Effects of BRAF V600E and NRAS mutational status on the progression-free survival and clinicopathological characteristics of patients with melanoma

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Abstract. Hotspot mutations of the BRAF and NRAS genes are the most common genetic alterations in invasive cutaneous melanoma; however, the prognostic significance of BRAF and NRAS co-mutations remains controversial. The present study aimed to determine the association between NRAS and BRAF mutation status and the clinicopathological characteristics of patients with stage IA-IIC melanoma. A total of 118 patients who underwent surgical treatment for stage IA-IIC melanoma at the Riga East University Hospital between 2012 and 2018 were retrospectively enrolled in the present study. BRAF and NRAS mutation status was assessed by digital droplet PCR using the BRAFV600, NRAS Q61 and NRAS G12/G13 Screening Assays. The association between mutation status and clinicopathological features and progression-free survival (PFS) was then analyzed. The BRAF V600 mutation was detected in 67 out of 118 patients (56.8%). The PFS did not differ between patients with BRAF wild-type and BRAF-mutant melanoma. NRAS mutations were detected in 35 out of 118 patients (29.6%). The NRAS mutational status was associated with Breslow thickness (P=0.035), tumor type (P=0.020; χ^2=0.20), mitotic rate (P=0.025) and lymphovascular invasion (P=0.02; χ^2=0.20). Patients with NRAS-mutant melanoma had significantly worse PFS compared with NRAS wild-type melanoma (HR=12.30; 95% CI=5.78-26.21, P<0.0001). Furthermore, BRAF and NRAS co-mutant melanoma was associated with a significantly worse PFS compared with BRAF-mutant melanoma (HR=6.30; 95% CI=3.10-12.70, P<0.0001). In conclusion, NRAS-mutant and NRAS/BRAF co-mutant stage IA-IIC melanoma was associated with worse PFS compared with NRAS wild-type and BRAF-mutant melanoma. The assessment of NRAS mutation status in melanoma in routine clinical practice may be beneficial for the risk stratification of disease progression for primary non-metastatic malignant melanoma.

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Introduction

The incidence of malignant melanoma has increased worldwide over recent years and it is currently a significant public health problem (1,2). Ultraviolet radiation, which directly damages DNA, is the significant risk factor for the pathogenesis of melanoma (1,3). The early detection of melanoma and evaluation of melanoma tissue biomarkers are important for patient risk stratification, personalized diagnostics and treatment (4,5).

The current World Health Organization (WHO) classification of skin tumors subdivides melanoma on the basis of solar elastosis assessed by dermal elastic fibers, and measures cumulative sun damage (CSD) (3). According to WHO classification, there are currently 3 classes of melanomas: Those associated with high CSD, those associated with low CSD and nodular melanomas (3,6). Solar elastosis is usually apparent in superficially spreading and lentigo malignant melanoma, the so-called high CSD melanoma. Desmoplastic melanoma is associated with increased solar elastosis. The most common subtype of high CSD melanoma is superficially spreading melanoma, which usually begins with early radial growth followed by vertical growth and invasion of the dermis (3). Acral, mucosal, uveal and spitzoid melanomas are not associated with CSD, or are characterized by low CSD. Nodular melanomas are usually characterized as a low CSD type with early progression to vertical growth (3).

While the advent of novel personalized treatments of melanoma based on BRAF inhibitors and immunotherapies have reduced mortality rate over the last decade, advanced and metastatic melanomas still remain difficult to treat (7-10). Therefore, early diagnostic and risk stratification for the progression of melanoma is of particular importance. However,

rare melanoma histopathological subtypes can make diagnosis challenging (3).

Therefore, the biomarkers of early-stage melanoma for the prediction of melanoma clinical behavior is of particular importance. It has been shown that such clinicopathological characteristics such tumor size, tumor type, tumor invasiveness (Breslow thickness, Clark level, lymphovascular invasion, neurotropisms), ulceration and tumor mitosis activity are significant prognostic factors for the development and progression of melanoma (3,6,11). In addition, it has been demonstrated that tumor-infiltrating lymphocytes could stratify melanoma with low and high risk progression (12-14).

The development of melanoma is closely related to somatic and epigenetic changes. Different mutations have been implicated in its pathogenesis and evolution. Recent genomic classification subdivides melanoma into 4 major subtypes based on the pattern of the most prevalent significantly mutated genes: mutant BRAF, RAS, NF1 and triple-WT (wild type) (15). Advances in molecular pathology and assessment of genetic biomarkers are increasingly used in clinical practice for diagnosis, personalized treatment and the prognosis of melanoma. Modern treatment guidelines are focused on the assessment of genetic biomarkers of melanoma (1,16,17).

The assessment of the BRAF gene mutation is of particular importance (3). BRAF mutations are observed in 40-60% of all primary malignant melanoma cases (16-20). The BRAF mutation is usually observed in younger patients, in non CSD skin and in superficial spreading melanoma, whereas NRAS mutational melanoma were characterized for nodular subtype and CSD skin (1,16-20). The BRAF gene is located on chromosome 7 and encodes a cytoplasmic serine-threonine kinase. BRAF plays a role in MAP-kinase (MAPK) pathway activation, contributing to cellular growth, differentiation, survival and proliferation (21). The mutations of the BRAF gene are generally located in codon 600 of the BRAF gene. The most common mutation observed in up to 90% of cases, resulted of transversion of T to A at nucleotide 1,799 position (T1799A). Less common mutations included substitutions of V for lysine (V600K), arginine (V600R) and leucine, V600M (22). Previous studies showed that the BRAF V600E mutation is usually observed in younger patients and at the body extremities, whereas V600K mutations are associated with older patients and usually found at the head and neck (14-19,23).

The RAS gene family includes genes that encode the G proteins which are responsible for: cell growth, proliferation and differentiation. RAS gene family consists of 3 main genes-NRAS, KRAS, and HRAS (15,19). The NRAS gene is most frequently mutated at hotspots in exon 2 (codons 12 and 13) and exon 3 (codon 61) (15,19). Recent evidence showed that in up to 20-30% of cases, NRAS mutations co-existed with BRAF mutations. Patients with both BRAF and NRAS mutations had poorer prognoses than those with the BRAF mutant melanoma alone (24-26). Generally, NRAS mutations are independent of BRAF mutations, but dual expression has been reported (25). The association of NRAS mutations with the degree of solar elastosis suggests that NRAS is closely related to the mutations induced by UV irradiation. Previous studies showed that the NRAS mutation is also associated with decreased immune responses in peritumoral melanoma tissue and a more advanced tumor stage (26). However, the association of NRAS mutational status with histopathological characteristics in early-stage melanoma is still poorly understood.

The current study's objective was to compare the NRAS and BRAF mutation status with the clinicopathological characteristics of patients with Stage IA-IIC melanoma.

Patients and methods

Design of the study. 118 patients who underwent melanoma stage IA-IIC surgical treatment (excision) at Riga East University Hospital, Latvian Centre of Oncology Riga, Latvia, in 2012-2018 were retrospectively enrolled in the study. Only patients with primary cutaneous nodular and superficial spreading malignant invasive melanoma were studied. Patients with nodular and superficial spreading melanoma were defined based on gross and histopathological examination.

Ethics. The study protocol was approved by the Central Medical Ethics Committee of Latvia, Riga, Latvia (approval no. 01-29.1/2016-1-1 from January 2016) and the Ethical Committee of Institute of Cardiology and Regenerative Medicine, the University of Latvia (approval no. 12/2019; from September 2019). The study was conducted according to The Declaration of Helsinki and Oviedo Convention. All patients signed an informed consent to participate in the study.

Exclusion criteria. Patients with lentigo maligna, acral lentiginous melanomas, non-cutaneous and metastatic melanoma as well as patients who had stage III and IV melanoma or who had undergone neoadjuvant treatment were excluded from the study.

Clinical characteristics. The clinical characteristics of melanoma patients such as age, gender, lesion location and size were analyzed. Various clinical factors-age, gender, length of follow-up after surgery, recurrence or metastasis-were obtained from medical records. Progression-free survival time was defined as local, regional or systemic metastasis, or death from the date of surgical excision of tumor and was estimated to be from the surgical resection date to the first loco-regional or systemic metastasis or death without any type of relapse. The patients were follow-up until 1 March 2022. During follow-up, the disease progression was estimated with at least one of these features being observed-local recurrence, regional lymph node metastasis and distant metastasis.

Histopathological characteristics. The histopathological characteristics of melanoma were reviewed by 2 expert pathologists (T.Z. and S.I.) according to the current WHO (World Health Organization) and CAP (College of American Pathologists) guidelines (8). Such characteristics as tumor type, ulceration, peritumoral lymphocytes, Clark invasion level, Breslow invasion level, lymphovascular invasion, neurotropism, regression and mitotic activity was assessed. In addition, the excision lines and distance from the tumor were recorded. The pTNM staging was determined on the basis of histopathological assessment.

Evaluation and scoring of peritumoral lymphocytes. Peritumoral lymphocytes were defined as the lymphocytes surrounding the tumor mass. The peritumoral lymphocyte

infiltration (TIL) was scored from 0 to 3 by a previously described method (14). The scoring was defined as follows: 0=absence of TIL within the tumor tissue, 1=TIL infiltrate less than 25% of the tissue, 2=TIL infiltrate 25 to 50% of the tissue, and 3=TIL infiltrate more than 50% of the tissue.

BRAF and NRAS mutations evaluation. Genomic DNA was isolated from 10 μm sections, cut from formalin-fixed paraffin-embedded tissues using *GeneRead*™ DNA FFPE kit (Qiagen, Germany). The melanoma BRAF and NRAS mutation status were assessed by digital droplet PCR (ddPCR) using BRAF V600 (#12001037), NRAS Q61 (#12001006) and NRAS G12/G13 (#12001627) Screening Assays (all Bio-Rad, USA) as per the manufacturer's instructions. In addition, BRAF V600 positive samples were tested for the presence of the BRAF V600E mutation using the BRAFV600E Mutation Assay Kit (#1863100, Bio-Rad, USA). Droplets were generated using the Biorad QX200 Droplet Generator and analyzed with a QX200 Droplet Reader (Bio-Rad, USA). Absolute quantifications of mutant and wild-type alleles were estimated by modeling a Poisson distribution using QuantaSoftTM analysis software version 1.7 (Bio-Rad, USA).

Statistical analysis. The results were reported as median (range). Histopathologic and clinical characteristics were analyzed using the χ^2 or Mann-Whitney U test. Association of the mutation status with clinical and histopathological characteristics for categorical variables was analyzed by using Pearson χ^2 and by Mann-Whitney U test for continuous variables to calculate statistical significance. Progression-free survival (PFS) was estimated with the Kaplan-Meier method with the log-rank test. Time was defined as the event of disease progression or last follow-up visit (censored). Statistical calculations were performed with SPSS version 21.0 (SPSS Inc., Chicago, Illinois, USA). P-values of less than 0.05 were considered statistically significant.

Results

General characteristics. Altogether, 118 patients were enrolled in the study. 12 patients had stage IA, 20 patients had stage IB, 18 patients had stage IIA, 32 patients had stage IIB, and 36 patients had stage IIC melanoma. The median age was 67 years (range 24-86). 50 patients were males and 68 patients were females. Primary tumor localization was head/neck, limbs, and trunk in 18.0, 40.0, and 42.0% of patients, respectively (Table I).

BRAF mutational status and its correlation with clinicopathological characteristics. All tissues were analyzed for BRAF mutational status, with the BRAF V600 mutation being found in 67 out of 118 patients (56.8%) (Fig. 1). From those, 63 patients had BRAF V600E mutation and 4 patients had another undefined V600 mutation. The associations of BRAF V600 mutational status and Breslow thickness (P=0.030), patient gender (P=0.035; χ^2=0.030), peritumoral lymphocytes infiltration and TIL (P=0.0008) was observed (Table II). However, any association between the disease stage, patient age, solar elastosis, mitotic activity, Clark level of invasion and BRAF mutational status was not demonstrated.

Table I. Clinicopathological characteristics of enrolled study subjects.

Variable	Value
Median age, years (range)	67 (24-86)
Sex, male/female	50/68
Median Breslow thickness, mm (range)	2.4 (0.1-20)
Median Clark level, n (range)	3 (1-5)
Ulceration, present/absent	48/70
LVI, present/absent	76/42
Neurotropism, present/absent	6/112
Solar elastosis, n (range)	1 (0-3)
Median tumor size, cm (range)	1.5 (0.2-20.0)
Median mitotic count, /10 HPF (range)	2 (1-18)
Median TIL, score (range)	2 (0-3)
BRAF mutational status, V600 mutant/wild type	67/51
NRAS mutation status, mutant/wild type	35/83
BRAF/NRAS co-mutant melanoma/BRAF mutant	26/67
Stage IA, n	12
Stage IB, n	20
Stage IIA, n	18
Stage IIB, n	32
Stage IIC, n	36

TIL, peritumoral lymphocyte infiltration; LVI, lymphovascular invasion.

The obtained results showed that the BRAF V600E mutation is closely related to melanoma growth, since the Breslow thickness is a major characteristic of melanoma, also incorporated in melanoma TNM classification. In addition, a BRAF mutational status association with peritumoral lymphocyte infiltration could link the immune system response and tumor progression.

BRAF mutation status and PFS. All 118 patients were clinically followed up, and there were 29 incidences of locoregional recurrence or systemic metastasis. The PFS did not differ between wild type and BRAF mutant melanoma (HR=1.10; 95% CI=0.40-2.50, P=0.20).

NRAS mutational status and its correlation with clinicopathological characteristics. All tissues were analyzed for NRAS mutational status (Fig. 2). NRAS mutation was found in 35 out of 118 patients (29.6%). 26 melanoma samples (75%) were both NRAS and BRAF co-mutant. 26 patients had NRAS Q61 mutation and 9 patients had NRAS G12, G13 mutations.

The NRAS mutational status was associated with Breslow thickness (P=0.035), tumor type (P=0.02; χ^2=0.20), mitotic activity (P=0.025) and lymphovascular invasion (P=0.020; χ^2=0.200). However, any association between the disease stage, Clark level of invasion, solar elastosis, TIL, patient age, patient gender and NRAS mutational status was not demonstrated (Table III).

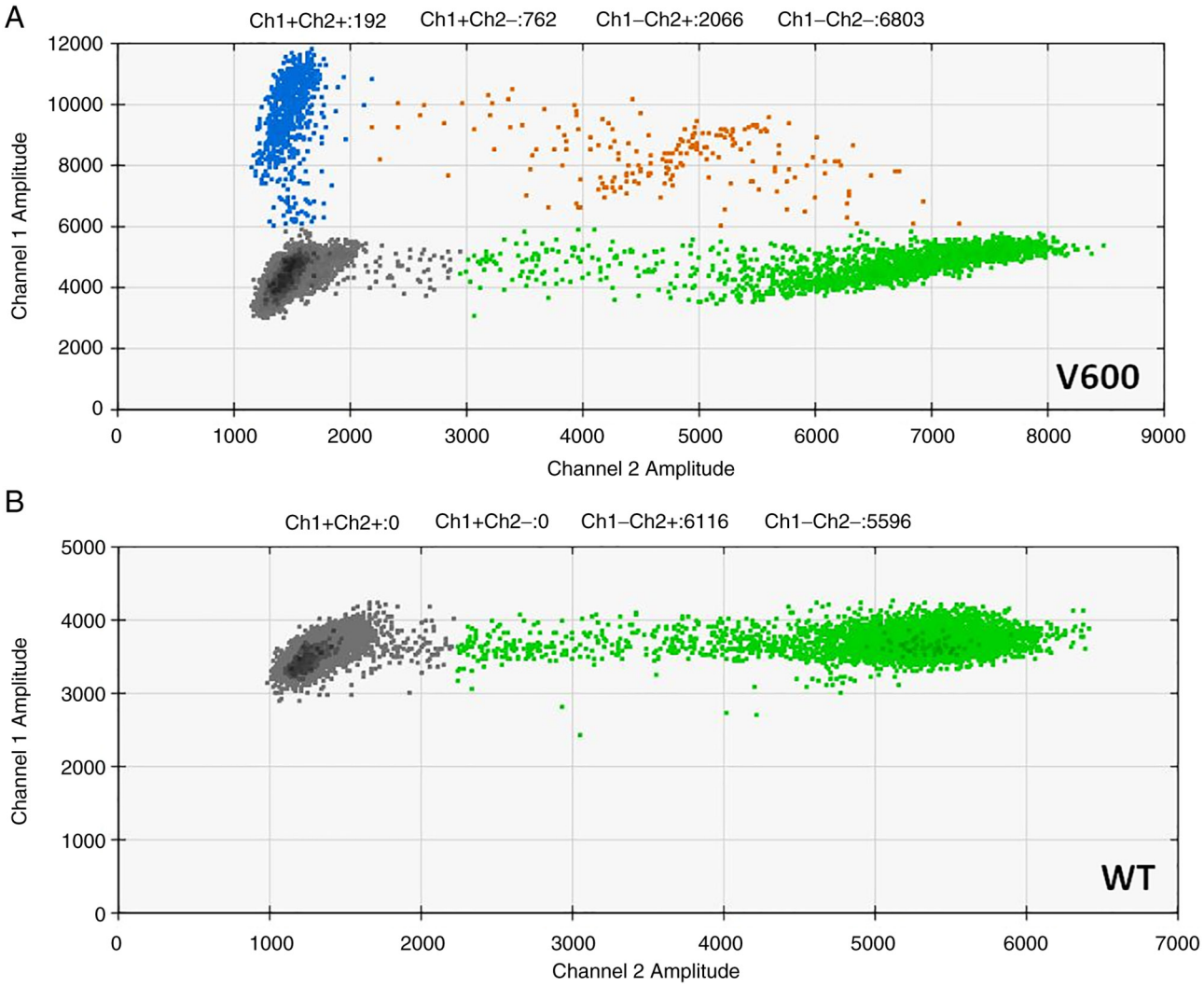

Figure 1. Two-dimensional droplet digital PCR plots. Plots of representative (A) BRAF V600-mutant and (B) WT tumors. WT, wild-type.

The obtained results showed that NRAS mutational status was closely related to tumor growth, evaluated histopathologically by Breslow thickness, mitotic activity and tumor type and commonly detected clinically by gross examination. The association of NRAS mutational status and lymphovascular invasion could indicate that NRAS mutant melanoma has higher metastatic potential compared to that of NRAS wild type melanoma.

NRAS mutation status and PFS. Patients with NRAS mutant melanoma had significant poorer PFS compared to NRAS wild melanoma (HR=12.30; 95% CI=5.78-26.21, P<0.0001). Furthermore, the BRAF and NRAS co-mutant melanoma had significant poorer PFS compared to the BRAF mutant melanoma (HR=6.30; 95% CI=3.10-12.70, P<0.0001) (Fig. 3).

Discussion

In the current study, genotype-phenotype associations were assessed in 118 patients with Stage IA-IIC malignant invasive melanoma, according to the AJCC classification. Histopathological examination of melanoma is currently a gold standard for the diagnosis of melanoma. In addition, such histopathological characteristics of invasive cutaneous melanoma as tumor size and type, lymphovascular invasion, ulceration, Breslow thickness, Clark invasion level, mitotic rate and disease are well established powerful prognostic and predictive factors for melanoma (3,6). The development of melanoma is closely related to somatic and epigenetic changes. Activated mutations of the oncogenes BRAF and NRAS are of particular importance in melanoma progression (15-20). BRAF personalized treatment of melanoma significantly improved patients' prognosis (18-20), however, the prognostic value of BRAF and NRAS mutation and its association with clinical and histopathological characteristics is still controversial, especially in early-stage melanoma.

Our study showed that BRAF mutations and NRAS mutations were identified in 56.8 and 29.6% of cases respectively. Furthermore, while BRAF mutational status was not associated with PFS, the NRAS mutational status did significantly correlate with PFS. In patients with BRAS and NRAS co-mutant melanoma, the PFS was significantly poorer compared to the BRAF mutant melanoma.

BRAF mutations in primary melanomas have been observed at a rate of 22-72% (14-20). In our study, the frequency of BRAF mutation falls within this range. Over 90% of the

Table II. Association analysis of BRAF mutation with clinicopathological characteristics.

Variable	BRAF (mutant and wild)	BRAF (wild)	BRAF (mutant)	P-value
Median age, years (range)	67 (24-86)	68 (44-86)	62 (24-78)	0.120[a]
Sex, male/female	50/68	24/27	26/41	0.035[b]
Median Breslow thickness, mm (range)	2.4 (0.10-20.0)	1.90 (0.1-20.0)	3.0 (0.2-18.0)	0.030[a]
Median Clark level (range)	3.0 (1.0-5.0)	3.0 (2.0-5.0)	3.0 (1.0-5.0)	0.220[a]
Ulceration, present/absent	48/70	26/25	22/45	0.120[b]
LVI, present/absent	76/42	24/27	52/15	0.280[b]
Median solar elastosis (range)	1.0 (0.0-3.0)	1.0 (0.0-2.0)	2.0 (0.0-3.0)	0.090[a]
Median tumor size, cm (range)	1.5 (0.2-20.0)	1.8 (0.7-5.0)	1.5 (0.3-20.0)	0.065[a]
Median mitotic count (range)	2.0 (1.0-18.0)	3.0 (1.0-7.0)	2.0 (1.0-18.0)	0.580[a]
Median TIL, score (range)	2 (0.0-3.0)	1.0 (0.0-3.0)	2 (0.0-3.0)	0.0008[a]
Tumor type, nodular/superficial spreading	68/50	30/21	38/29	0.460[b]

TIL, peritumoral lymphocyte infiltration; LVI, lymphovascular invasion. [a]Mann-Whitney U test, P<0.05; [b]Pearson's χ^2 test, P<0.05.

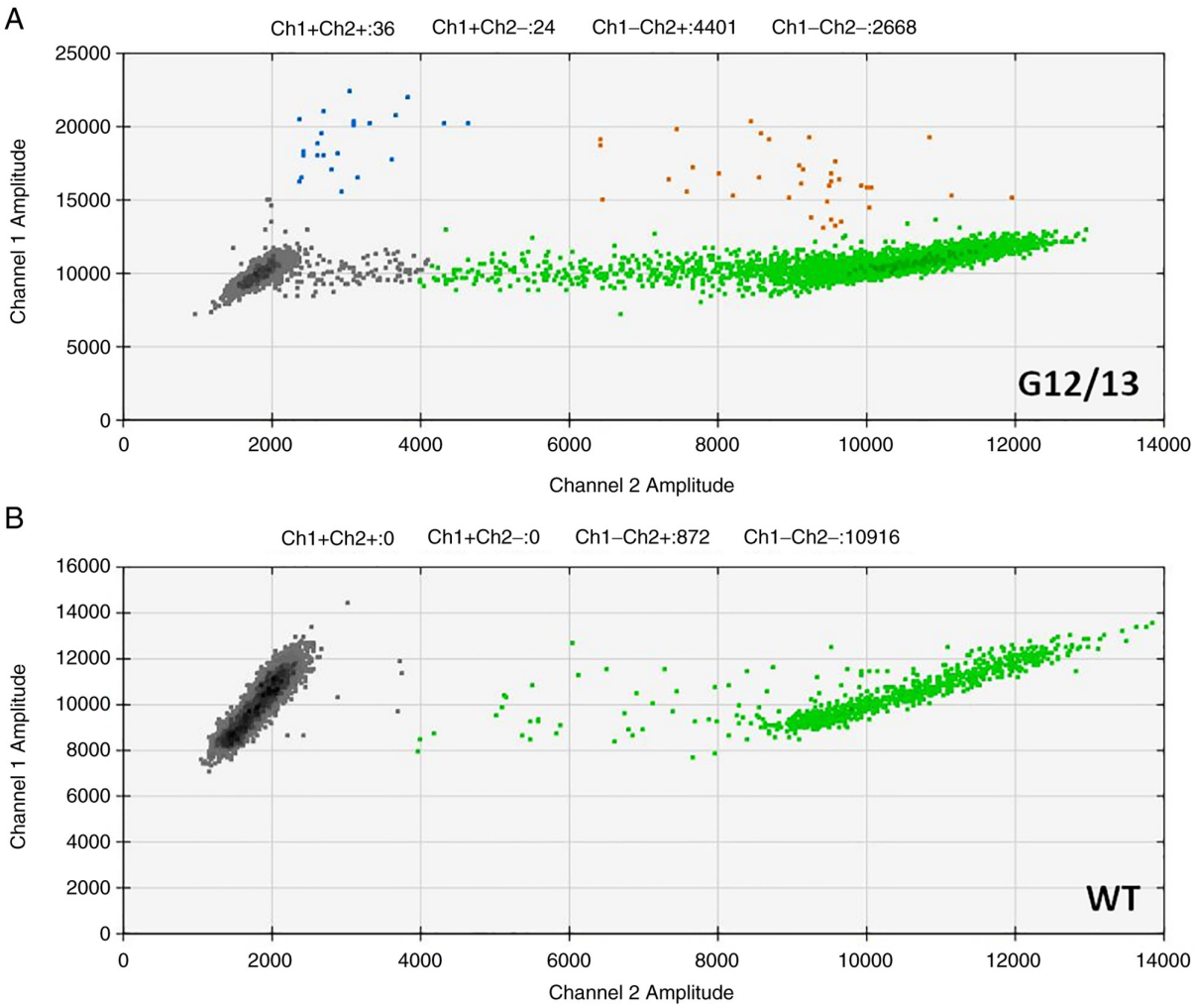

Figure 2. Two-dimensional droplet digital PCR plots. Plots of representative (A) NRAS-mutant at G12_13_codons and (B) WT tumors. WT, wild-type.

mutations in BRAF result in substitution of the valine at position 600, resulting in activation of the downstream effectors of the RAS-RAF-MEK-MAPK pathway (1). The associations of BRAF V600 mutational status and Breslow thickness, patient gender, Breslow thickness and peritumoral lymphocytes infiltration was revealed, supporting our previous evidence (14).

Table III. Association analysis of NRAS mutation with clinicopathological characteristics.

Variables	NRAS (wild and mutant)	NRAS (wild)	NRAS (mutant)	P-value
Median age, years (range)	67 (24-86)	66 (24-83)	68 (30-86)	0.760[a]
Sex, male/female	50/68	35/48	15/20	0.960[b]
Median Breslow thickness, mm (range)	2.4 (0.10-20.0)	1.5 (0.1-20.0)	3.5 (0.2-20.0)	0.035[a]
Median Clark level (range)	3.0 (1.0-5.0)	3.0 (2.0-5.0)	3.0 (1.0-5.0)	0.220[a]
Ulceration, present/absent	48/70	27/56	21/14	0.400[b]
LVI, present/absent	76/42	45/38	31/4	0.020[b]
Median solar elastosis (range)	1.0 (0.0-3.0)	1.0 (0.0-3.0)	1.0 (0.0-3.0)	0.720[a]
Median tumor size, cm (range)	1.5 (0.2-20.0)	1.5 (0.2-7.0)	1.6 (0.3-20.0)	0.076[a]
Median mitotic count (range)	2.0 (1.0-18.0)	2.0 (1.0-8.0)	4.0 (1.0-18.0)	0.025[a]
Median TIL, score (range)	2 (0.0-3.0)	2.0 (0.0-3.0)	1.0 (0.0-3.0)	0.38[a]
Tumour type, nodular/superficial spreading	68/50	41/42	27/8	0.020[b]

TIL, peritumoral lymphocyte infiltration; LVI, lymphovascular invasion. [a]Mann-Whitney U test, P<0.05; [b]Pearson's χ^2 test, P<0.05.

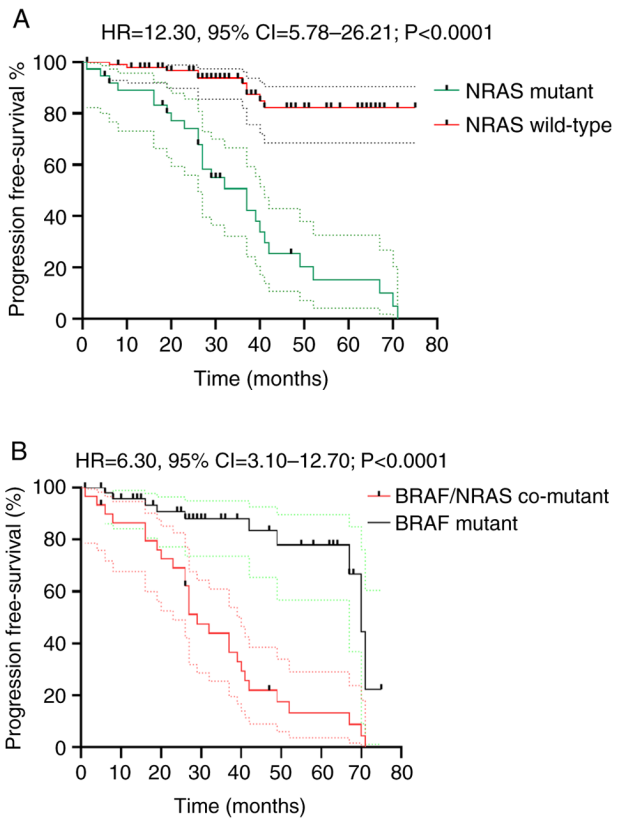

Figure 3. Progression-free survival in patients with melanoma. (A) NRAS-mutant and NRAS wild-type melanoma. Kaplan-Meier plot was analyzed using a log-rank (Mantel-Cox) test. P<0.0001, NRAS-mutant vs. NRAS wild-type melanoma. (B) BRAF and NRAS co-mutant melanoma and BRAF-mutant melanoma. Kaplan-Meier plot was analyzed using a log-rank (Mantel-Cox) test. P<0.0001, BRAF/NRAS co-mutant melanoma vs. BRAF-mutant melanoma.

Previous studies showed the different value of BRAF mutational status in association with clinicopathological characteristics and PFS of melanoma (27-33). Our study did not find any association between the BRAF mutational status and PFS of melanoma.

It has been demonstrated that BRAF mutational status correlates to younger age groups and females (34,35). However, some studies also demonstrated associations with the male gender (29). In our study BRAF mutation correlated to female gender and older age. This observation could be explained by the fact that only early-stage melanoma patients were enrolled in our study. Some studies showed the importance of immunological tolerance mechanisms in the development of BRAF mutant melanoma (14,36). This study confirmed our previous results which showed that TIL infiltration is associated with BRAF mutational status (14,37). It seems that the assessment of TIL is beneficial for the risk stratification of melanoma and therefore it should be included in the routine histopathological assessment of melanoma.

Previous studies of BRAF and NRAS com-mutant melanoma have been discordant. NRAS gene mutation was found in 15-25% of melanoma cases (27,38). In our study NRAS mutation was observed in up to 30% of melanoma cases.

We assume therefore that the high prevalence of NRAS mutations could be explained by the older median age of the enrolled patients in our study, e.g., 67 years. It has been demonstrated that patients with NRAS mutant melanoma compared with BRAF mutant melanoma were usually older (>55 years) with a previous history of UV exposure. NRAS mutant melanoma is commonly found in upper extremities and characterized by increased Breslow tumor thicknesses (1,27,38).

These results showed that NRAS mutations are associated with Breslow thickness, nodular melanoma tumor type, mitotic activity and lymphovascular invasion. It was assumed that NRAS mutations in primary stage IA-IIC melanoma could have potentially relevant predictive value. The NRAS gene is most frequently mutated at hotspots in exon 2 (codons 12 and 13) and exon 3 (codon 61) (38). The NRAS mutation characteristic for nodular melanoma is localized in sun-damaged skin (39).

Nevertheless, the value of NRAS mutation on disease progression and prognosis is still controversial. Some studies showed that the NRAS mutation was associated with a favorable prognosis (40). In contrast, other studies demonstrated that NRAS gene mutation was associated with a poorer

prognosis (38,41,42). Other studies did not find any significant association between the NRAS mutation and the prognosis of melanoma (37,43,44).

Similarly in stage IV melanoma, the data for the NRAS mutation is also controversial. While one study suggested that the NRAS-mutated tumor genotype in metastatic Stage IV melanoma was associated with increased overall survival compared to the BRAF-mutated and WT tumor genotypes (40). Other studies had the opposite results and did not support this evidence (43,44).

It has been demonstrated that NRAS mutation status was an independent predictor of shorter survival after a diagnosis of stage IV melanoma (45). It could be suggested that molecular mechanisms involving NRAS genetic pathway could be different between metastatic Stage IV and early-Stage IA-IIC melanoma. These results, in line with previous studies, demonstrated that NRAS mutations are associated with higher Breslow's thickness and poor disease prognosis (38,41,42).

In addition, our study showed that NRAS mutations are associated with increased mitotic activity of the tumor and lymphovascular invasion, which could be one of potential explanations of the aggressive behavior of those tumors which carried a NRAS mutation. Furthermore, it was demonstrated that NRAS mutational status in primary Stage IA-IIC melanoma is a powerful predictive factor, significantly associated with progression free survival.

NRAS personalized treatment of melanoma is challenging. Lonafarnib and tipifarnib have been studied for NRAS mutant melanomas (1). In addition, selective MEK inhibitors could have potential benefit in the treatment of NRAS mutant melanoma (45). However, the potential significant value of our study is in the finding of a significant predictive value of NRAS mutations for Stage IA-IIC melanoma. Therefore, routine assessment of NRAS mutations in Stage IA-IIC melanoma could be potentially beneficial for the prediction of disease progression. It should be stressed that our study subjects included only those with local disease, e.g., at the time of diagnosis patients did not have local recurrence, regional lymph node metastasis or distant metastasis.

The value of the current study is in the demonstration that Stage I and II NRAS mutant melanoma is characterized by poorer progression free survival and is associated with histopathological characteristics responsible for tumor growth such as Breslow thickness, mitotic activity and lymphovascular invasion. Further research for patients with advanced and metastatic melanoma should evaluate the role of BRAF and NRAS mutational status in disease progression.

Several limitations of our study should be mentioned. A significantly higher number of case-cohort with equal gender distribution would be beneficial. At the same time, the strength of the present study was the demonstration of significant role of NRAS mutational status in patients with early-stage IA-IIC non-metastatic melanoma. All patients were enrolled from the single oncology hospital, which treats up to 85% of all melanoma cases in Latvia.

In our study BRAF and NRAS mutation status was assessed by digital droplet PCR (ddPCR) using BRAFV600, NRAS Q61 and NRAS G12/G13 Screening Assay. The PCR testing is the gold standard for BRAF and NRAS mutation testing according to ASCO and CAP protocols. The immunohistochemistry is cost-effective method compared to PCR and the value, specificity, and sensitivity of BRAF and NRAS immunohistochemistry should be addressed in future studies for general melanoma testing.

In conclusion, the patients with NRAS and NRAS/BRAF co-mutant Stage IA-IIC melanoma had poorer progression free survival when compared to the NRAS wild and BRAF mutant melanomas. The NRAS assessment in melanoma in routine clinical practice is beneficial for the risk stratification of disease progression. Our results highlighted the value of NRAS personalized treatment in patients with invasive melanoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors have contributed to and agreed on the content of the manuscript. TZ and SI analyzed the histopathological slides and data. DP and MK performed genetic analysis. TZ, SI and DP wrote the manuscript. TZ and SI participated in the patient enrollment, and data collection and analysis. SI and DP supervised the project. All authors read and approved the final manuscript. TZ, SI and DP confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study protocol aimed to enroll at least 150 patients with cutaneous invasive melanoma from 2012 until 2021. The study protocol was approved by the Central Medical Ethics Committee of Latvia (approval no. 01-29.1/2016-1-1; January 2016) and the Ethical Committee of the Institute of Cardiology and Regenerative Medicine, University of Latvia (approval no. 12/2019; September 2019). The study was conducted according to The Declaration of Helsinki and Oviedo Convention. All subjects signed informed consent to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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