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Intragenic *NF1* deletions in sinonasal mucosal malignant melanoma.

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ABSTRACT

Mucosal malignant melanoma (MMM) is a rare and aggressive tumor, most frequently originating in the oral and sinonasal cavities. Despite effective local therapies, tumor recurrence and metastasis remain frequent. The genetics of MMM remain incompletely understood. Fifteen MMM samples were analyzed by next-generation and Sanger sequencing. Gene copy number alterations were analyzed by MLPA. Mutation status was correlated with pERK and Ki67 expression and follow-up data. Inactivating mutations and intragenic deletions in *NF1* were identified in 3 and 2 cases, respectively (in total 5/15, 33%) and activating mutations in *NRAS* and *KRAS* (3/15, 20%) cases. Other mutated genes included *CDKN2A, APC, ATM, MITF, FGFR1* and *FGFR2*. *BRAF* and *KIT* mutations were not observed. Cases with *NF1* alterations tended to have worse overall survival. The mutational status was not associated with pERK or Ki67 immunostaining. MMM carry frequent gene mutations activating the MAPK pathway, similar to cutaneous melanoma. In contrast, however, *NF1* is the most frequently affected gene. Intragenic *NF1* deletions have not been described before and may go undetected by sequencing studies. This finding is clinically relevant as *NF1* mutated melanomas usually may have worse survival and could benefit from targeted therapy with immune checkpoint and MEK inhibitors.

Keywords: sinonasal cancer; mucosal melanoma; next-generation sequencing; mutation; intragenic deletion; NF1

INTRODUCTION

Mucosal malignant melanomas (MMM) are rare neoplasms that arise most frequently (55%) in the head and neck region.[1] Approximately 70% of these affect the sinonasal cavities and 20% in the oral cavity. Tumors are equally distributed between men and women and the mean age of onset lies between 60-70 years old, although it can occur in any age group.[2] MMM is a very aggressive and highly metastatic neoplasm with a 5-year overall survival rate of 20-40%.[3] Despite technological developments in surgery, radiation therapy and systemic modalities, no increased survival is achieved.[4]

The etiological factors remain unknown. Studies using exome or whole-genome sequencing have shown that MMM are similar to acral melanomas (AM), having a lower tumor mutation burden and more frequent structural chromosomal variants than cutaneous melanomas. In addition, mutations in MMM are not related to UV radiation or any other known carcinogen.[2] Most frequent mutations in cutaneous melanoma (CM) occur in *BRAF, CDKN2A* and *NRAS*, in AM these are *BRAF* and *NF1*, and in MMM *KIT*, *NRAS* and *SF3B1*. Mutations affecting the *TERT* promoter are frequent in all melanomas.[5]

During transformation and neoplastic progression, both melanomas acquire aberrant activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway.[6-7] Activating mutations in *NRAS, HRAS, KRAS, BRAF* and inactivating mutations in *NF1* lead to upregulation of transcription factors ERK1 and ERK2, thus promoting cell growth, survival and invasion. A framework for genomic classification of CM has been proposed by the Cancer Genome Atlas Network (TCGA) based on exome and genome sequencing studies. The four subtypes have been defined based on the mutational pattern in either *BRAF, RAS* or *NF1*, or none of these, the so-called triple-wild-type group.[1] Cirenajwis and coworkers demostrated that *NF1* tumors have significantly worse overall and disease free survival as compared to the other genomic subtypes, even after adjustment for age, gender and tumor type.[8] For MMM a possible prognostic value of this classification has not yet been described.

This study aimed to analyze actionable genetic alterations in a series of 15 sinonasal MMM. The main novelty was that *NF1* is frequently mutated, also by intragenic deletions that hitherto have not been described in melanoma or indeed any other cancer type.

MATERIAL AND METHODS

Patients

MMM from 15 patients treated between 1994 and 2018 were included in this study. Tumor samples, 14 primary and 1 metastasis, were recollected from the biobank archives of hospitals in Oviedo Spain, Hradec Kralove Czech Republic and Pisa Italy. All patients had signed an informed consent for the collection, analysis and storage of their biological material and the study was approved by the ethical committee (approval number 07/16 for project CICPF16008HERM). The mean age at diagnosis was 72 years, 7/15 (47%) patients were male and 8/15 (53%) were female. The major site of origin was the nasal cavity with 12/15 (80%) cases, 2/15 (13%) were located in the paranasal sinuses and 1/15 (7%) in the oral cavity. According to the AJCC staging system[9], 4/15 (27%) tumors were stage III, 8/15 (53%) stage IVa and 3/15 (20%) stage IVb. Treatment and follow-up data for all cases are listed in Table 1.

DNA extraction

Of 10 cases, tumor DNA was extracted from fresh frozen tissue samples using the Qiagen tissue extraction kit (Qiagen GmbH, Hilden, Germany). In 8 of these cases, also germline DNA from peripheral blood samples could be isolated with the Roche High Pure Template Preparation Kit (Roche Diagnostics GmbH, Manheim, Germany), following the supplier´s guidelines. Of 5 cases, tumor DNA was extracted from paraffin-embedded tissue samples with the QIAmp DNA Mini KIT (Qiagen GmbH, Hilden, Germany), using an elaborate deparaffinization and lysis protocol published previously.[10]

Mutation analysis

Sequence data from both tumor and corresponding germline DNA were obtained in 8 cases and in 2 cases only tumor DNA could be analyzed. Next-generation sequencing (NGS) using the SureSelect QXT Target Enrichment Kit for Illumina Multiplexed Sequencing (Agilent Technologies, Santa Clara CA, USA) was performed as described previously[11] using a panel covering all exons of the following 120 cancer-related genes: *AKT, AKT1, AKT3, ALK, APC, AR, ARAF, ATM, ATR, AURKA, BAP1, BCL2L1, BCR-ABL1, BCR-JAK2, BRAF, BRCA1, BRCA2, BRD4, CBL, CCND1, CCNE1, CDK4, CDK6, CDKN1A, CDKN1B, CDKN2A, CDKN2B, CDKN2C, COL1A1-PDGFRB, CRLF2, CSF1R, CSF3R, CTNNB1, DDR2, DNMT3A, EGFR, EPHA2, ERBB2, ERBB3, ERBB4, ERCC1, ERS1, EZH2, FBW7, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXA1, FOXL2, FOXP1, GNA11, GNAQ, HGF, HRAS, IDH1, IDH2, IGF1, IGF1R, IGF2, IL10, IL7R, INPP4B, JAK1, JAK2, JAK3, KIT, KMT2A, KMT2B, KMT2C, KMT2D, KRAS, LRP1B, MAP2K1, MAP2K2, MAP2K4, MCL1, MDM2, MET, MGMT, MITF, MLL, MPL, mTOR, MYCN, MYD88, NF1, NF2, NFKB1, NFKB2, NOTCH1, NOTCH2, NOTCH3, NPM1, NRAS, NTRK1, OTX1, OTX2, PALB2, PDGFRA, PIK3CA, PIK3R1, PIK3R2, PML-RARA, PRDM2, PRDM9, PRDM14, PTCH1, PTEN, RAC1, RAF1, RB1, RET, RET-PTC1, ROS1, SETD1A, SETD1B, SETD2, SH2B3, SMO, SOCS1, STAG2, STK11, TMPRSS2-ERG, TMPRSS2-ETV1, TP53, TSC1, TSC2*. Libraries in 16-plex pools were sequenced in a MiSeq system (Illumina Inc.) at the sequencing service of IMOMA (Oviedo, Spain). The coverage of the sequencing was between 212 and 420X.

For bioinformatic analysis the bioinformatics software HD Genome One certified with IVD/CE-marking (DREAMgenics, Oviedo, Spain) was applied as described by Sanchez et al.[11] In short, sequence variants with a minor allele frequency >1% in the normal population were filtered out and only non-synonymous changes with an impact on the sequence of the protein were considered. The 8 matched tumor/germline sequencing gave unequivocal results on the somatic status of the variants and only those with an allelic frequency >10% of the total reads in the tumor sample were considered relevant. With respect to the 2 tumor-only cases, only variants with > 50 reads were taken into consideration to avoid artifacts. Gene copy number information from a 120 gene NGS panel were used only when

indicating deletions of 0 copies or gains of ≥ 4 copies. The datasets generated in the study are in the process of depositing in a publicly available repository.

In 5 cases, mutations in *KRAS* exon 2 (codons 12 and 13), *HRAS*, *NRAS* and *BRAF* were analyzed by PCR Sanger sequencing. Amplification was carried out in a Simpliamp Thermal Cycler VXA24811 in standard conditions using the following primers: *KRAS* exon 2 Forward:TACTGGTGGAGTATTTGATAGTG / Reverse:CTGTATCAAAGAATGGTCCTG, *NRAS* exon 3 Forward:GGCAGAAATGGGCTTGAATA / Reverse:TCGCCTGTCCTCATGTATTG, *HRAS* exon 3 Forward:AGAGGCTGGCTGTGTGAACT / Reverse:TGGTGTTGTTGATGGCAAAC, *BRAF* exon 15 Forward:CTTCATAATGCTTGCTCTGATAGG / Reverse:GCATCTCAGGGCCAAAAAT, *NF1* exon 2 Forward:TGTGGTTGATGCAGTTTTCC / Reverse:GTGAGGCCGCTTATAACCAA, *NF1* exon 36 Forward:TCAAAACTGGTCAAATCAATGG / Reverse:CAAGGTGGCAGCAGGTAGTT, *NF1* exon 37 Forward:TCCTAGGGCAATCAGTCTTTC / Reverse:CCTACCGTAAACTCGGGTCA, *NF1* intron 49 Forward:TGTTCAGCATTTGGTGAGTACC / Reverse:GTGTTGGCCTGAGAAGGTTG. The PCR conditions were 5min/95°C + 32 cycles (30'' 95°C /45'' 58°C / 1min 72°C) + 7min/72°C + 4°C. PCR products were purified with Exo-BAP Mix (EURx Ltd, Gdansk, Poland) in accord with the manufacturer's specifications, and analyzed by Sanger sequencing using the ABI PRISM 3100 and 3730 Genetic Analyzer, (Applied Biosystems, Foster City CA).

NF1 gene copy number analysis

Multiplex Ligation-dependent probe amplification (MLPA) was performed as described in detail previously[12] using probemix P081-D1 *NF1* mix 1 and P082-C2 *NF1* mix 2 (MRC-Holland, Amsterdam, The Netherlands) that together contain probes for all 58 exons of *NF1* as well as one upstream (8 kb before exon 1) and one downstream probe (5 kb after exon 58). This mix allows the detection of aberrant copy numbers of specific exons of *NF1*. Tumor versus normal DNA ratios of <0.75 of two or more consecutive probes was interpreted as deletion and ratios of >1.25 of two or more consecutive probes was interpreted as gain.

Immunohistochemistry

Immunohistochemistry (IHC) on whole tissue sections was performed on an automatic staining workstation (Dako Autostainer Plus; DakoCytomation, Glostrup, Denmark) with antigen retrieval by EnVision FLEX+Mouse. The antibodies used for IHC were: pERK (MAPK) rabbit anti-phospho-p44/42 clone D13.14.4E, (Cell Signaling Technology, Cambridge, UK) at a 1:500 dilution and ready to use Ki67 mouse anti-Ki-67 clone MIB-1 (DAKO, Glostrup, Denmark). Nuclear and/or cytoplasmic pERK staining and nuclear Ki67 staining was evaluated by two investigators (CR and VBL) and scored as the percentage of positive tumor cells (0-100%).

Statistical analysis

Kaplan-Meier curves were plotted to assess the relation of *NF1* and *NRAS* status to overall survival, using the log-rank-test. P-values < 0.05 were considered to indicate statistical significance. Statistical analysis was carried out with the use of SPSS Base, version 15.0 and SPSS Advanced models, version 15.0 (SPSS Inc., Chicago, IL, USA) software.

RESULTS

All patients were treated surgically, 8/15 (53%) by nasal endoscopic surgery and 7/15 (47%) by open approaches, 5/15 (33%) patients received complementary radiotherapy. Local recurrence was observed in 9/15 patients (60%) with a mean time to recurrence of 19 months, 5/15 (13%) developed metastases, 3 of which were distant metastases. The mean overall survival was 57 months and the mean disease free survival 20 months (Table 1).

NGS showed one or more non-synonymous sequence variants with effect on the amino acid sequence in 5/8 paired tumor-germline cases, with an average of 2 (range: 1-4) per tumor. Three

tumors did not harbor somatic mutation in any of the 120 analyzed genes. The most frequently altered gene was *NF1* in 3/8 cases, two with a truncating or splice mutation and one with a partial gene copy number loss. Other pathogenic or likely pathogenic gene mutations affected *NRAS* in two cases, and *CDKN2A, APC, ATM*, each occurring in one case.

NGS of the 2 tumor-only cases yielded respectively 9 and 15 mutations, however, somatic status could not be ascertained for these variants. According to the Varsome prediction algorithm, variants in *NF1, KRAS, MITF, FGFR1* and *FGFR2* (all in one case) were pathogenic or likely pathogenic (Supplementary Table 1).

The MAPK pathway mutations in *NF1, NRAS* and *KRAS* observed by NGS in 6/10 tumors were confirmed by PCR Sanger sequencing (Figure 1). Five additional tumor samples derived from FFPE tissue blocks were analyzed but yielded no mutations in *NRAS, KRAS HRAS* or *BRAF*. MLPA analysis confirmed the partial loss of *NF1* in case 4, with tumor/normal ratio of 1.03 for exons 1-30 and ratio 0.08 for exons 31-58, indicating homozygous deletion. Analysis of the other 14 tumors revealed one additional tumor (case 13) carrying partial loss of *NF1*, with tumor/normal ratio of 0.67 for exons 1-14 and exons 41-58, and ratio 0.25 for exons 15-40 (Figure 2). In sum, 7/15 (47%) of cases harbored MAPK pathway mutations *in NF1, NRAS* or *KRAS*. A case-by-case list of all variants in 15 cases is presented in Supplementary Table 1. Kaplan Meier survival analysis showed a tendency for worse overall survival for *NF1* mutated cases and best overall survival for *NRAS* mutated cases (Supplementary Figure 1).

Ki67 and pERK protein expression levels were evaluated as markers of MAPK pathway activity. Positivity of Ki67 and pERK ranged between 5-40% and 0-90% respectively. Immunohistochemical staining of both markers was not related to MAPK pathway mutations. Supplementary Figure 2 shows the pERK and Ki67 staining of 4 cases with different MAPK mutation status.

DISCUSSION

Over recent years CM has been genetically well characterized and shown to present MAPK pathway gene mutations in over 90% of cases, up to 50% of which affecting *BRAF*, close to 30% *NRAS* and approximately 15% *NF1*.[5,8,13-14] Also some 50-90% of MMM carry alterations in the MAPK pathway, although with a different distribution among these genes. Reports have indicated mutations at an incidence of 6% in *BRAF* (range 0-32%), 20% in *NRAS* (range 5-41%) and 15% in *NF1* (range (4-37%), while also *KIT, SF3B1* and *SPRED* mutations are observed in 15% (range 3-47%).[5,8,15-23] In the present study on 15 MMM, *BRAF* and *KIT* mutations were absent, while *NRAS* and *NF1* mutations occurred in 13% and 33% of cases, respectively. These percentages fall within the published ranges. One of the cases had both *NF1* and *KRAS* mutation. Simultaneous *NF1/RAS* mutations have been described before and may indicate a more robust MAPK activation.[14] Other mutations seen in only one of the cases concerned *CDKN2A, APC, ATM, MITF, FGFR1* and *FGFR2*. With the exception of *FGFR1*, all have been reported previously in NGS studies on CM and MMM.[2,5,13-14]

A new finding in this study was that, aside from three cases with inactivating mutations, two tumors carried inactivating intragenic losses comprising several exons of *NF1*. An in-depth search of the literature on all types of melanoma yielded reports on gross deletions including the whole *NF1* gene, but none on intragenic deletions, also using the terms exonic or partial deletions.[2,5-8,13-24] This is probably due to the genetic techniques generally used. Sanger sequencing generates information on mutations but not on gene copy number. Also next-generation sequencing, often performed using a reduced gene panel and without co-sequencing the germline DNA, does not produce copy number information. Eight cases in this cohort were analyzed by a 120 gene panel NGS analyzing both tumor and germline DNA, and in one case bioinformatic analysis suggested partial deletion in *NF1*, reason why MLPA was applied for confirmation. MLPA showed homozygous deletion for exons 31-58, and subsequent analysis of the other 14 cases revealed one more case with a deletion of exons 15-40 (Figure 2).

Intragenic deletions and duplications of the *NF1* gene have been described in the germline of Neurofibromatosis Type 1 (NF1) patients, affecting different exons of the gene.[6,25-26] Hsiao et al. found that germline *NF1* intragenic alterations vary in size, location, and rearrangement mechanisms, and suggested a mechanism involving fork stalling and template switching (FoSTeS), and microhomology-mediated break-induced replication (MMBIR).[26] It is unclear if somatic intragenic deletions such as in the present study are caused in the same way and/or if they reflect structural chromosomal rearrangements. In CM the *NF1* mutation subtype has been shown biologically different with a stronger correlation with UV radiation mutagenesis and a higher tumor mutational burden. It is generally believed that UV radiation does not play a role in MMM, although in a recent paper Newell et al. claimed that this may be true for lower body mucosal sites, but not for all cases arising in the oral and nasal cavities.[2] Still it is unlikely that UV radiation is involved in somatic partial deletions of *NF1*. Proliferation rate and pERK expression did not appear associated with *NF1, NRAS* or combined *NF1/NRAS* status (Supplementary Figure 2). This finding is similar to CM where *BRAF/NRAS* mutations and the ERK phosphorylation status also no correlation between found in cutaneous melanomas.[27]

Cirenajwis et al. reported that *NF1* subtype is a distinct clinical entity of CM, affecting older male patients with worse overall and disease free survival.[8] To date, such a relation has not been reported in MMM. Amit and coworkers found that mutation status of *NRAS, BRAF* and *KIT* did not affect survival.[18] The series of 15 cases in this paper is too small for relevant statistical analyses, however, Kaplan Meier survival analysis showed a tendency toward worse overall survival for the 5 patients with *NF1* alterations, while the 2 patients with *NRAS* mutation had a very favorable overall survival (Supplementary Figure 1). Aside from being an adverse prognostic marker, *NF1* status can be a marker for response to immunotherapy by its association with high tumor mutational burden. In addition, both *NF1* and *NRAS/KRAS* status may be indicators for therapy with MAPK pathway inhibitors.[2,8,24] The introduction of immune checkpoint and MEK inhibitors have improved the treatment of CM and the growing knowledge on genetic alterations indicates their relevance for MMM

as well**.** Considering the high metastatic rate of MMM in spite of relatively good local control, the combination of surgery with adjuvant targeted inhibitors could have significant survival benefit.

In conclusion, *NF1* mutations occur frequently in MMM and may indicate worse prognosis but also a options for targeted therapy. The fact that two of the five *NF1* alterations in this study concerned intragenic deletions which are generally overlooked in sequencing studies suggest an even greater role for this gene in MMM and possibly also in CM.

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AUTHOR CONTRIBUTIONS

M.A.H., C.R. and J.L.L. contributed to the study conception and design. J.L., A.F., F.L., and J.L.L. were responsible for tissue sample and clinical data collection. Material preparation, experiments and data analysis were performed by C.R., R.C.M., L.S.F., R.G.M., V.N.C., and V.B.L. The first draft of the manuscript was written by C.R., R.C.M. and M.A.H. and all authors commented on the draft versions of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Mutations in MAPK pathway genes. Activating missense mutations are seen in *NRAS* (A,B) and *KRAS* (C), and nonsense, splice, frameshift and inframe deletion mutation in *NF1* (respectively D, E, F and G).

Figure 2. MLPA copy number analysis of the *NF1* gene. Case 13 shows deletion of exons 15 to 40 with a mean tumor/normal ratio of 0.25 versus 0.67 in the other exons (A). Case 4 shows deletion of exons 31 to 58 with a mean tumor/normal ratio of 0.08 versus 1.03 in the other exons (B).

Table 1: Clinical and follow-up data of all tumors.

VARIABLE	NUMBER (%)
Gender	
Male	7/15 (47%)
Female	8/15 (53%)
Localization	
Nasal cavity	12/15 (80%)
Paranasal sinuses	2/15 (13%)
Oral cavity	1/15 (7%)
Tumor stage	
III	4/15 (27%)
IVa	8/15 (53%)
IVb	3/15 (20%)
Surgery	
Endoscopic	8/15 (53%)
Open	7/15 (47%)
Adjuvant treatment	6/15 (40%)
Radiotherapy	5/15 (33%)
Immunotherapy*	1/15 (6%)
Follow-up	
Local recurrence	9/15 (60%)
Distant metastasis	3/15 (20%)
Lymph node metastasis	2/15 (13%)
Both distant and lymph node metastasis	2/15 (13%)
Patient status	
Alive	6/15 (40%)
Died of disease	4/15 (27%)
Died of other causes	5/15 (33%)

Legend: *This patient received Interferon-gamma

Figure 1

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Figure
Riobello Figure 1.jpg

Figure 2

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Figure
Riobello Figure 2.jpg

Supplementary data to:

Intragenic *NF1* deletions in sinonasal mucosal malignant melanoma.

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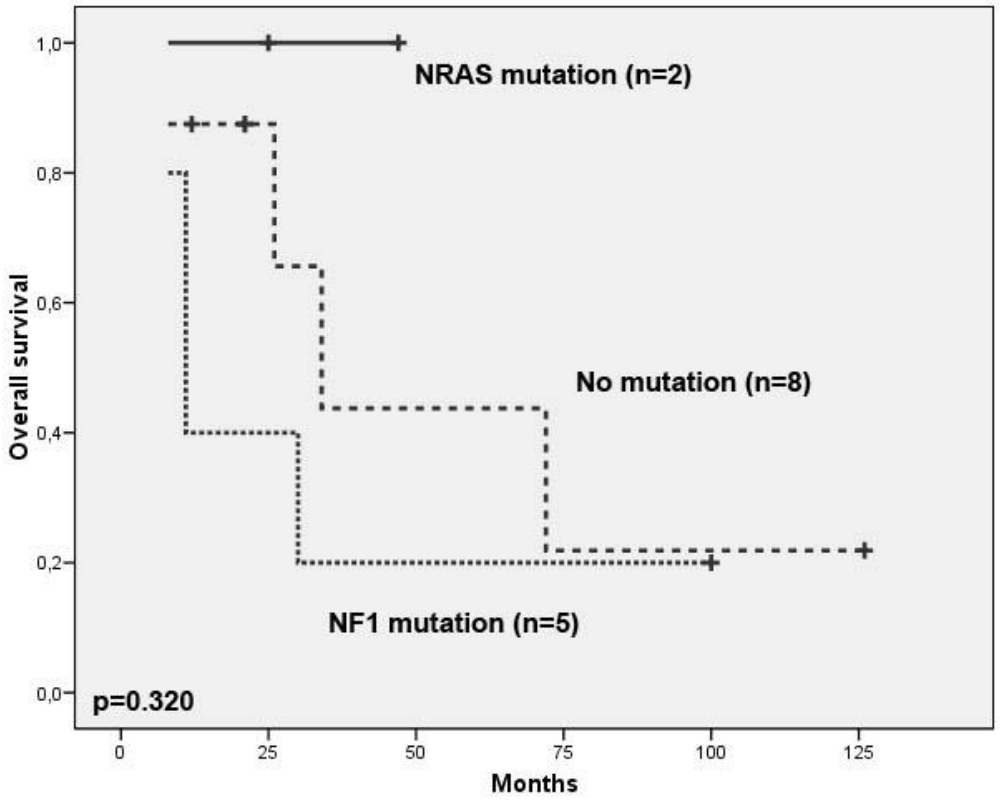
Supplementary Table 1. Case-by-case description of mutations*, deletions and p-ERK and Ki-67 protein expression.

Case	Gene	c.Hgvs	p.Hgvs	Germline DNA variant frequency	Tumor DNA variant frequency	NF1 MLPA	p-ERK protein expression	Ki-67 protein expression
1	*NF1*	c.4923G>A	p.Trp1641*	0	0,32731377	wt	20%	40%
2	*NRAS*	c.183A>T	p.Gln61His	0	0,435294118	wt	40%	10%
3	-	-	-	-	-	wt	0%	15%
4	*CDKN2A*	c.143C>T	p.Pro48Leu	0,053977273	0,413294798		50%	20%
	APC	c.1186dupG	p.Asp396Glyfs*2	0,052050473	0,726190476			
	ATM	c.332G>A	p.Arg111Lys	0,041958042	0,779411765			
	NF1	-	-	-	-	Partial loss exons 31-58		
5	-	-	-	-	-	wt	NE#	NE#
6	-	-	-	-	-	wt	50%	5%
7	-	-	-	-	-	wt	10%	25%
8	*NRAS*	c.181C>A	p.Gln61Lys	0	0,313609467	wt	1%	25%
9	*NF1*	c.7395-1G>A	-	0	0,303468208	wt	60%	35%
10	*MITF*	c.1255G>A	p.Glu419Lys	not available	0,561170213	wt	90%	10%
	FGFR1	c.1375G>T	p.Gly459Trp	not available	0,110320285			
	FGFR2	c.301A>G	p.Ile101Val	not available	0,876373626			
	KRAS	c.38G>A	p.Gly13Asp	not available	0,369565217			
	NF1	c.73dupA	p.Thr25Asnfs*13	not available	0,470967742			
	NF1	c.5371_5373del	p.Phe1791del	not available	0,203910615			
11ǂ	-	-	-	-	-	wt	70%	40%
12ǂ	-	-	-	-	-	wt	0%	20%
13ǂ	*NF1*	-	-	-	-	Partial loss exons 15-40	20%	20%
14ǂ	-	-	-	-	-	wt	0%	20%
15ǂ	-	-	-	-	-	wt	305	40%

Legend. *: Only mutations regarded as pathogenic or likely pathogenic according to the Varsome prediction. ǂ: Sequenced for *KRAS/NRAS/HRAS/BRAF* hotspots; MLPA: multiplex ligation-dependent probe amplification; NE: Not evaluable due to excessive presence of melanin.

Supplementary Figure 1. Overall survival according to *NF1* and *NRAS* mutation status.



Supplementary Figure 2. Immunohistochemical stainings of p-ERK and Ki-67 in relation to MAPK pathway gene mutations in 4 cases.