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KIT, *NRAS* and *BRAF* mutations in sinonasal mucosal melanoma: a study of 56 cases

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Background: Mucosal melanomas in the head and neck region are most frequently located in the nasal cavity and paranasal sinuses. Sinonasal mucosal melanoma (SNMM) comprises <1% of all melanomas. The aim was to determine the *KIT*, *NRAS* and *BRAF* mutation frequencies in a large series of primary SNMMs.

Methods: Laser capture microdissection was used to isolate tumour cells from 56 formalin-fixed paraffin-embedded tumours. The tumour cells were screened for *KIT*, *NRAS* and *BRAF* mutations by direct sequencing.

Results: Overall, 21% (12 out of 56) of SNMMs harboured *KIT*, *NRAS* or *BRAF* mutations. Mutations in these oncogenes occurred in a mutually exclusive manner. Both *KIT* and *BRAF* mutations were identified at a similar frequency of 4% each (2 out of 56), whereas *NRAS* mutations were detected in 14% (8 out of 56) of the SNMMs. Four of the *NRAS* mutations were located in exon 1. Mutations in these oncogenes were significantly more common in melanomas located in the paranasal sinuses than in nasal cavity ($P=0.045$). In a multivariate analysis, patients with melanomas in the nasal cavity had a significantly better overall survival than those with tumours in the paranasal sinuses ($P=0.027$).

Conclusion: Our findings show that *KIT* and *BRAF* mutations, which are accessible for present targeted therapies, are only rarely present in SNMMs, whereas *NRAS* mutations seem to be relatively more frequent. The data show that majority of SNMMs harbour alterations in genes other than *KIT*, *NRAS* and *BRAF*.

Approximately 1–2% of all melanomas originate from the mucosal membranes in the digestive, respiratory and genitourinary tracts (Clifton *et al*, 2011; The National Board of Health and Welfare (1960-2009)). Mucosal melanomas in the head and neck region are most frequently located in the nasal cavity, followed by paranasal sinuses and oral cavity (Jethanamest *et al*, 2011). Primary sinonasal mucosal melanoma (SNMM), however, comprises <1% of all melanomas (Clifton *et al*, 2011), and conversely, SNMMs amount to only 1–9% of all malignant lesion of the nasal tract (Harbo *et al*, 1997; Norlander *et al*, 2003).

The incidence of SNMM in Sweden has increased significantly from 1960 through 2000, although not at the same pace as that of cutaneous melanoma (Jangard *et al*, 2013). For women, the incidence has doubled and for men it almost tripled comparing 1960–1964 *vs* 1995–2000. Patients with SNMM have a poor prognosis with 5-year survival rates of 20–28% (Lund *et al*, 2012; Jangard *et al*, 2013).

The mitogen-activated protein kinase and phosphatidylinositol-3 kinase-Akt pathways have critical roles in the pathogenesis of melanoma. Activation of these pathways in cutaneous and mucosal melanomas commonly occur through activating mutations in the *BRAF, NRAS* and *KIT* genes (Jovanovic *et al*, 2008; Omholt *et al*, 2011). However, mucosal melanomas have a distinct genetic background compared with cutaneous melanomas. For example, the frequency of *BRAF* mutation is significantly higher in melanoma arising in the trunk and skin without chronic sun damage than in mucosal melanomas (Curtin *et al*, 2005; Ellerhorst *et al*, 2011; Lee *et al*, 2011). On the other hand, *NRAS* mutations are frequently detected in melanomas located in extremities and skin with chronic sun damage (Ellerhorst *et al*, 2011; Lee *et al*,

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2011). Mucosal melanomas frequently harbour mutations and/or amplifications of the *KIT* gene, but very rarely contain *BRAF* mutations (Curtin *et al*, 2006; Beadling *et al*, 2008). Approximately 50 and 20% of cutaneous melanomas harbour *BRAF* and *NRAS* mutations, respectively (Davies *et al*, 2002; Omholt *et al*, 2003; Edlundh-Rose *et al*, 2006; Lee *et al*, 2011), whereas *KIT* mutations are detected in about $<2\%$ of melanomas in skin without chronic sun damage (Curtin *et al*, 2006; Handolias *et al*, 2010).

Interestingly, in a recent study of mucosal melanomas from several different sites, we found a significantly higher frequency of *KIT* mutations in vulvar melanomas compared with tumours of other sites (35% *vs* 10%), suggesting that the *KIT* mutation rate in mucosal melanomas varies with anatomical site (Omholt *et al*, 2011). So far, all of the published studies have analysed a small number of SNMM samples and the reported frequencies of mutations in SNMM vary considerably between these studies; *KIT*, 0–40%; *NRAS*, 22–60% and *BRAF* 0–6% (Cohen *et al*, 2004; Beadling *et al*, 2008; Carvajal *et al*, 2011; Schoenewolf *et al*, 2012; Turri-Zanoni *et al*, 2012).

Although most of the primary SNMMs are localised at diagnosis, radical surgical resection is difficult (Bradley, 2006) and therefore effective, alternative treatment options are essential for patients with these tumours. Molecular targeted therapy is now available for patients with malignant melanomas. A phase III trial has shown that vemurafenib, a selective BRAF inhibitor, improves both progression-free and overall survival compared with standard systemic chemotherapy (Chapman *et al*, 2011). Phase II trials and case reports have shown promising effects of targeted therapy with imatinib and dasatinib for patients with *KIT* mutant melanomas (Woodman and Davies, 2010; Carvajal *et al*, 2011). Very recently, a phase II trial showed that patients with *NRAS* mutant melanomas might benefit from treatment with MEK1/2 inhibitor (Ascierto *et al*, 2013). These novel therapeutic advances stress the importance of investigating the mutations in these oncogenes in patients with SNMM.

Given the rarity of SNMM, the frequency of *KIT*, *NRAS* and *BRAF* mutations has not been well characterised in these tumours. The purpose of the current study was to evaluate a large number of primary SNMMs in order to better define the frequency of *KIT*, *NRAS* and *BRAF* mutations.

MATERIALS AND METHODS

Tumour samples. Archival materials of formalin-fixed paraffin-embedded blocks of 61 SNMMs were collected from pathology departments throughout Sweden. Patients were diagnosed between 1986 and 2011 and were reported to the Swedish National Cancer Registry. All clinical records and pathological reports were collected and reviewed. We retrieved information on diagnosis, classification, disease site, overall survival and clinical features such as clinically reported pigmentation of tumours and reports of ulceration in pathological assessment. When data could not be appropriately determined, they were coded as missing. Five samples were excluded because the sections contained too few tumour cells. Thus, overall 56 primary SNMMs were included and 12 of these cases were part of a previously published data set (Omholt *et al*, 2011). This study was approved by the Research Ethics Committee, Karolinska Institutet, Stockholm, Sweden.

Laser capture microdissection and DNA extraction. Sections of 5 μm thickness were cut from formalin-fixed paraffin-embedded blocks and placed on plain slides. Sections were deparaffinised with two washes of xylene, rehydrated in increasing concentrations of ethanol, rinsed with deionised water, shortly stained with haematoxylin, rinsed with deionised water and dehydrated in decreased concentrations of ethanol and two washes of xylene. Tumour cells were microdissected from sections by laser capture microdissection (LCM) using the Arcturus PixCell LCM System (Arcturus Engineering, Mountain View, CA, USA) according to the manufacturer's recommendations. Samples were incubated overnight with proteinase K-enriched digestion buffer (PicoPure DNA Extraction KIT, Arcturus Engineering) to extract the DNA from the dissected cells. Proteinase K was then inactivated by heating samples at 95 °C for 10 min.

Mutation analysis. Genomic DNA was subjected to first and nested PCR to amplify *BRAF* (exon 15), *NRAS* (exons 1 and 2) and *KIT* (exons 11, 13 and 17) genes. In the first PCR, the DNA was amplified in a 10 μl mixture reaction containing 2.5 mM deoxynucleotide triphosphate, 5 U μl^{-1} platinumTaq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 50 pmol μl^{-1} of each primer, 10 × PCR buffer, 50 mM $MgCl_2$ and 10 $\mu g\,\mu l^{-1}$ bovine serum albumin. Two microlitres of the first PCR reaction was used as DNA template for the nested PCR. The DNA was extracted and purified from agarose gels by using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Sequencing reactions were performed in a final volume of 20 μl using BigDye Terminator V1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were purified by ethanol precipitation, and automated DNA sequencing was performed by ABI PRISM3130xl Genetic Analyzer (Applied Biosystems). All mutations were confirmed by a second independent PCR and sequencing reaction. The primers used for amplification and sequencing are described in Supplementary Table 1.

Statistical analysis. Fisher's exact test was used to correlate the mutation status with clinicopathological features such as gender, ulceration, anatomical site and pigmentation. Age at diagnosis was compared between the mutated and wild-type group using Wilcoxon rank-sum test. Overall survival was estimated from the date of diagnosis to the date of death or last follow-up (1 November 2012). Patients who were alive at end of the study were censored. Survival data were available for all patients. Multivariate Cox regression model, Log-rank test and Kaplan–Meier graphs were used to assess the association of anatomical site with overall survival. All *P*-values were two-sided. *P*-values <0.05 were considered statistically significant.

RESULTS

Clinicopathological characteristics. Patient and tumour characteristics are listed in Table 1. Overall, there were 35 females and 21 males with a median age at diagnosis of 76 years. Thirty-four tumours were located in the nasal cavity and 22 in the paranasal sinuses (10 in the maxillary sinuses, 6 in the ethmoid sinuses and 6 tumours invaded the surrounding structures: 4 involved the orbit; one the skull base and another one spread to the retromaxillary infratemporal fossa).

Mutation analysis. Of the 56 primary SNMMs analysed, 12 (21%) harboured *KIT*, *NRAS* or *BRAF* mutations and 44 (79%) were wild type. Mutations in *KIT*, *NRAS* and *BRAF* occurred in a mutually exclusive manner. The difference between *KIT*, *NRAS* and *BRAF* mutation frequencies in SNMMs was borderline significant ($P=0.058$).

KIT mutations were detected in 2 of the 56 SNMMs (4%). Both tumours with *KIT* mutations contained the hotspot mutation L576P in exon 11 (Table 2). No mutations were observed in exons 13 and 17. In our previous study, we identified a much higher frequency of *KIT* mutations in vulvar melanomas, with mutations in 8 of 23 tumours (35%; Omholt *et al*, 2011). Thus, in our material the difference between the updated results on *KIT* mutation

frequency in SNMMs and that previously presented for vulvar melanomas is statistically significant ($P = 0.001$).

Table 1. Patient and tumour characteristics

Characteristics	Total $n = 56$
Age at diagnosis, year	
Median	76
Mean	74
Range	52–97
Gender, *n* (%)	
Male	21 (37.5)
Female	35 (62.5)
Anatomical site, *n* (%)	
Nasal cavity	34 (60.7)
Paranasal sinus	22 (39.3)
Ulceration, *n* (%)	
Present	34 (60.7)
Absent	14 (25.0)
Data missing	8 (14.3)
Pigmentation, *n* (%)	
Present	23 (41.1)
Absent	25 (44.6)
Data missing	8 (14.3)
Ballantyne staging	
I	52 (92.8)
II	1 (1.8)
III	1 (1.8)
Data missing	2 (3.6)
Median survival, month (range)[a]	32 (2–230)
Alive, *n*	10
Dead, *n*	46

[a]Last updated on 1 November, 2012.

NRAS mutations were identified in 8 (14%) and *BRAF* mutations in 2 (4%) of the 56 SNMMs. Among the identified *NRAS* mutations, four were found in exon 1 (G12C, G12D, G12A and G13D) and four in exon 2 (Q61K, Q61R and Q61H ($n = 2$)). The *BRAF* mutations consisted of one V600E and one V600K change. Both *BRAF* mutated tumours were located in maxillary sinuses (Table 2).

Association of mutations with clinicopathological features. As the number of mutations identified was small, we compared the clinicopathological features between tumours with *KIT*, *NRAS* or *BRAF* mutations and those lacking these mutations. Tumours with mutations were more likely to be located in the paranasal sinuses, whereas the wild-type lesions were more often found in the nasal cavity and the difference was statistically significant ($P = 0.045$, Table 3). There was no difference between the mutated and wild-type group with respect to age at diagnosis, gender, ulceration and tumour pigmentation.

Survival. In univariate analysis, the age, anatomical site and clinical stage were significantly associated with overall survival. Patients with melanoma in the nasal cavity had a significantly better prognosis than those with a tumour in the paranasal sinuses (median survival, 39 *vs* 16 months; Log-rank $P = 0.027$; Figure 1). This effect remained significant in a multivariate analysis after adjusting for age at diagnosis, gender, ulceration, pigmentation and clinical stage ($P = 0.001$). The mutation status of the tumours showed no association with the overall survival.

DISCUSSION

Mutational data on SNMM are rare and there are only a few published reports with limited number of tumours (listed in Table 4). In this study, which is the largest of its kind, to our knowledge, we screened primary SNMM for mutations in some of the most commonly mutated oncogenes in cutaneous melanoma. We identified *KIT*, *NRAS* and *BRAF* mutations in 4%, 14% and 4% of tumours, respectively. The finding of *KIT* mutations in only 2 of 56 SNMMs suggests that *KIT* mutations differ between mucosal melanomas at different sites, and that they are very rare in this subtype of mucosal melanomas. Altogether, the present results and those of our previous study on mucosal melanomas from several different sites show that the *KIT* mutation frequency in SNMM is

Table 2. Summary of mutations identified in primary SNMM ($n = 56$)

Case	Gender	Age	Anatomical site	Gene	Exon	Nucleotide change	Amino-acid change
1	F	63	Nasal cavity	*KIT*	11	c.1727T>C	p.L576P
2	M	65	Maxillary sinus	*KIT*	11	c.1727T>C	p.L576P
3	M	88	Maxillary sinus	*NRAS*	1	c.34G>T	p.G12C
4	F	66	Maxillary sinus	*NRAS*	1	c.35G>A	p.G12D
5	M	78	Ethmoid sinus	*NRAS*	1	c.35G>C	p.G12A
6	F	97	Nasal cavity	*NRAS*	1	c.38G>A	p.G13D
7	M	70	Nasal cavity	*NRAS*	2	c.181C>A	p.Q61K
8	F	58	Maxillary sinus	*NRAS*	2	c.182A>G	p.Q61R
9	M	68	Maxillary sinus	*NRAS*	2	c.183A>C	p.Q61H
10	F	82	Nasal cavity	*NRAS*	2	c.183A>C	p.Q61H
11	F	80	Maxillary sinus	*BRAF*	15	c.1799T>A	p.V600E
12	F	52	Maxillary sinus	*BRAF*	15	c.1798GT>AA	p.V600K

Abbreviations: F = female; M = male; SNMM = sinonasal mucosal melanoma.

Table 3. Association of mutation status with clinical features in primary SNMMs ($n = 56$)

	Mutated[a] $n = 12$	Wild type[b] $n = 44$	*P*-value
Median age, year (range)	69 (52–97)	76 (52–93)	0.347
Gender, *n* (%)			0.748
Male	5 (41.7)	16 (36.4)	
Female	7 (58.3)	28 (63.6)	
Anatomical site, *n* (%)			
Nasal cavity	4 (33.3)	30 (68.2)	0.045
Paranasal sinus	8 (66.7)	14 (31.8)	
Ulceration, *n* (%)			0.656
Present	6 (50.0)	28 (63.6)	
Absent	1 (8.3)	13 (29.6)	
Data missing	5 (41.7)	3 (6.8)	
Pigmentation, *n* (%)			0.189
Present	3 (25.0)	20 (45.4)	
Absent	8 (66.7)	19 (43.2)	
Data missing	1(8.3)	5 (11.4)	

Abbreviation: SNMM = sinonasal mucosal melanoma.
[a]Mutated in *KIT*, *NRAS* or *BRAF*.
[b]Wild type in *KIT*, *NRAS* and *BRAF*.

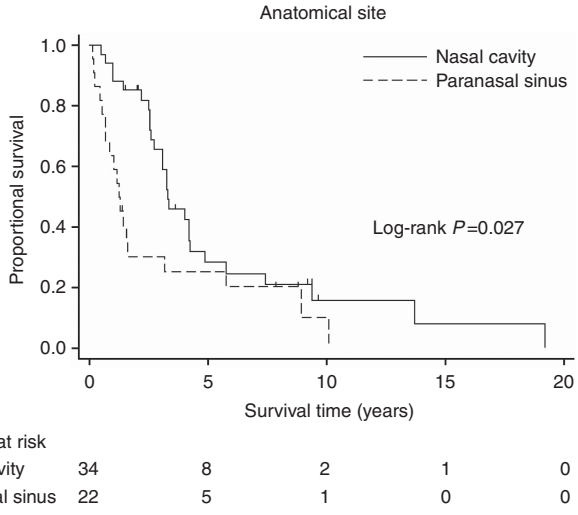

Figure 1. Overall survival of patients with SNMM located in the nasal cavity and paranasal sinuses.

significantly lower than that in vulvar melanomas (Omholt *et al*, 2011). Our results are also supported by Schoenewolf *et al* (2012), who in a recent study on sinonasal and vulvovaginal melanomas found no *KIT* mutations in 12 sinonasal tumours compared with 5 mutations in 11 vulvovaginal tumours (45%). Beadling *et al* (2008) also found a lower frequency of *KIT* mutations in melanomas of the head and neck (3 of 36; 8%) compared with melanomas of the anorectum/vulva/vagina (4 of 9; 44%).

In contrast to *KIT* mutations, the frequency of *BRAF* mutations is generally low in mucosal melanomas and does not seem to vary significantly between different sites (Omholt *et al*, 2011). In the current study, *BRAF* mutations were identified in 4% of SNMMs, which is similar to that detected in mucosal melanomas from other sites such as the vulva, vagina and anorectum (Curtin *et al*, 2006; Omholt *et al*, 2011).

The frequency of *NRAS* mutations that we identified in SNMM (14%) seem to be similar to that seen in cutaneous melanomas (Omholt *et al*, 2003; Edlundh-Rose *et al*, 2006; Lee *et al*, 2011). Interestingly, however, the types of *NRAS* mutations that we detected in SNMM differ from the types that predominate in cutaneous melanomas. In cutaneous melanomas, substitutions of glutamine for either arginine or lysine at codon 61 (Q61R and Q61K) represent the two most common *NRAS* mutations (Hocker and Tsao, 2007). In the current study, only two of eight *NRAS*-mutated tumours contained either of these mutations, whereas two had other alterations at codon 61 and four tumours contained mutations at codon 12 or 13 in exon 1. This indicates that *NRAS* mutations in mucosal melanomas, as opposed to cutaneous melanomas, are present in exon 1 and 2 with similar frequencies (Omholt *et al*, 2011; Turri-Zanoni *et al*, 2012). The *NRAS* mutations at codon 12 and 13 also predominate in other malignancies such as haematological cancers (Ward *et al*, 2012). The different pattern of *NRAS* mutations in mucosal melanoma, compared with cutaneous melanoma, possibly indicate an aetiology hitherto unknown but different from UV-radiation.

Interestingly, we found that mucosal melanomas located in the sinuses have a higher frequency of *KIT*, *NRAS* or *BRAF* mutations than those located in the nasal cavity. We also found that patients with disease emerging from the sinuses have a worse prognosis compared with those with tumours originating from the nasal cavity. This has also been observed in other studies (Liétin *et al*, 2010; Jethanamest *et al*, 2011). In the current study, the poor prognosis might be the result of more advanced tumour stage because in six cases the paranasal tumours invaded the surrounding structures. It remains to be addressed whether the adverse prognosis is associated with more aggressive biology and whether this is linked to the presence of oncogene mutations. Here we found no difference in overall survival between patients with mutated melanomas and those with wild-type melanomas;

Table 4. Summary of *KIT*, *NRAS* and *BRAF* mutations in SNMM

Reference	SNMM	*KIT*	*NRAS*	*BRAF*
Cohen *et al* (2004)	17	—	—	5.9% (1/17)
Beadling *et al* (2008)	29	8.4% (3/36)[a]	[b]	0.0% (0/29)
Carvajal *et al* (2011)	5	40.0% (2/5)	60.0% (3/5)	0.0% (0/5)
Schoenewolf *et al* (2012)	12	0.0% (0/12)	—	—
Turri-Zanoni *et al* (2012)	32	12.5% (4/32)	21.9% (7/32)	3.1% (1/32)
Current study	56	3.6% (2/56)	14.3% (8/56)	3.6% (2/56)
Total	151	7.8% (11/141)	19.3% (18/93)	2.9% (4/139)

Abbreviation: SNMM = sinonasal mucosal melanoma; '—' = not determined.
[a]29 melanomas were sinonasal and 7 were oral melanomas.
[b]The *NRAS* mutation frequency was not specified by anatomical site.

however, the number of mutations identified are too small, which can skew the results. In our previous study, we found that *KIT* mutations as well as *NRAS* mutations associated with poor survival in univariate but not in multivariate analysis (Omholt *et al*, 2011). In contrast to our results, a recent Chinese study showed that *KIT* mutations adversely affected survival (Kong *et al*, 2011); however, in this report mucosal melanomas were combined with cutaneous melanomas and a multivariate analysis was not performed.

The frequencies of *KIT* and *BRAF* mutations in SNMMs suggest that only a minority of patients with SNMM may benefit from treatment with KIT and BRAF inhibitors. The higher proportion of *NRAS*-mutated tumours suggest that it may be worthwhile to perform studies using MEK inhibitors, which have shown promising phase II results in cutaneous melanoma with *NRAS* mutations (Ascierto *et al*, 2013). It would be intriguing to investigate whether tumours with codon 12–13 activating mutations have similar therapeutic outcome as cutaneous melanomas with codon 61 mutations. Mutation analysis might yield positive results particularly in tumours from paranasal sinuses, as our results indicate that tumours from these areas more probably harbour mutations in *KIT*, *NRAS* or *BRAF* than the tumours from the nasal cavity. Still, a majority of SNMM has other unknown underlying oncogenic driver mutations that need to be addressed in future studies. Very recently, a high frequency of somatic mutations have been discovered in the promoter of telomerase reverse transcriptase (*TERT*) in cutaneous melanoma, resulting in increased transcriptional activity at the *TERT* promoter that might act as driver mutations (Horn *et al*, 2013; Huang *et al*, 2013). Presence of mutations in *TERT* promoter is still waiting to be determined in mucosal melanomas.

In conclusion, our results show that *KIT*, *NRAS* and *BRAF* mutations occur at low frequencies in SNMM, and confirm our recent findings that the frequency of *KIT* in mucosal melanoma mutations vary significantly between different anatomical sites.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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