research article

Prevalence of BRAF, NRAS and c-KIT mutations in Slovenian patients with advanced melanoma

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Background. BRAF, NRAS and c-KIT mutations are characteristics of tumour tissues that influence on treatment decisions in metastatic melanoma patients. Mutation frequency and their correlation with histological characteristics in Slovenian population have not been investigated yet.

Patients and methods. In our retrospective analysis we analysed mutational status of BRAF, NRAS and c-KIT in 230 pathological samples of patients who were intended to be treated with systemic therapy due to metastatic disease at the Institute of Oncology Ljubljana between 2013 and 2016. We collected also histological characteristics of primary tumours and clinical data of patients and correlated them with mutational status of tumour samples.

Results. The study population consisted of 230 patients with a mean age 59 years (range 25−85). 141 (61.3%) were males and 89 (38.7%) females. BRAF mutations were identified in 129 (56.1%), NRAS in 31 (13.5%) and c-KIT in 3 (1.3%) tissue samples. Among the 129 patients with BRAF mutations, 114 (88.4%) patients had V600E mutation and 15 (11.6%) had V600K mutation. Patients with BRAF mutations tended to be younger at diagnosis (52 *vs.* 59 years, $p < 0.05$), patients with NRAS mutations older (61 *vs.* 55 years, $p < 0.05$). Number of c-KIT mutations were too low for any statistical correlation, but there was one out of 3 melanoma located in mucus membranes.

Conclusions. The analysis detected high rate of BRAF mutations, low NRAS mutations and low c-KIT mutations compared to previously published studies in Europe and North America. One of the main reasons for this observation is specific characteristics of study population.

Key words: BRAF; NRAS; c-KIT; prevalence

Introduction

Melanoma incidence is on the 6th place among all the cancers in Slovenia and it is constantly rising during last period.[1,2] Although early melanoma has a good prognosis, melanoma with distant metastasis carries a high mortality rate.[3] Until recently there was a lack of successful treatment approach in metastatic melanoma. Nowadays we are experiencing a new era in this field since there are several options available: immunotherapy, target therapy and chemotherapy. Still a proper adjustment of the treatment is needed according to tumour and patient characteristics.[4]

Mechanisms of melanoma development and progression are complex. There are several mutations identified, some are recognized as causative "driver" mutations (BRAF, NRAS, c-KIT, GNAQ/GNA11), others are bystander "passenger" mutations (MET, AKT3, PTEN, ...).[5] In majority of melanoma MAPK (Ras-Raf-MEK/ERK) signalling pathway is constitutively activated due to mutation in BRAF or NRAS.[5]

The most prevalent mutations in melanoma are BRAF mutations with a frequency between 40–70%[6,7], among them BRAF V600E 80–90%, BRAF V600K 5–12% and other less frequent.[6,8,9] Second most common mutations are NRAS mutations with a frequency around 15–30%.[6,9,10] BRAF and NRAS mutations are mutually exclusive. C-KIT mutations present in 5–10%.[11]

BRAF, NRAS and c-KIT mutations were correlated to pathological and clinical characteristics of melanoma.[10,12,13] Melanomas with BRAF mutations are more common in younger patients, in superficial spreading melanoma and on a skin without chronical UV skin damage.[14,15,16,17] NRAS mutations appear more frequent in older patients, in nodular melanoma and on a skin with chronical UV damage.[18,19] Majority of c-KIT mutation are found in acral lentiginous and mucosal melanomas.[11,12]

Several clinical studies confirmed the link between certain mutation status and treatment response, therefor many guidelines already recommend standard testing for BRAF, NRAS and c-KIT muatation.[4]

However, prevalence of mutation and their correlation with pathological and clinical characteristic in Slovenian patients has not been investigated till now.

Patients and methods

In retrospective study we included 230 patients with metastatic melanoma who were planned to be treated with systemic therapy between 2013–2016 at Institute of Oncology Ljubljana, the only cancer centre for treating metastatic melanoma in the country.

Patient characteristics

All data, such as patient demographics (age, gender), details of primary melanoma (date of primary diagnosis, Clark, Breslow, ulceration, mitotic rate, histological subtype, anatomic site, stage) and clinical course were obtained from archived patient medical records at the Institute of Oncology Ljubljana and from the Cancer Registry of Republic of Slovenia.

The primary melanomas were categorised as cutaneous, mucosal, uveal or occult. Anatomical site was coded as: head and neck, trunk, extremities, uveal, mucosal or occult. Histological subtypes of cutaneous melanomas were grouped for analysis as superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanomas (LMM), acral lentigo maligna (ALM), other specified and no other specified (NOS).

The study was conducted according to the Declaration of Helsinki and was approved by the medical ethics committee of Institute of Oncology Ljubljana and National Ethics Committee (approval number 46/09/16).

Tumour tissue and molecular testing

The tumour tissues (44.8% from primary and 55.2% from metastatic lesion) were recollected from archived paraffin-embedded samples. Molecular testing was performed using RT-PCR BRAF Mutation Analysis Kit II (EntroGen, Inc.), RT-PCR NRAS Mutation Analysis Kit (EntroGen, Inc.), RT-PCR RAS c.59/117 Mutation Detection Kit (EntroGen, Inc), and c-KIT Mutation Detection Kit (EntroGen, Inc.), according to manufacturer's instructions. Molecular testing for BRAF mutation was completed on all 230 samples, but for NRAS and c-KIT only on 205 samples due to the lack of tissue material.

Statistical analysis

Categorical data are described using absolute numbers and percentages, continuous by mean, minimum and maximum. For all patients, clinical and pathological features were tested for association with BRAF, NRAS and c-KIT mutation using simple cross tabulation and Pearson's χ^2 test. All the statistical analysis was performed using SPSS software, version 22.0. For all analysis, two-tailed $p < 0.05$ was considered statistical significant.

Results

Patient demographic data are shown in Table 1. A total of 230 patients with melanoma were included in the study. Mean age was 59 years (range 25–85). There were 141 (61.3%) males and 89 (38.7%) females. Location of primary melanoma lesion was skin in 167 (72.6%) cases, mucus membranes in 7 (3.0%) and uveal in 11 (4.8%). In 45 (19.6%) cases no primary tumour was found. Most common anatomical primary site of cutaneous melanoma was trunk in 91 (39.6%) cases, extremities in 52 (22.6%) cases, head and neck in 24 (10.4%) cases.

Among primary cutaneous melanomas the most common histological subtype was superficial spreading melanoma in 61 (36.6%) cases, nodular

melanoma in 45 (26.9%) cases, lentigo maligna melanoma in 4 (2.4%) cases and acral lentigo melnoma in 1 (0.6%) case, 2 (1.2%) other rare types. There were 54 (32.3%) cases of unclassified type or not otherwise specified.

The overall mutation frequency in samples analysed for all mutation (N = 205) was 146 (71.2%); for BRAF 129 (54.6%), NRAS 31 (15.1%) and c-KIT 3 (1.5%). Wild type frequency for all tested mutation was 59 (28.8%). In our study population BRAF, NRAS, c-KIT were mutually excluded in all cases.

In 25 cases only analysis of BRAF mutation was carried out, due to lack of appropriate material for additional molecular testing.

BRAF was mutated in 129 samples out of 230; 114 (49.6%) samples had V600E, 15 (6.5%) samples V600K and none had V600D mutation.

Patients with BRAF mutations tended to be younger at diagnosis compared to non-mutated (52 *vs.* 59 years old, $p<0.05$). Among BRAF mutated the oldest were those with V600K mutation compared to patients with V600E mutation (60 *vs.* 51 years old). We didn't find any statistical significant correlation between BRAF mutation and gender, anatomical location or any histological feature. We also didn't find more BRAF mutation in a group with primary metastatic patients (Table 4).

NRAS was mutated in 31 out of 205 samples. NRAS mutated patients were older at diagnosis compared to non-mutated (61 *vs.* 55 years old, $p<0.01$). We didn't find any statistical significant correlation between NRAS mutation and gender, anatomical location or any histological feature. We also didn't find more NRAS mutations in a group with primary metastatic patients (Table 4).

C-KIT was mutated in 3 (1.5%) patients, one was located on mucus membrane and two were nodular melanomas of the skin. The sample was too small to carry out further statistical analysis (Table 4).

TABLE 1. Patient demographic and clinical characteristics of primary melanoma

		Number of patient (N = 230)	% of all patient
Gender			
male		141	61.3
female		89	38.7
Age at the time of diagnosis (years)			
< 50		78	33.9
50 – 59		58	25.2
60 – 69		55	23.9
> 69		39	17.0
Location of primary tumour			
cutaneous			
	trunk	91	39.6
	extremities	52	22.6
	head and neck	24	10.4
uveal		11	4.8
mucusal		7	3.0
occult		45	19.6
Tumour stages at diagnosis			
in situ		1	0.4
localised		67	29.1
regional		116	50.5
distant		46	20.0

Discussion

Prevalence of BRAF, NRAS and c-KIT mutation varies across different regions in the world. There are several studies that have examined the prevalence of BRAF, NRAS and c-KIT and their association with tumour characteristics.[20,21,23] However, until now, we lacked detailed information about the situation in our region.

In a present study, we recorded high prevalence of BRAF mutation (56.1%) compared to majority of studies published.[9-12,16-19,23,25] During interpretation of our results we need to be aware that any direct comparison to a single study is difficult since several differences among studies exists (different study population, methods,…). To overcome some of these barriers two meta-analyses on prevalence of BRAF mutation were performed.[20,21] Their final results estimate the prevalence of BRAF mutation to around 40% in white population and even lower 19.5% in Asian.

Our results revealed that our study group does not represent general population of patient with melanoma, as well as not the most common group of patients in the majority of studies. Our cohort consists of patients with advanced melanoma, with their own characteristics (mixed clinical subgroup, all M stages) and specific tumour features (higher rate of nodular melanoma (26.9%), worse histopathological features of primary melanoma) that had led to metastatic spread.

The evidences of a higher BRAF mutation rates in a metastatic disease already exist.[9,10,18,21,23] In a

TABLE 2. Histopathological characteristic of cutaneous melanoma (N = 167)

	Number of patient (N = 167)	% of all patient	Mean	Range
Melanoma subtype				
SSM	61	36.6		
NM	45	26.9		
ALM	1	0.6		
LMM	4	2.4		
NOS	54	32.3		
Other	2	1.2		
Clark			3.8	(2.0–5.0)
Breslow			4.8	(0.2–48.0)
Mitotic index			8.4	(0.0–60.0)
Ulceration	81	48.5		

ALM = acral lentigo maligna; LMM = lentigo maligna melanomas; NOS = not other specified; NM = nodular melanoma; SSM = superficial spreading melanoma

study where researchers were comparing paired samples of primary and metastatic lesion they detected differences between BRAF mutations in metastatic lesion as high as in 53% compared to the primary samples in 43%.[9] The mutation analyses in our analyses were performed from metastatic lesion in more than half of them.

We also need to be aware of the impact of various diagnostic methods used in distinct studies, their detection limit and the influence of DNA quality in formalin-fixed paraffin embedded samples.[22] The detection limit of the methods used in our study ranges from 0.25 – 3.0% of mutated DNA in the background of wild type DNA.

Among all BRAF mutations (N = 129) we observed similar distribution of V600E mutation in 88.4% and V600K mutation in 11.6% compared to results published in other studies.[18,23]

However according to higher prevalence of BRAF mutation, we have detected low frequency of NRAS (15.1%) mutation and c-KIT (1.5%) mutation compared to similar studies. All of tested mutations were mutually excluding in our study group.

In order to demonstrate the association between mutational status (BRAF, NRAS and c-KIT) and clinico-pathological characteristics we completed a correlation analysis between several data, but only association between BRAF and NRAS mutation and age reached the statistical significance of $p<0.05$. Patients with BRAF mutation were statistically significantly younger than those without BRAF mutation, patients with NRAS mutation were older than those without NRAS mutation at the time of diagnosis. This association was reported already in previously published data[23,24], but was not confirmed by metaanalysis.[20]

We have found no statistically significant association between BRAF or NRAS mutations and gender or pathological features (Breslow thickness, ulceration, regression, mitotic index).

According to anatomical tumour location, the prevalence of BRAF mutation was highest in a trunk (48.8%), followed by other locations and

TABLE 3. Mutation of BRAF, NRAS and c-KIT

	Number of wild type (%)	Number of mutation (%)	Type
BRAF (N = 230)	101 (43.9%)	129 (56.1%)	
		114 (49.6%)	V600E: Val600Glu (c.1799T>A)
		15 (6.5%)	V600K: Val600Lys (c.1798_1799GT>AA)
NRAS* (N = 205)	174 (84.9%)	31 (15.1%)	
		10 (4.9%)	c.181C>A p.(Gln61Lys)
		14 (6.7%)	c.182A>G p.(Gln61Arg)
		2 (1.0%)	c.182A>T p.(Gln61Leu)
		1 (0.5%)	c.34G>T p.(Gly12Cys)
		3 (1.5%)	c.37G>C p.(Gly13Arg)
		1 (0.5%)	c.183A>C p.(Gln61His)
c-KIT* (N = 205)	202 (98.5%)	3 (1.5%)	
	2 (1.0%)	c.1676T>C p.(Val559Ala)	
	1 (0.5%)	c.1727T>C p.(Leu576Pro)	

* in 25 cases NRAS and c-KIT analysis was not completed due to inadequact tissue samples

TABLE 4. Correlation of BRAF, NRAS, c-KIT mutation and clinico-pathological features of melanoma, all patients (N = 230)

		BRAF			NRAS			c-KIT		
		mutation	wild type	P	mutation	wild type	P	mutation	wild type	P
		129 (56.1%)	101 (43.9%)		31 (15.1%)	174 (84.9%)		3 (1.5%)	202 (98.5%)	
Age (years; mean)		52.3	59.3	**< 0.05**	61.4	54.7	**< 0.05**	63.4	54.9	**N.A.**
Gender	male	82 (63.6%)	59 (58.4%)	**0.43**	19 (61.3%)	113 (64.9%)	**0.62**	2 (66.7%)	130 (64.4%)	**N.A.**
	female	47 (36.4%)	42 (41.6%)		12 (38.7%)	61 (35.1%)		1 (33.3%)	72 (35.6%)	
Histological subtypes*										
	SSM	40 (31.0%)	21 (20.8%)	**< 0.05***	6 (19.3%)	46 (26.4%)	**0.59***	0 (0.0%)	52 (25.7%)	**N.A.**
	NM	22 (17.1%)	23 (22.8%)		10 (32.3%)	33 (19.0%)		2 (66.7%)	41 (20.3%)	
	ALM	0 (0.0%)	1 (1.0%)		0 (0.0%)	1 (0.6%)		0 (0.0%)	1 (0.5%)	
	LMM	1 (0.8%)	3 (3.0%)		0 (0.0%)	3 (1.7%)		0 (0.0%)	3 (1.5%)	
	other	3 (2.3%)	11 (10.9%)		2 (6.5%)	9 (5.1%)		1 (33.3%)	10 (5.0%)	
	NOS	63 (48.8%)	42 (41.5%)		13 (41.9%)	82 (47.2%)		0 (0.0%)	95 (47.0%)	
Site of primary										
	head and neck	12 (9.3%)	12 (11.9%)	**0.46**	1 (3.2%)	21 (12.1%)	**0.56**	0 (0.0%)	22 (10.9%)	**N.A.**
	trunk	63 (48.8%)	28 (27.8%)		10 (32.2%)	70 (40.2%)		1 (33.3%)	79 (39.1%)	
	extremities	25 (19.4%)	27 (26.7%)		12 (38.8%)	33 (19.0%)		1 (33.3%)	44 (21.7%)	
	unknown	27 (20.9%)	18 (17.8%)		8 (25.8%)	35 (20.1%)		0 (0.0%)	43 (21.3%)	
	mucosal	0 (0.0%)	7 (6.9%)		0 (0.0%)	7 (4.0%)		1 (33.3%)	6 (3.0%)	
	uveal	2 (1.6%)	9 (8.9%)		0 (0.0%)	8 (4.6%)		0 (0.0%)	8 (4.0%)	
Initially metastatic disease										
	Yes	36 (27.9%)	25 (24.8%)	**0.59**	10 (32.2%)	47 (27.0%)	**0.73**	1 (33.3%)	56 (27.7%)	**N.A.**
	No	93 (72.1%)	76 (75.2%)		21 (67.8%)	127 (73.0%)		2 (66.7%)	146 (72.3%)	

* due to low number of specified groups results should be interpreted carefully

ALM = acral lentigo maligna; LMM = lentigo maligna melanomas; N.A. = not applicable, NOS = not other specified; NM = nodular melanoma; SSM = superficial spreading melanoma

NRAS on extremities (38.8%). Associations we have observed between anatomical location and histological subtypes with mutations have been described before and are consistent with meta-analysis results.[20,21,23]

In our study group we could also notice a trend to a higher frequency of BRAF mutation in superficial spreading melanoma and higher frequency of NRAS mutation in nodular melanoma (Figure 1), but results of statistical analysis were weak due to low sample number in some of the subgroups.

Correlations of clinical-pathological features with c-KIT mutation were not performed because of a small number of cases.

Conclusions

Today we are aware, that there are distinct sets of melanoma, with several genetic alterations that lead to molecular pathways dysregulation and influence the cell growth, proliferation and differentiation. Discovery of the drugs that target those mutations significantly changed every day clinical practise. Testing for BRAF mutation is today already recommended as a standard diagnostic test before starting systemic treatment in advanced melanoma. In patients without BRAF mutation some cancer centres perform additional testing, for other less frequent mutation as NRAS and c-KIT,

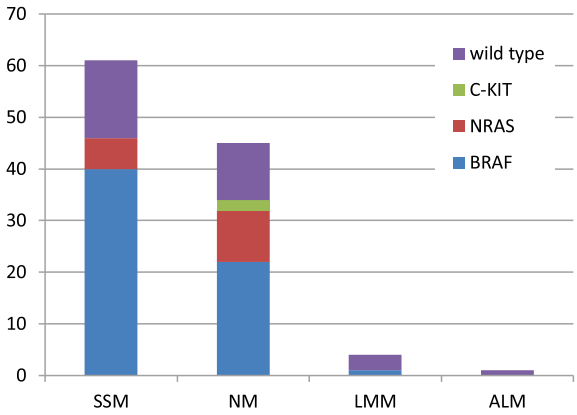

FIGURE 1. Distributions of BRAF, NRAS and c-KIT mutation according to common histological subtypes in cutaneous melanoma.

ALM = acral lentigo maligna; LMM = lentigo maligna melanomas; NOS = not other specified; NM = nodular melanoma; SSM = superficial spreading melanoma

that can further assist at how to individually adjust most appropriate systemic treatment.[25,26]

The main purpose of our study was to determine the frequency of most common mutations in melanoma and their correlation with histological characteristics in the Slovenian population. Our analysis detected higher rate of BRAF mutation, lower rate of NRAS and c-KIT mutation compared to previously published studies in Europe and North America. Explanation for such results is complex, mostly due to specific characteristic of our study group. The main consequence of high rate of BRAF mutation in our population will be a higher consumption of BRAF inhibitors. At the same time low c-KIT mutation among our population raise a question about the role and cost-benefit of implementation a c-KIT as a standard testing in our region.

Our study has limitations and results should be interpreted carefully. Our study group consist of patient with specific clinical and tumour tissue characteristics and do not represent general population. We have had also relatively small sample size and therefore some planned statistical analyses were not applicable. Therefore, the results may not be applicable for the general population of patients with melanoma in Slovenia.

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