and representing potential therapeutic targets pending further validation. However, despite this, the mutation *BRAF* V600E was the only marker that could be reliably used to select personalized therapy, and the pharmacological markers cannot yet be used in medical practice, although there is potential for predicting the effectiveness of therapy.

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Conflict of interest

The authors declare no competing interests.

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СОМАТИЧНІ ГЕННІ ВАРІАНТИ В КЛІТИНАХ НЕРЕЗЕКТАБЕЛЬНОЇ МЕЛАНОМИ ШКІРИ ТА ВИЯВЛЕННЯ ФАРМАКОГЕНОМНИХ МАРКЕРІВ: ВПЛИВ НА СТРАТЕГІЮ ЕФЕКТИВНОГО ЛІКУВАННЯ РАКУ

Метою дослідження було виявити клінічно значущі генні варіанти в зразках нерезектабельної меланоми шкіри українських пацієнтів та дослідити низку фармакогеномних маркерів за допомогою технології NGS для розробки стратегій лікування раку. **Матеріали та методи.** У дослідженні було використано 30 зразків українських пацієнтів віком 28—76 років, які мали нерезектабельну меланому шкіри різної локалізації та ступеня диференціації. Для виявлення генетичних змін використовувалася технологія Ion Torrent NGS таргетного секвенування генів (панелі Custom AmpliSeq™ Cancer Hotspot і Pharmacogenomic), які класифікувалися за базою даних Franklin by Gennox і власним фармакогеномним пайплайном у Ion Reporter Software. **Результати.** За допомогою панелі Cancer Hotspot було виявлено 148 різних генетичних змін у 40 генах (SNV, MNV, INDEL), причому виявлені мутаційні патерни відповідали міжнародним даним. Однак існують помітні розбіжності, такі як високий рівень мутацій *KRAS* (29,3%), які переважно зустрічалися в пухлинах III стадії, що вказує на їхню роль в агресивності та прогресуванні пухлин. Ми виявили часті мутації *TP53* та *BRAF*, серед яких найпоширенішою є *BRAF* V600E, та спостерігали вищу поширеність мутацій *BRAF* у жінок. Мутації *TP53* є поширеними (59,3%) і варіюються залежно від віку та статі, хоча їх прогностичне значення потребує подальшої перевірки. Другим важливим новим відкриттям стало виявлення мутацій *FLT3* у 22,2% зразків, із статистично достовірно вищою поширеністю на IV стадії захворювання, що вказує на потенційну роль *FLT3* у прогресуванні меланоми та вимагає подальшого дослідження як прогностичного біомаркера та потенційної мішені для існуючих інгібіторів *FLT3*. Зрештою, наш пілотний аналіз фармакогеномних маркерів підкреслює їх потенційну клінічну корисність для прийняття рішень щодо персоналізованого лікування. Наприклад, виявлення пацієнта з алелем rs35599367 (G/A) ризику побічної реакції на ліки підкреслює цінність використання фармакогенетичних даних для вибору між затвердженими методами лікування. **Висновки.** Це дослідження є вперше проведеним комплексним аналізом соматичних мутацій та фармакогеномних маркерів у зразках нерезектабельної меланоми шкіри у виборці українських пацієнтів. Ми показали, що поруч із загальними варіантами соматичних мутацій, що відповідають глобальним трендам, виявляються й унікальні мутаційні патерни, зокрема висока частота мутацій *KRAS*, асоційована з певними стадіями захворювання, та висока частота мутацій *FLT3*, переважно в разі пухлинного процесу в стадії IV. Подальша валідація на більших вибірках та більш ретельний аналіз із застосуванням альтернативних методів дозволять всебічно обґрунтувати одержані дані, що сприятиме підвищенню ефективності лікування хворих на меланому.

Ключові слова: NGS, клінічна значущість, варіанти генів, меланома, фармакогеномні маркери, ефективне лікування раку.