

Karakterizacija globalnih proteomskih profila stanica raka debelog crijeva čovjeka u ovisnosti o statusu gena BRAF

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UNIVERSITY OF RIJEKA
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Graduate programme
Biotechnology in Medicine

Veronika Horvat
Characterisation of global proteome profiles of human colon cancer cells
depending on the status of the BRAF gene
Graduate thesis

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Sažetak

Protein BRAF je signalna serin/treonin protein kinaza uključena u aktivaciju MAPK signalnog puta koji utječe na staničnu diobu, rast, diferencijaciju i preživljenje. Mutacije BRAF, kao što je BRAF V600E, dodatno aktiviraju MAPK signalni put što pojačava proliferaciju i preživljenje tumora. BRAF V600E mutirani kolorektalni karcinom predstavlja podskup kolorektalnog karcinoma karakteriziran lošim cjelokupnim preživljenjem i ograničenim odgovorom na kemoterapiju i ciljanu terapiju. Unatoč brojnim studijama koje ukazuju na pozitivan odgovor pacijenata oboljelih od kolorektalnog karcinoma s BRAF mutacijom na kombiniranu inhibiciju BRAF/EGFR, BRAF/MEK i BRAF/EGFR/MEK, ukupna stopa odgovora i medijan preživljenja bez progresije nisu značajni, što dodatno naglašava potrebu za učinkovitijim terapijama za ovu specifičnu populaciju pacijenata.

Stoga je svrha ovog diplomskog rada proširiti postojeće znanje o signalnim putevima i staničnim procesima koje regulira BRAF V600E mutacija u stanicama kolorektalnog karcinoma čovjeka. U tu svrhu, provedeno je globalno proteomsko profiliranje u rasponima pH 3-10 NL i 4-7 staničnih linija kolorektalnog karcinoma s divljim tipom BRAF (HTC116, SW620) i BRAF V600E mutacijom (RKO, HT29) uporabom dvodimenzionalne poliakrilamidne gel elektroforeze i MALDI-TOF/TOF masene spektrometrije. Pri tome smo identificirali dvadeset proteina sa statistički značajnom ($p < 0.05$) promjenom razine ekspresije u BRAF V600E mutiranim u odnosu na divlji tip BRAF stanica kolorektalnog karcinoma. Većina identificiranih proteina uključena je u stanične procese koji su važni za progresiju tumora uključujući staničnu komunikaciju, stanični ciklus i rast.

U ovom istraživanju otkriveno je nekoliko novih proteina biomarkera potencijalno povezanih s agresivnim fenotipom BRAF-mutiranog kolorektalnog karcinoma. Ti proteini uključuju proteine s potencijalnom onkogenom ulogom, kao što je protein 5 koji sadrži MORN ponavljanje, protein cinkovog prsta 230, eukariotski elongacijski faktor 2 kinaza i serin/treonin protein kinaza 40, kao i one koje mogu djelovati kao tumorski supresori, uključujući stanični receptor virusa hepatitisa A2 i Ras-povezan protein Rab-40A. Potrebne su daljnje imunohistokemijske analize kako bi se utvrdila uloga ovih proteina u patogenezi BRAF V600E-mutiranog kolorektalnog karcinoma i procijenila njihova prognostička relevantnost. Specifično ciljanje eukariotskog elongacijskog faktora 2 kinaze i serin/treonin protein kinaze 40 se može razviti kao nova strategija za kontrolu BRAF mutiranih kolorektalnih stanica i povećanje njihove osjetljivosti na kemoterapijske lijekove.

Ključne riječi:

MAPK signalni put, BRAF V600E mutirani kolorektalni karcinom, 2-DE, MALDI-TOF/TOF MS.

Summary

The BRAF protein is a signalling serine/threonine protein kinase involved in the MAPK signalling pathway that regulates cell division, growth, differentiation and survival. BRAF mutations, such as BRAF V600E, activate the MAPK signalling pathway which enhances proliferation and cancer cell survival. BRAF V600E mutated colorectal cancer represents a subset of colorectal cancer characterized by poor overall survival and limited response to chemotherapy. Despite multiple studies showing evidence of response to combined BRAF/EGFR, BRAF/MEK and BRAF/EGFR/MEK inhibition, overall response rates and median progression-free survival are modest, which further underlines the necessity for more effective therapies for this specific patient population.

The major goal of this graduate thesis was to extend the existing knowledge of signalling pathways and cellular processes regulated by the BRAF V600E mutation in colon cancer cells. For this purpose, global proteomic profiling of BRAF V600E mutant (RKO, HT29) *versus* wild-type BRAF (HTC116, SW620) colon cancer cells was carried out in the pH ranges 3-10 NL and 4-7 by means of two-dimensional gel electrophoresis followed by MALDI-TOF/TOF mass spectrometric analysis. In this way, we were able to identify twenty proteins with statistically significant ($p < 0.05$) changes in expression levels in BRAF V600E mutated in comparison to wild-type BRAF colon cancer cells. The majority of identified proteins were involved in cellular processes important for cancer progression including cellular communication, cell cycle and growth.

Importantly, this study revealed several novel protein biomarkers potentially associated with aggressive phenotype of BRAF mutated colorectal cancer. These include proteins with potential oncogenic role such as MORN repeat-containing protein 5, zinc finger protein 230, eukaryotic elongation factor 2 kinase and serine/threonine-protein kinase 40, as well as those that may act as tumour suppressors including Hepatitis A virus cellular receptor 2 and Ras-related protein Rab-40A. Further immunohistochemical analyses are required to ascertain the role of these proteins in the pathogenesis of BRAF V600E mutated CRC and to assess their prognostic relevance. In addition, targeting eukaryotic elongation factor 2 kinase and serine/threonine-protein kinase 40 may prove as a novel strategy to control behaviour of BRAF mutated CRC cells and to increase their sensitivity to chemotherapy.

Keywords:

BRAF kinase, MAPK signalling pathway, BRAF V600E mutated colorectal cancer, 2-DE, MALDI-TOF/TOF MS.

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1. INTRODUCTION

1.1. MAPK signalling pathway

The MAPK (mitogen-activated protein kinase) signalling pathway is one of the best characterized and investigated signalling pathways in cell biology. Proteins involved in this signal cascade transmit the signal from the extracellular space to the cell nucleus, which stimulates the expression of genes responsible for cell growth, mitosis and differentiation. Activation of the MAPK signalling pathway facilitates wound healing, and promotes cell migration and angiogenesis. Therefore, aberrations in the MAPK signalling pathway that regulates basic cell functions lead to the development of many tumours (1,2).

Activation of the MAPK signalling pathway begins with ligand binding in the extracellular space to the transmembrane tyrosine kinase receptor. The most studied receptors of the MAPK pathway are the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR). Binding of the ligand promotes oligomerization of the tyrosine kinase receptor resulting in the activation of the cytoplasmic kinase domain and transphosphorylation. The adaptor protein Grb2 binds the tyrosine kinase receptor and allows further binding of protein factors such as SOS-1 and CDC25. Protein factors can then interact with RAS proteins resulting in conformational change from the inactive GDP-bound state (guanosine diphosphate) of the RAS protein to the active GTP-bound (guanosine triphosphate) RAS protein (1). Downstream targets of activated RAS proteins are RAF proteins. Activated RAF proteins further phosphorylate and activate MEK1 and MEK2 kinases. Downstream in the MAPK signalling pathway, MEK kinases phosphorylate and activate ERK1 and ERK2 kinases. Activation of the ERK protein regulates many cellular processes. ERK1 and ERK2 phosphorylate and activate many cytosolic and nuclear proteins including kinases and transcription factors such as c-Myc, c-Ets1, c-Ets2, p90RSK1, MNK1 and MNK2. Activation of transcription factors ELK1 and c-JUN promotes expression of genes responsible for cell proliferation, while activation of Tob protein inhibits cell proliferation (1,3). Activation of ERK protein also regulates cellular processes such as cell migration through RHO GTPases, apoptosis *via* BCL-2, JNK and p38 proteins and survival through HIPPO signalling pathway (Figure 1.) (4). Since MAPK signalling pathway regulates many cellular processes, it has been identified as a critical regulator of carcinogenesis of many cancers, including colorectal cancer, and is therefore a major target for the development of anti-cancer therapy (5).

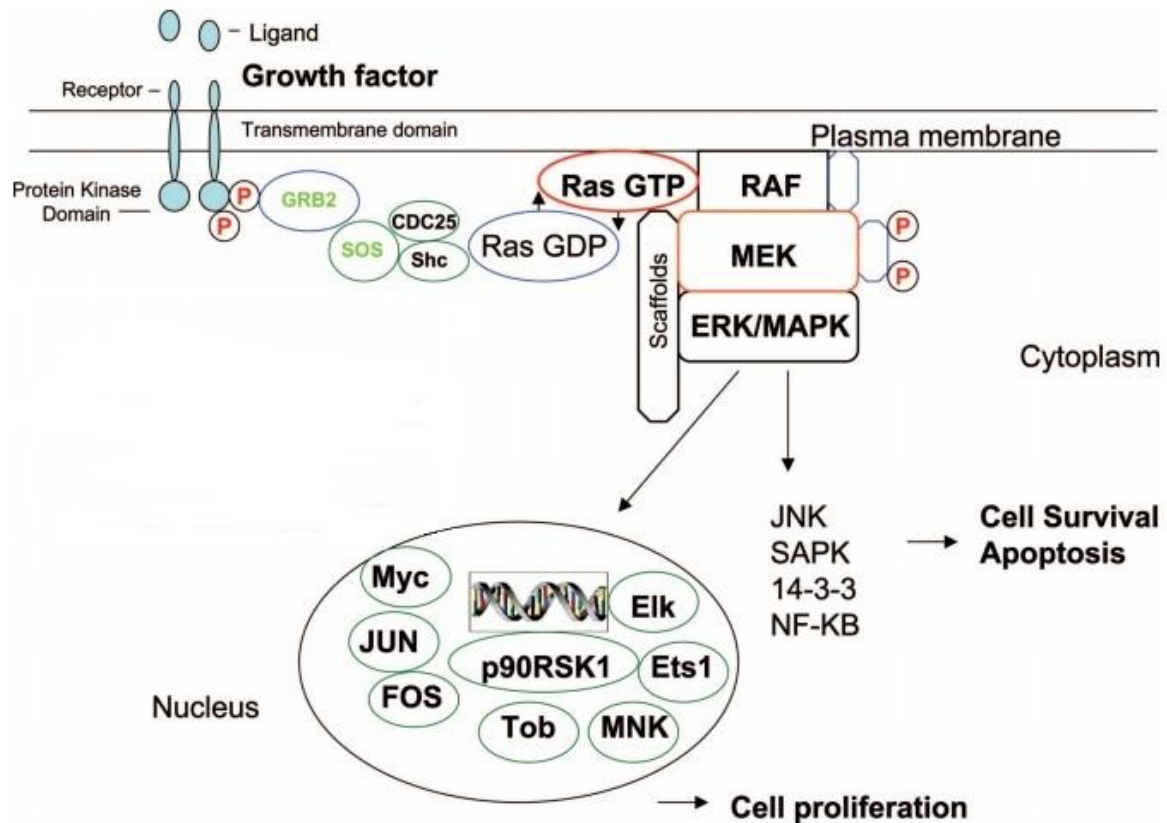


Figure 1. The MAPK signalling pathway involved in the process of cell proliferation and survival. Adapted from (1).

1.2. BRAF kinase

RAF proteins are key signalling molecules in the MAPK signalling pathway belonging to the serine/threonine kinase family and include ARAF, CRAF and BRAF proteins. (1,5) BRAF is an important oncogene and is the best characterized RAF protein isoform. The most common BRAF mutation, identified in approximately 90% of all BRAF mutated colorectal carcinomas, is missense mutation T1779A or nucleotide change of thymidine to adenine at position 1799 in the BRAF gene kinase domain, resulting in amino acid substitution of V600E, valine to glutamate in the protein structure at position 600. This specific mutation mimics phosphorylation and the BRAF protein is therefore in the state of increased activation. The mutated BRAF protein then deregulates downstream signalling and increases activation and phosphorylation of the MEK kinase. Thus, the mutated BRAF protein promotes MAPK pathway activity and cell proliferation independently of extracellular signals leading to tumour development. It is considered that the activity of the mutated BRAF V600E protein is ten times larger in comparison with wild type BRAF (2,5).

The BRAF protein kinase in the MAPK signalling pathway plays an important role in controlling cell proliferation, survival and inducing carcinogenesis. Excessive MAPK signalling eventually interferes with programmed cell death or apoptosis *via* cytosolic caspase that inhibits the release of cytochrome c from the mitochondria. Mutated BRAF kinase regulates the activity of cyclin D1 protein whose concentrations are then reduced and the cyclin-dependent kinase p27^{Kip1} inhibitor whose concentrations are increased in BRAF mutated cell lines (6). It has also been shown that BRAF V600E mutation reduces the expression of CDX2 (caudal type homeobox 2), a transcriptional factor and tumour suppressor protein involved in the regulation of intestinal epithelial differentiation, cell adhesion and polarity. Decreased expression of CDX2 is involved in the metastatic process and may contribute to poor prognosis for patients with BRAF mutated colorectal cancer (7). In the later stages of cancer, activation of the Wnt/ β -catenin signalling pathway followed by the inactivation of tumour suppressor proteins p53 and p16 greatly affects further progression of cancer (4).

1.3. KRAS kinase

RAS proteins are GTP-binding and low molecular weight proteins that include isoforms HRAS, NRAS and KRAS in human cells (1). Research into RAS proteins has shown that RAS proteins, as well as RAF proteins, play an important role in regulating cancer cell behaviour. More than 40% of colorectal cancers include activating missense KRAS mutations, most of which are G12D, G12V, G12C, G12A and G13D in codons 12 and 13 in GDP/GTP binding and hydrolytic domains (3).

The KRAS isoform of the protein is synthesized in the cell as an inactive propeptide that undergoes a series of post-translational modifications (e.g., farnesylation) on the carboxy terminus which increase protein hydrophobicity and allow its localization in the phospholipid layer of the cell membrane. Incorporated in the cell membrane, the KRAS protein cycles between an inactive GDP-bound state and an active GTP-bound state. GTP and GDP binding forms have highly different conformations. Therefore KRAS protein in its active GTP-bound state interacts with various effector molecules and regulatory proteins such as RAF and MAPK proteins and thus functions as a switch (1,3).

If mutation in the GDP/GTP binding and hydrolytic domain of KRAS proteins occurs, interaction between KRAS protein and its GTPase activator is reduced. KRAS protein then prefers its active GTP-bound state instead of returning to its inactive GDP-bound state, which increases the activation of the MAPK signalling pathway independent of the extracellular signal. Thus, mutations in KRAS proteins and increased protein activation lead to continuous cell proliferation and development of cancer (1).

Research has shown that simultaneous BRAF and KRAS mutations in colorectal carcinoma in humans are mutually exclusive (5,7). Since BRAF

and KRAS mutations both induce activation of the MAPK signalling pathway and have an equivalent effect on carcinogenesis, co-mutations in the same cascade do not offer a selective advantage for tumour development.

1.4. Molecular and clinical features of BRAF V600E mutant colorectal cancer

BRAF mutations, specifically V600E mutation, are detected in 7%-10% of all metastatic CRC (7). Mutations in BRAF have also been identified in several other human tumours such as melanoma (50-60%), papillary thyroid carcinoma (30-70%) and low-grade serous ovarian carcinoma (30%) (6). Different studies have shown strong correlation between V600E mutated BRAF CRC and increased risk of mortality with reduced median overall survival of CRC patients.

Patients with mutated BRAF have rapidly progressing disease with a lower chance to receive the second-line treatment unlike those with BRAF wild-type cancer (6). Despite the treatment with combination chemotherapy and targeted therapies, the median survival rate of BRAF mutant CRC patients determined in most studies is one year compared to two to three years in BRAF wild-type patients (3,7). BRAF mutation status is therefore considered as a prognostic indicator of survival.

BRAF mutant colorectal cancer represents a distinct subset of colorectal cancer with specific molecular, pathological and clinical features. BRAF V600E mutated CRC is associated with female sex, older age, location on the right side of the colon (proximal colon), high-grade histology and reduced response to anti-EGFR therapy (3,7). BRAF mutations are linked with a specific metastatic pattern with higher likelihood of spreading to peritoneum and lymph nodes and lower chances of lung metastases (7). An identical pattern of BRAF mutation has been recorded in both primary tumours and related metastasis with a concordance rate of around 90% (6).

Jang et al. (8) have found several factors that correlate with a negative prognosis of BRAF mutant CRC. They demonstrated that, in contrast to BRAF wild-type CRC, BRAF mutant CRC was more frequently presented with histologic appearance of mucinous, signet ring cells, infiltrative borders, tumour budding and poorly differentiated histology, all of which was commonly associated with a worse prognosis. Several studies have confirmed low tumour grading, i.e. poor tumour differentiation. Therefore, colorectal cancer is more commonly classified as advanced 3rd and 4th stage rather than early first and second (3,7).

BRAF V600E mutated CRC is also associated with microsatellite instability (MSI). Microsatellite instability is a form of genetic instability caused by defects in DNA mismatch repair (MMR) system which is characterised by an abnormal (increased or decreased) length of microsatellite repeats (repetitive DNA). Defective insertions, deletions and DNA substitutions are not fixed during replication due to DNA mismatch repair (MMR) deficiency resulting in the emergence of high rate mutations

(4). Research has shown a significant difference in the frequency of BRAF mutations between MSI-high and microsatellite stable (MSS) tumours. Specifically, BRAF mutation occurs in 60% of MSI-high tumours and in only 5-10% of MSS tumours (5). Nonetheless, both MSI-high and MSS BRAF mutated CRCs have poor overall survival (6,9).

Inflammatory reactions of the host against the cancer are commonly observed in various types of cancers. Several studies of CRC have revealed the anti-tumour effect of tumour-infiltrating lymphocytes (TILs), especially CD8+ T cells, and discovered their positive effects on survival. Jang et al. (8) found that BRAF mutated CRC more frequently exhibited peritumoral lymphoid reactions than BRAF wild type CRC independently of the MSI status, but those observed reactions did not have a prognostic relevance in their study. They have highlighted the immune microenvironment as a new marker of prognostic prediction in CRC. On contrary, Zlobec et al. (10) showed that BRAF mutation correlated with the lack of peritumoral lymphocytic reaction what might explain the poor prognosis of patients with BRAF mutated CRC. Therefore, additional large population studies are needed to determine the exact relationship between BRAF mutation status and tumour-associated inflammatory reactions (8). By identifying all common clinical characteristics of V600E BRAF mutated CRC, it will be possible to improve early diagnosis and determine the best course of treatment (7).

An international consortium dealing with CRC classification systems proposed four consensus molecular subtypes with different features and identified BRAF mutations as frequent mutations in CMS1 (microsatellite instability immune) subtype that includes tumours which are hypermutated, microsatellite unstable, hypermethylated and have strong immune activation (11). In line with previous studies showing worse prognosis of patients with MSI and BRAF mutated CRCs that recur, patients classified into the CMS1 group had very poor survival rate after relapse.

Based on specific gene expression patterns, Barras et al. (9) have propounded classification of BRAF mutant CRC into two distinct subtypes called BM1 and BM2. Their analysis has revealed that BM1 is associated with changes in EMT-related processes (EMT, apical junction, myogenesis) and immune response, whereas BM2 displays deregulation of the cell cycle. Thus, KRAS/AKT pathway activation and mTOR/4EBP deregulation were found in BM1 subtype. EMT-related processes were increased in BM1 and repressed in BM2. Cell cycle-related processes and glycolysis were not only increasingly activated in BM2 compared with BRAF wild type CRC and KRAS mutant CRC but were concomitantly repressed in BM1. Cell cycle-related proteins such as CDK1, cyclin D1, ATM and E2F as well as genes involved in G2 to M transition were also differentially expressed between these two subtypes which further confirms cell cycle misregulation in BM2 subtype. WNT activation was decreased in both subtypes in contrast to BRAF wild type and KRAS mutant CRC, although BM1 has higher WNT activity than BM2. Significantly different EGFR expression levels between BM1 and BM2 were not found. BM1 subgroup was associated with a stronger immune profile and enhanced inflammatory response that includes increased

activation of IL2/STAT5 and TNF- α signalling pathways *via* NF- κ B and IL6/JAK/STAT3. Process of angiogenesis was also enriched in BM1 compared to BM2. BM1 tumours were more significantly infiltrated by macrophages and monocytes rather than resting dendritic cells, while BM2 displayed more mast cell content (9).

1.5. Treatment options in BRAF mutated CRC

Due to poor prognosis of patients with BRAF mutated CRC, it is important to identify which combination of different drugs and which different pathway inhibitions are particularly effective. The first line of treatment in BRAF V600E mutated CRC includes chemotherapy FOLFOX (folinic acid/leucovorin, 5-fluorouracil and oxaliplatin), FOLFIRI (folinic acid/leucovorin, 5-fluorouracil and irinotecan) or FOLFOXIRI (folinic acid/leucovorin, 5-fluorouracil, oxaliplatin and irinotecan) (12). Anti-EGFR monoclonal antibodies (cetuximab and panitumumab) have been added to standard treatment of BRAF mutated CRC due to their positive results in the RAS wild type population (2).

Tol et al. (13) conducted an analysis in patients with metastatic CRC comparing chemotherapy, bevacizumab and cetuximab (CBC regimen) in comparison with chemotherapy and bevacizumab alone (CB regimen). They found that median progression-free survival (mPFS) was shorter among patients treated with CBC regimen than among patients treated with CB regimen. Patients with BRAF mutated tumours had a significantly shorter mPFS and mOS compared to patients with wild-type-BRAF tumours, both in the CB group and in the CBC group. No difference in response rates was observed. They concluded that BRAF mutation is a negative prognostic marker in patients with metastatic CRC, independent of the chemotherapy and the biological agent used.

Bevacizumab is an antiangiogenic monoclonal antibody that targets the ligand vascular endothelial growth factor A (VEGF-A) and prevents its binding to VEGF receptor 2. Studies compared the benefit of FOLFOXIRI and bevacizumab versus FOLFIRI and bevacizumab in patients with BRAF V600E mutated CRC (6,7,12). Although results lacked the statistical significance, a trend towards the benefit of FOLFOXIRI and bevacizumab was shown. This evidence suggests that an intensive treatment with FOLFOXIRI and bevacizumab could be a suitable first line treatment option.

BRAF kinase inhibitors have been developed and tested in clinical trials and approved by the Food and Drug Administration (FDA) for the treatment of cancers with BRAF mutations. Some of the BRAF inhibitors include sorafenib, encorafenib, dabrafenib and vemurafenib (3). Vemurafenib is a small molecule that potently and selectively inhibits mutated BRAF protein. It has been shown to inhibit cell proliferation and tumour growth in BRAF V600E mutant CRC cell lines and xenograft models (12). Because of its high activity and efficacy in reducing or eliminating tumours, it is used for the treatment of advanced BRAF mutated melanoma. Similar efficacy was

expected in the treatment of BRAF mutated metastatic CRC. However, unlike melanoma, single-agent vemurafenib in the treatment of BRAF mutated metastatic CRC was shown to be ineffective in reducing or eliminating cancer (4,6,12). This finding has spurred further research into the mechanisms of resistance to BRAF inhibition in CRC.

1.6. Mechanisms of resistance to BRAF inhibitors

1.6.1. Mechanism of primary resistance

Mechanisms of resistance to BRAF inhibition in melanoma have been extensively studied and include MEK1, MEK2 and NRAS activating mutations, BRAF amplification, excessive expression of COT, PDGFR and EGFR, and secondary mutations in RAF genes. However, those findings cannot be directly translated into BRAF mutated CRC because responses to BRAF inhibitors are very different between these two types of cancer (4). Research has shown that the reason for these differences is the rapid development of resistance in CRC involving the reactivation of MAPK and activation of PI3K/AKT and Wnt signalling pathways. New strategies are being developed involving simultaneous inhibition of these pathways (6).

It has been shown that primary resistance of BRAF V600E mutated CRC involves rapid reactivation of EGFR by the feedback mechanism (7). In BRAF V600E mutated CRC phosphorylated and activated ERK kinase negatively regulates EGFR signalling. However, when tumour cells are treated with vemurafenib, several molecular changes occur in cellular MAPK signalling that affect the development of resistance. Such changes include the inhibition of phosphorylated ERK, KRAS and MEK1 mutations, KRAS, BRAF and CRAF amplification, formation of RAF protein dimers, CRAF-mediated reactivation of MAPK signaling, all of which promotes EGFR signaling and leads to MAPK signaling reactivation and consequently tumour cell proliferation, tumour growth and progression (2,4). It is considered that such mechanism of resistance is specific for CRC since most melanoma cells do not increase EGFR expression level after treatment with BRAF inhibitors, from which it can be concluded that BRAF V600E mutation differently regulates protein interactions between these two types of cancer (6). Despite the inhibition of BRAF kinase, reactivation of MAPK signalling pathway in BRAF V600E mutated CRC explains the inefficacy of BRAF inhibitors in these patients (7).

New treatment strategies are therefore developed that prevent EGFR reactivation in combination with BRAF inhibitors such as vemurafenib or dabrafenib. Research has shown that dual inhibition of BRAF and EGFR is effective in MAPK signalling suppression *in vitro* and in xenograft models of BRAF mutant CRC (7). Clinical trials have evaluated the combination of BRAF and EGFR inhibitors including vemurafenib and panitumumab, vemurafenib and cetuximab, vemurafenib and erlotinib, encorafenib and cetuximab, and dabrafenib plus panitumumab. Combined EGFR and BRAF

inhibition in clinical trials showed modest tumour regression with 4–23% response rates with main adverse events being vomiting and fatigue (2,7).

To improve the clinical benefit of combined EGFR and BRAF inhibition, Yang et al. (14) evaluated combinations of vemurafenib with different drugs including capecitabine, bevacizumab, irinotecan, erlotinib and cetuximab in BRAF mutated CRC cell lines and xenografts. All doublet combinations achieved greater antitumor activity and survival than monotherapy, but the highest rate of tumour growth inhibition was achieved with triple therapy including vemurafenib, cetuximab and irinotecan. Based on these results, Phase I and II (SWOG 1406) clinical trials with this specific triple combination therapy were carried out and showed that both survival outcomes and response rates were higher in patients receiving triplet therapy than in patients receiving vemurafenib monotherapy and irinotecan-cetuximab doublet therapy. (2) In the SWOG 1406 study, the addition of vemurafenib significantly prolonged mPFS in comparison with irinotecan and cetuximab alone (4.4 vs 2.0 months). (7) The triplet therapy was well tolerated with the most common adverse effects being fatigue, diarrhoea, nausea, anaemia and rash with no observed increase in skin toxicity. (7,12) Therefore, the combination of vemurafenib, cetuximab and irinotecan is an emerging standard care treatment option.

Because MEK plays an important role in resistance to BRAF inhibition, studies investigated the combination of dabrafenib, a BRAF inhibitor, with trametinib, a MEK inhibitor. The combination showed only a limited improvement in patients with BRAF V600E mutant CRC with 12% partial responses, 51% stable disease and one durable complete response (6). Combined BRAF and MEK inhibition reduced pERK levels by 47% what indicates that additional targets must be inhibited to effectively block MAPK signalling. (7) Although no increase in EGFR phosphorylation was detected, this combination could be improved by adding EGFR inhibitor to target feedback reactivation of the MAPK pathway (2).

So, to further achieve sustained MAPK pathway inhibition, recent studies have investigated combination of BRAF inhibitor dabrafenib, MEK inhibitor trametinib and EGFR inhibitor panitumumab in patients with BRAF V600E mutated CRC (6,7,12). The response rate to dabrafenib plus panitumumab was lower compared to triplet combination. Triplet therapy produced more potent inhibition of pERK than either dabrafenib and panitumumab doublet or dabrafenib and panitumumab doublet. These results point to combined targeted therapies as a therapeutic option for BRAF mutant CRC (Figure 2.).

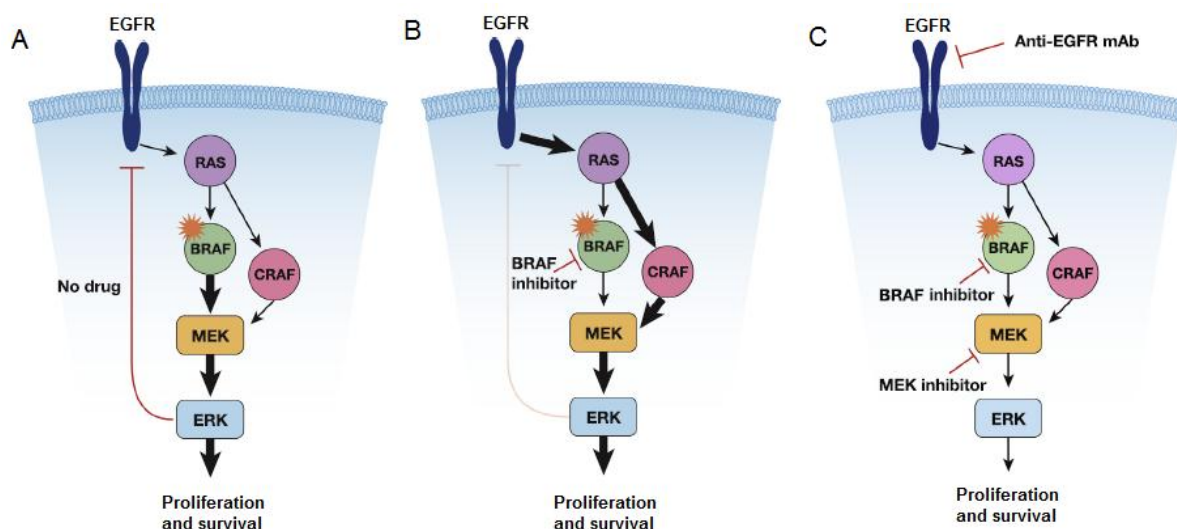


Figure 2. A) Negative feedback mechanism in BRAF mutated CRC. Mutated BRAF protein increasingly activates and phosphorylates MEK kinase which promotes MAPK signalling (bold line), while ERK kinase negatively regulates EGFR signalling. B) Primary resistance mechanism after BRAF inhibition. ERK-dependent negative feedback is reduced but MAPK signalling reactivation via CRAF protein (bold line) occurs. C) Targeted inhibition of MAPK signalling pathway with BRAF, MEK inhibitors and anti-EGFR monoclonal antibody (mAb). Adapted from (7).

1.6.2. Alternative mechanisms of resistance

Alternative mechanisms that explain the resistance of BRAF mutant CRC to BRAF inhibitors have been partially identified. Research in BRAF V600E mutated CRC cell lines has shown increased activation of PI3K/AKT signalling pathway (phosphoinositide 3-kinase/protein kinase B) in relation to melanoma (3). RAS kinases activate PI3K type 1 *via* the catalytic domain of the protein. Activation of PI3K consequently leads to phosphorylation and activation of AKT kinases that stimulate cell proliferation, survival and growth. Other mutations in BRAF V600E mutated CRC have been identified and include mutations in PTEN (tumour suppressor protein) and PI3CA (catalytic subunit PI3K) genes that activate PI3K/AKT signalling pathway and lead to cellular survival and proliferation (Figure 3.) (5,7). Results suggest that elevated activation of MAPK and PI3K signalling pathways leads to limited therapeutic effect of monotherapy with BRAF inhibitors. Combination therapy with BRAF, EGFR and PI3K inhibitors was therefore proposed as a solution for patients with BRAF V600E mutated CRC (2).

Preclinical data confirmed that simultaneous inhibition of BRAF, EGFR and PI3K resulted in synergistic anti-proliferative effects *in vitro* and tumour regression in xenograft models (12). Therefore, a clinical study was performed in which patients were treated with combination therapy including BRAF inhibitor encorafenib and EGFR inhibitor cetuximab with or

without alpelisib, a potent inhibitor of the alpha subunit of PI3K (2,6,12). Although Phase I trial showed promising results with disease control rate of 93% for the triplet versus 77% for doublet therapy, Phase II trial showed no statistically significant differences between triplet and doublet therapy. Additionally, toxicity increased in triplet combination with high rates of grade III and IV adverse effects (2).

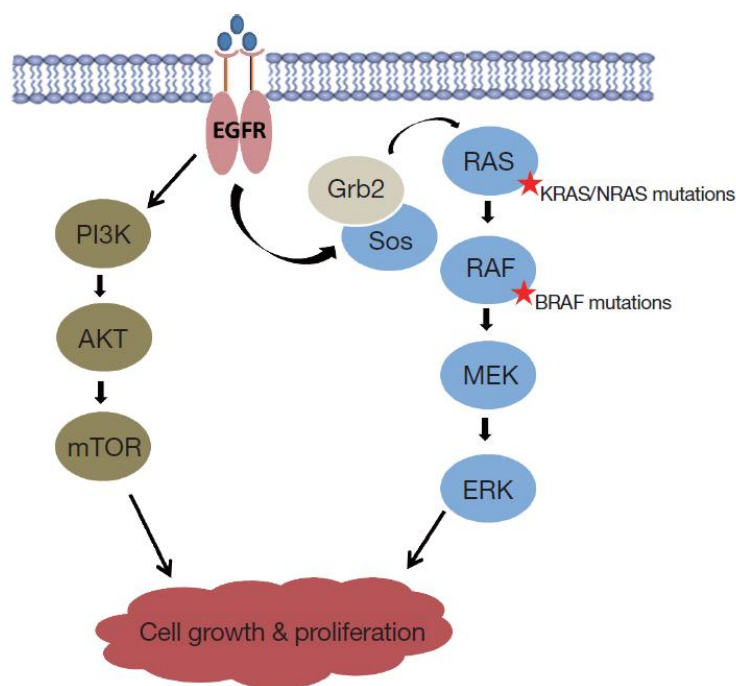


Figure 3. Activation mechanism of the PI3K/AKT signaling pathway. PI3K kinase phosphorylates and activates AKT kinase which promotes cell proliferation, survival and growth. Adapted from (5).

Another alternative resistance mechanism includes an evolutionally conserved Wnt/ β -catenin signalling pathway involved in the regulation of cell proliferation, differentiation and survival (7). ERK kinase phosphorylates and activates Wnt co-receptor LRP6 which regulates the transcription of c-Myc proto-oncogene. Studies have shown the presence of mutations in Wnt/ β -catenin signalling pathway in patients with BRAF V600E mutated CRC (2,7). Thus, signalling pathways MAPK and Wnt/ β -catenin act synergistically in the control of cell proliferation and are therefore important in the development of many cancers and resistance to drugs. Therefore, a combined therapy targeting MAPK and Wnt signalling pathway is another option for the treatment of BRAF mutated CRC (2).

In order to improve the survival and quality of life in patients with BRAF mutated CRC, it is necessary to better understand the molecular mechanisms regulating cell response to BRAF inhibitors. This knowledge will contribute to the development of new treatment strategies based on inhibition of MAPK signalling pathway and alternative signalling pathways (Figure 4.) (6,7).

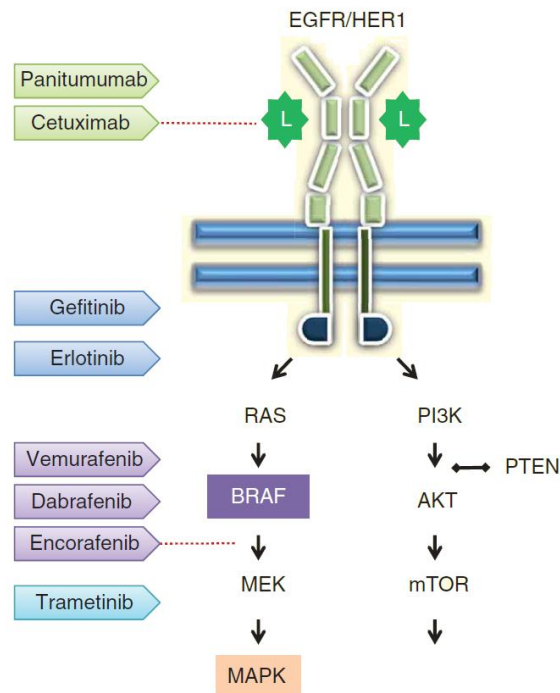


Figure 4. Therapeutic possibilities for multiple inhibition of MAPK signalling pathway including EGFR inhibition (monoclonal antibodies panitumumab and cetuximab, inhibitory molecules gefitinib and erlotinib), inhibition of BRAF kinase (inhibitory molecules vemurafenib, dabrafenib or encorafenib) and inhibition of MEK kinase (inhibitor molecule trametinib). Adapted from (6).

1.7. Cell lines

Four colorectal carcinoma cell lines were used in this study. RKO and HT-29 cell lines harbour BRAF V600E mutation, whereas HCT-116 and SW620 cell lines are BRAF wild-type. Some genetic features of these cell lines are presented in Table 1.

Earlier research explored the antitumor activity of BRAF inhibitor vemurafenib and combined therapies in preclinical models of BRAF V600E mutant CRC. (14) Antitumor effect of vemurafenib was observed in the HT29 xenograft model. However, efficacy observed during clinical studies of single-agent vemurafenib is modest. Paradoxically, vemurafenib induced ERK and MEK phosphorylation in BRAF wild type HCT116 cells. RKO cell line harbours a mutation in the PIK3CA gene, resulting in constitutive activation of the PI3K–AKT signaling pathway. RKO cells do not respond to the antiproliferative effect of vemurafenib but combination of vemurafenib with the AKT inhibitor is efficient to induce blockade of both pathways. Even though the addition of AKT inhibitor to vemurafenib was efficient, it did not produce complete regression of tumours.

Table 1. Colorectal carcinoma cell lines classified by the mutational status of critical genes and MSI status. Abbreviations: MSI – microsatellite instability, MSS – microsatellite stable, wt - wild type. Adapted from (15).

Cell line	MSI status	KRAS	BRAF	PIK3CA	TP53
RKO	MSI	wt	V600E	H1047R	wt
HT-29	MSS	wt	V600E	P447T	R273H
SW620	MSS	G12V	wt	wt	R273H, P309S
HCT-116	MSI	G13D	wt	H1047R	wt

2. OBJECTIVE

The aim of this study was to broaden current knowledge and to better understand signalling pathways and cellular processes specifically regulated by BRAF V600E mutation in colorectal cancer. For that purpose, we performed global comparative proteomic analyses of human colorectal cancer cells bearing wild-type BRAF and BRAF V600E mutation. Using 2-DE and MALDI-TOF/TOF MS, we set out to identify specific molecular differences that could account for aggressive phenotype of BRAF mutated colorectal carcinoma. We expect that obtained results will reveal specific proteomics signatures with potential implications in the BRAF V600E-dependant regulation of colon cancer cell behaviour.

3. MATERIALS AND METHODS

3.1. Cell culture

Cells were cultured in plastic cell culture flasks (Falcon, USA) at 37°C and 5% CO₂ in culture medium DMEM (Dulbecco's Modified Eagle Medium, GIBCO Invitrogen, USA) previously completed with FBS (foetal bovine serum, GIBCO Invitrogen, USA) to final volume ratio of 10% and L-glutamine (Sigma, Germany) to final concentration of 2 mM. In order to prevent microbial growth, antibiotics penicillin and streptomycin (GIBCO Invitrogen, USA) were added to final concentrations of 100 units/mL and 100 µg/mL, respectively. RKO cell line was grown in completed EMEM (Eagle's minimal essential medium, GIBCO Invitrogen, USA) liquid culture media. Cells were grown until 100% confluence.

3.2. Cell preparation and lysis

Medium was removed and confluent cells were washed with PBS (phosphate buffered saline). To detach cells from the flask surface, 0.25% trypsin solution (GIBCO Invitrogen, USA) was added and cells were incubated for 5 minutes in an incubator. Confluent cells were collected and transferred into falcon tubes and centrifuged for 5 min at 4 °C and 1200 rpm. Cell pellet was washed three times using 16x diluted PBS (0.18 mM KCl, 9.133 mM NaCl) to remove salts that could potentially interfere with isoelectric focusing (IEF). All remaining PBS was removed and cell lysis buffer (7M urea, 2M thiourea, 4% CHAPS and 1% DTT) supplemented with a cocktail of protease inhibitors (Roche, Switzerland) was added onto the cell pellet. Cell pellet was briefly resuspended and vortexed until it was fully dissolved. Samples were incubated in thermoblock for 1 hour at 30°C at constant shaking (1000 rpm). To remove cell debris, samples were centrifuged in an ultracentrifuge (Sorvall™ MTX 150 Micro-Ultracentrifuge, Thermo Fischer Scientific, USA) at 4 °C and 40 000 rpm for 30 minutes, after which supernatant containing proteins was gently transferred into new Eppendorf tube and stored at -80°C until further analysis.

Concentration of proteins in samples was determined using Qubit™ Protein Assay Kit (Invitrogen, Thermo Fischer Scientific, USA) according to the manufacturer instructions and Qubit™ fluorimeter (Invitrogen, Thermo Fischer Scientific, USA).

3.3. Two-dimensional gel electrophoresis (2DE)

A total of 400 µg of proteins was loaded onto 17 cm 3-10 pH nonlinear gradient IPG strips, and 800 µg of total proteins was loaded onto 17 cm 4-7 pH IPG strips. After overnight protein precipitation with ice-cold acetone, samples were centrifuged at 4 °C, 15 minutes and 14 000 rpm. Acetone was removed and protein pellet was dissolved in 330 µL of cell lysis buffer (7M urea, 2M thiourea, 4% CHAPS and 1% DTT). After gentle resuspending

and vortexing, samples were placed into ultrasonic bath for 15 minutes and briefly centrifuged to remove residual protein clusters. Protein samples were loaded onto the focusing tray, IPG strips were placed onto samples and kept for 15 minutes in the focuser (PROTEAN i12™ IEF system, Bio-Rad, USA), after which 4 mL of mineral oil was added onto each IPG strip. Active rehydration for 12 hours at 50V and 20 °C was applied. After rehydration, the programme was paused and wetted paper wicks were placed between the IPG strip and both electrodes. Isoelectric focusing was started with the programme details shown in Table 2. The same programme was used for both types of IPG strips with current limit of maximum 50 µA per strip.

Table 2. Running conditions for isoelectric focusing

STEP	Voltage ramp	Voltage	Duration
01	Rapid	250	0:15 h
02	Rapid	500	1:00 h
03	Gradual	10 000	3:00 h
04	Rapid	10 000	50 000 Vh
05	Hold	50	

After IEF, IPG strips were reduced in 100 mM DTT (dithiothreitol) equilibration buffer and alkylated in 2.5% IAA (iodoacetamide) equilibration buffer. For SDS-PAGE, IPG strips were shortly washed in 1x running buffer and immediately placed on the top of 1.0 mm 12% polyacrylamide gel, and layered with agarose solution (0.5% Agarose, 0.0002% Bromophenol blue, 10x running buffer and ultrapure water). Electrophoresis was run using PROTEAN II xi Cell (Bio-Rad, USA) at 30 mA for 30 minutes in the first step and 50 mA until the protein front reached the end of the gel in the second step. Gels were stained with Coomassie dye (0.1% Coomassie Brilliant Blue G-250 (Sigma-Aldrich), 2% orthophosphoric acid, 10% ammonium sulphate) and methanol in the ratio of 8:2. After destaining with demineralized water, gels were stored in 1% acetic acid solution at 4 °C until image analysis.

3.4. 2DE gel image analysis

Gel images were acquired using ChemiDoc XRS+ system (Bio-Rad, USA) with 600 dpi resolution. For the image analysis, three gels were taken, each displaying a separate cell lysate. 2DE gel image analysis was performed using the Progenesis SameSpots software v4.0 (Nonlinear Dynamics, UK) by Dr. Tania Gamberi from the University of Florence, School of Human

Health Sciences, Department of Experimental and Clinical Biomedical Sciences "Mario Serio". The univariate data analysis was performed as one-way ANOVA on each spot individually. Then, multivariate statistical analysis was applied on all the ANOVA p-values by the False Discovery Rate (FDR) correction method (q-value) (16). In addition, a power analysis was carried out in order to determine the number of sample replicates that need to be analysed to confidently discover differentially abundant proteins. We considered statistically different protein expression for spots having ANOVA p-value <0.05, q-value <0.1 and power >0.8.

Strict criteria of protein selection had to be applied in order to minimize the identification of proteins whose expression is connected with cell line characteristic, not the BRAF mutational status. Therefore, when looking for differences between the BRAF mutant and BRAF wild type cells, differentially expressed protein should be either expressed or absent in both cell lines sharing the same BRAF status. All spots matching these criteria were carefully excised from 2D gels and stored in demineralised water at -80°C until mass spectrometry analysis.

3.5. Protein identification with MALDI-TOF/TOF MS

Proteins from Coomassie-stained gel spots were prepared for digestion. In order to remove Coomassie dye and SDS, repeating gel rehydration and dehydration was performed. Rehydration was achieved with 100 mM ammonium bicarbonate swelling solution. Dehydration was achieved with a shrinking solution of 50 mM ammonium bicarbonate in 60% acetonitrile. Gel spots were incubated on shaker and after each successive step, the supernatant was discarded. The protocol was performed in the following steps:

1. Swelling solution was added and incubated for 15 minutes.
2. Shrinking solution was added and incubated for 30 minutes.
3. Swelling solution was added and incubated for 20 minutes.
4. Shrinking solution was added and incubated for 30 minutes.
5. Steps 3 and 4 were repeated.

The protein samples were completely dried in the speed-vac for 30 minutes. 10 ng/μl of trypsin solution was added to cover the gel pieces (about 3x volume of the gel piece, approximately 20 μl) and then rehydrated at 4 °C for 30 minutes. Digestion of proteins took place overnight at 37°C. After protein digestion, the aqueous phase was transferred into a clean Eppendorf tube. Gel pieces were then repeatedly washed and the supernatant was collected into the same Eppendorf tube. Gel washing was performed in the following steps:

1. The solution of 65% acetonitrile and 5% formic acid was added to the gel pieces and vortexed for 30 minutes, shortly spinned and sonicated for 5 minutes. Supernatant was collected.
2. Demineralized water was added to the gel pieces and incubated for 15 minutes. Supernatant was collected.
3. Gels pieces were again washed with solution of acetonitrile and formic acid, then with demineralized water (repeat steps 1 and 2).
4. 100% acetonitrile was added to the gel pieces and incubated for 20 minutes. Supernatant was collected.

Each collected protein sample was completely dried to a white pellet in the speed-vac and dissolved in 0.1% trifluoroacetic acid (TFA). Samples were then purified and concentrated using Milipore Ziptips with C18 resin (Milipore, Sigma-Aldrich, USA) according to the manufacturer instructions. The sample was mixed with the MALDI matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid in 50% ACN and 0.5% TFA in 1:1 ratio. Mass spectra were recorded by MALDI-TOF/TOF (UltrafleXtreme, Bruker, USA) in the positive reflector mode in the mass range of 700-3500 Da. The spectra were analysed in FlexAnalysis (version 3.0) and protein identification was performed by MASCOT search engine (Matrix Science, London, UK) through the UniProtKB database. The following parameters were used: taxonomy - *Homo sapiens*; a mass tolerance - 50 ppm; variable modifications - alkylation of cysteine by carbamidomethylation and methionine oxidation.

3.6. Bioinformatics functional analysis

Differentially expressed proteins identified by MALDI-TOF/TOF were then analysed on the basis of the Gene Ontology (GO) biological processes using PANTHER classification system. Proteins were classified into functional categories and categories of cellular localization.

4. RESULTS

4.1. Two-dimensional gel electrophoresis using pH 3-10 non-linear immobilised pH gradient (IPG) strips

Two dimensional electrophoresis (2DE) analyses were performed in order to identify differences in protein expression profiles between BRAF V600E mutant (RKO, HT-29) and BRAF wild-type (SW620, HCT-116) colon cancer cell lines. Firstly, we carried out 2-DE in 3-10 NL pH range as a preliminary screening to examine robust differences at global proteome level (Figures 5A-D). We visually observed that marked differences in protein expression could be detected in the central region of the gels. This initial observation prompted us to further zoom-in on the central portion in the 2-DE pattern. For this reason, we next performed 2-DE using narrow-range IPG strips.

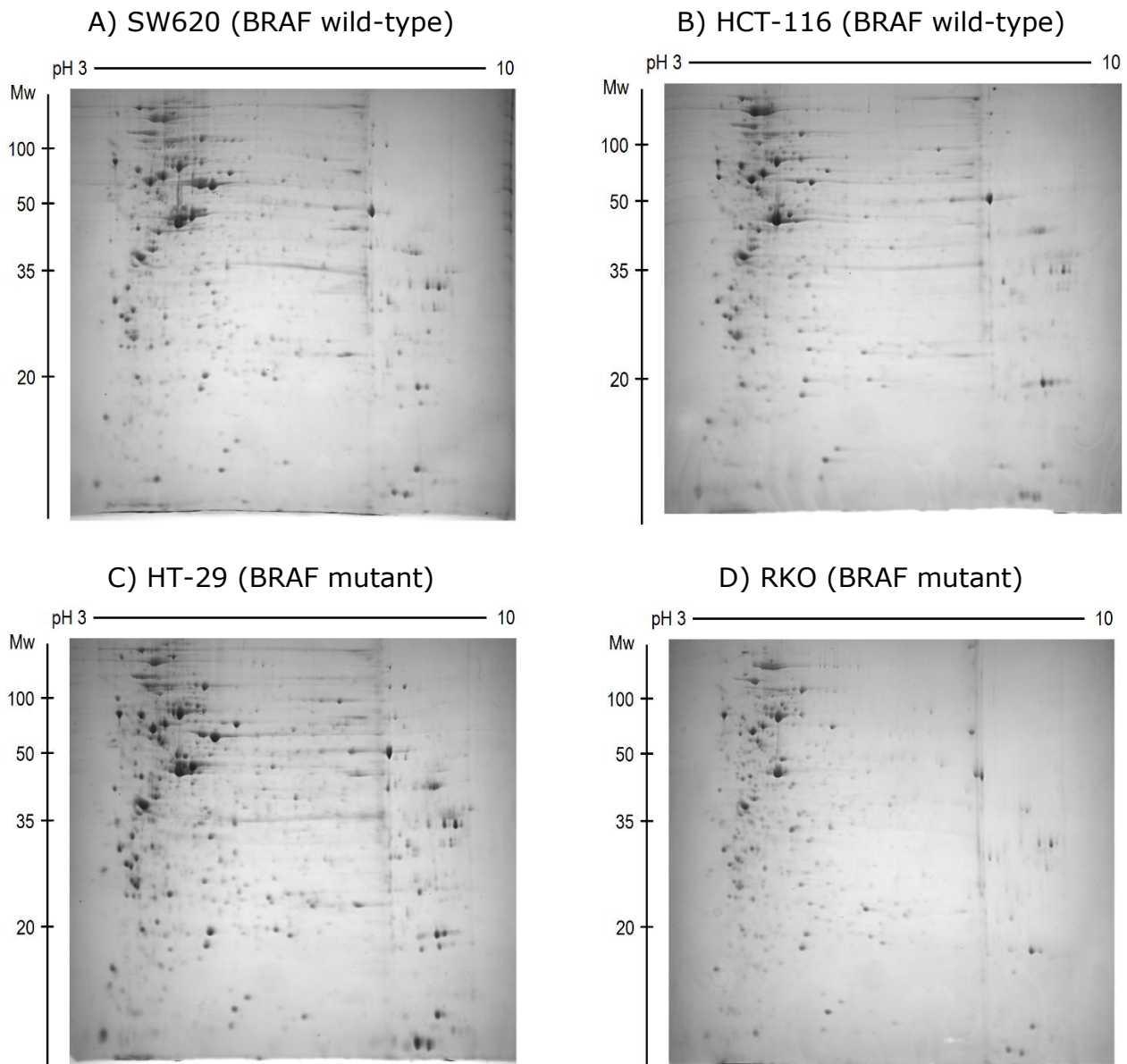


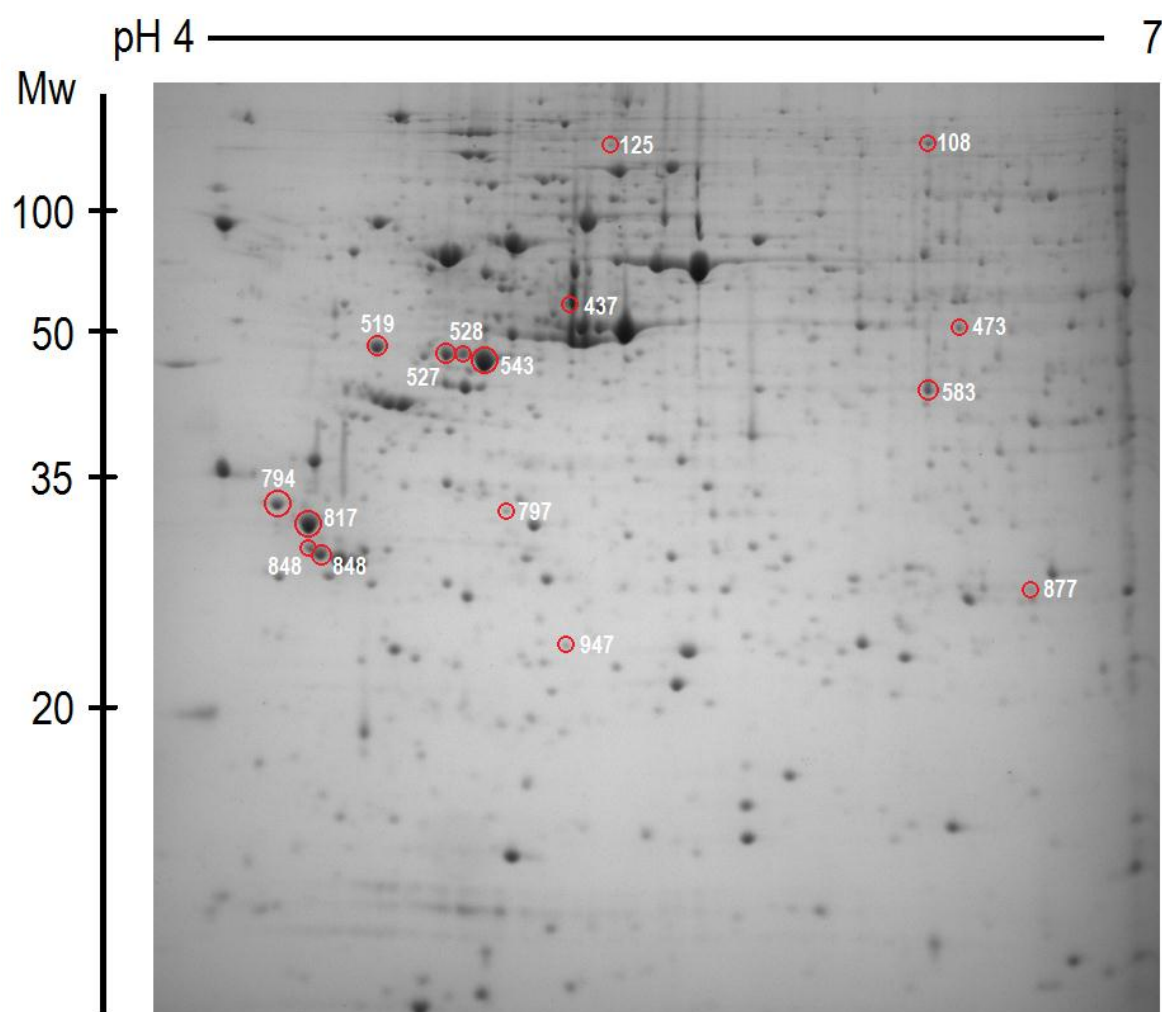
Figure 5. 2-DE gel images obtained in the pH range 3-10 NL.

4.2. Two-dimensional gel electrophoresis using narrow range pH 4-7 IPG strips

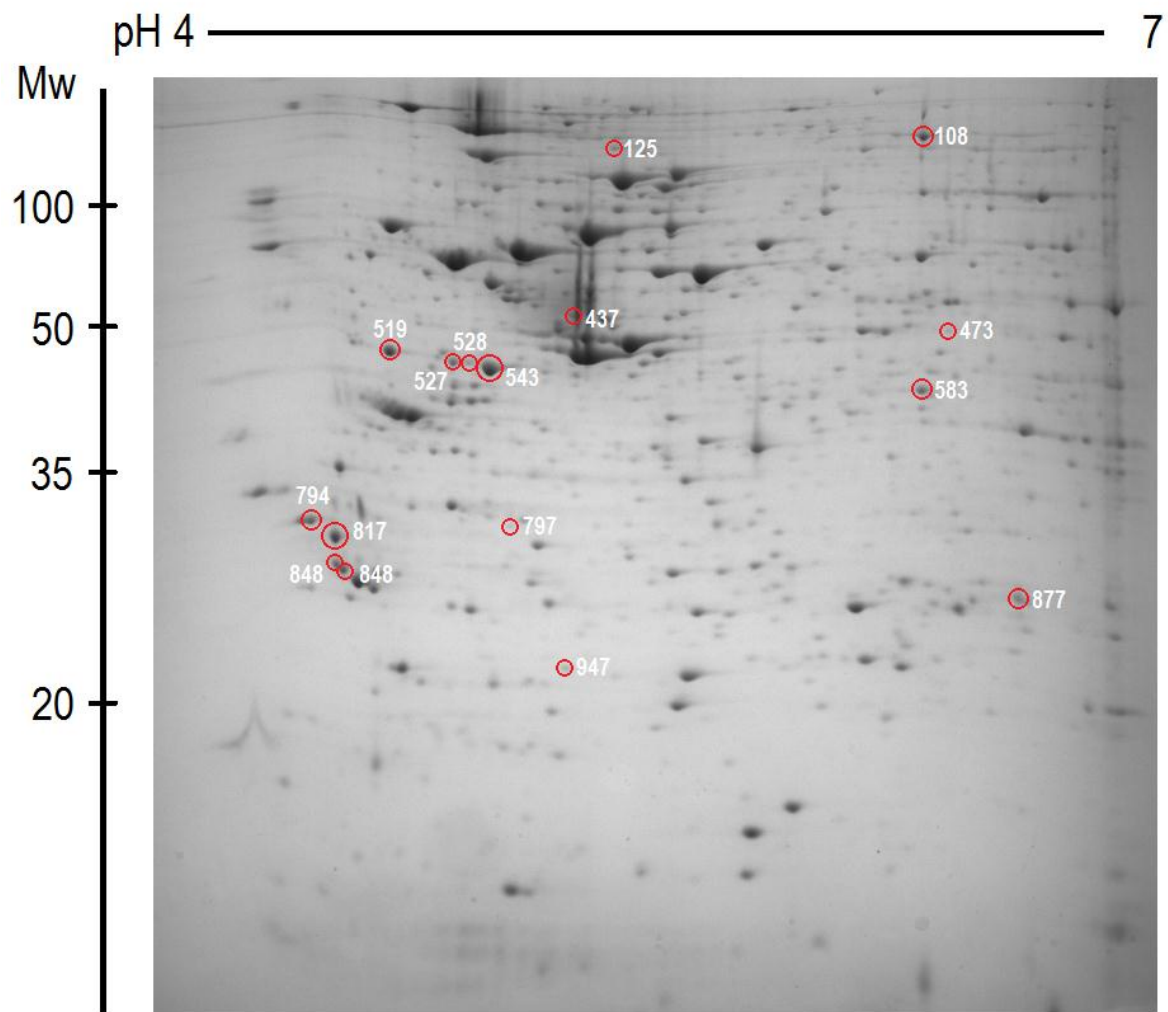
Further proteomics studies were aimed to capture more subtle changes in the protein expression between BRAF mutant and wild-type cells and to identify low-abundant proteins that are otherwise difficult to detect in wide pH range. Resulting 2-DE gel images obtained using pH 4-7 IPG strips are presented in Figure 6. The gel image with the highest number of spots and best resolution was selected as the reference image (Figure 7.) and its spots were then matched across all gels. This reference image was used to quantify and normalize the spot volumes in all gel images. Image analysis revealed 99 differentially expressed protein spots between BRAF V600E mutant (RKO, HT-29) and BRAF wild-type (SW620, HCT-116) colon cancer

cell lines, among which only 20 protein spots had statistically significant ($p < 0.05$) differences in the expression levels (Table 3). Out of these, 16 and 4 were downregulated and upregulated, respectively, in BRAF mutant in comparison with BRAF wild-type cells with the average fold change of 2.4. Identity of these proteins was further revealed by MALDI-TOF/TOF mass spectrometric analysis and indicated in Table 3.

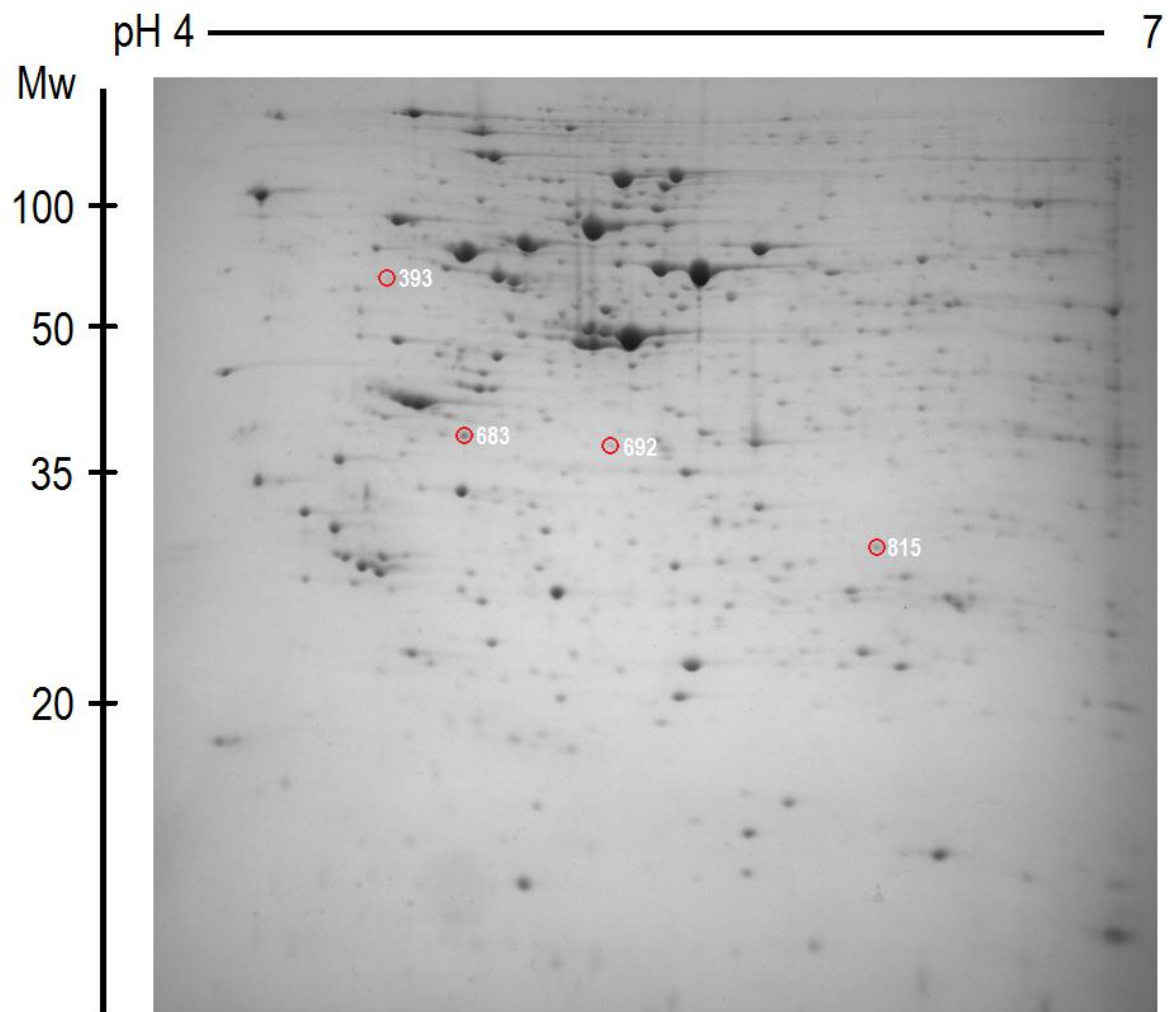
A) BRAF wild type cell line (SW620)



B) BRAF wild type cell line (HCT116)



C) BRAF mutant cell line (HT29)



D) BRAF mutant cell line (RKO)

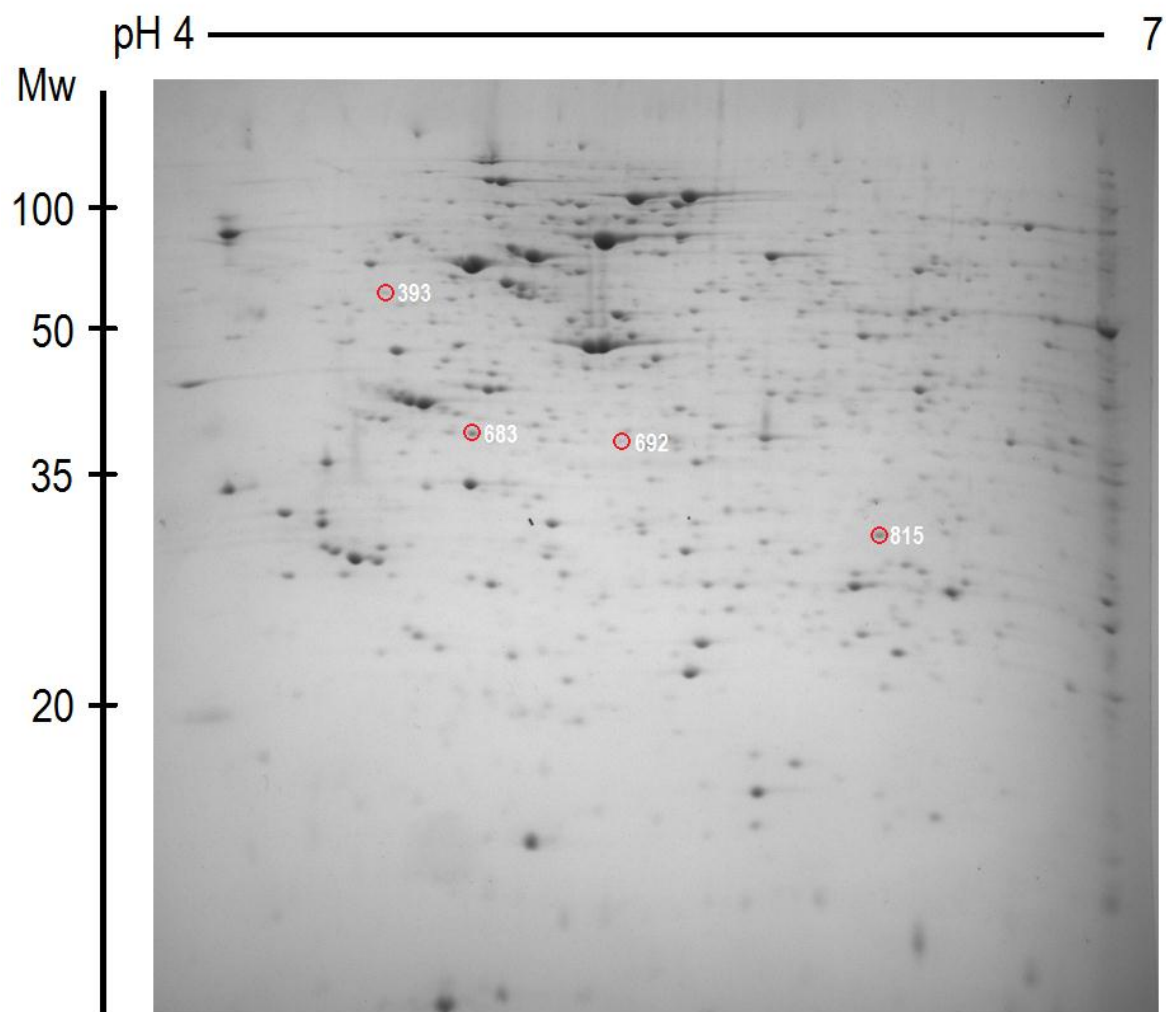


Figure 6. 2-DE gel images obtained in 4-7 pH range. Statistically significant spots are represented with red circles and their spot number.

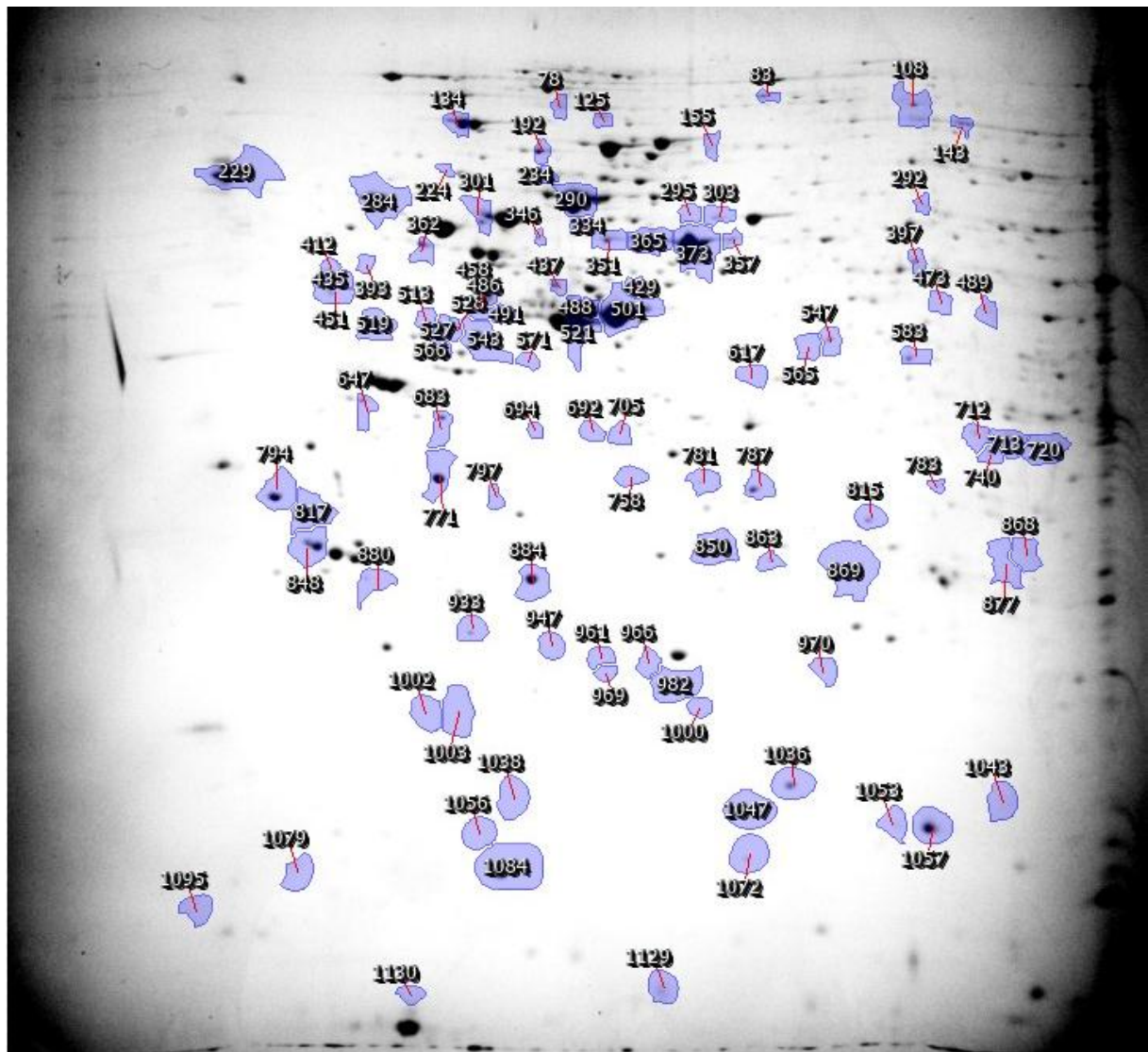
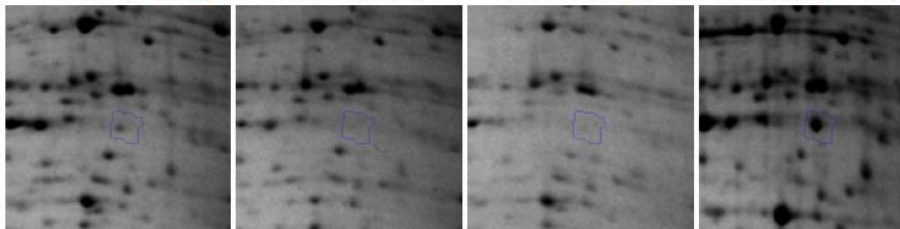
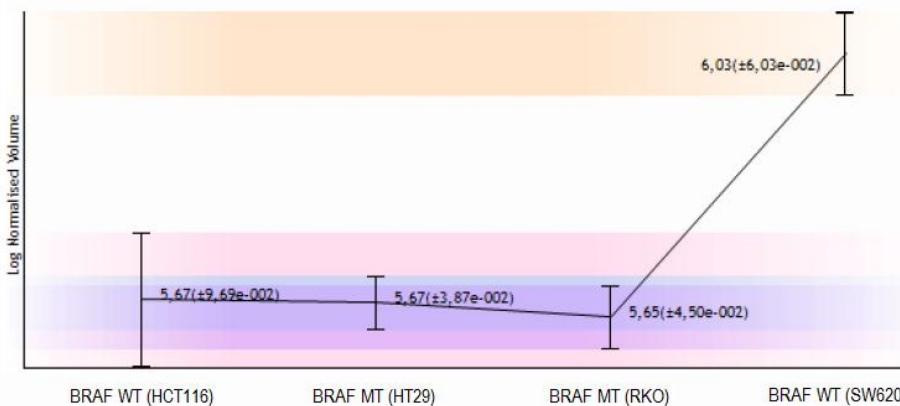
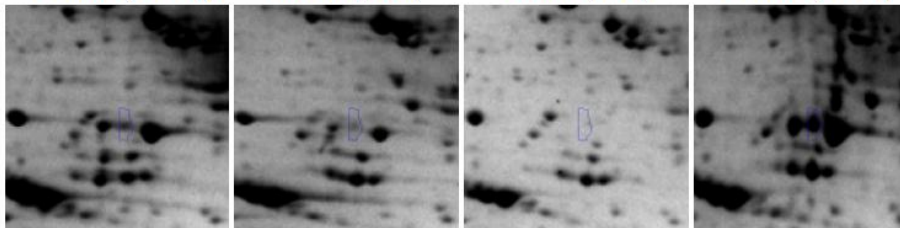
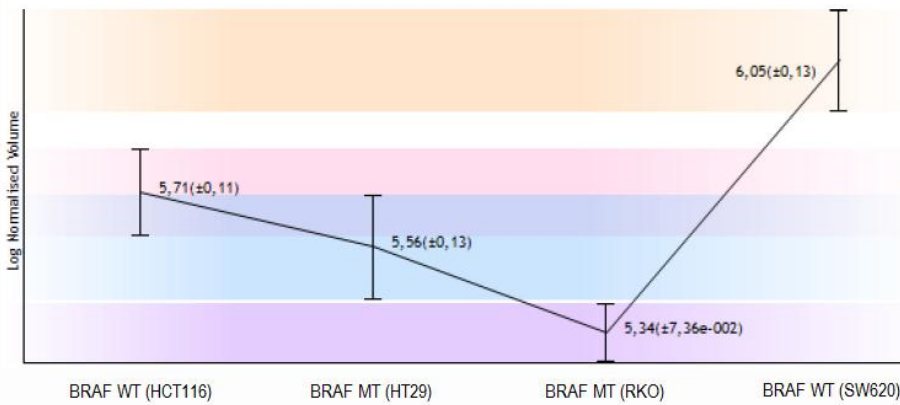


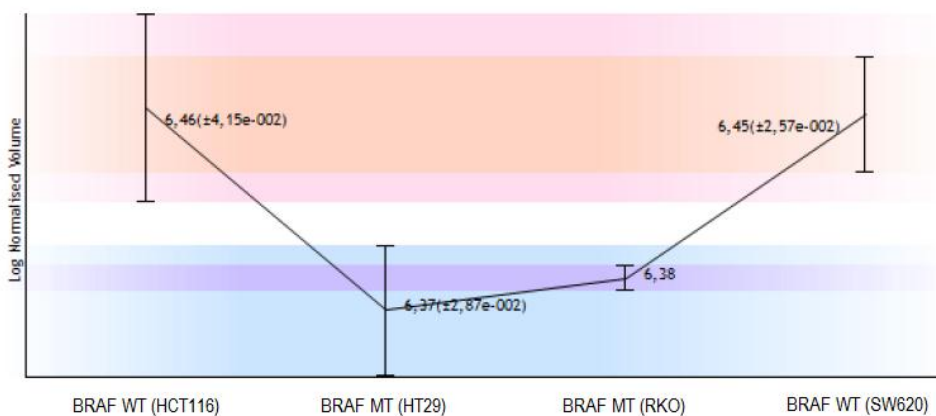
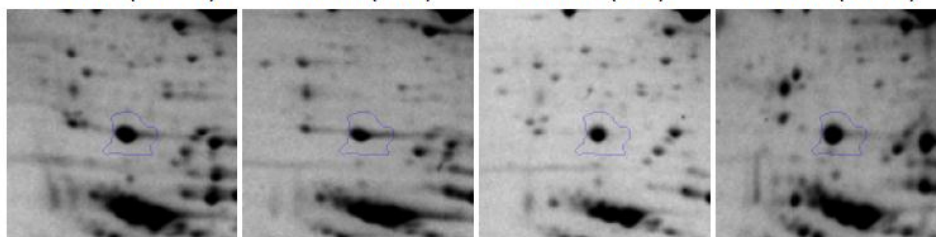
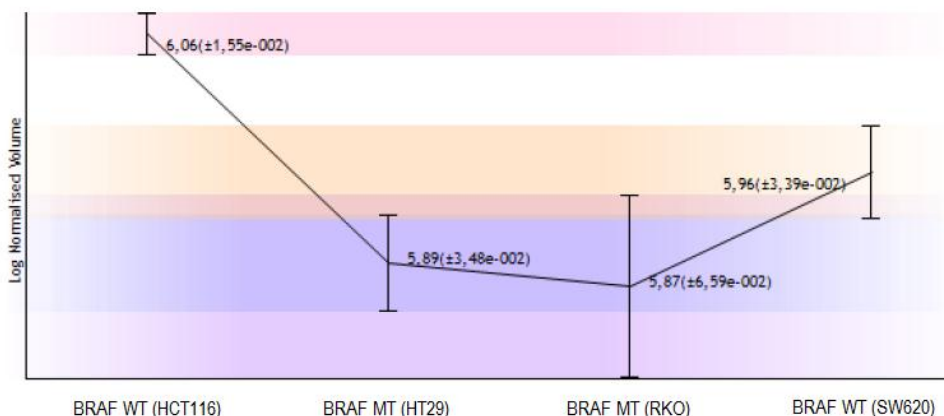
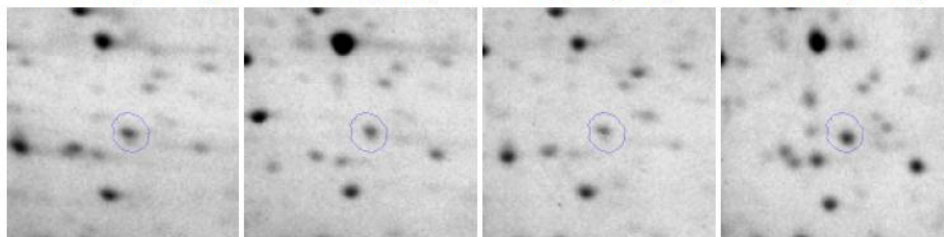
Figure 7. Reference image with 99 differentially expressed proteins and their spot numbers.

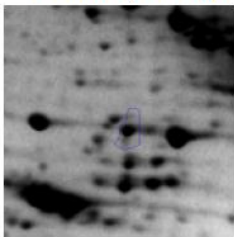
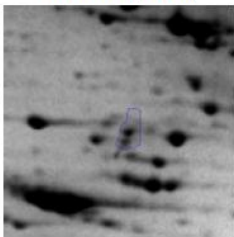
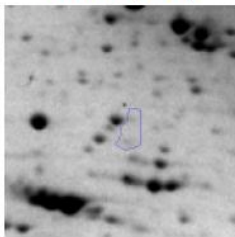
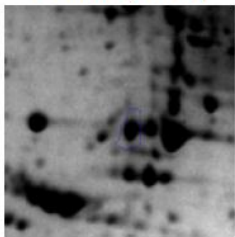
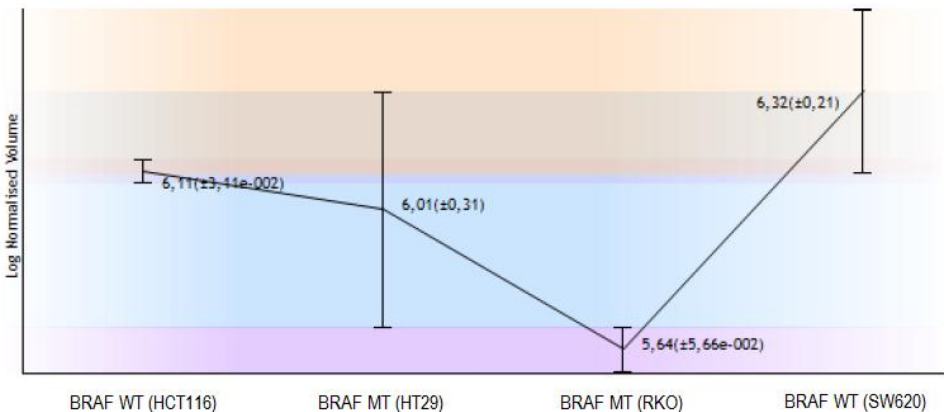
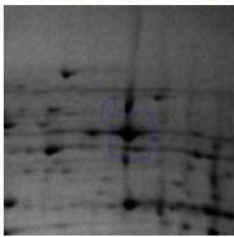
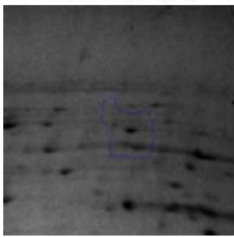
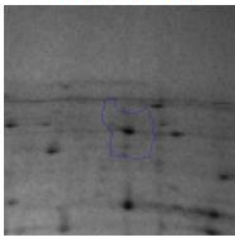
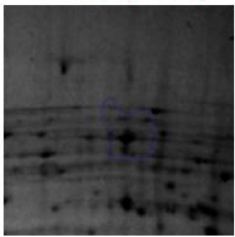
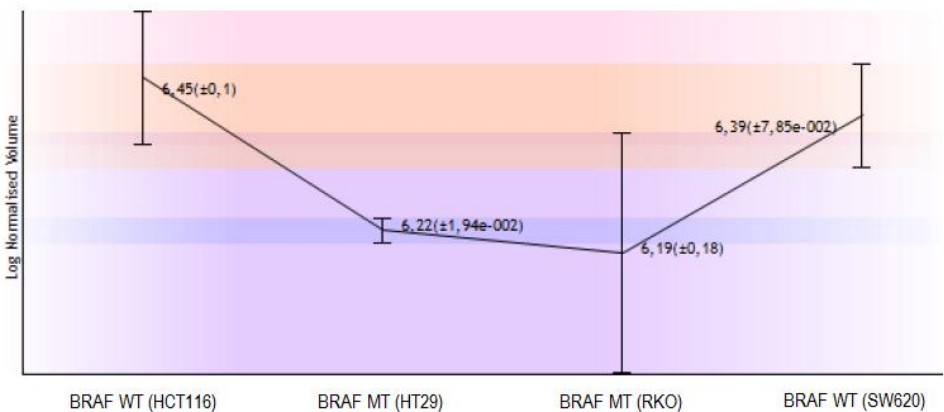
Table 3. Differential protein spots between BRAF mutated and wild-type cells with statistically significant changes in the expression level.

Proteins downregulated in BRAF mutant CRC cell lines													
Spot number	p-value												
473	5.82*10 ⁻⁴	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)								
													
		 <table><tr><th>Cell Line</th><th>Log Normalised Volume (Mean ± SE)</th></tr><tr><td>BRAF WT (HCT116)</td><td>5,67(±9,69e-002)</td></tr><tr><td>BRAF MT (HT29)</td><td>5,67(±3,87e-002)</td></tr><tr><td>BRAF MT (RKO)</td><td>5,65(±4,50e-002)</td></tr><tr><td>BRAF WT (SW620)</td><td>6,03(±6,03e-002)</td></tr></table>				Cell Line	Log Normalised Volume (Mean ± SE)	BRAF WT (HCT116)	5,67(±9,69e-002)	BRAF MT (HT29)	5,67(±3,87e-002)	BRAF MT (RKO)	5,65(±4,50e-002)
Cell Line	Log Normalised Volume (Mean ± SE)												
BRAF WT (HCT116)	5,67(±9,69e-002)												
BRAF MT (HT29)	5,67(±3,87e-002)												
BRAF MT (RKO)	5,65(±4,50e-002)												
BRAF WT (SW620)	6,03(±6,03e-002)												
528	9.09*10 ⁻⁴	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)								
													
		 <table><tr><th>Cell Line</th><th>Log Normalised Volume (Mean ± SE)</th></tr><tr><td>BRAF WT (HCT116)</td><td>5,71(±0,11)</td></tr><tr><td>BRAF MT (HT29)</td><td>5,56(±0,13)</td></tr><tr><td>BRAF MT (RKO)</td><td>5,34(±7,36e-002)</td></tr><tr><td>BRAF WT (SW620)</td><td>6,05(±0,13)</td></tr></table>				Cell Line	Log Normalised Volume (Mean ± SE)	BRAF WT (HCT116)	5,71(±0,11)	BRAF MT (HT29)	5,56(±0,13)	BRAF MT (RKO)	5,34(±7,36e-002)
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BRAF MT (RKO)	5,34(±7,36e-002)												
BRAF WT (SW620)	6,05(±0,13)												

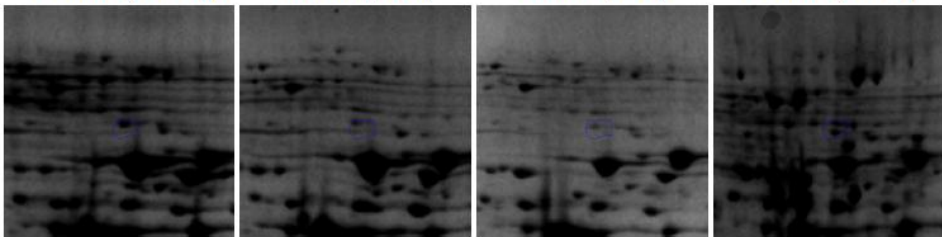
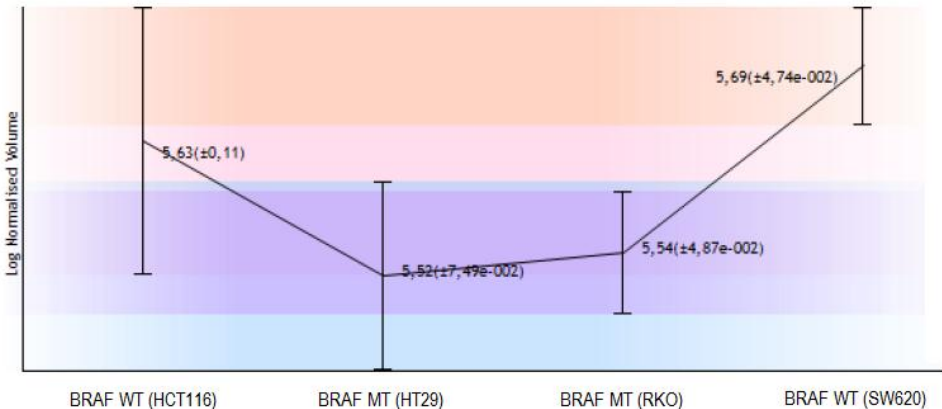
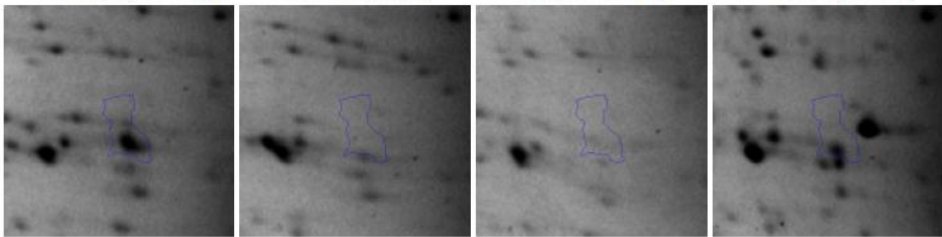
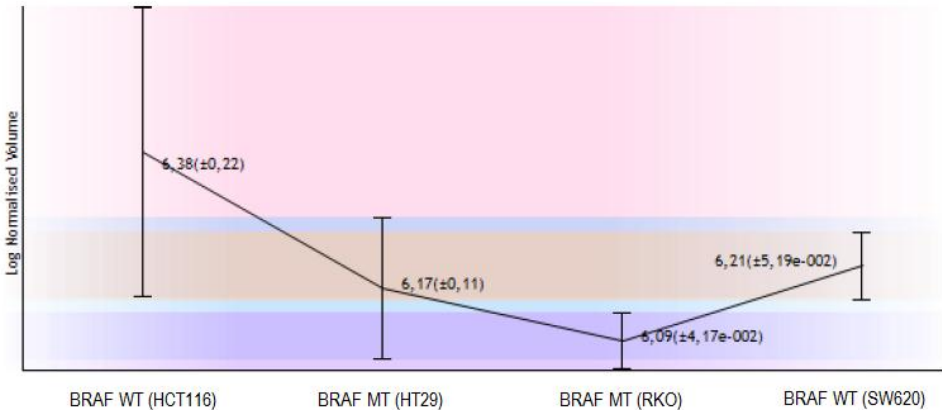
Proteins downregulated in BRAF mutant CRC cell lines				
Spot number	p-value			
437	0.001	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)
		BRAF WT (SW620)		
		<p>Log Normalised Volume</p> <p>6.2($\pm 9.41e-002$)</p> <p>6.04($\pm 2.98e-002$)</p> <p>5.82($\pm 4.46e-002$)</p> <p>5.81(± 0.13)</p> <p>BRAF WT (HCT116) BRAF MT (HT29) BRAF MT (RKO) BRAF WT (SW620)</p>		
817	0.001	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)
		BRAF WT (SW620)		
		<p>Log Normalised Volume</p> <p>6.8($\pm 1.88e-002$)</p> <p>6.62(± 0.1)</p> <p>6.5</p> <p>6.58($\pm 2.90e-002$)</p> <p>BRAF WT (HCT116) BRAF MT (HT29) BRAF MT (RKO) BRAF WT (SW620)</p>		

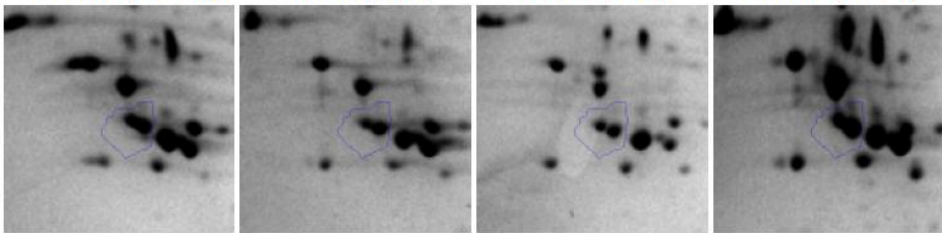
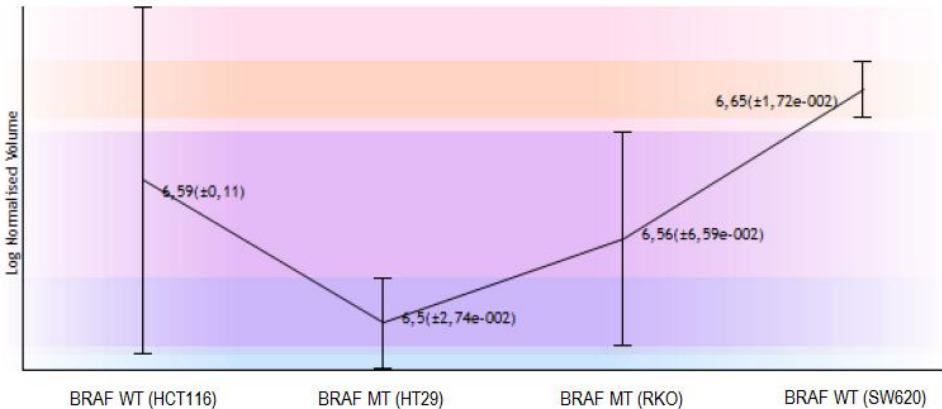
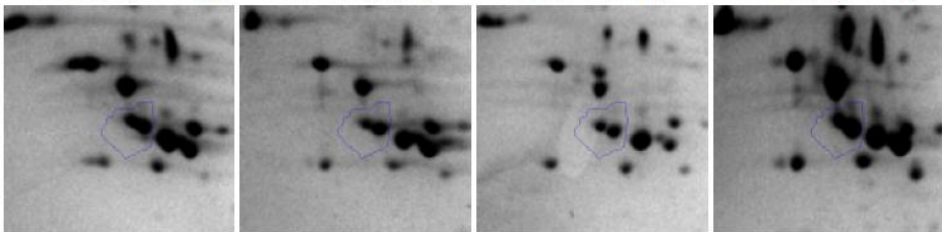
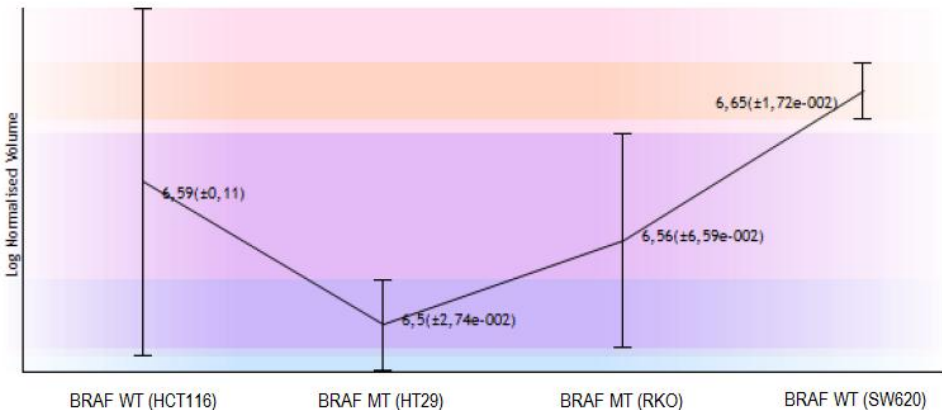
Proteins downregulated in BRAF mutant CRC cell lines				
Spot number	p-value			
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		BRAF WT (SW620)		
519	0.005	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)
		BRAF WT (SW620)		



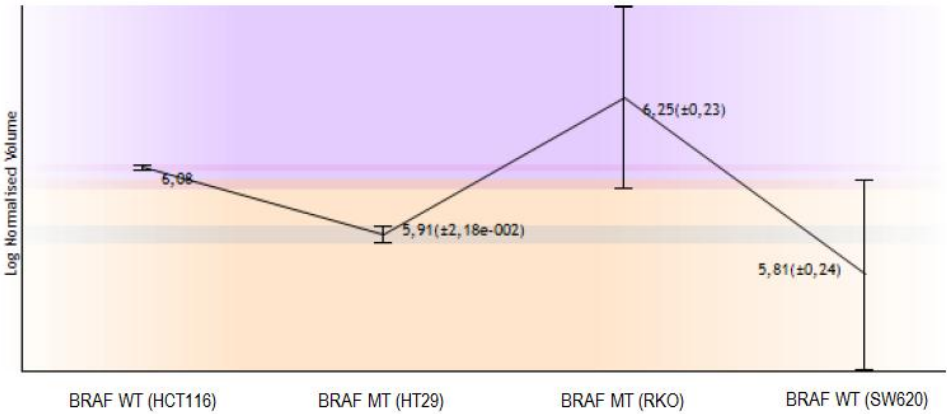
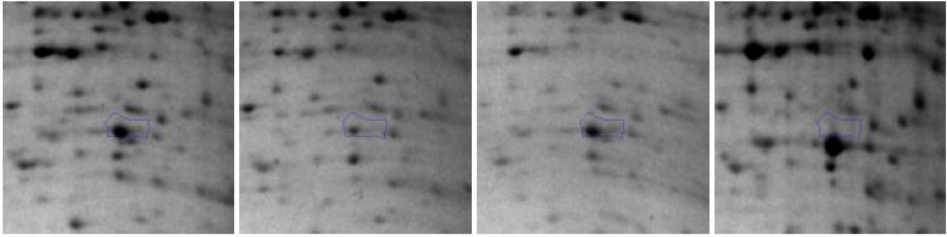
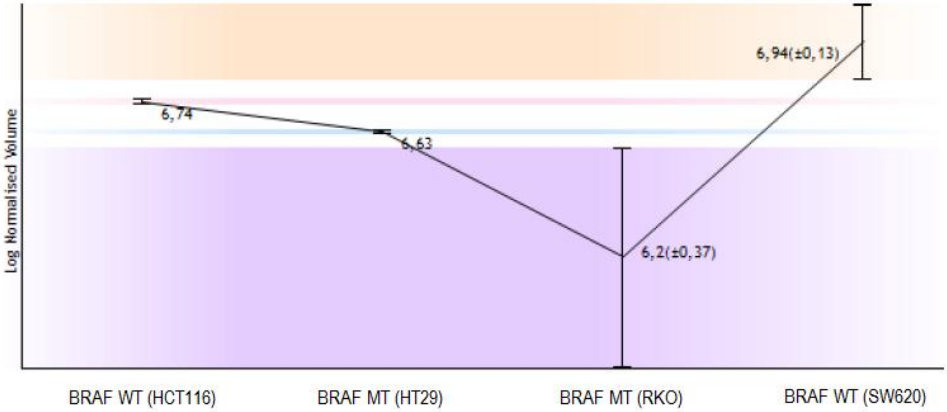
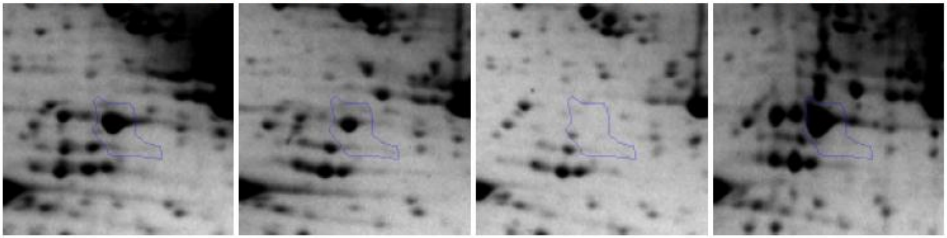
Proteins downregulated in BRAF mutant CRC cell lines					
Spot number	p-value				
527	0.007	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					
108	0.017	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					

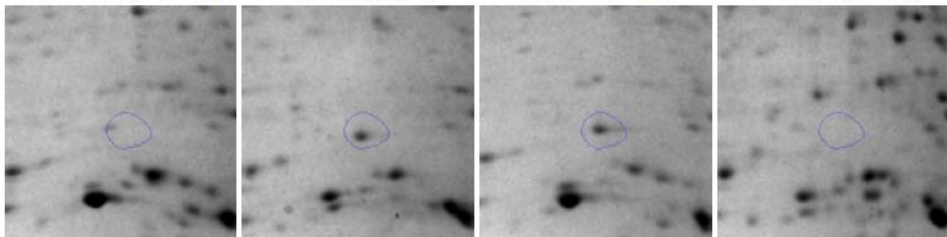
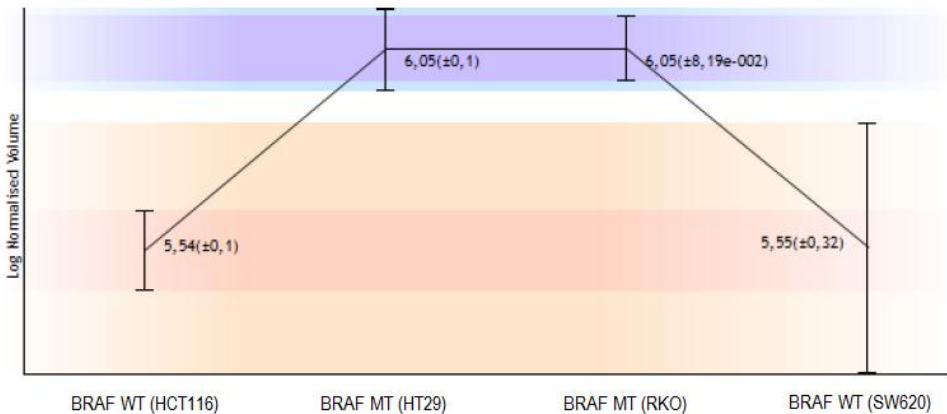
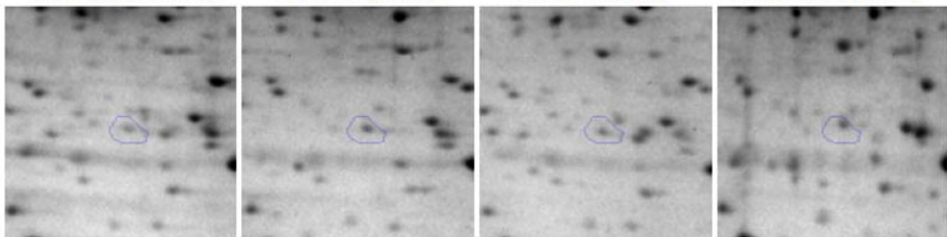
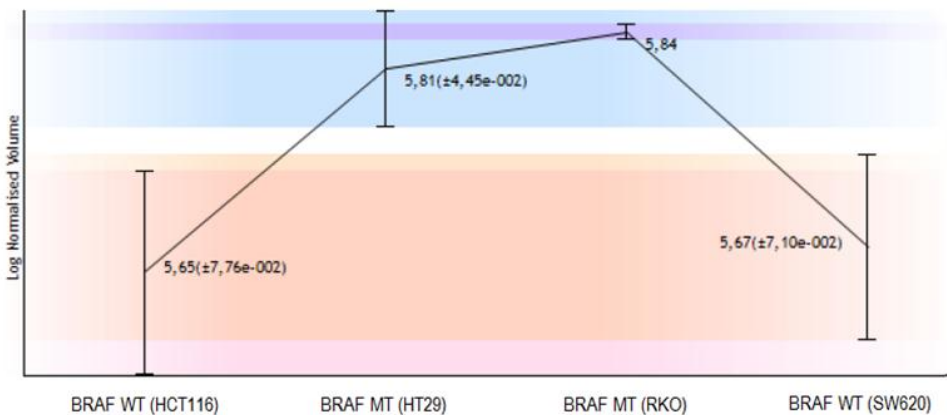
Proteins downregulated in BRAF mutant CRC cell lines																		
Spot number	p-value																	
797	0.019	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)													
		<table><thead><tr><th>Cell Line</th><th>Log Normalised Volume</th><th>Error</th></tr></thead><tbody><tr><td>BRAF WT (HCT116)</td><td>5,67</td><td>(±1,09e-002)</td></tr><tr><td>BRAF MT (HT29)</td><td>5,57</td><td>(±9,42e-002)</td></tr><tr><td>BRAF MT (RKO)</td><td>5,64</td><td>(±8,62e-002)</td></tr><tr><td>BRAF WT (SW620)</td><td>5,76</td><td>(±5,04e-002)</td></tr></tbody></table>				Cell Line	Log Normalised Volume	Error	BRAF WT (HCT116)	5,67	(±1,09e-002)	BRAF MT (HT29)	5,57	(±9,42e-002)	BRAF MT (RKO)	5,64	(±8,62e-002)	BRAF WT (SW620)
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BRAF MT (HT29)	5,57	(±9,42e-002)																
BRAF MT (RKO)	5,64	(±8,62e-002)																
BRAF WT (SW620)	5,76	(±5,04e-002)																
794	0.020	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)													
		<table><thead><tr><th>Cell Line</th><th>Log Normalised Volume</th><th>Error</th></tr></thead><tbody><tr><td>BRAF WT (HCT116)</td><td>6,52</td><td>(±3,13e-002)</td></tr><tr><td>BRAF MT (HT29)</td><td>6,38</td><td>(±3,98e-002)</td></tr><tr><td>BRAF MT (RKO)</td><td>6,38</td><td>(±3,69e-002)</td></tr><tr><td>BRAF WT (SW620)</td><td>6,45</td><td>(±0,11)</td></tr></tbody></table>				Cell Line	Log Normalised Volume	Error	BRAF WT (HCT116)	6,52	(±3,13e-002)	BRAF MT (HT29)	6,38	(±3,98e-002)	BRAF MT (RKO)	6,38	(±3,69e-002)	BRAF WT (SW620)
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BRAF WT (SW620)	6,45	(±0,11)																

Proteins downregulated in BRAF mutant CRC cell lines					
Spot number	p-value				
125	0.023	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					
877	0.035	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					

Proteins downregulated in BRAF mutant CRC cell lines					
Spot number	p-value				
848	0.037	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					
848	0.037	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					

Proteins downregulated in BRAF mutant CRC cell lines				
Spot number	p-value			
543	0.006	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)
		BRAF WT (SW620)		
583	0.017	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)
		BRAF WT (SW620)		



Proteins upregulated in BRAF mutant CRC cell lines					
Spot number	p-value				
815	0.005	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					
692	0.005	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					

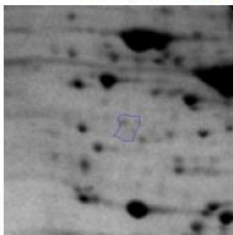
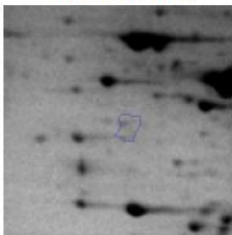
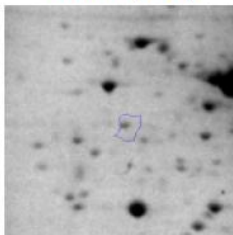
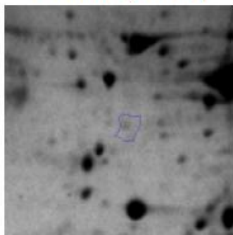
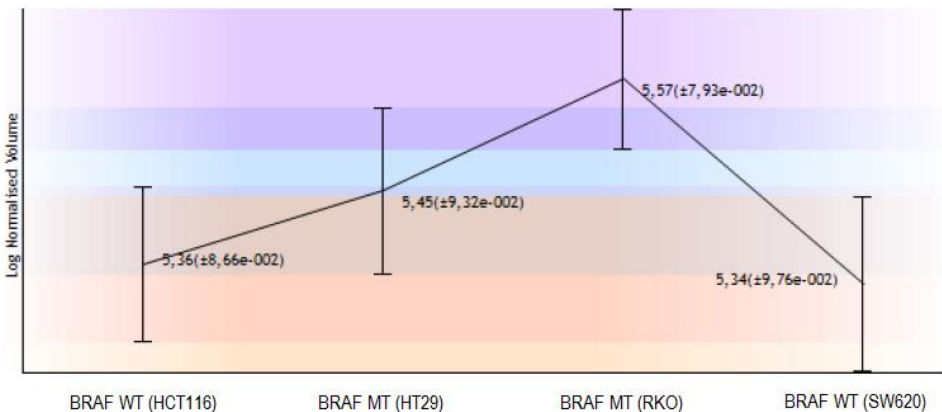
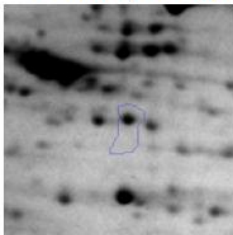
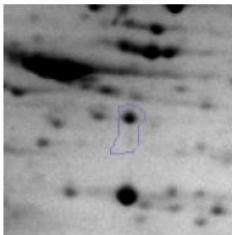
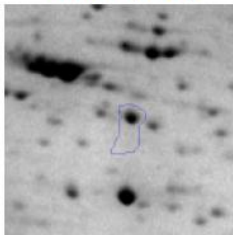
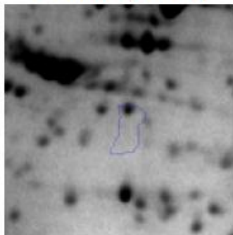
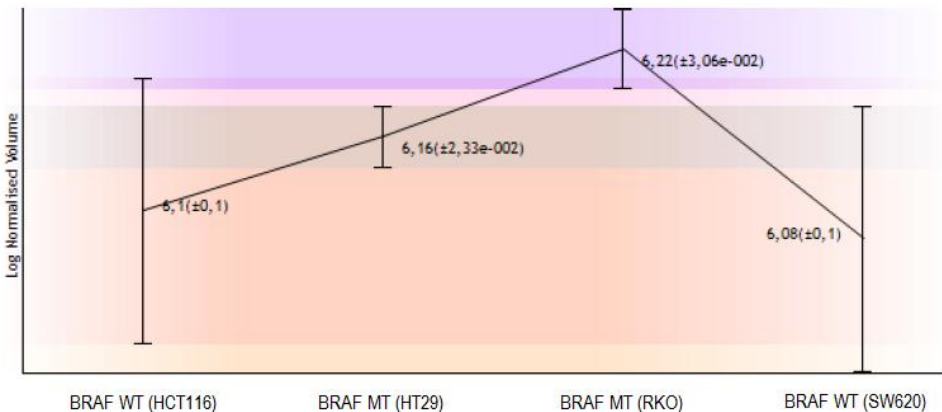
Proteins upregulated in BRAF mutant CRC cell lines					
Spot number	p-value				
393	0.018	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					
683	0.046	BRAF WT (HCT116)	BRAF MT (HT29)	BRAF MT (RKO)	BRAF WT (SW620)
					
					

Table 4. The list of differentially expressed proteins between BRAF V600E mutated and wild-type colon cancer cells with statistically significant ($p < 0.05$) changes in the expression level.

Proteins downregulated in BRAF mutant CRC cell lines				
Spot Number	Accession ID	Protein name	Subcellular localization	Biological role
473	CDKL3_HUMAN	Cyclin-dependent kinase-like 3	Cytoplasm	Cellular protein modification process Regulator of cell cycle
528	RIPOR2_HUMAN	Rho family-interacting cell polarization regulator 2	Plasma membrane, cytoskeleton	Cell adhesion and chemotaxis Cellular response to chemokine
437	HNRPM_HUMAN	Heterogeneous nuclear ribonucleoprotein M	Nucleus	RNA metabolic processes Fibroblast growth factor receptor signalling pathway
817	CL060_HUMAN	Uncharacterized protein C12orf60	Intracellular	-
947	GBG13_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13	Plasma membrane	Modulator in various transmembrane signalling systems
519	PYRD1_HUMAN	Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1	Nucleus	Cellular response to oxidative stress
527	WISP3_HUMAN	WNT1-inducible-signaling pathway protein 3	Secreted	Cell adhesion and cell-cell signalling Regulation of cell death and growth Signal transduction
108	GSLG1_HUMAN	Golgi apparatus protein 1	Golgi apparatus membrane	Leukocyte migration Regulation of protein processing and transforming growth factor beta receptor signalling pathway
797	YPEL4_HUMAN	Protein yippee-like 4	Nucleus	Metal ion binding

Proteins **downregulated** in BRAF mutant CRC cell lines

Spot Number	Accession ID	Protein name	Subcellular localization	Biological role
794	ICA1L_HUMAN	Islet cell autoantigen 1-like protein	-	-
125	HXK1_HUMAN	Hexokinase 1	Mitochondrion outer membrane	Glycolysis and carbohydrate phosphorylation Cellular glucose homeostasis
877	SAM10_HUMAN	Sterile alpha motif domain-containing protein 10	-	-
848	RB40A_HUMAN	Ras-related protein Rab-40A	Plasma membrane	Intracellular signal transduction Protein ubiquitination
848	HAVR2_HUMAN	Hepatitis A virus cellular receptor 2	Plasma membrane	Cellular response to lipopolysaccharide Inflammatory response
543	SPC24_HUMAN	Kinetochores protein Spc24	Nucleus, kinetochores	Cell cycle and division
583	BAP31_HUMAN	B-cell receptor-associated protein 31	Endoplasmic reticulum	Antigen processing and presentation of peptide antigen <i>via</i> MHC class I Apoptosis Intracellular protein transport

Proteins **upregulated** in BRAF mutant CRC cell lines

Spot Number	Accession ID	Protein name	Subcellular localization	Biological role
815	STK40_HUMAN	Serine/threonine-protein kinase 40	Nucleus	Glycogen metabolic process Regulation of apoptosis and gene expression Regulation of MAPK cascade
692	ZN230_HUMAN	Zinc finger protein 230	Nucleus	Transcription
393	EF2K_HUMAN	Eukaryotic elongation factor 2 kinase	Cytoplasm	Cellular response to anoxia and ischemia Negative regulation of apoptosis Regulation of protein autophosphorylation
683	MORN5_HUMAN	MORN repeat-containing protein 5	-	-

4.3. Functional classification of identified proteins

To better understand their biological roles, identified proteins were classified into functional categories on the basis of the Gene Ontology (GO) biological processes using PANTHER. As can be seen from Table 5 and Figure 8, the most represented proteins were those involved in different cellular processes including cell communication, cell cycle and cell growth. All of these processes could be associated with aggressive phenotype of BRAF mutated CRC. Further classification according to cellular compartment revealed that the majority of identified proteins were localized to either specific cell part (intracellular, plasma membrane, nuclear outer membrane-endoplasmic reticulum membrane network) or organelle (Table 6, Figure 9). The least represented were extracellular matrix proteins and those found in the extracellular region.

Table 5. Functional classification of differentially expressed proteins between BRAF mutated and wild-type colon cancer cells. Identified proteins were sorted into functional categories according to the Gene Ontology biological processes using PANTHER.

cellular process (GO:0009987); 25.0% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>HXK1_HUMAN</i> , <i>WISP3_HUMAN</i> , <i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i> , <i>ICA1L_HUMAN</i>)	cell communication (GO:0007154); 60.0% (<i>WISP3_HUMAN</i> , <i>RB40A_HUMAN</i> , <i>ICA1L_HUMAN</i>)
	cell cycle (GO:0007049); 20.0% (<i>CDKL3_HUMAN</i>)
	cell growth (GO:0016049); 20.0% (<i>CDKL3_HUMAN</i>)
biological regulation (GO:0065007); 17.9% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>HXK1_HUMAN</i> , <i>WISP3_HUMAN</i> , <i>RB40A_HUMAN</i>)	regulation of biological process (GO:0050789); 80.0% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>WISP3_HUMAN</i> , <i>RB40A_HUMAN</i>)
	homeostatic process (GO:0042592); 20.0% (<i>HXK1_HUMAN</i>)
metabolic process (GO:0008152); 14.3% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>HXK1_HUMAN</i> , <i>RB40A_HUMAN</i>)	
response to stimulus (GO:0050896); 10.7% (<i>HNRPM_HUMAN</i> , <i>WISP3_HUMAN</i> , <i>RB40A_HUMAN</i>)	
localization (GO:0051179); 7.1% (<i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i>)	
developmental process (GO:0032502); 7.1% (<i>CDKL3_HUMAN</i> , <i>WISP3_HUMAN</i>)	
multicellular organismal process (GO:003250); 7.1% (<i>CDKL3_HUMAN</i> , <i>ICA1L_HUMAN</i>)	
biological adhesion (GO:0022610); 3.6% (<i>WISP3_HUMAN</i>)	

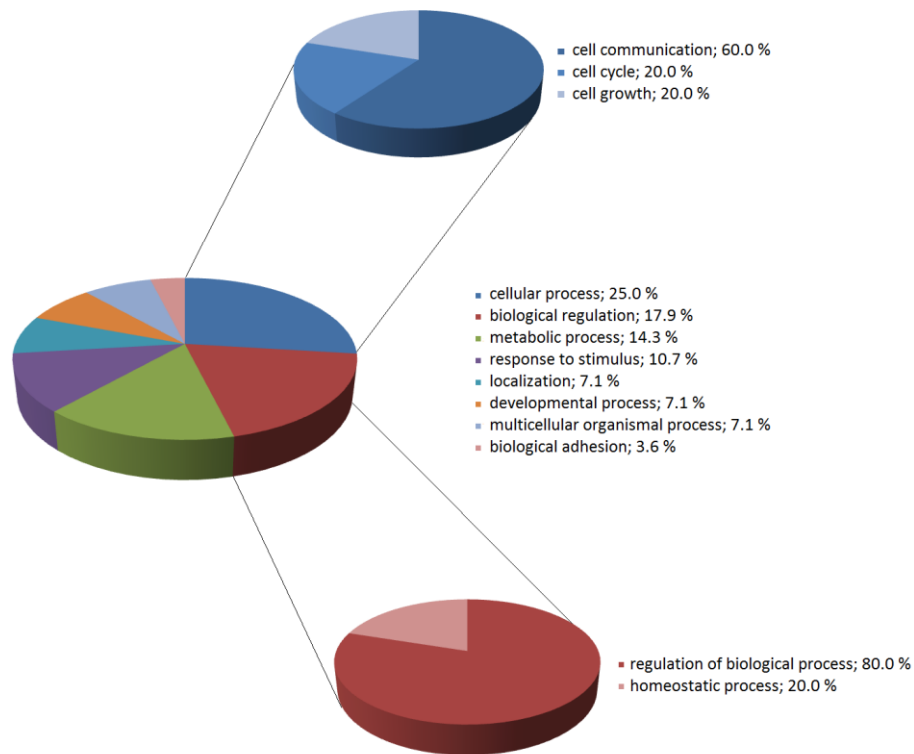


Figure 8. Pie chart representing the distribution of identified proteins across functional categories.

Table 6. Classification of identified proteins according to Gene Ontology cellular localization.

cell part (GO:0044464); 33.3% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>HXK1_HUMAN</i> , <i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i>)	intracellular (GO:0005622); 55.6% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>HXK1_HUMAN</i> , <i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i>)
	plasma membrane (GO:0005886); 22.2% (<i>BAP31_HUMAN</i> ; <i>RB40A_HUMAN</i>)
	nuclear outer membrane-endoplasmic reticulum membrane network (GO:0042175); 22.2% (<i>BAP31_HUMAN</i> ; <i>RB40A_HUMAN</i>)
organelle (GO:0043226); 26.7% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i> , <i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i>)	
macromolecular complex (GO:0032991); 13.3% (<i>CDKL3_HUMAN</i> , <i>HNRPM_HUMAN</i>)	
membrane (GO:0016020); 13.3% (<i>BAP31_HUMAN</i> , <i>RB40A_HUMAN</i>)	
extracellular matrix (GO:0031012); 6.7% (<i>WISP3_HUMAN</i>)	
extracellular region (GO:0005576); 6.7% (<i>WISP3_HUMAN</i>)	

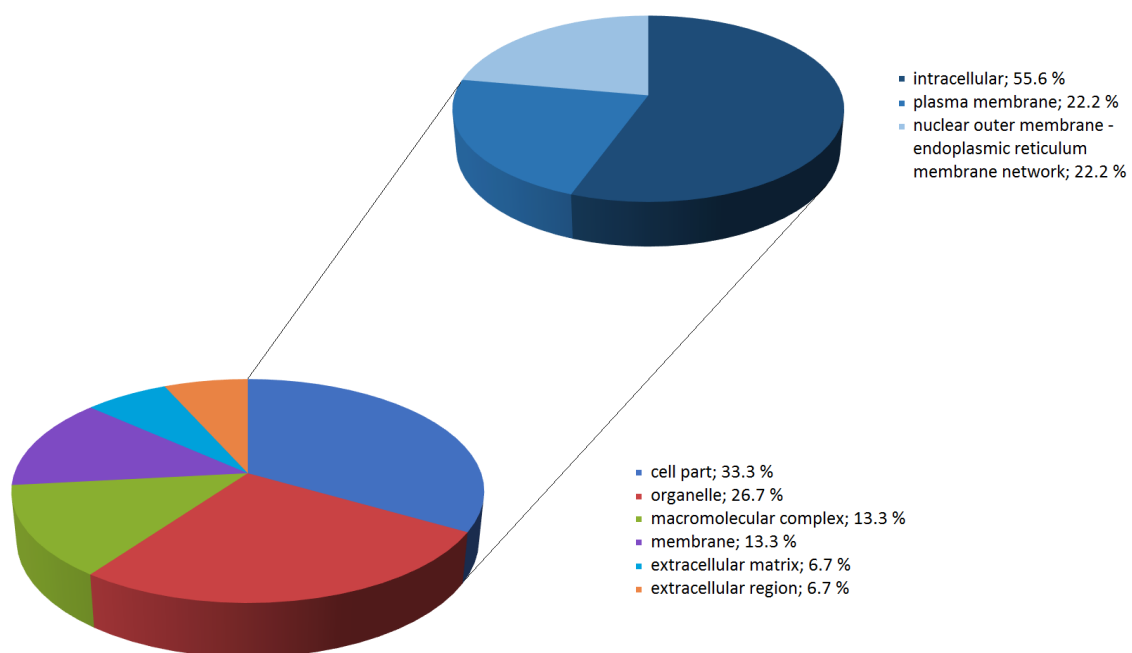


Figure 9. Pie chart showing cellular and extracellular distribution of identified proteins.

5. DISCUSSION

The main goal of this study was to identify specific proteomic changes driven by oncogenic BRAF V600E mutation in human colorectal cancer cells. Towards this aim, we applied 2-DE coupled with MALDI-TOF/TOF mass spectrometric analysis, which revealed 20 differentially expressed proteins with statistical significance between BRAF mutated and wild-type BRAF colorectal cancer cells. Majority of identified proteins are involved in the cellular processes pertinent to cancer development including cell communication, cell cycle and growth, but we were also able to identify some novel proteins whose function has not been elucidated yet.

Among identified proteins, three have previously demonstrated role in tumorigenesis of CRC, namely WNT1-inducible-signaling pathway protein 3 (WISP3), hexokinase 1 (HXK1) and eukaryotic elongation factor 2 kinase (EF2K). WISP3 is involved in many cancer-related processes including epithelial-mesenchymal transition, cell death, invasion and metastasis, and is considered a tumour suppressor. We identified WISP3 as downregulated in BRAF-mutant CRC cell lines. Loss of WISP3 expression was identified in 21% of CRCs (17). Lu et al. (18) confirmed that WISP3 is highly expressed in a subset of CRCs with a better prognosis i.e. less aggressive phenotype and a longer overall survival time compared with those with lower WISP3 expression. Our results are in good agreement with literature data because we found WISP3 to be indeed downregulated in more aggressive BRAF-mutant cells. Furthermore, Lee et al. (17) found that the loss of expression was more common in those with MSI-high CRCs than with microsatellite stable/low MSI. The fact that BRAF mutation occurs in 60% of MSI-high tumours further supports the link between BRAF V600E mutation and downregulation of WISP3 in CRC. In addition, data from the aforementioned study indicates that WISP3 may play a role in tumorigenesis of CRC with MSI-H like BRAF-mutant CRC through inhibition of its tumour suppressor functions. This mechanism has not been described yet, but study in breast cancer cells indicated that WISP3 may act *via* Smad-independent TAK1/p38 pathway (19), modulation of E-cadherin activity (20) and attenuation of insulin-like growth factor 1 receptor signalling (21). Downregulation of WISP3 can therefore have similar effects on CRC cells as seen in breast cancer cells.

We have identified hexokinase 1 (HXK1) as downregulated in BRAF mutant CRC cell lines. Tseng et al. (22) investigated the effects of HXK1 knockdown in human cervical carcinoma cells, breast cancer cells and human prostate cancer cells, and found that knockdown of HXK1 induces several changes in cell regulation. Firstly, a typical EMT change is induced that promotes cancer malignancy with an increased cancer cell metastasis and proliferation both *in vitro* and *in vivo*. Secondly, enhanced levels of HXK2 and LDH1 are detected, which points to an altered energy metabolism. Thus, downregulation of HXK1 observed in our study could, at least partially, account for aggressive nature of BRAF-mutant CRC.

Similarly, Hutton et al. (23) analysed metabolic differences between BRAF mutant CRC cell line RKO and BRAF wild-type/KRAS mutant CRC cell line DLD-1, both of which display Warburg phenotype. However, these authors failed to detect significantly altered proteins associated with metabolic reprogramming using global proteomic profiling approach as they detected only modest impact of BRAF V600E mutation on cell proteome with no detectable alterations associated with cell metabolism. By using next ion monitoring technique based on high-resolution and high-precision mass spectrometry, the so-called parallel reaction monitoring, these authors demonstrated that BRAF mutant CRC notably changes its metabolism to improve glycolysis, phosphoserine biosynthesis, glutamine metabolism and the non-oxidative pentose phosphate pathway. More specifically, these authors detected an upregulation of HXK2 in BRAF-mutant cell line compared with wild-type BRAF cell line, which was consistent with an increased glycolytic activity. Thus, it is plausible to believe that downregulation of HXK1 in BRAF mutant CRC leads to an upregulation of HXK2 and consequently to increased Warburg effect and tumour malignancy.

Eukaryotic elongation factor 2 kinase (EF2K) is a protein that negatively modulates protein synthesis. High expression of EF2K was found in several types of malignancies. Similarly, we showed EF2K to be upregulated in BRAF mutant CRC. Fu et al. (24) suggested that EF2K acts as an oncogene; it may inhibit apoptotic process in cancer, promote cancer cell survival by regulating autophagy and increase proliferation in malignant cells. Several other studies have suggested that EF2K promotes cell migration (25–29) and may thereby play important roles in facilitating cancer progression.

In contrast, a study supporting the role of EF2K in suppressing tumorigenesis was carried out in colon cancer cells HT-29 and HCT116 (30). This study found that EF2K suppresses autophagy and suggested that silencing of EF2K promotes autophagic survival in colon cancer cells. Wang et al. (31) showed that mTORC1 pathway and oncogenic MAPK pathway cooperate to inactivate EF2K activity and concluded that EF2K is regulated by a network of oncogenic signalling pathways. Thus, EF2K is likely to perform two opposite functions in either promoting or inhibiting protein synthesis, autophagy and cancer growth in cell type-dependent manner.

We found serine/threonine-protein kinase 40 (STK40) to be upregulated in BRAF mutant CRC cells. Previous study demonstrated that STK40 depletion decreased cell viability and colony formation and induced apoptosis of triple negative breast cancer cells (TNBC) (32). In addition, STK40 knockdown impaired growth *in vitro* and slowed TNBC tumour growth *in vivo*. Thus, one cannot rule out the possibility that STK40 plays an oncogenic role in CRC as well and that its expression is associated with activating mutations in BRAF. It would be interesting to further investigate if pharmacologic inhibition or genetic knockdown of STK40 could inhibit proliferation of BRAF mutated CRC cells and increase their response to clinically approved BRAF inhibitors such as vemurafenib.

Furthermore, we found Hepatitis A virus cellular receptor 2 (HAVR2) to be downregulated in BRAF mutant CRC cells. HAVR2 is a member of T cell immunoglobulin and mucin domain family involved in multiple diseases including cancer (33). Zhou et al. (34) analysed the role of HAVR2 in HT29, SW620 and HCT116 human CRC cell lines. In contrast to our findings, they found higher expression of HAVR2 in BRAF mutated CRC cell line HT-29 in comparison with BRAF wild type cell lines SW620 and HCT116. They also found that the expression of HAVR2 was significantly higher in tumour tissues than in normal tissues, and that its expression in CRC tissues was in correlation with CRC lymphatic metastasis. On the other hand, Zhang *et al.* (35) found significantly lower HAVR2 levels in CRC patients with poorer prognosis. Having in mind that BRAF mutant CRC patients have significantly lower overall survival when compared to those with wild-type tumours, further studies in tissues from CRC patients are warranted to validate our findings and to investigate the association between HAVR2 expression and BRAF V600E mutation in CRC, particularly in relation with patients' prognosis.

Several proteins with altered expression in BRAF mutant CRC cells seem to be associated with MAPK signalling, which raises the possibility of their involvement in BRAF V600E mutated protein-dependent signal transduction. For example, guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13 (GBG13), also known as G γ 13 protein, known modulator in various transmembrane signalling systems, was downregulated in BRAF mutant CRC cells in this study. Direct role of protein GBG13 in tumorigenesis has neither been investigated nor demonstrated, and the possible role of this protein can only be speculated. GBG13 is part of larger functional unit G $\beta\gamma$ shown to initiate MAPK signalling (36). Yippee-like 4 (YPEL4), located at the centrosome, adjacent to nucleolus and mitotic apparatus, is likely to have a role in the cell cycle (37). Although its role in tumorigenesis has not been shown, previous data have demonstrated the link between YPEL4 and MAPK signalling pathway (38,39).

We identified Ras-related protein Rab-40A (RAB40A) as downregulated in BRAF mutant CRC cell lines. RAB40A belongs to the family of Rab proteins, small GTP-binding proteins that regulate vesicular trafficking and intracellular signal transduction behaving as membrane-associated molecular switches. Since RAB proteins are one of key regulators of these essential processes, studies have found that Rab proteins are connected with human cancers (40). Several expression studies have suggested that they could play both an activating and an inhibiting role in tumour progression. For example, RAB25 has been documented as a tumour suppressor gene for colon cancer (41). Thus, it is plausible to believe that RAB40A may have similar function in BRAF mutated CRC.

Finally, we found two proteins to be upregulated in BRAF mutant CRC in comparison with BRAF wild type CRC cells, and these include MORN repeat-containing protein 5 (MORN5) that may be involved in TGF- β signalling pathway (42) and zinc finger protein 230 (ZN230) whose function is unknown. Our study appears to be the first to demonstrate the involvement of these proteins in colorectal cancer pathogenesis.

6. CONCLUSION

This study has revealed several novel protein biomarkers potentially associated with aggressive phenotype of BRAF mutated colorectal cancer. These include proteins with potential oncogenic role such as MORN repeat-containing protein 5, zinc finger protein 230, eukaryotic elongation factor 2 kinase and serine/threonine-protein kinase 40, as well as those that may act as tumour suppressors including Hepatitis A virus cellular receptor 2 and Ras-related protein Rab-40A.

Since we studied two cell lines for each genetic status (two BRAF mutant and two BRAF wild type cell lines), we have increased the chance of identifying proteins whose expression levels are directly associated with BRAF mutation status. However, these preliminary data obtained *in vitro* warrant further validation. In particular, immunohistochemical analyses are required to ascertain the role of these proteins in the pathogenesis of BRAF V600E mutated CRC and to assess their prognostic relevance in this population of CRC patients.

In addition, targeting eukaryotic elongation factor 2 kinase and serine/threonine-protein kinase 40 may prove as a novel strategy to control the behaviour of BRAF mutated CRC cells. Future efforts should be directed towards specific pharmacological inhibition of these kinases to investigate their role in BRAF mutant colon cancer cell survival and their response to BRAF inhibitor vemurafenib.

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8. CURRICULUM VITAE

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UNIVERSITY EDUCATION

- | | |
|---------------|--|
| 2016. – 2019. | University of Rijeka
Department of Biotechnology
Biotechnology in Medicine
Graduation thesis " <i>Characterization of Global Proteome Profiles of Human Colon Cancer Cells Depending on the Status of the BRAF gene</i> " |
| 2014. – 2016. | University of Rijeka
Department of Biotechnology
Biotechnology and Drug Research
Baccalaurea of Biotechnology (Graduated <i>Cum Laude</i>) |
| 2012. – 2014. | University of Zagreb
Faculty of Food Technology and Biotechnology
Biotechnology |

HIGH SCHOOL EDUCATION

- | | |
|---------------|--|
| 2008. – 2012. | Gimnazija Josipa Slavenskog
Mathematics and Science class |
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WORK EXPERIENCE:

- | | |
|-------|------------------------------|
| 2015. | Practice in ADRIALAB Company |
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AWARDS AND RECOGNITIONS

- Scholarship of the city of Čakovec (2012.-2018.) (Scholarship awarded to top 10% first year students)

OBTAINED CERTIFICATES:

- General Safety Instruction
- Certificate of attendance at seminar: Applications of the Cell-Based Luciferase Report Assays (HiBit Technology, NanoBIT Technology, NanoBRET Technology)

WORK SKILLS:

- Laboratory work (working with cell cultures, working with optical microscope, two dimensional electrophoresis, Western blot, Mass spectrometry)

SOFTWARE SKILLS:

- OS Windows, Microsoft Office
- Statistical analysis programs: Statistica, MedCalc
- *In silico* programs for visualization and analysis of molecular structures and reactions (Avogadro, Chimera, PyMol, NAMD, VMD, Vina, Spark, APBS, Gamess, MacMolPlt)

LANGUAGES:

- Mother tongue Croatian
- Fluent in English (reading, speaking and writing)

DRIVER'S LICENSE

B Category