Title

Improved sensitivity in BRAFV600E detection in combined tissue and extracellular vesicles-based liquid biopsy in melanoma

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Running title

BRAFV600E detection in combined tissue and liquid biopsy

Keywords

BRAF, Melanoma, Biomarkers, Oncology, Tumor markers, Cancer

Letter Test

To the editor, cutaneous melanoma in its early stages is a curable disease by surgery of skin lesions. However, intermediate stages display variable outcomes. BRAF V600 mutations are the most frequent alterations in melanoma ocurring in 35-50% of patients (Luke et al., 2017) and remain informative for guiding first-line treatment election (Robert et al., 2019).

Extracellular vesicles (EV) secretion is a cell-to-cell communication process (Tkach and Théry, 2016) that it is enhanced during melanoma progression (Peinado et al., 2012). EV-associated DNA is an emerging biomarker in liquid biopsy with similar or even enhanced specificity and sensitivity than cell free DNA (cfDNA) (Allenson et al., 2017, Figueroa et al., 2017, García-Silva et al., 2020, Yang et al., 2017). However, the use of EVs in liquid biopsy studies is still a matter of study. In this work, we have combined different sources of biological material for testing BRAF mutation in melanoma patients. We found that analysis of BRAFV600E mutation both in tissue and plasma cfDNA/EVs enhances the accuracy and prognostic value in patients upon their visit at the dermatologist.

We have carried out a prospective study in melanoma patients to investigate if the use of EV-based liquid biopsy in plasma samples could improve or complement clinical diagnosis based on tissue BRAF status. We have evaluated a cohort of 22 patients for whom routine clinical BRAF mutation analysis in resected tissue took place after the first dermatologist visit using cobas® BRAF V600 Mutation Test (Table 1). Patients were recruited between April 2014 and August 2021 and median age was 53 years. The inclusion criteria were: 1) requirement of a signed informed consent, 2) availability and quality of plasma sample. Exclusion criteria were 1) insufficient plasma volume for performing the liquid biopsy test and 2) absence of metastasis during the time of study. At the time of tissue biopsy, 27,3 % displayed lymph node metastases and 18,2 % of patients presented distal metastases (Table 1).
Plasma samples were acquired at variable time points regarding initial tissue biopsy. Median time gap between analyses was 2 months and at that time 54,4% and 40,1% of patients presented lymph node and distal metastases respectively (Table 1). For plasma analysis, we have applied a combined cfDNA and EV-associated nucleic acid approach followed by an allele-specific PCR for BRAFV600E mutation (García-Silva et al., 2019). Collection and freezing of plasma were performed in EDTA-tubes in less than 1-2 hours. Isolation of exosomal RNA and DNA along with any cfDNA was performed using ExoLution Plus Extraction kit (Exosome Diagnostics). This approach provides increased sensitivity than standard cfDNA analysis (Castellanos-Rizaldos et al., 2018, Krug et al., 2018). The limit of detection of the EV-based test was 0.001%. The total isolated nucleic acids were subjected to reverse transcription and preamplification of the BRAF exon 15 followed by a quantitative PCR reaction for mutant BRAF allele. Volume sample oscillated between 0.5 and 1.3 ml and BRAFV600E allelic frequency in positive samples was 3.43-0.01% (Table 1).

We observed that BRAF status determined by combining information from tissue and liquid biopsy was not clinically meaningful of patient overall survival, although there was a clear trend for better survival in BRAF WT patients (P = 0.1345 by Log-rank test) (Figure **1a**). However, this integrative analysis significantly discriminated patients with poor overall survival within the two first years after last analysis (P = 0.0184 by Log-rank test) (Figure **1b**). Of note, neither individual BRAF status information by tissue nor by liquid biopsy reached prognostic significance on its own (P=0.3366 and P = 0.0611 by Log-rank test respectively) (Figure **1c**, **d**). Integration of BRAF mutation data from tissue and liquid biopsy could help to identify patients progressing in less than approximately two years. Interestingly, our test

showed a poorer survival for mutant BRAF patients after 5-years follow up, although it did not reach significance, suggesting that treatment variability or other variables could influence patient outcome in the long term.

This work suggests that EV-based liquid biopsy tests for BRAF status can contribute to enhanced prognostic accuracy in patients at their first visits to the dermatologist if evaluated in addition to routine tissue sampling followed by BRAFV600E assay. Our findings showed that mutant BRAF copies were identified in plasma samples of three patients diagnosed as wild type BRAF by tissue biopsy. This fact indicates that false negatives in tissue sampling due to tumor heterogeneity, undiagnosed additional melanomas or technical issues could be uncovered by subsequent liquid biopsy analysis. This test analyzes cfDNA and DNA/RNA in EVs showing improved performance than cfDNA analysis (Castellanos-Rizaldos et al., 2018, Krug et al., 2018). However, it did not completely overlap with tissue data, highlighting the sensitivity challenge that still faces liquid biopsy regarding early stages and/or small tumor burden.
Our test is particularly useful for identification of patients at risk of progression within the first 2 years after their visit to the dermatologist. Importantly, an accurate determination of BRAFV600 status in liquid and tissue biopsy could extend the use of targeted therapy to a broader number of patients even in an adjuvant setting.
This study favors the consideration of the presence of BRAF mutation as a negative prognostic factor for short term survival in melanoma patients. In fact, the incorporation of BRAF status into melanoma staging has been proposed for stages IIIB and IIIC (Barbour et al., 2014), although its general prognostic significance still remains a controversial issue (Ny et al., 2020). Interestingly, the presence of this mutation in lymphatic drainage after lymphadenectomy is also indicative of minimal residual disease and fast progression in stage III melanoma patients (García-Silva et al., 2019). Overall, our data indicate that systematic evaluation of BRAF mutation both in tissue and plasma increases the detection rate of mutant BRAF and the efficiency of prognosis, at least in the short term, for high-risk patients. As such, BRAF status could be considered as an additional clinical factor for adjuvant therapy. Nevertheless, the establishment of BRAFV600 mutation as independent prognostic factor will require more detailed studies with larger cohorts.

Data Availability Statement

Data sets related to this article can be found at https://data.mendeley.com/datasets/6x5rhvs32y/1, hosted at Mendeley Data repository (Garcia-Silva, Susana; Peinado, Hector (2023), “Survival based on BRAF detection in combined tissue and liquid biopsy in melanoma”, Mendeley Data, V1, doi: 10.17632/6x5rhvs32y.1).

Conflict of Interest Statement

Johan Skog has patents for exosome-based technologies and is an employee and shareholder of Bio-techne. J. Aquiles Sanchez is an employee of Bio-techne. Lisa Meyer, Daniel Enderle and Mikkel Noerholm are former employees of Bio-techne. Authors declare no additional conflicts of interest.

Acknowledgments

We would like to thank Carmen María García, Sara Sánchez-Redondo, Vanesa Santos and Juan García-Agulló for technical support. This work has been funded by Fundación AECC (LABAE19027PEIN) (H.P.), Grupo Español de Melanoma (H.P.), Fundación BBVA (H.P.), FIS PI-17/00957 (P.L.O-R) and Centro de Excelencia "Severo Ochoa" (CEX2019-000891-S) (S.G-S.).

CRediT Statement

Conceptualization: SG-S, PLO-R, HP; Data Curation: SG-S, CV-A, LM, DE, JAS, MMO, MN, JS, PLO-R, HP; Formal Analysis: SG-S, HP; Funding Acquisition: JS, PLO-R, HP; Investigation: SG-S, CV-A, LM, DE, MN; Methodology: SG-S, CV-A, LM, DE, JAS, MMO, MN, PLO-R, HP; Project Administration: MN, JS, PLO-R, HP; Resources: JLR-P, MN, JS, PLO-R, HP; Supervision: CV-A, MN, JS, PLO-R, HP; Validation: SG-S, CV-A, LM, DE, MN; Visualization: SG-S; Writing - Original draft preparation: SG-S, HP; Writing – Review and Editing: SG-S, DE, MN, PLO-R, HP.

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Ethical statement

This study was approved by the institutional ethical review board (CEIC) of Hospital 12 de Octubre (Madrid, Spain) and institutional review board information is 04.625 version September 26th 2018 on Act 20/18 dated by November 13th 2018. Written informed consent was obtained from all participants.

Funding sources: Fundación AECC **(** LABAE19027PEIN) (H.P.), Grupo Español de Melanoma (H.P.), Fundación BBVA (H.P.), FIS PI-17/00957 (P.L.O-R) and Severo Ochoa Excellence Program (S.G-S.).

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Patient ID	Age	Sex	Tumor stage	Follow-up after liquid biopsy (days)	Patient Outcome	LN mets at first biopsy	Distal mets at first biopsy	LN mets at plasma analysis	Distal mets at plasma analysis	Time gap between tests (days)	BRAF V600E detected by Cobas® in tissue	Plasma BRAF V600E/K detected	BRAF V600E (copies/ml)	Mutant allele fraction [%]
1	65	Female	T4b	2096	Alive	Yes	No	Yes	No	22	negative	negative	ND	0,0
2	55	Male	T4b	2205	Alive	No	No	No	No	29	negative	negative	ND	0,0
3	97	Female	T4a	923	Died	No	No	No	No	10	negative	negative	ND	0,0
4	77	Male	T3a	2474	Alive	No	No	No	No	630	negative	negative	ND	0,0
5	53	Female	T1x	2206	Alive	No	No	No	No	0	negative	negative	ND	0,0
6	52	Female	Tx	177	Died	Yes	Yes	Yes	Yes	75	negative	V600E	72	3,43
7	52	Male	T4b	143	Died	No	No	No	Yes	87	negative	V600E	<10	0,01
8	32	Male	T3b	123	Died	No	No	Yes	Yes	660	V600E	negative	ND	0,0
9	52	Male	T2x	709	Died	No	No	Yes	No	749	negative	negative	ND	0,0
10	39	Male	T3a	2323	Alive	Yes	Yes	No	Yes	1	V600E	V600E	22	3,0
11	47	Male	T2b	563	Died	No	No	Yes	Yes	670	V600E	negative	0	0,0
12	61	Female	Tx	860	Alive	Yes	No	Yes	Yes	722	negative	negative	0	0,0
13	41	Male	T2a	1012	Alive	No	No	Yes	No	1	V600E	negative	ND	0,0
14	46	Female	T3a	1141	Alive	No	No	Yes	No	764	not done	negative	ND	0,0
15	40	Male	T4b	1120	Alive	No	Yes	Yes	Yes	182	negative	V600E	1,1	0,21
16	57	Male	T2	155	Died	Yes	No	No	Yes	519	V600E	V600E	1.847	0,76
17	79	Male	T4b	366	Died	No	No	No	No	11	V600E	V600E	1,01	0,03
18	69	Male	T4b	1013	Alive	No	No	No	No	0	V600E	negative	ND	0,0
19	67	Male	T2a	1046	Alive	No	No	No	No	0	V600E	V600K	0,24	0,0
20	67	Female	T3a	441	Alive	Yes	No	Yes	No	0	V600E	negative	ND	0,0
21	52	Male	T4b	371	Alive	No	No	Yes	No	1	V600E	V600K	0,25	0,02
22	85	Female	Tx	1015	Alive	Yes	Yes	Yes	Yes	3	negative	negative	ND	0,0

Table legend

Table 1. Clinical characteristics of melanoma patients and BRAF status information for liquid biopsy test compared to tissue analysis. ND, not detected; <10 copies, sample positive but below limit of quantification.

Figure legend

Fig 1. Overall survival data for melanoma patients according to BRAF status. (a) Kaplan Meier curve displaying overall survival in melanoma patients considering to BRAF V600E mutation status analyzed by Cobas test performed on tumor tissue together with BRAF status on corresponding plasma samples. (b) Kaplan Meier curve displaying 2-years overall survival in patients according to BRAF status in tissue and liquid biopsy performed in plasma samples (c) Kaplan Meier curve displaying 2-years overall survival in patients according to BRAF status in tissue biopsy samples. (d) Kaplan Meier curve displaying 2-years survival in patients based on liquid biopsy samples. N=22 patients, P-values represent Log-rank tests.

Figure 1

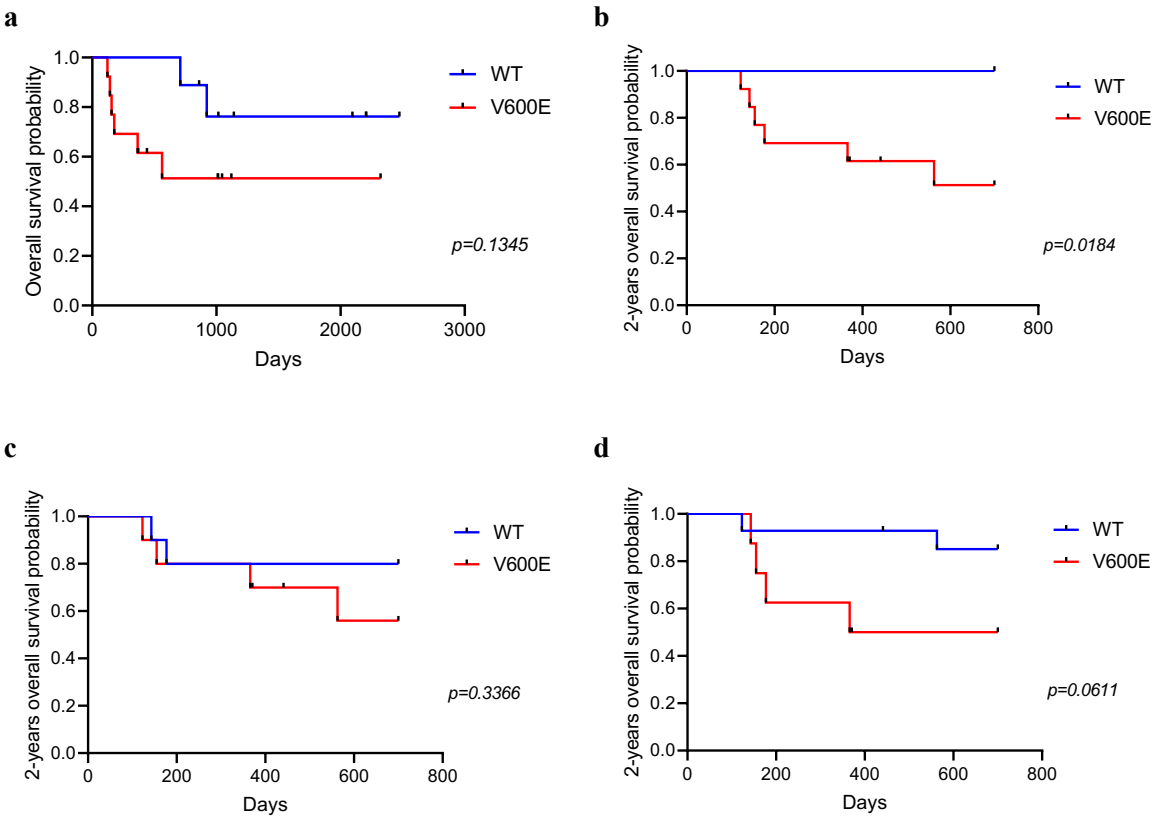

Figure 1. Overall survival data for melanoma patients according to BRAF status. (a) Kaplan Meier curve displaying overall survival in melanoma patients considering BRAF V600E mutation status analyzed by Cobas test performed on tumor tissue together with BRAF status on corresponding plasma samples. (b) Kaplan Meier curve displaying 2-years survival in patients according to BRAF status in tissue and liquid biopsy performed in plasma samples (c) Kaplan Meier curve displaying 2-years overall survival in patients according to BRAF status only in tissue biopsy samples. (d) Kaplan Meier curve displaying 2-years overall survival in patients based only in liquid biopsy samples. N=22 patients, P-values represent Log-rank tests.