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Time and tumor type (primary or metastatic) do not influence the detection of *BRAF/NRAS* mutations in formalin fixed paraffin embedded samples from melanomas

Abstract

Background: *BRAF* and *NRAS* mutation detection is
crucial for advanced melanoma treatment. Our aim was to
evaluate how different characteristics from formalin-fixed
paraffin-embedded (FFPE) samples, age of the block or
DNA concentration could influence the success of *BRAF*
and *NRAS* mutational screening.

Methods: DNA was obtained from 144 FFPE samples (62
primary melanoma, 43 sentinel lymph nodes [SLN] and 39
metastasis). *BRAF* and *NRAS* were sequenced by Sanger
sequencing.

Results: Complete sequencing results were obtained from
75% (108/144) of the samples, and at least one gene was
sequenced in 89% (128/144) of them. *BRAF* was mutated
in 55% (29/53) and *NRAS* in 11% (5/45) of the primary
melanomas sequenced. DNA concentration correlated
with the tumor area used for DNA extraction (mm^2) (adj
p-value<0.01, r=0.73). The age of the block did not affect
sequencing success. In 60% of samples kept for more
than 10 years, both *BRAF* and *NRAS* were successfully
sequenced.

Conclusions: Preserving sufficient tumor area in FFPE
blocks is important. It is necessary to keep the FFPE
blocks, no matter their age, as they are necessary to decide
the best treatment for the melanoma patient.

Keywords: BRAF; FFPE; melanoma; NRAS; somatic
mutations.

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Introduction

Melanoma is the most deadly of the common skin cancers.
Melanoma mortality is around 20%, but when the tumor
is diagnosed with a thickness exceeding 4 mm, the mor-
tality rises to 60% [1]. Targeted therapy has become one
of the most important strategies for the treatment of meta-
static patients [2].

The MAPK-ERK pathway, which includes the RAF,
MEK and ERK protein kinases and the small G protein RAS,
plays an important role in melanoma development and
progression [3]. Mutations in *BRAF* are found in approxi-
mately 50% of melanomas [4]. *BRAF* p.V600E is found in
up to 90% of *BRAF* mutated tumors [5], which allowed the
development of targeted therapies against this mutated

protein. BRAF-inhibitors now play an important role in targeted therapy in melanoma [6]. *NRAS* is the second most commonly mutated MAPK-related gene in melanoma tumors (13–22%) [7]. To date it has been very difficult to target this molecule. Furthermore, *NRAS* mutations can mediate BRAF-inhibitor resistance [8]. New primary melanomas, *BRAF* wild type, can be developed during BRAF inhibition [9–12]. Thus, the detection of mutations in *BRAF* and *NRAS* genes in melanoma tumors is crucial to decide the best treatment for metastatic patients and in the analysis of new tumors developed during BRAF inhibition therapy.

Formalin fixation and paraffin embedding (FFPE) is the conventional method for preserving melanoma tumors [13]. We hypothesize that several characteristics of the block and the sample could influence the capacity to correctly detect *BRAF* and *NRAS* mutations in melanoma tumors. The aim of this project was to assess whether different parameters evaluated on hematoxylin-eosin stained slides and the age of the block, could affect DNA extraction and sequencing success.

Materials and methods

Patients and samples

The study initially included 165 FFPE samples belonging to 96 melanoma patients from four groups: stage II free of relapse, stage II with relapse, stage III free of relapse and stage III with relapse, respectively (Figure 1). Melanoma subtype from pathology reports was available in 86% of the samples and Breslow thickness in 95% (Table 1). Samples were collected from January 1994 to December 2011. The study was approved by the Ethical Committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

Different parameters were evaluated on Hematoxylin-Eosin stained slides: the percentage of tumor present in the whole section, tumor cells in tumor area, immune cells in tumor area, stroma cells in tumor area, other cells in tumor area and total tumor area used for DNA extraction (mm^2). According to these parameters, the number of sections needed per DNA extraction to obtain 50 mm^2 of tumor area with at least 50% of tumor cells was estimated.

The age of the block was also recorded. All tissues had been routinely fixed in 4% buffered neutral formalin and embedded in paraffin. Formalin fixation was overnight except for weekend and holidays
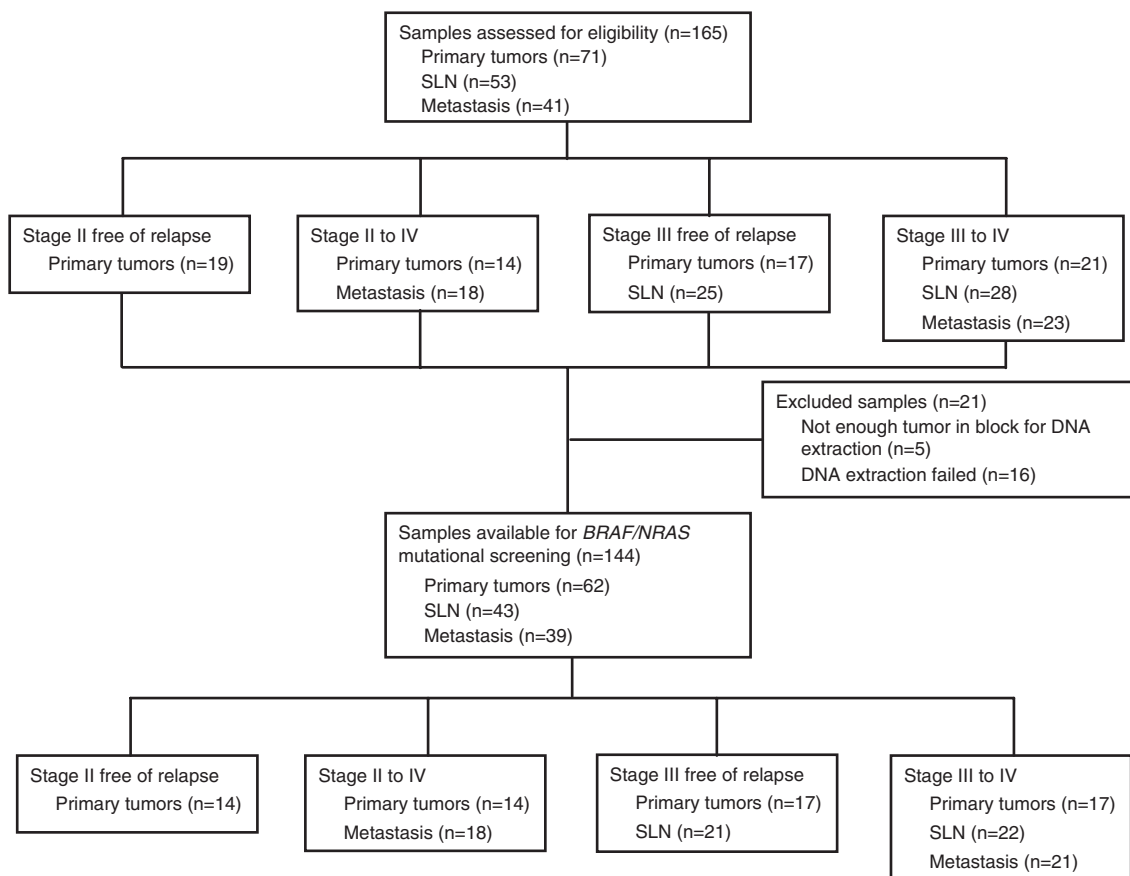

Figure 1: Flowchart of samples included in the study.

Table 1: Primary tumors description.

Melanoma subtype	Stage II		Stage III		Total n (%)
	Free of relapse n (%)	With relapse n (%)	Free of relapse n (%)	With relapse n (%)	
SSM	13 (76%)	5 (63%)	15 (88%)	8 (42%)	41 (67%)
NM	4 (24%)	1 (12%)	2 (12%)	8 (42%)	15 (25%)
ALM	–	2 (25%)	–	1 (5%)	3 (5%)
LMM	–	–	–	1 (5%)	1 (1.5%)
Unclassifiable	–	–	–	1 (5%)	1 (1.5%)
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Breslow (mm)	3.22 (1.59)	5.4 (3.89)	1.72 (1.12)	4.31 (4.54)	3.53 (3.29)

Unknown subtype: 2 Stage II free of relapse, 6 Stage II with relapse and 2 Stage III with relapse.
SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma; LMM, lentigo malignant melanoma.

where fixation could last 2–3 days. FFPE samples were stored at room temperature.

BRAF and *NRAS* mutational screening

DNA extraction was performed using the QIAmp DNA FFPE Tissue Kit (Qiagen, USA). DNA concentration was calculated using the Nanodrop (Thermo Fisher Scientific Inc., USA) absorbance measure method.

BRAF exons 11 and 15, and *NRAS* exons 2 and 3 were amplified by PCR using the PCR Master Mix (Promega, USA) with the primers and conditions described in the Supplementary Material. For samples that did not amplify in the first PCR, internal primers were designed for each exon to perform nested PCR (Supplementary Material).

The same primers were used for sequencing. The Sanger sequencing process was carried out by Macrogen (Macrogen Europe, The Netherlands) and sequences were analyzed using the SeqPilot Software (JSI Medical Systems GmbH, Germany). If sufficient DNA was available after *BRAF* and *NRAS* sequencing, a fluorescent allele-specific PCR followed by capillary electrophoresis was performed to detect the *BRAF* p.V600E mutation, as previously described [14]. The sample was considered positive for p.V600E only after this variant was confirmed at least twice. The limitation of this study is that the number of repeats needed to obtain the sequence for each sample was not considered.

Statistical analyses

Different characteristics of the sample were evaluated according to sample type, age of the block (<5 years, 5 to 10 years, >10 years), sequencing success defined as having a sequence that could be clearly evaluated (both *BRAF* and *NRAS* sequenced – if at least exon 15 of *BRAF* and exon 3 of *NRAS* were correctly sequenced it was considered successful – only one gene sequenced, neither gene successfully sequenced) and mutational status of the samples (*BRAF/NRAS* WT vs. mutated). Sequencing success and mutational status was also evaluated according to the age of the block. To compare means, non-parametric tests (Kruskal-Wallis) were used as continuous variables did not follow a Normal distribution. The Pearson's correlation and

the two-sided Fisher's exact test were used for continuous or categorical variables analyses, respectively. All p-values were corrected using the false discovery rate method (http://www.sdmproject.com/utilities/?show=FDR).

Results

Mutational analyses

Of the 165 total samples assessed for eligibility, DNA was correctly extracted in 87% (144/165), DNA extraction failed in 10% (16/165) and 3% (5/165) samples had not enough tumor in the block (Figure 1). From samples in which DNA extraction failed, 6/16 were primary tumors, 9/16 SLN and 1/16 a metastasis. The age of block did not affect the DNA extraction success (Table 2). The median area used for DNA extraction was significantly smaller in these samples (3.8 mm) compared to the ones with DNA available (6.67 mm) (adj. p=0.01).

From the 144 samples with DNA available, *BRAF* sequencing results were obtained from 87% (125/144). Overall, 55% (29/53) of primary tumors sequenced, 49% (20/41) of SLN and 42% (13/31) of metastases had mutations in *BRAF*. We identified the p.V600E mutation in 98% (61/62) of mutant *BRAF* samples and p.V600M in

Table 2: DNA extraction success according the age of block.

Age of block	Succeeded n (%)	Failed n (%)	Adjusted p-value
<5 years	37 (25.7)	4 (25.0)	
5 to 10 years	73 (50.7)	9 (56.3)	1.000
>10 years	34 (23.6)	3 (18.8)	
Total	144 (90.0)	16 (10.0)	–

the remaining case. We did not detect mutations in exon 11 of *BRAF*. We sequenced *NRAS* in 80% (115/143) of all samples. We detected *NRAS* mutations in 11% (5/45) of the primary tumors, 12% (5/41) SLN and 14% (4/29) metastasis. p.Q61R was the most frequent *NRAS* mutation (35.7%, 5/14) followed by p.Q61K (28.6%, 4/14), p.Q13R (21.4%, 3/14) and both p.Q61H and p.G60R were detected in one sample each.

We assessed the concordance of the mutational results between samples from the same patient (Figure 2). There was a concordance between the genetic result of the primary tumor and the SLN or metastasis in 77% (17/24) and 85% (11/13) of cases, and between SLN and metastasis in 44% (4/9) of cases, respectively.

We did not detect any statistically significant differences between the different parameters assessed in FFPE samples according to sequencing results or age of the block (Table S1). The area used for DNA extraction was higher in metastasis, which also had a higher amount of tumor cells (adj. p<0.01) and a higher DNA concentration (adj. p=0.02), than primary tumor or SLN. We did not find statistically significant differences between the age of the block and the success of the sequencing process or the capacity to detect mutations in *BRAF* and *NRAS* (Table 3). DNA concentration correlated with total tissue area used for DNA extraction (adj. p<0.01, r=0.73), % of tumor in whole section (adj. p<0.01, r=0.40) and a non-significant tendency was observed in % of tumor cells in tumor area (adj. p=0.06, r=0.19).

Discussion

Here we have evaluated how different characteristics of FFPE samples, including DNA concentration, influence the success of mutational screening of *BRAF* and *NRAS*, key genes for choosing appropriate targeted therapy treatment in advanced melanoma. We selected 165 FFPE samples, including primary melanomas, SLN and metastasis that were paired in some cases. DNA was obtained in 87% of samples. DNA extraction failure was associated with a smaller tumor area but not with the age of the block. Furthermore, the parameter that best correlated with DNA concentration was the tumor area used for DNA extraction. Thus, when possible, we should preserve large tumor areas in the FFPE blocks.

From 144 samples with DNA, *BRAF* was sequenced in 87% of samples and *NRAS* in 80%. The frequency of genomic mutations detected in primary tumors correlated with that previously reported [4, 15]. Shitara et al. showed that laser microdissection does not alter the frequency of *BRAF* and *NRAS* mutations [15]. We observed discordances between mutations found in the primary melanoma and the SLN and/or the metastasis in some cases. This evidence has already been reported [16]. As previously observed [15, 17–19], in some samples we have detected co-occurrence of *BRAF* and *NRAS* mutations. At the single cell level, mutations in *BRAF* or *NRAS* are mutually exclusive [17]. Supporting this hypothesis, in the paired SLN of a primary tumor with *BRAF* p.V600E and *NRAS* mutation co-occurrence, only a *NRAS* mutation was detected.
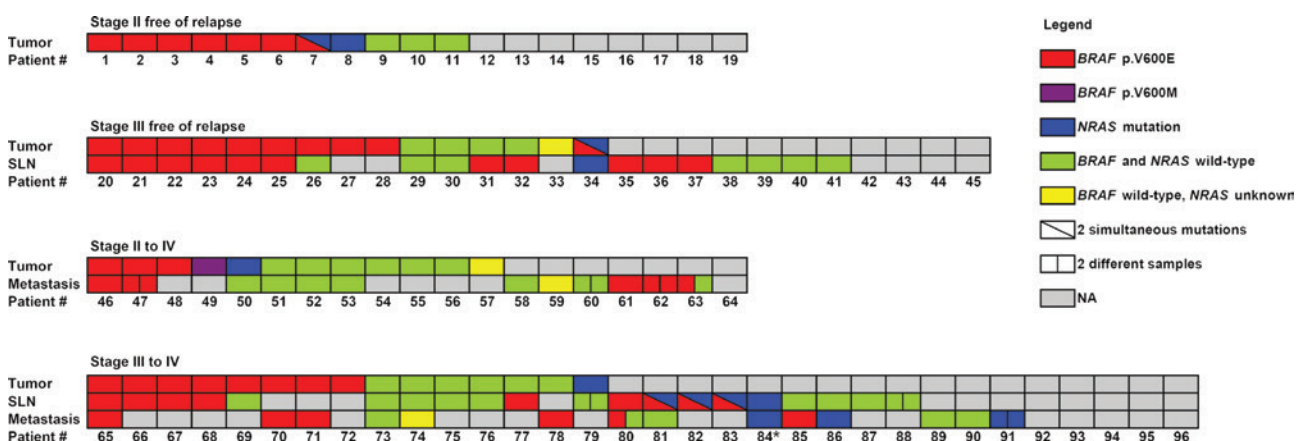

Figure 2: Genetic results by patient and samples.
The Figure shows the genetic results detected by sample type and patient to illustrate the mutation concordance between samples from the same individual. Patients are distributed according to the different staging groups: stage II free of relapse, stage III free of relapse, stage II that progressed to stage IV and stage III that progressed to stage IV. *NRAS* mutations detected were: p.Q13R, p.G60R, p.Q61R, p.Q61K, p.Q61H. *In Patient #84 different *NRAS* mutations were detected in the SLN and the metastasis. Patient #: Number given to each patient. SLN, sentinel lymph node. NA: indicates samples with no genetic results (includes samples assessed for eligibility but excluded, those in which the sequence could not be properly analyzed and those blocks that were not available for the study).

Table 3: Sequencing results according to the age of the block.

	<5 years (n=37) n (%)	5–10 years (n=73) n (%)	>10 years (n=34) n (%)	Adjusted p-value
Sequencing success				
All genes	31 (83.8)	59 (80.8)	21 (61.8)	0.13
One gene	4 (10.8)	5 (6.8)	8 (23.5)	
None	2 (5.4)	9 (12.3)	5 (14.7)	
BRAF status				
WT	20 (57.1)	34 (54.0)	9 (33.3)	0.27
mutated	15 (42.9)	29 (46.0)	18 (66.7)	
NRAS status				
WT	27 (84.4)	54 (90.0)	20 (87.0)	0.76
mutated	5 (15.6)	6 (10.0)	3 (13.0)	

The sequencing success is defined as having a sequence that could be clearly evaluated. Adjusted p-values were calculated using the false discovery rate method.

It is recommended to perform the molecular screening on tumor tissue, preferably derived from the metastasis [20]. However, as *BRAF* inhibitors may induce metastasis in *RAS* mutant tumors [21], it would be advisable to test both the primary melanoma and the different metastasis tissue available to ensure that there are no different driver mutations within samples that could influence the treatment's success.

Even though more pathology colleges/societies recommend archiving FFPE tumors for at least 10 years [22–24] or even 30 years [25], it is not regulated by law in most Countries. There is no single regulation that covers FFPE tissue specimens in the US [26] and in Spain the requirement is of 5 year storage after the last visit of the patient in the institution as part of the clinical record (Spanish Law 41/2002, 14th November). However, a melanoma patient may develop metastasis 10 or more years after the primary tumor diagnosis and having the FFPE specimen preserved could be essential for the selection of the most appropriate treatment. A previous study with FFPE samples from other solid tumors showed that macromolecules could be extracted from FFPE samples, and in terms of quality and quantity did not differ between the samples archived for 1–2 years, 5–7 years or 11–12 years [27]. However, that study did not assess the applicability of those macromolecules obtained for molecular diagnosis. To determine whether the blocks should be kept for a long time for molecular diagnosis in melanoma, we evaluated whether the age of the block affected the success of the sequencing process and the capacity to detect mutations. Using a representative number of blocks with ages <5 years old, between 5 and 10 years or over 10 years, we did not detect a diminished capacity to correctly perform the mutational screening of *BRAF* and *NRAS*. Although a non-significant decreased percentage of samples with both genes correctly sequenced was observed, we could obtain the sequencing results of *NRAS* and *BRAF* in more than 60% of samples kept for more than 10 years in FFPE blocks.

The optimization of the sequencing process, the existing drug combinations and the development of new drugs will play an important role in the future of melanoma therapy [28]. Some strategies have been developed to cryo-preserve melanoma samples in bio-banking with well-established mapping protocols using imaging techniques [29], but these types of strategies are not common. Thus, keeping the blocks for more than 10 years is useful for any future genetic analysis that may be required if a patient develops metastasis, to better determine the appropriate treatment.

In conclusion, it is important to preserve FFPE blocks in order to look for mutations that could be targeted by existing or new therapies. Furthermore, the age of the block seems not to influence mutation detection according to the results of this study, even when they have been stored for more than 10 years.

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