

# **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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## Abstract

In comparison to other human cancer types, malignant melanoma exhibits the greatest amount of heterogeneity. After DNA-based detection of the BRAF V600E mutation in melanoma patients, targeted inhibitor treatment is the current recommendation. This approach, however, does not take the abundance of the therapeutic target, i.e., the B-raf V600E protein, into consideration. As shown by immunohistochemistry, the protein expression profiles of metastatic melanomas do clearly reveal the existence of inter- and intra-tumor variability. Nevertheless, the technique is only semi-quantitative. To quantitate the mutant protein there is a fundamental need for more precise techniques that are aimed at defining the currently non-existent link between the levels of the target protein and subsequent drug efficacy. Using cutting-edge mass spectrometry combined with DNA and mRNA sequencing, the mutated B-raf protein within metastatic tumors was quantitated for the first time. B-raf V600E protein analysis revealed a subjacent layer of heterogeneity for mutation-positive metastatic melanomas. These were characterized into two distinct groups with different tumor morphologies, protein profiles and patient clinical outcomes. This study provides evidence that a higher level of expression for the mutated protein is associated with a more aggressive tumor progression. Our study design that is comprised of surgical isolation of tumors, histopathological characterization, tissue biobanking, and protein analysis may enable the eventual delineation of patient responders/non-responders and the subsequent therapy of malignant melanoma.

**Key words:** malignant melanoma; BRAF V600E mutation; proteomics; mass spectrometry genetics; heterogeneity; prognosis

## 1. Introduction

Malignant melanoma (MM) is one of the most aggressive and heterogeneous of all human cancer types [1]. The melanoma subtypes differ in origin, location and mutational profile [2]. At the genetic level, BRAFmut, RASmut, NF1mut, and triple WT subgroups exist; while at transcriptomic level, low and high immune cell-infiltrated prognostic subtypes have emerged. These are reflected in the pathological categorization of brisk and non-brisk patterns of tumor-infiltrating lymphocytes [3]. With respect to the variation in clinical symptoms, appearance, and the biology in patients combined with the morphological and molecular variation of an individual tumor; malignant melanoma is one of the most heterogeneous of all diseases [4].

Most cases of malignant melanoma are diagnosed at an early stage where surgical excision is curative [5]. The management of patients with disseminated disease, however, is troublesome. For instance, checkpoint immunotherapy with monoclonal antibodies targeting CTLA-4 and PD-1 have provided clinically-important benefits for only a subset of melanoma patients. This treatment modality was developed in parallel with targeted mitogen-activated protein kinase (MAPK) pathway inhibitor therapies such as vemurafenib and dabrafenib alone, or when combined with trametinib and cobimetinib [6–8]. In BRAF V600E-mutated melanomas, such therapeutical approaches inhibit the activity of key members of the MAPK pathway such as BRAF and MEK. The treatment has resulted in a significantly higher response rate in reducing the bulky tumor mass, however, efficacy still varies; and after a period free from disease advancement, most responsive patients develop resistance to the therapy and lethally progress [9]

In the clinical setting, the golden standard are FDA-approved PCR-based DNA tests that selectively amplify the mutant BRAF gene [10]. These tests reveal the presence or absence of a specific BRAF gene mutation; however, there is no indication as to whether the BRAF gene

is transcribed and translated into the B-raf protein. Therefore, it is still unclear whether BRAF regulates expression at the mRNA level or the protein level. The therapeutic target of the clinically-administered drugs is the protein. Therefore, it is important to evaluate the relationship between drug efficacy and protein expression level. Thus, there is a current lack of crucial information that could aid clinical decisions.

Proteomics is a highly-promising field of research that can assist in identifying therapeutic targets and cancer biomarkers [11–15]. At the core of proteomics is the technique of mass spectrometry (MS) that provides sensitive analysis of complex mixtures of proteins and peptides. In addition, MS provides a means of undertaking unsurpassed challenges that exist in genomics; including protein identification, studying post-translational modifications, and determining the relative abundance of protein products [16].

The identification and quantitation of B-raf V600E protein by mass spectrometry is challenging. To date, only one study has attempted to address the issues of heterogeneity that is inherent to expression of WT and V600E BRAF [17]. Using immunoenrichment-based techniques following a targeted liquid chromatography multiple reaction monitoring (LC-MRM) approach, both proteins were identified and quantitated in complex biological samples comprised of colorectal carcinoma CRC cell lines and tissue specimens. Thus, identification and quantitation by mass spectrometry of the V600E B-raf protein remains a understudied topic for most cancerous tissues including melanoma.

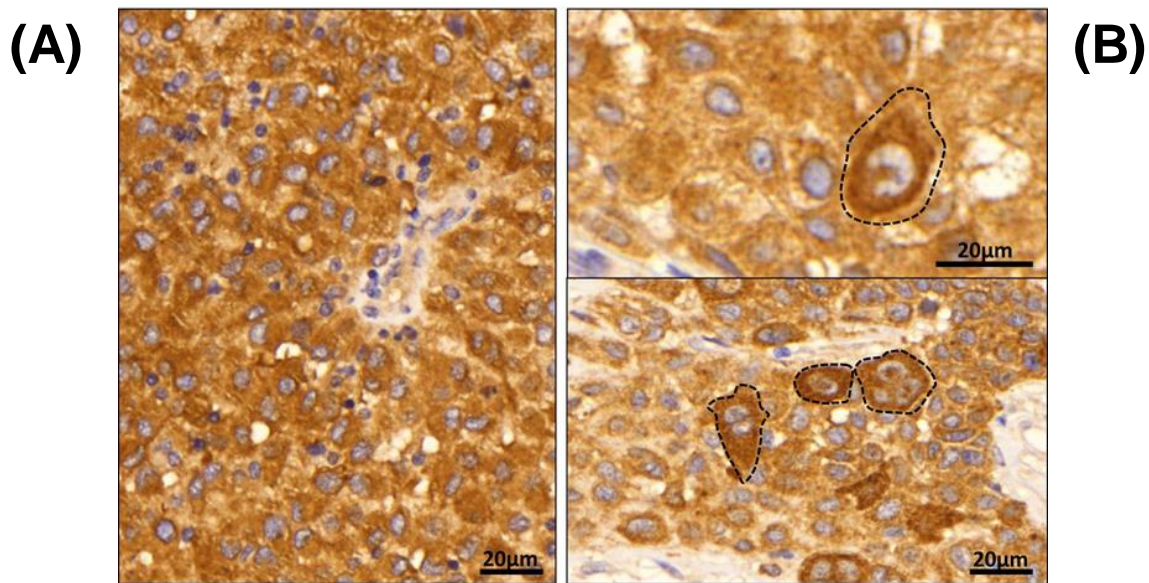
In the current work, the first mass spectrometry/proteomic study was performed on a set of malignant melanoma samples to identify and quantify the B-raf V600E mutant protein. The samples were also screened for the mutation at the DNA and mRNA level. Our study revealed a subjacent layer of heterogeneity in V600E BRAF-mutated melanomas. Two distinct groups associated with different clinical outcomes and molecular features were apparent. The data indicated that patients with tumors displaying a higher level of B-raf

V600E expression had a poorer prognosis than patients with a lower level of the mutated protein. For both tumor groups, morphological differences in histological images and protein differential expression profiles supported the novel findings of distinctive tumor phenotypes. When combined with optimized, high-quality biobanking protocols and technology-driven protein analytical approaches, our results illustrated that surgically-removed and histologically well-characterized tumor tissues can provide important new insights into the expression of key protein mutations. Targeted inhibitor therapies are currently directed towards the protein and not the gene. Thus, such unique information may ultimately impact the management of advanced-stage melanoma.

## **2. Results**

### **2.1. B-raf V600E mutant protein expression is a heterogeneous event.**

Although DNA and RNA sequencing indicate the presence of mutated BRAF, there is no obvious correlation between this information and the inter- and intra-tumoral abundance and distribution of the mutant protein. With immunohistochemistry it is possible to map the distribution of B-raf V600E within the tumor (Figure 1). The first example shows a tumor with homogeneous expression of the mutant protein throughout the tumor tissue (Figure 1A). Conversely, an example of intra-tumor heterogeneity is illustrated by two different regions within the same tumor (Figure 1B). This example highlights the pathological heterogeneous expression pattern that can eventuate for the mutated B-raf V600E protein; and is epitomized by highly-irregular cellular assignment.

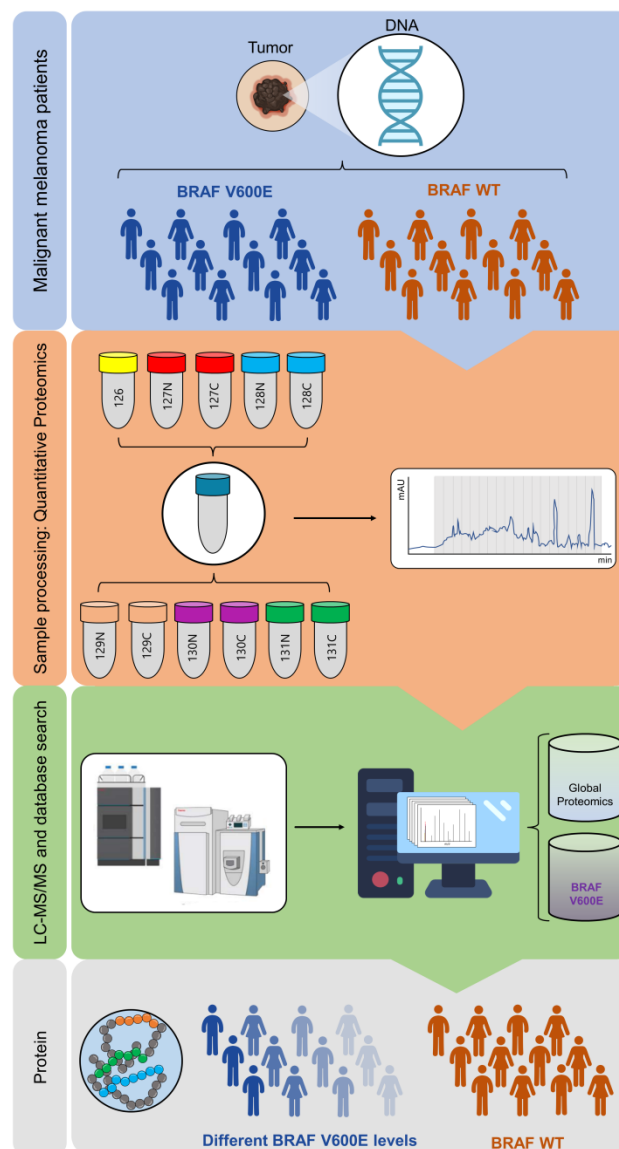


**Figure 1.** Immunohistochemical images of mutated B-raf V600E displayed from two patients with malignant melanoma. A) a patient with homogeneous B-raf expression, B) two IHC images generated from two different areas of the same tumor with heterogeneous and disperse B-raf expression highlighted by brown colorimetric reaction. Note the grouping cells (dashed lines) with more pronounced B-raf V600E expression pattern by brown discoloration of the HRP-DAB reaction. /IHC, OM 112x/

In routine daily practice, conventional immunohistochemistry is readily available to identify the mutated B-raf V600E protein. With the VE1 clone, a fairly specific staining can be observed that is comparable to a genetic analysis; however, there are no accurate cut-off values for cases with positive heterogeneity. Therefore, there are still some instances where there is limited potential for accurate microscopic assessment, e.g., when only focal sparse positivity is observed, or when intra-tumoral heterogeneity is apparent with more pronounced, focal B-raf V600E expression (Figure 1B, dashed lines). Due to the intrinsic limitations of the technique, inter-tumor comparison of mutated protein levels is only qualitative. Mass spectrometry not only provides a means of discriminating between B-raf WT and V600E; but also, to accurately quantitate the proteins.

## 2.2.B-raf V600E mutant and B-raf WT protein identification

The BRAF mutational status was studied in 56 tumor samples from metastatic melanoma patients. Tumors were firstly characterized by histopathology and frozen sections were analyzed using a semi-automated proteomic workflow (Materials and Methods and Figure 2). The methodology is comprised of automatic protein extraction from tumors, automated denaturation and tryptic digestion of proteins on a robotic micro-chromatographic platform, peptide labeling with TMT 11-plex reagents, HpH RP-HPLC fractionation, LC-MS/MS analysis of collected fractions; and finally, protein identification and quantitation.

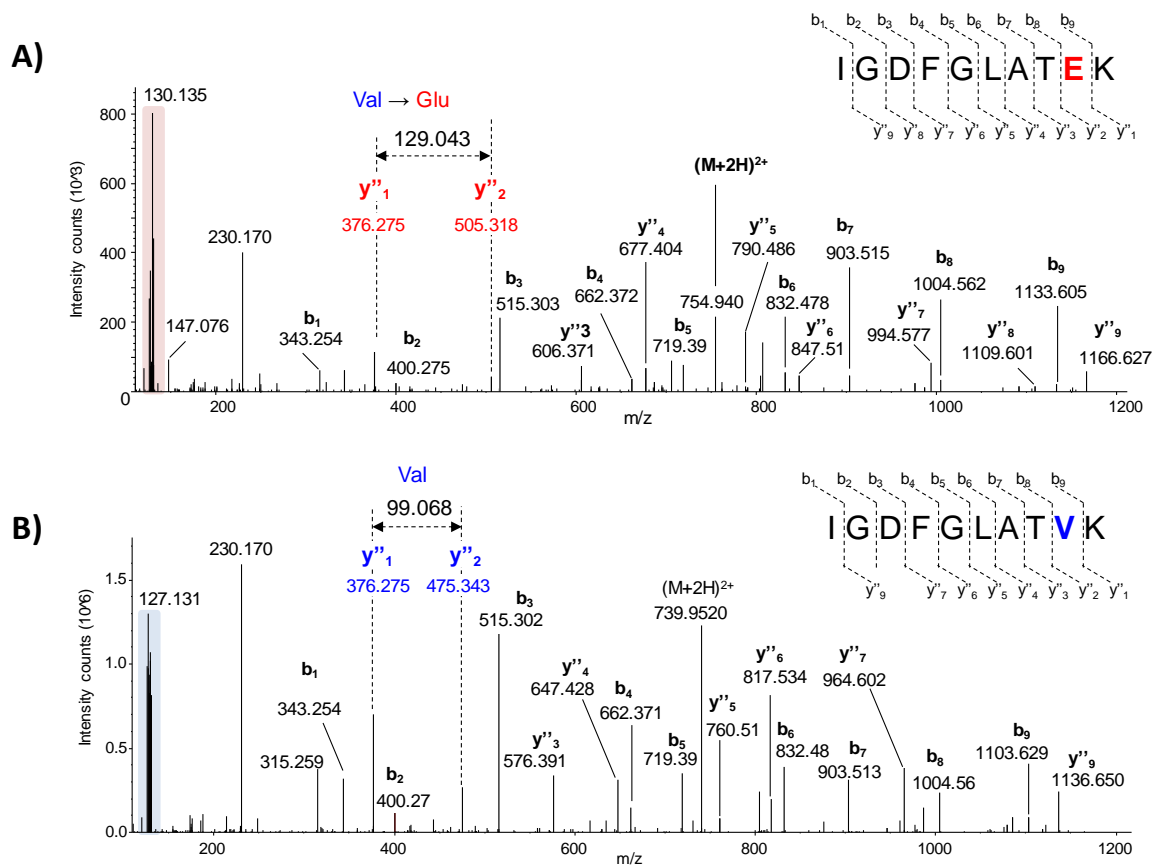




**Figure 2.** *General schematic of the workflow followed for identification and quantitation of B-raf V600E from metastatic melanoma patients. Sample processing consisted of: automatic generation of protein extracts from patient tumor samples, protein denaturation and tryptic digestion on a robotic micro-chromatographic platform, TMT 11-plex labelling of generated peptides, and peptide fractionation by high pH RP-HPLC. Fractions were analyzed by LC-MS/MS and the processed data searched against the human protein database (see Materials and Methods). Identification and quantitation of the B-raf V600E mutation and the proteins expressed by individual melanoma tumors (global proteomic analysis) including WT B-raf was then performed.*

With this strategy, the mutated B-raf V600E was identified and quantitated for the first time by mass spectrometry in malignant melanoma tissue samples. Corresponding to the simultaneous analysis of ten MM samples, Figure 3 shows the assigned MS/MS spectra of TMT-labeled peptides for the mutated B-raf (IGDFGLATEK, Figure 3A) and the WT protein (IGDFGLATVK, Figure 3B).

The BRAF mutational status of 52 of the 56 samples was previously determined at DNA and mRNA levels. The results obtained were in agreement for 50 of the cases (Tables 1 and 2 supplementary information). The mRNA study, however, was performed on the same region of the tumor that had been selected for proteomics and it detected a higher number of samples containing the BRAF V600E mutation; thus this data provided a more valuable measure against which the mass spectrometry results could be compared.



**Figure 3.** Identification of peptides from B-raf V600E and B-raf WT by mass spectrometry.

(A) Assigned MS/MS spectrum of the TMT-labeled peptide IGDFGLATEK from B-raf V600E. Interpretation of the data showed the substitution of valine residue for glutamic acid (Glu,  $m/z_{theo}=129.0425$ ) that corresponds to the mutation. (B) Assigned MS/MS spectrum of the TMT-labeled peptide IGDFGLATVK from WT B-raf. Interpretation of the data showed the presence of the expected valine residue (Val,  $m/z_{theo}=99.0684$ ). The low  $m/z$  region is highlighted in both MS/MS spectra to indicate the presence of TMT 11-plex reporter ions that are used to relatively quantify the protein to provide a measure of protein expression.

**Table 1.** Summary of the BRAF mutational status results obtained by DNA, RNA and mass spectrometry for melanoma metastases.

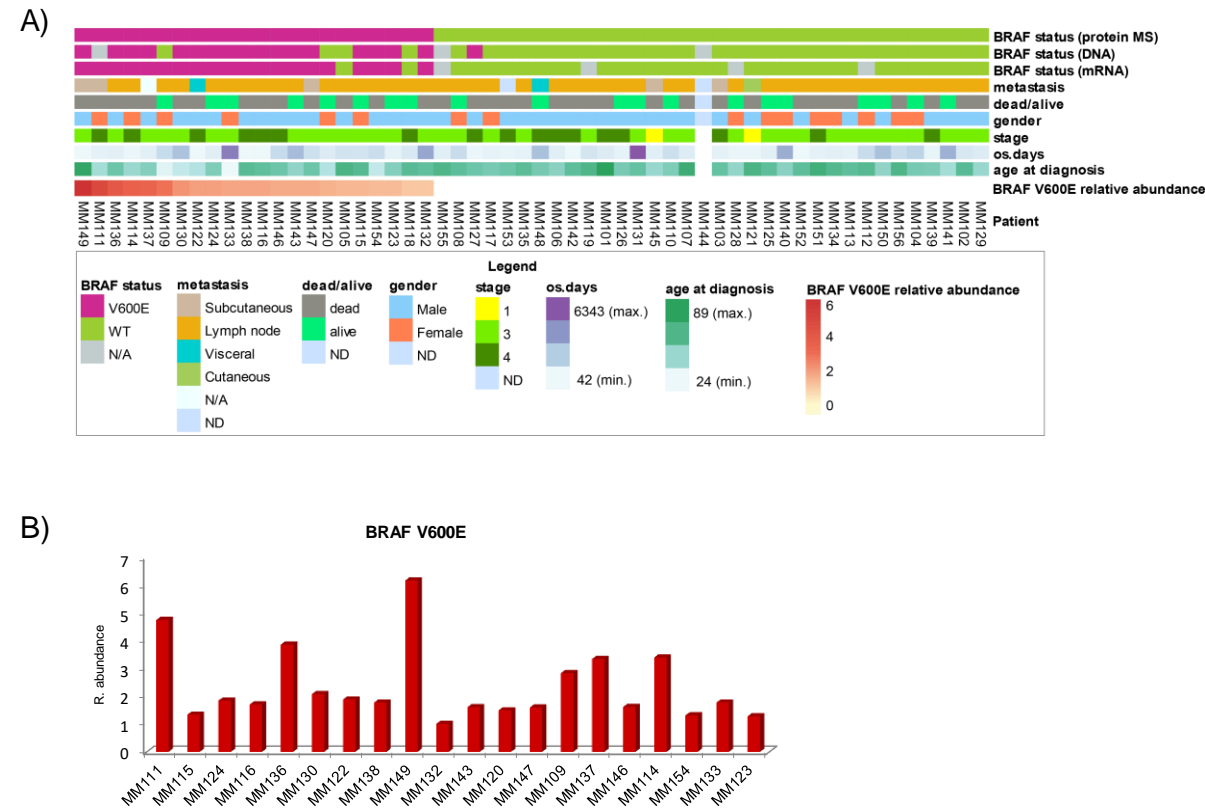
BRAF V600E status	Positive	Negative	Sensitivity	Specificity
DNA	18	34		
mRNA	20	32		
Protein (MS)	22	34	100%	91%

By proteomics, the B-raf mutational status was determined for all 56 tumor samples. Of these, 22 were B-raf V600E positive and; except for two instances, were in agreement with the mRNA-based study (Table 1 and Table 1 supplementary information). In addition, the WT B-raf protein was observed in all the tumor samples. Overall, the proteomic results aligned with the data obtained by genomics in 50 from 52 samples.

**2.3.B-raf V600E expression and correlation with patient survival and tumor phenotype**

The clinical data from the metastatic melanoma patients and the BRAF status data from genomic and mass spectrometric determination are combined in Figure 4A. Noticeably, this heat map representation captured the limited information provided by both genomic studies regarding BRAF V600E mutation compared to the more complex picture offered by the protein expression. As determined by mass spectrometry, the relative abundance of the B-raf V600E protein revealed a high degree of variability with a coefficient of variation of 57% across the twenty mutation-positive metastatic melanomas that were also verified at mRNA level. Some metastatic tissues had high levels of the mutant protein and appeared to be

responsible for most of the observed variability. Other tissues had lower, but similar, levels of mutant protein expression (Figure 4B).

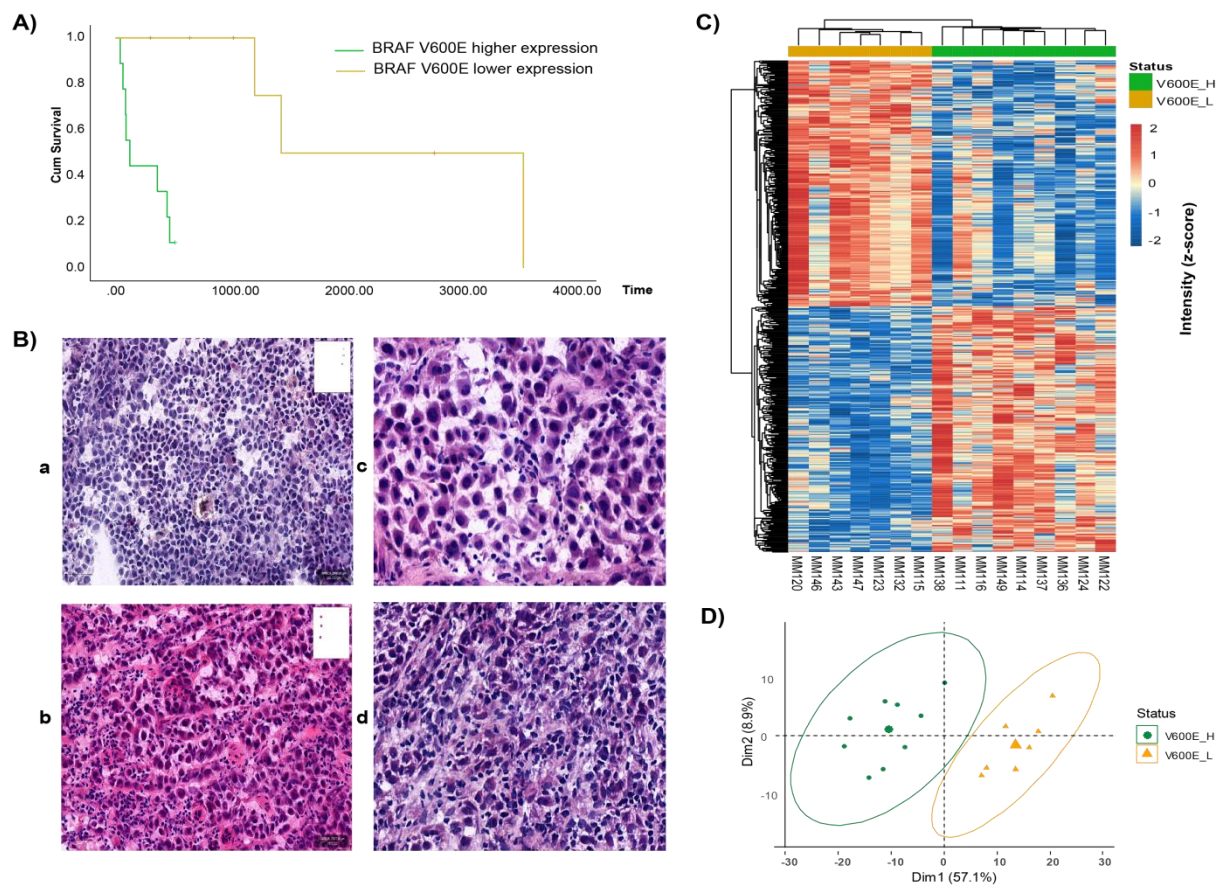


**Figure 4.** Patient clinical data and BRAF status for metastatic melanoma. (A) Heat map representation of patient clinical data and BRAF determination by genomic and proteomic techniques. (B) Plot of relative abundance of B-raf V600E protein against a reference sample.

To ascertain if there was a correlation between the relative abundance of the B-raf V600E protein and survival, four patients < 40 years of age at diagnosis were excluded from the analysis. This limit was decided because several studies have shown that survival is higher below this age [18–20]. The data revealed that B-raf V600E protein expression is significantly negatively-correlated with patient overall survival ( $r=-0.58$ ,  $p=0.048$ ). Univariate analysis generated two groups of patients with distinct differences in survival; and significantly-reduced survival was associated with a high expression of the B-raf V600E mutated protein (Figure 5A). The median overall survival for the two groups was markedly

different; 248 days (<9 months) for the 9 patients with the highest B-raf mutation levels and 2,460 days (>6 years) for the 7 patients with a lower expression of the B-raf mutation. Notably, all patients with high levels of B-raf V600E-expressing tumors did not survive beyond 18 months. This result suggests that protein expression of the B-raf V600E mutation in the tumor could be a significant risk factor for poorer prognosis of patients with stage 3/4 malignant melanoma.

Next, histological images of mutation-positive metastatic melanoma samples were examined to determine if any apparent morphological relationships exist between the high and low B-raf V600E mutant-expressing groups (Figure 5B and Figure 2 supplementary information). For tumors that expressed high levels of B-raf V600E, an increased vascularization was apparent. In addition, the cells were generally smaller but heterogeneous in size and had incohesion pattern (Figure 5B (a and b) images). Conversely, the cells from tumors with a lower expression of the B-raf V600E protein were less heterogeneous. This group was comprised of larger cells that often displayed multinucleation, a deeper cytoplasmic color, cell grouping, and connective tissue septa (Figure 5B (c and d) images). Based on the above-described features, a heterogeneity score (0-4) was calculated. The total score was equal to the tumor cell size variation + vascularization + discohesion + multinucleation. In order to accept a feature for the group, > 55% of cases had to display a specific property.



**Figure 5.** *B-raf V600E expression correlated with patient survival and tumor phenotype (A) Overall survival (OS) of malignant melanoma patients according to B-raf V600E mutation levels (log-rank  $p=0.001$ , Breslow  $p=0.002$  and Tarone Ware  $P=0.001$ ). (B) Histological images of mutation-positive metastatic melanoma samples: (a and b) tumors MM114 and MM111 with high expression of the B-raf V600E mutated protein; and tumor (c and d) tumors MM147 and MM120 with low expression of the B-raf V600E mutated protein. For all the images the magnification and scale were 10x and 50  $\mu\text{m}$ , respectively. (C) Hierarchical clustering heat map of 697 differentially-expressed proteins between the two groups of mutation-positive metastatic melanomas. (D) PCA of the two groups of mutation-positive metastatic melanomas based on the differentially-expressed proteins. Tumor samples from each group are highlighted in common colors: high B-raf V600E expression (V600E\_H, green), low B-raf V600E expression (V600E\_L, yellow).*

**Table 2.** Histopathological evaluation of tumors with B-raf V600E mutation

Group	cell size variation (7µm<)	neo- vascularization	discohesive pattern	mutli- nucleation	heterogeneity score (0-4)
B-raf V600E high	5/9 (56%)	7/9 (77%)	6/9 (66%)	3/9 (33%)	3
B-raf V600E low	3/7 (43%)	1/7 (14%)	2/7 (29%)	5/7 (71%)	1

*Tumors expressing high or low levels of B-raf V600E (rows) displayed heterogeneous properties with respect to tumor cohesion, vasculature, and cellular morphology (columns). The individual factors can be summed to give the heterogeneity score (final column).*

Whether the expression of the BRAF V600E mutation could define distinct molecular phenotypes for the two groups of mutation-positive metastatic melanomas was also explored. The 697 differentially-expressed proteins determined from the high and low B-raf V600E-expressing tumors were used to direct a hierarchical clustering at the level of protein quantitation. As expected, clear differences in protein abundance were apparent with the data dividing into two major clusters corresponding to the two groups (Figure 5C). This was also evident in the PCA plot (Figure 5D). A strong discrimination between high and low B-raf V600E-expressing tumors was observed in both analyses.

**2.4. Protein profiles associated with B-raf V600E expression**

Functional analysis of the 697 differentially-expressed proteins with the Ingenuity IPA system provided interesting insights. The overrepresented molecular functions included RNA post-transcriptional modification, cell-to-cell signaling and interaction, cellular development and cellular growth and proliferation. The top canonical pathways included B cell

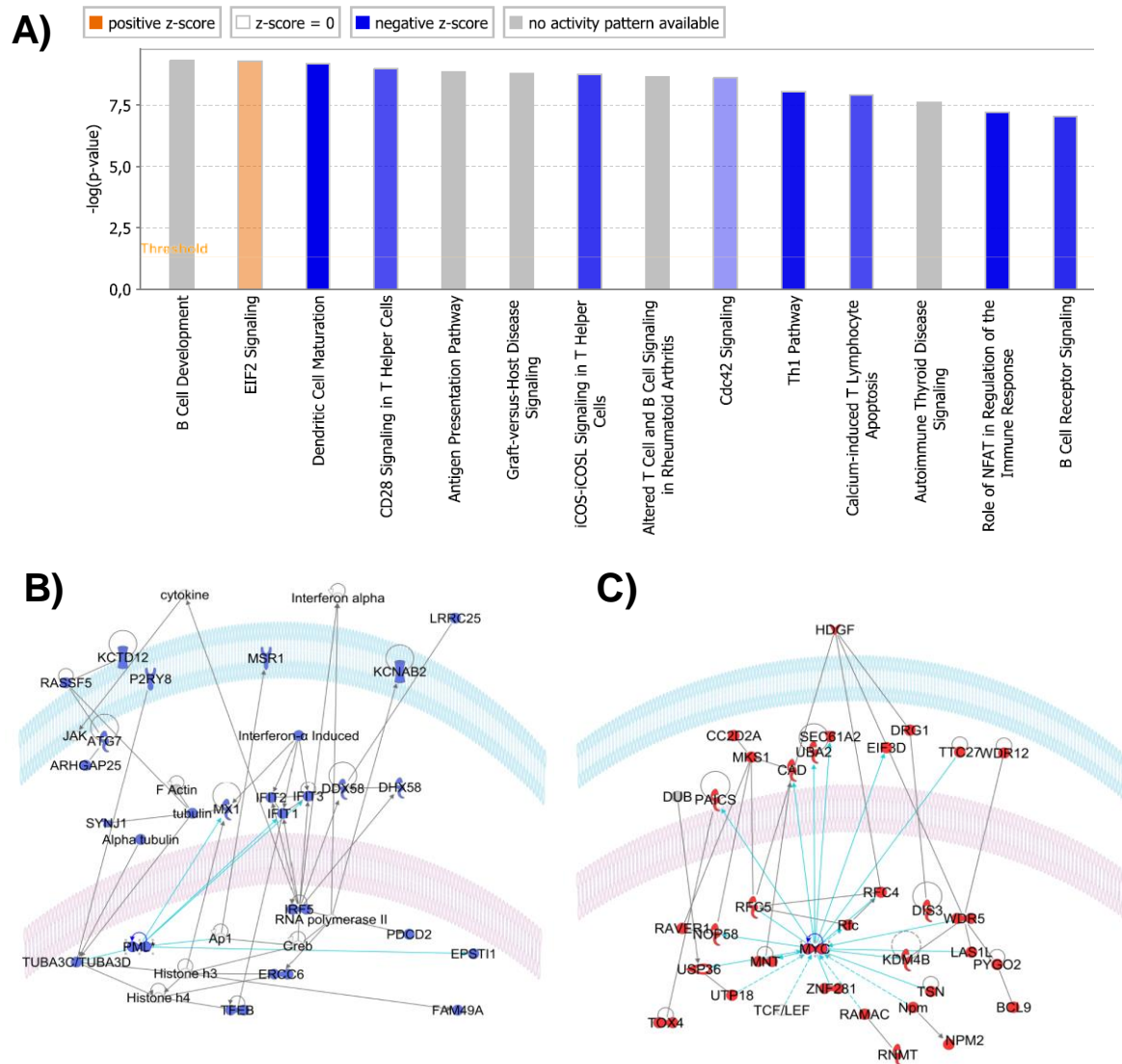


development, EIF2 signaling, antigen presentation pathway, B cell receptor signaling, natural killer cell signaling, and actin cytoskeleton signaling (see Figure 6).

When the IPA was performed only on the proteins that were up- and down-regulated in the samples with a high expression of B-raf V600E, revealing information emerged. The down-regulated proteins in the B-raf V600E\_high samples were significantly related to inflammatory response, cell-to-cell signaling and interaction, cellular growth and proliferation. Up-regulated proteins in the B-raf V600E\_high samples were significantly related to RNA post-transcriptional modification, protein synthesis, gene expression and cell death and survival. These relationships tend to reflect the proliferative nature of the B-raf V600E\_high tumors (poor prognosis) and the activated state of the immune response pathways in the B-raf V600E\_low tumors (better prognosis).

IPA-derived relational networks reflect possible roles of known oncogenes, e.g., MYC and HDGF (REF: PMID 27543492), overexpressed in B-raf V600E\_high samples (Figure 6C), and tumor suppressors, e.g., PML and toll-like receptors (PMID: 30127747, 29416846), downregulated in B-raf V600E\_high samples (Figure 6B).





**Figure 5.** (A) Canonical pathways overrepresented by proteins differentially-expressed in samples with low and high expression of B-raf V600E. Blue: on average, pathway members have a lower expression in B-raf V600E\_high samples. Red/orange: on average, pathway members have a higher expression in B-raf V600E\_high samples. (B) One of the top IPA relational protein subnetworks (blue) significantly-downregulated in samples with a high expression of B-raf V600E. The tumor suppressor PML is highlighted. (C) One of the top IPA relational protein subnetworks (red) significantly-upregulated in samples with a high expression of B-raf V600E. The oncogene MYC is highlighted.

### 3. Discussion

B-raf is a key regulatory protein in malignant melanoma that has a disparate and irregular mechanism of expression. This element is a major factor in understanding melanoma pathobiology. To verify the BRAF mutational status of a tumor, standard molecular pathology procedures are based on DNA sequencing. Diagnosis of BRAF mutations with this approach have not always been successful and sometimes failures in verification has led to major clinical consequences for the patients [21].

To date, the direct analysis of the protein in melanoma tumors has been achieved by immunohistochemistry [22–24]. In comparison to DNA-based assays, these studies have shown high sensitivity and specificity in detecting BRAF mutants, with a study reporting B-raf V600E protein expression as a novel prognostic marker in primary melanoma [25]. At present, however, the main limitations of the immunohistochemical approach are related to the possibility of false negatives because of the high degree of heterogeneity, and the subjective differences between pathologists when interpreting the results [26–28].

In the course of this research we found only one mass spectrometry-based study that identified and quantified the mutated B-raf protein in tumor tissues [17]. The mutation was detected in two colorectal carcinoma tissues with 2 mg of total protein extract used as the starting material. This indicates that the challenge of observing the B-raf V600E protein is most probably associated with low expression of the variant.

In this study, the B-raf V600E mutant was identified by mass spectrometry in metastatic melanoma samples for the first time. Compared to previous studies, the frequency of the BRAF mutation in our metastatic melanoma samples was slightly lower (35% c.f. 43%) [29]. When compared to the mRNA-based study performed on the same tissue samples, the identification of the B-raf V600E protein achieved 100% sensitivity and 90% specificity (Table 1). Discrepancies in the status BRAF V600E mutation using different techniques are

not uncommon [30]. This was also apparent from mutational status determinations at DNA and mRNA levels (Table 1). Taken together, such differential results may reflect the high degree of intra-tumoral heterogeneity often observed with BRAF V600E mutations. This aspect has been noted in previous studies [31–33].

The quantitation of the B-raf V600E protein variant exposed new insights into melanoma that were characterized by the high degree of variability and the increased level of expression of the mutated protein in one group of tumors (Figure 1A). In contrast, fewer dynamic changes were apparent with the other group of metastatic tumors that expressed the B-raf V600E mutation at lower, yet similar, levels. The different levels of B-raf V600E expression could subsequently be linked to the kinase activity of the protein that it is known to increase due to the mutation [34,35], and altogether provide information that is beyond the reach of genomics techniques.

WT B-raf was also identified in this study; however quantitation of the protein remains a challenge. Here, the relative abundance of the protein can only be determined based on unique tryptic peptides that ensured unambiguous quantitation of WT B-raf, RAF-1 and ARAF proteins. These proteins share more than 40% sequence homology. The V600E mutation, however, is located in a region that generates a tryptic peptide that is identical for all three proteins. Therefore, quantitation of the WT B-raf with our current data and experimental design was not attempted.

For the past few decades, the prognostic factors for melanoma in clinical practice have remained unaltered. Pathological staging that primarily focuses on tumor thickness is still applied as an estimate of the clinical behavior of the primary melanoma [36]. The mitotic score at the primary site is also often assessed [37,38]. Nevertheless, the prognostic value of As a consequence of the diverse and contradictory information surrounding patient survival

when analyzing both primary and metastatic melanomas the prognostic value of BRAF mutations is still under discussion [25,29,39–43].

Albeit in a small sample set, by focusing the analysis of B-raf V600E on protein quantitation rather than mutational status, our data suggests that the B-raf V600E expression level could be a prognostic factor for metastatic melanoma. Compared to metastatic tissue from patients with lower expression levels of B-raf V600E, higher levels were associated with a worse overall survival. Similar observations have been made in an immunohistochemistry study of primary tumors [25]. The authors showed that survival was significantly-reduced for patients with a strongly-positive B-raf V600E expression compared to the group with weakly-positive expression. To the best of our knowledge and although at different stages of the disease, that and our study are the only investigations that have analyzed B-raf V600E protein expression and reached the same correlation with clinical outcome. Nevertheless, when the disease is progressing and giving rise to metastases, whether in the lymph nodes or any distant organ, it is subject to evolutionary changes [44,45], and it may differ from the primary tumor in certain characteristics, e.g. morphology, tumor mutational burden, and eventually BRAF status [46,47]. At the time of the actual treatment, the latest information source is key to the optimal choice of therapy, thus, metastases shall be sampled and analyzed at time of progression [48,49]. Our study therefore further extends the existing knowledge on the role played by the BRAF V600E mutation in malignant melanoma.

Histological images of high- and low-expressing B-raf V600E tumors showed different features. For instance, tumors expressing high levels of B-raf V600E displayed a heterogeneous small-to-medium cell size, discohesion and largely devoid of connective tissue. Based on features such as tumor cell size variation, neovascularization, incoherence, and multinucleation, a heterogeneity score was calculated. Although the evaluation was performed on a limited number of samples, these features revealed that tumors that expressed

higher levels of the B-raf V600E protein had a higher degree of heterogeneity compared to the tumors expressing lower levels of B-raf V600E.

The tumor group that had a lower level of B-raf V600E protein showed a tendency towards multinuclear and larger cells; characteristics that have been associated with senescence [50–52]. Interestingly, a previous study has shown that B-raf V600E-expressing melanocytes display classical hallmarks of senescence. Thus suggesting that oncogene-induced senescence represents a genuine protective physiological process [53]. On the contrary, the tendency of the tumor group expressing high levels of B-raf V600E towards neovascularization is indicative of rapid cancer cell proliferation, tumor invasion and cancer progression in general [54].

The protein profiles associated with the high and low levels of B-raf V600E had striking functional features. For the proteins that were significantly more abundant in the B-raf V600E\_low samples, there was a clear over-representation of proteins involved in immune response pathways, e.g., PML, toll-like receptors, and HLA antigens [55,56]. Indeed, it is a well-appreciated and known fact that the activation of the immune response correlates with an improved prognosis.

Conversely, proteins that were significantly more abundant in the B-raf V600E\_high samples were clearly related to the proliferative nature of these tumors (and poor prognosis) and include several known oncogenes tumor drivers, e.g., the transcription factor MYC [57], and the growth factor HDGF [58]. Also, functional analysis of these proteins highlighted the over-representation of ribosome biogenesis proteins; thus reflecting the proliferative nature of an aggressive tumor type [59]. This preliminary data provides indications of a correlation between the levels of the B-raf V600E protein variant and cancer-related molecular pathways.

#### **4. Materials and Methods**

See supplemental material for details.

#### **4.1. Tissue specimens**

The study samples was approved by the Regional Ethical Committee at Lund University, Southern Sweden, approval numbers: DNR 191/2007, 101/2013 and 2015/266, 2015/618. All patients included in the study provided written, informed consent. The malignant metastatic tissues used in the study had been deposited in the Biobank located at the Department of Biomedical Engineering (Lund University, Sweden). Samples corresponded to metastatic tissue from melanoma patients undergoing surgery at Lund University Hospital, Sweden. The composition of the research tissue was assessed by a board-certified pathologist [15].

#### **4.2. Patient characteristics**

A total of 56 patients diagnosed with metastatic melanoma were evaluated in the study (Table 1 and Tables 1 and 2 supplementary information). Only two received targeted B-raf treatment with vemurafenib. There were 40 men and 16 women among the investigated cases. Average age $\pm$ standard deviation (range) at diagnosis of metastases was 64.1 $\pm$ 11.7 (24–89) years. The overall survival was 2.9 $\pm$ 3.5 (0.1–17.4) years. The majority of metastatic tissue studied were from the lymph nodes (82%), while the remainder were cutaneous, subcutaneous and visceral.

#### **4.3. BRAF V600E mutation testing**

BRAF V600E analysis results at DNA level were obtained from melanoma patient records stored at Lund University Hospital (Sweden) and were obtained following procedures detailed before [29,60].

Direct sequencing cDNA (ds-cDNA) was performed as previously described [14]. Briefly, mRNA was extracted from frozen melanoma tissue specimens and cDNA was synthesized.

Using a pair of BRAF-specific primers, BRAF cDNA was amplified by polymerase chain reaction (PCR). After purification of the PCR products, BRAF mutation status was determined by Sanger sequencing

#### **4.4. Protein extraction, digestion and automated C18 desalting workflow**

The protocols for protein extraction and digestion have been previously described [61]. Protein extraction was performed on sectioned, fresh-frozen human metastatic tissue (10 µm) using the Bioruptor plus, model UCD-300 (Dieagenode). Protein digestion and peptide desalting were performed on the AssayMAP Bravo (Agilent Technologies) platform with the urea solution digest and peptide cleanup v2.0 protocols.

#### **4.5. TMT 11-plex labeling and off line high pH fractionation**

Peptides were labeled with TMT 11-plex reagents according to the instructions provided by the manufacturer. The labeled peptides were mixed together and then purified and concentrated on a C-18 Sep-Pak cartridge (Waters). TMT-11 labelled peptides were fractionated by high pH RP-HPLC using a Phenomenex Aeris Widepore XB-C8 (3.6 µm, 2.1 × 100 mm) column on an 1100 Series HPLC (Agilent). Ninety-eight fractions were collected at 1 min intervals and further concatenated to 24 or 25 fractions.

#### **4.6. nLC-MS/MS analysis**

nLC-MS/MS analysis of peptide fractions were performed on an Ultimate 3000 HPLC coupled to a Q Exactive HF-X mass spectrometer (Thermo Scientific, San Jose, CA).

#### **4.7. Histopathological evaluation**

After each set of slides were submitted for molecular analysis, step-wise sectioning of the tissues was performed. Thus, on average, three sections were evaluated by a board-certified pathologist. Two to three  $\mu\text{m}$ -thick, frozen tissue sections were placed on glass slides, stained with hematoxylin and eosin, and then placed in an automated slide scanner system (Zeiss Mirax, Germany). The slides were then evaluated for tissue content as previously published [62]. Taking into account features that could be further captured based on morphology, all slides from each patient were evaluated and the properties scored as present or not present (0/1). To derive a yield of tissue heterogeneity, a score (0-4) was calculated and incorporated into a sum equaling tumor cell size variation + vascularization + discohesion + multinucleation. To accept a feature for the entire group, > 55% of the cases must display the specific property.

#### **4.8. Immunohistochemistry**

For immunohistochemistry, 4  $\mu\text{m}$ -thick, paraffin-embedded tissue sections were firstly placed on silanized slides. An automated immunohistochemical protocol was then performed using a BOND Autostainer and Polymer Refine Detection system (Leica Biosystems Inc., IL, USA). Anti-B-raf V600E antibody (clone VE1, Spring Bioscience Corp. CA, USA) was used at a dilution of 1:100 for 1 h at room temperature (RT). HIER made in BOND target epitope retrieval solution 2 (pH=9). The intensity of the brown colorimetric reaction was visualized by DAB.

#### **4.9. Data analysis**

Data were processed with Proteome Discoverer 2.3 (Thermo Fisher Scientific, San José, CA, USA) and searched against the Homo sapiens UniProt revised database (2018-10-01) and the B-raf V600E mutant protein sequence using the Sequest HT search engine. The search results



were directly imported into Perseus software [63] to perform data normalization and filtering of missing values.

To enable comparison across the entire sample set, relative protein abundances were calculated as the ratio between the protein intensity in the sample and the intensity of the protein in the reference. The protein intensities were calculated from the TMT 11-plex reporter ions, log<sub>2</sub>-transformed and normalized by subtracting the median intensity in each sample. The relative abundances were obtained by anti-log transformation after subtracting the normalized intensity of the protein from the reference sample.

For statistical analysis, SPSS 25 (SPSS Inc, Chicago, IL, USA) was used. Two groups of tumor samples were created according to the levels of mutated B-raf (high expression N=9, and low expression N=7). Kaplan-Meier survival analysis with log-rank, and Breslow and Tarone Ware testing were used for univariate analysis between Groups 1 and 2. P-value < 0.05 was considered statistically-significant and the B-raf expression values for patients reported as alive (N=5) were censored.

Differentially-expressed proteins between V600E\_H and V600E\_L were determined by Student t-test (two-tails). In this case, a p-value < 0.01 was considered significant. PCA and heat maps were generated in R [64,65] using the packages 'FactoMineR' and 'pheatmap' respectively. For functional analysis of the differentially-expressed proteins, the Ingenuity IPA Core Analysis was performed (Ingenuity, Qiagen).

## 5. Conclusions

The identification and relative quantitation of the B-raf V600E-mutated protein in malignant melanoma by mass spectrometry and the subsequent direct link of this information to a clinical outcome is pioneering work. To our knowledge, this is the first time that the

heterogeneous translation of BRAF V600E to the level of the mutated protein in tumors from metastatic melanoma that exhibit disparate or similar expressions has been verified. The study showed that a higher level of expression for the mutated protein is associated with more aggressive tumor progression. This revealed a subjacent layer of heterogeneity in V600E BRAF-mutated melanomas comprised of two distinct subtypes with different molecular features and associations with different clinical outcomes.

The results demonstrated the potential of proteomic techniques for molecular profiling and monitoring key mutations. Validation of our results in a larger cohort of metastatic melanoma patients will be beneficial for researchers, clinicians in the field. Quantitating the expression of the B-raf V600E protein/mutant will hopefully aid prognosis of the disease; and improve care for melanoma patients by providing relevant information that can potentially impact treatment regimes. In conclusion, as the drugs that have been developed target the protein and not the corresponding gene, such data is mandatory clinical information.

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