The Hidden Story on Heterogeneous B-raf V600E Mutation Quantitative Protein Expression in Metastatic Melanoma – Association with Clinical Outcome and Tumor Phenotypes

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Supplementary information

Materials and Methods

Protein extraction, digestion and automated C18 desalting workflow

Protein extraction was performed on sectioned, fresh-frozen human MM lymph node metastasis tissues (10 µm) using the Bioruptor plus, model UCD-300 (Dieagenode). A total of 56 MM tissue samples were lysed in 100 µL lysis buffer containing 4 M urea and 100 mM ammonium bicarbonate. After briefly vortexing, samples were sonicated in the Bioruptor for 40 cycles at 4°C. Each cycle consisted of 15 s at high power and 15 s without sonication. The samples were then centrifuged at 10,000 ×g for 10 min at 4°C. A pool of MM lysates was also prepared as the reference sample. The protein content in the supernatant was determined using the colorimetric micro BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL).

Protein digestion was performed on the AssayMAP Bravo (Agilent Technologies) platform using the digestion v2.0 protocol. Proteins were reduced with 10 mM DTT for 1 h at room temperature (RT) and sequentially alkylated with 20 mM iodoacetamide for 30 min in the dark at RT. To decrease the urea concentration, the samples were then diluted approximately seven times with 100 mM ammonium bicarbonate. Digestion was performed in two steps. Proteins were first incubated with Lys-C at a 1:50 (w/w) ratio (enzyme:protein) for 5 h and then trypsin was then added at a 1:50 (w/w) ratio and the mixture incubated overnight at RT. The reaction was quenched by adding 20% TFA to a final concentration of ~1%. Peptides were desalted on the AssayMAP Bravo platform using the peptide cleanup v2.0 protocol. C18 cartridges (Agilent, 5 µL bed volume) were primed with 100 µL 90% acetonitrile (ACN) and equilibrated with 70 µL 0.1% TFA at a flow rate of 10 µL/min. The samples were loaded at 5 µL/min, followed by an internal cartridge wash with 0.1% TFA at a flow rate of 10 µL/min. Peptides were eluted with 30 µL 80% ACN, 0.1% TFA and dried in speed vac prior to TMT labeling.

TMT 11-plex labeling

The peptide content in each sample was determined using the quantitative colorimetric peptide assay kit (Thermo Fisher Scientific, Rockford, IL) to ensure equal amounts of material in each TMT channel. TMT labeling was performed according to manufacter's instructions. Samples were resuspended in 100 µL of 200 mM TEAB and individual TMT 11-plex reagents were dissolved in 41 µL of dried ACN. Peptides were labeled by mixing the peptide solution with TMT 11-plex reagents for 1 hour at room temperature. Reaction was quenched by adding 1 µL of 5% hydroxylamine and incubation at room temperature for 15 minutes. The, the labeled peptides were mixed in a single tube, the volume was reduced in a speed vac and the peptides were cleaned up using a C-18 Sep-Pak cartridge (Waters). The

eluted peptides were dried in a speed vac and finally resuspended in 20 mM ammonium formiate prior to high pH fractionation.

Off line high pH fractionation

Each batch of TMT-11 labelled peptide was fractionated using a Phenomenex Aeris Widepore XB-C8 (3.6 µm, 2.1 × 100 mm) column on an 1100 Series HPLC (Agilent) operating at 80 µL/min. The mobile phases were solvent A: 20 mM ammonium formate and solvent B: 80% ACN - 20% water containing 20 mM ammonium formate. Both solvents were adjusted to pH 10 with ammonium hydroxide. Separation was performed using the following gradient: 0 min 5% B; 1 min 20% B; 60 min 40% B; 90 min 90% B; 120 min 90% B. The column was operated at RT and the detection wavelength was 214 nm. Ninety-eight fractions were collected at 1 min intervals and further concatenated to 24 or 25 fractions, which were dried in a SpeedVac (Eppendorf).

nLC-MS/MS analysis

nLC-MS/MS analysis was performed on an Ultimate 3000 HPLC coupled to a Q Exactive HF-X mass spectrometer (Thermo Scientific, San Jose, CA). Peptides from each fraction (1 µg) were loaded onto a trap column (Acclaim1 PepMap 100 pre-column, 75 µm, 2 cm, C18, 3 mm, 100 Å, Thermo Scientific, San José, CA) and then separated on an analytical column (EASY-Spray column, 25 cm, 75 µm i.d., PepMap RSLC C18, 2 mm, 100Å, Thermo Scientific, San José, CA) using a 120 min ACN gradient with 0.1% formic acid at a flow rate of 300 nL/min and a column temperature of 45°C. Q Exactive HF-X mass spectrometer was set using the TMT node as follows: full MS scans at *m/z* 350-1400 with a resolution of 120000 at *m/z* 200, a target AGC value of 3×10^6 and IT of 50 ms, DDA selection of the 20 most intense ions for fragmentation in HCD collision cell with an NCE of 34 and MS/MS spectra acquisition in the Orbitrap analyzer at a resolution of 45000 (at *m/z* 200) with a

maximum IT of 86 ms, fixed first mass of 110 *m/z*, isolation window of 0.7 Da and dynamic exclusion of 30 s.

Data analysis

Data were processed with Proteome Discoverer 2.3 (Thermo Fisher Scientific, San José, CA, USA) using the Sequest HT search engine. The search was performed against the Homo sapiens UniProt revised database (2018-10-01) and the B-raf V600E mutant protein sequence. Cysteine carbamidomethylation was set as fixed modification while methionine oxidation and TMT 6plex at peptide N-terminus and lysine were set as variable modifications; peptide mass tolerance for the precursor ions and MS/MS spectra were 10 ppm and 0.02 Da, respectively. A maximum of two missed cleavage sites was accepted and FDR were set at 0.01 for identification at peptide level.

The search results were directly processed in Perseus software[1]. A filtering criterion was set to keep the identified proteins with the quantified values of all reporter ions (no missing value) in the final identification list. The protein intensities were log2–transformed and normalized by subtracting the median intensity in each sample. The relative abundance values were obtained by subtracting the intensity of the protein in the reference.

For statistical analysis samples were separated in two groups according to the levels of mutated B-raf. Group 1 (V600E_H) with B-raf >1.65 and Group 2 (V600E_H) B-raf ≤ 1.65. The cut-off value (1.65) was selected by a ROC curve where the ability of mutated B-raf to discriminate between long and short survivals (considering a three years' survival) was analyzed (Fig. 1 supplementary information). To make the ROC curve, patients with less than 40 years at the age of diagnosis were excluded. Kaplan-Meier survival analysis with log-rank, Breslow and Tarone Ware testing was used for univariate analysis between Groups 1 and 2. P value < 0.05 was considered statistically significant. These analyses were performed using SPSS 25 (SPSS Inc, Chicago, IL) software.

Differentially expressed proteins between V600E_H and 2 V600E_L were determined by Student t-test (two-tails). In this case, proteins quantified in at least three samples in each group were considered for the analysis and p-values < 0.01 dictated significant protein changes. Principal component analysis (PCA) was performed in R [2,3](‘FactoMineR’ package) to visualize the behavior of DEPs. These proteins were included in the bioinformatics analysis, including hierarchical clustering (‘pheatmap’ R package; distance: Euclidean; linkage: average) and heat map generation. For functional analysis of the DEPs, the Ingenuity IPA Core Analysis was performed (Ingenuity, Qiagen).

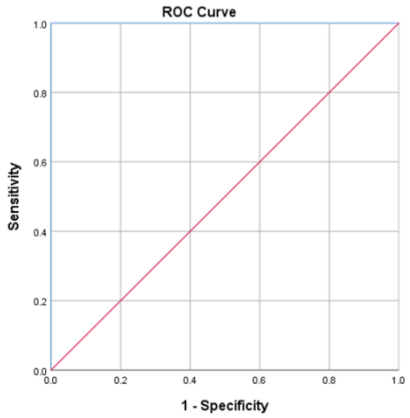


Figure 1. ROC curve analysis for the discrimination analysis between patients with long (more than 3 years) or short survival (less than 3 years) according to B-raf V600E relative abundances measured by mass spectrometry on melanoma tumors. The cut-off (relative abundance of B-raf V600E higher than 1.65) was determined considering 100% of sensitivity and specificity for long survival patients.

Table 1. Patient clinical data and of BRAF status results for mutation-positive melanoma metastases.

#	Sample	gender	stage	metastasis	age.diag	os.days	dead/alive	BRAF status (DNA)	BRAF status (mRNA)	B-raf status status (MS)	B-raf V600E (MS quatitation)	
1	MM149	Male	3	Subcutaneous	86	89	dead	V600E	V600E	V600E	6.165	
2	MM111	Female	4	Subcutaneous	50	368	dead	N/A	V600E	V600E	4.744	
3	MM136	Male	3	Lymph node	65	453	dead	V600E	V600E	V600E	3.857	
4	MM114	Female	4	Lymph node	52	126	dead	V600E	V600E	V600E	3.396	**High**
5	MM137	Male	3	NA	59	476	dead	V600E	V600E	V600E	3.338	**expression**
6	MM109	Female	3	Lymph node	29	1479	alive	WT	V600E	V600E	2.829	**group**
7	MM130	Male	3	Lymph node	39	2530	dead	V600E	V600E	V600E	2.076	
8	MM122	Male	4	Visceral	46	66	dead	V600E	V600E	V600E	1.881	
9	MM124	Male	3	Lymph node	60	523	alive	V600E	V600E	V600E	1.844	
10	MM133	Female	3	Lymph node	24	5005	alive	V600E	V600E	V600E	1.772	
11	MM138	Male	4	Lymph node	72	42	dead	V600E	V600E	V600E	1.772	
12	MM116	Male	4	Lymph node	69	93	dead	V600E	V600E	V600E	1.701	
13	MM146	Male	4	Lymph node	65	1455	dead	V600E	V600E	V600E	1.613	
14	MM143	Male	3	Lymph node	55	2800	alive	V600E	V600E	V600E	1.607	
15	MM147	Male	3	Subcutaneous	65	1222	dead	V600E	V600E	V600E	1.593	**Low**
16	MM120	Female	3	Lymph node	46	652	alive	WT	V600E	V600E	1.491	**expression**
17	MM105	Male	3	Lymph node	77	1097	dead	WT	WT	V600E	1.392	**group**
18	MM115	Female	3	Lymph node	73	1035	alive	V600E	V600E	V600E	1.337	
19	MM154	Male	3	Lymph node	39	1329	dead	V600E	V600E	V600E	1.31	
20	MM123	Male	3	Lymph node	59	307	alive	V600E	V600E	V600E	1.278	
21	MM118	Male	4	Lymph node	72	789	alive	WT	WT	V600E	1.023	
22	MM132	Male	3	Lymph node	69	3582	dead	V600E	V600E	V600E	1.009	

age. diag: age at diagnosis
os.days: overall survival
NA: Not analyzed
ND: No data available

Table 2. Patient clinical data and WT BRAF detection by different techniques.

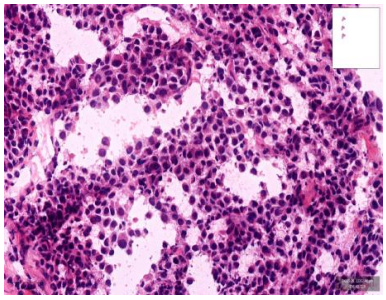
#	Sample	gender	stage	metastasis	age.diag	os.days	dead/alive	BRAF status (DNA)	BRAF status (mRNA)	B-raf status status (MS)
1	MM101	Male	4	Lymph node	89	638	dead	WT	WT	WT
2	MM102	Male	3	Lymph node	72	849	dead	WT	WT	WT
3	MM103	Male	4	Subcutaneous	68	392	dead	WT	WT	WT
4	MM104	Female	3	Lymph node	62	1779	alive	WT	WT	WT
5	MM106	Male	4	Lymph node	57	542	dead	WT	WT	WT
6	MM107	Male	3	Lymph node	82	574	dead	WT	WT	WT
7	MM108	Female	3	Lymph node	70	1406	alive	WT	WT	WT
8	MM110	Male	3	Lymph node	60	1260	alive	WT	WT	WT
9	MM112	Female	3	Lymph node	73	1379	alive	WT	N/A	WT
10	MM113	Male	3	Lymph node	70	399	dead	WT	WT	WT
11	MM117	Female	3	Lymph node	62	303	dead	WT	WT	WT
12	MM119	Male	3	Lymph node	75	970	dead	WT	N/A	WT
13	MM121	Male	1	Cutaneous	77	170	dead	WT	WT	WT
14	MM125	Female	3	Lymph node	70	424	alive	WT	WT	WT
15	MM126	Male	4	Lymph node	62	318	alive	WT	WT	WT
16	MM127	Male	4	Lymph node	65	165	dead	V600E	WT	WT
17	MM128	Female	3	Lymph node	54	174	alive	WT	NA	WT
18	MM129	Male	3	Lymph node	53	642	dead	WT	WT	WT
19	MM131	Male	3	Lymph node	68	6343	alive	WT	WT	WT
20	MM134	Female	3	Lymph node	74	461	dead	WT	WT	WT
21	MM135	Male	3	Lymph node	71	336	dead	WT	WT	WT
22	MM139	Male	4	Lymph node	61	275	dead	WT	WT	WT
23	MM140	Female	3	Lymph node	57	3363	alive	WT	WT	WT
24	MM141	Male	3	Lymph node	50	3206	alive	WT	WT	WT
25	MM142	Male	4	Lymph node	70	50	dead	WT	WT	WT
26	MM144	ND	ND	ND	ND	ND	ND	N/A	WT	WT

#	Sample	gender	stage	metastasis	age.diag	os.days	dead/alive	BRAF status (DNA)	BRAF status (mRNA)	B-raf status status (MS)
27	MM145	Male	1	Subcutaneous	53	511	dead	WT	WT	WT
28	MM148	Male	4	Visceral	63	2619	alive	WT	WT	WT
29	MM150	Male	3	Lymph node	55	2574	alive	WT	WT	WT
30	MM151	Female	4	Lymph node	80	386	dead	WT	WT	WT
31	MM152	Male	3	Lymph node	70	56	dead	WT	WT	WT
32	MM153	Male	4	ND	65	279	dead	WT	WT	WT
33	MM155	Male	3	Lymph node	75	519	dead	N/A	N/A	WT
34	MM156	Female	3	Lymph node	76	1401	dead	WT	WT	WT

age. diag: age at diagnosis
os.days: overall survival
NA: Not analyzed
ND: No data available

Figure 2. Histological images of mutation-positive metastatic melanoma samples. For all the images the magnification and scale were 10x and 50 µm, respectively.

High-expressing B-raf V600E tumors



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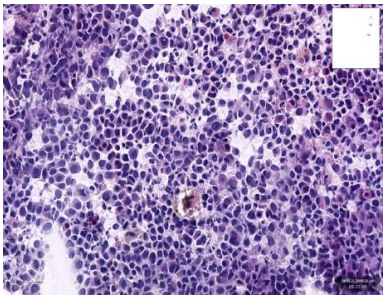

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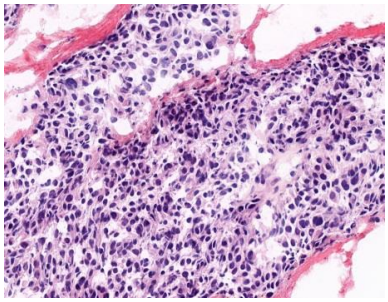

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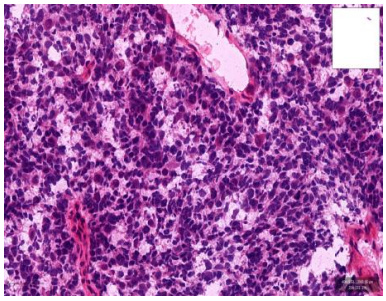

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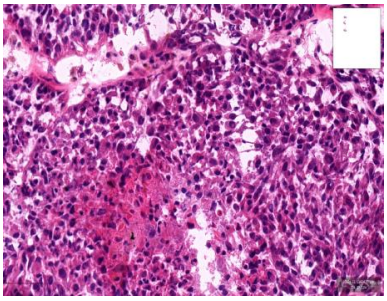

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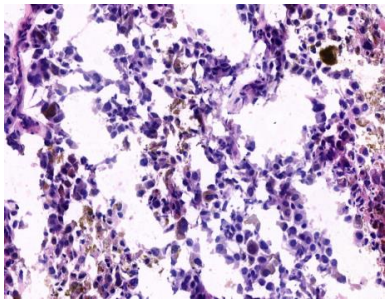

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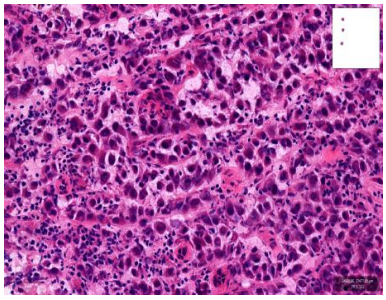

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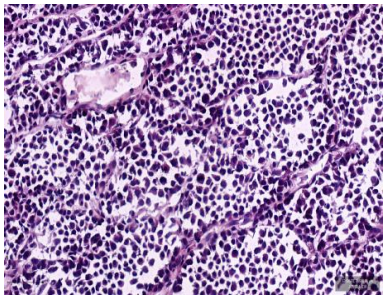

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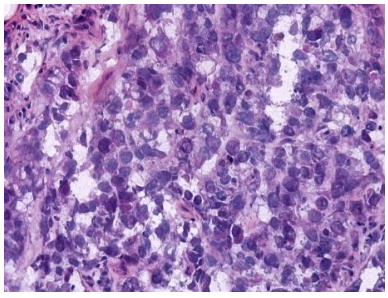
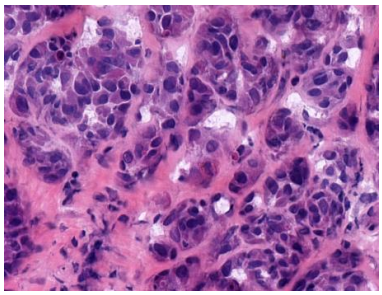


MM122

Low-expressing B-raf V600E tumors



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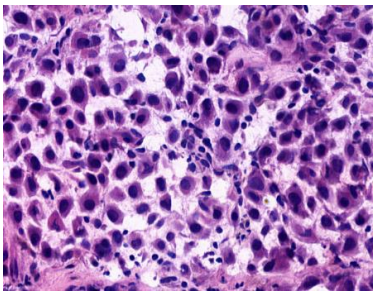

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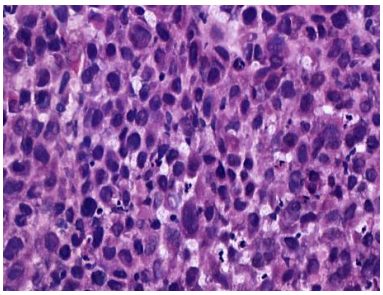

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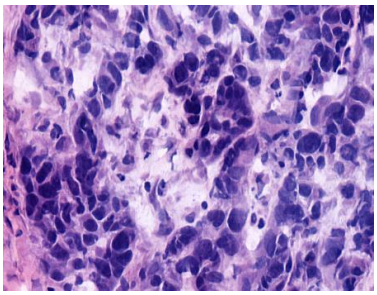

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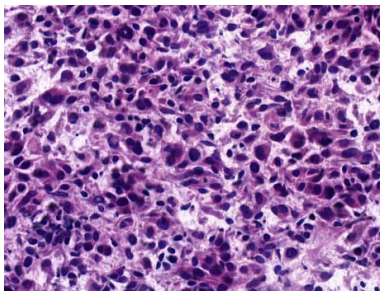

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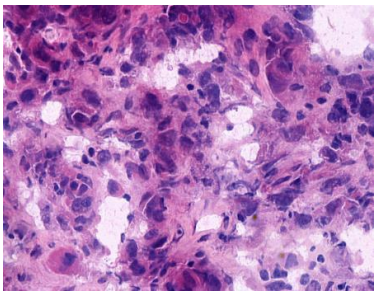

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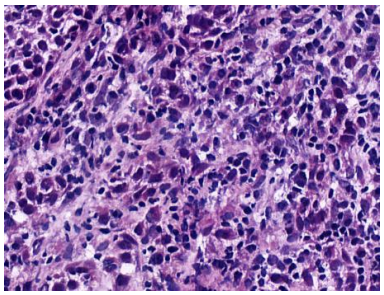

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