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Review

BRAF mutation: Current and future clinical pathological applications in colorectal carcinoma

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Summary. The aims are to review the relevance of the *BRAF* mutations in the clinical settings of colorectal carcinoma. All the literature concerning *BRAF* mutations and colorectal carcinoma published in PubMed from 2010 to 2018 was reviewed. Multiple variants of *BRAF* mutations exist in colorectal cancer, the most common type being V600E. The mutation is found in 5 to 15% of colorectal carcinomas and is less common in Asian populations. *BRAF* mutations are linked with older age, female gender, cigarette smoking and are more common in the right (proximal) portion of the large intestine. *BRAF* mutations are associated with carcinomas of high histological grade and advanced cancer stages. Often *BRAF* mutated carcinomas demonstrate adverse histological features such as lymphovascular invasion, perineural invasion, tumour budding and lymph node metastases. *BRAF* mutations are found in serrated polyposis syndrome and have a negative correlation with hereditary nonpolyposis colorectal cancer (HNPCC). An array of methods of detection of *BRAF* mutation in colorectal carcinoma are available, such as immunohistochemistry and next generation sequencing, etc. Combinatorial approaches involving anti-BRAF therapies targeting both MAPK signalling as well as the PI3K/mTOR pathway could be a new approach for treatment of metastatic colorectal carcinoma. To conclude, *BRAF* mutation is important in the pathogenesis of colorectal cancer. Further research on the detection method as well as its role in target therapy will help to improve the management of patients with colorectal cancer.

Key words: *BRAF*, Colorectal carcinoma, Pathology, Genetic

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Introduction

Colorectal cancer is the second largest cancer in Australia and accounts for 4,162 deaths per annum. Every year, there are 14,958 newly diagnosed cases of colorectal cancer in Australia, making colorectal cancer the third most common type of newly diagnosed cancer in the country (https://bowel-cancer.canceraustralia.gov.au/statistics). With the advent of improving biologic therapy and chemotherapy options, mortality rates are declining. Survival in metastatic colorectal cancer has more than doubled in the past two decades, and there has been increasing recognition of somatic mutations that may be prognostic or predictive markers of specific therapies (https://www.mycancergenome.org/content/disease/colorectal-cancer/).

Most colorectal cancer is colorectal carcinoma. Colorectal carcinomas arise from different molecular pathways including chromosomal instability pathway, the microsatellite instability pathway or the CpG island methylator phenotype pathway (Pino and Chung, 2010). Common mutations in colorectal carcinoma occur in genes *KRAS, BRAF, PIK3CA, AKT1, SMAD4, PTEN, NRAS* and *TDFBR2* (Fernandez-Peralta et al., 2005; Haigis et al., 2008; Sartore-Bianchi et al., 2009; Negri et

al., 2010; Papageorgis et al., 2011; De Roock et al., 2010; Baba et al. 2011; Dienstmann et al., 2011). Amongst these, *BRAF* is an important mutation, which plays an important part in the clinical management of patients with colorectal carcinoma. In this review, all recent literature (from years 2010 to 2108) concerning the mutation and colorectal cancer listed in PubMed was reviewed.

Genetics of *BRAF mutation*

BRAF is a RAF kinase with a gene located on chromosome 7. *BRAF* activating missense point mutations are in exons 11 and 15 of the gene (Haley et al., 2015). The T1799A transversion mutation in *BRAF* accounts for more than 80% of all known *BRAF* mutations and results in glutamic acid for valine substitution at codon 600 (V600E). 5-15% of colorectal cancers with *BRAF* V600E mutation are associated with biologically more aggressive colorectal cancer phenotypes (Ung et al., 2014).

Multiple variants of *BRAF* mutations exist; the most common types are V600E, followed by V600M. Other subtypes include G466V, G469A, G469E, G469, D594G, D594V and G596R. Identification of these variants is significant for the management of colorectal carcinoma as they may have distinctive features and respond differently to treatment (Haley et al., 2015). Morris and colleagues have shown that patients with D594G *BRAF* mutation may have less aggressive disease than those with V600E *BRAF* mutations by having a longer overall survival and higher incidence of co-existent RAS mutation (Morris et al., 2014).

BRAF mutated cases of colorectal cancer harbor a higher number of ERK expression; ligands bind the EGFR glycoprotein and induce dimerization of the receptor, causing autophosphorylation of intracellular tyrosine residue (Levidou et al., 2012; Rahman et al., 2013). This results in the transduction of a downstream signal via the MAP kinase and PI3k/AKT pathway and consequently leads to hyper-activation of the MAPK pathway (Pakneshan et al., 2013). EGFR glycoprotein is expressed in 60-80% of colorectal cancers. The protein leads to secretion of TGF-α, which activates transduction and promotes growth, invasion, metastasis and neovascularization.

BRAF and *KRAS* mutations are often mutually exclusive (Li et al., 2014). This is because the presence of both mutations confers negative selection for *BRAF*/*KRAS*-mutated cells that are more prone to senescence (Barras 2015). Despite this, recent studies suggest that co-occurrence of them in tumours happens at a rate of 0-0.4% (László, 2010) and has been described in *BRAF* variants 594G and 601E (Siraj et al., 2014). Other documented cases have been described harbouring *BRAF* V600E and *KRAS* G13D, *BRAF* V600M and *KRAS* G12V, *BRAF* G469V with both *KRAS* A146T and *KRAS* G13D (Hanna et al., 2013).

BRAF mutated carcinoma cells are able to proliferate in low glucose environments (whereas wild type *BRAF* cells require glucose supply for survival). In addition, *TP53* mutations are significantly less common in mutated *BRAF* tumours (Vakiani et al., 2012; Hirschi and Kolligs, 2013).

Epidemiological and clinical correlations of *BRAF* mutations

The prevalence of *BRAF* mutations in colorectal carcinoma varies in different populations (Table 1). In general, *BRAF* mutations in colorectal carcinoma appear to be less common in Asian populations. Sekal and colleagues reported that *BRAF* mutation of colorectal carcinoma is uncommon in Asian populations (Sekal et al., 2015). On the other hand, studies have shown that there is no difference in mutated *BRAF* frequencies in colorectal carcinomas between African Americans and Caucasians in USA and these mutations are not associated with overall survival disadvantages in either ethnicity (Sylvester et al., 2011; Kang et al., 2013). *BRAF* mutations in colorectal carcinoma are associated with older ages (Tie et al., 2011; Saridaki et al.,2013; Chen et al., 2014; Russo et al., 2014; Siraj et al., 2014) and are more prevalent in females (Rozek et al., 2010; Loupakis et al., 2015)

Smoking is not a significant cancer risk in the setting of *BRAF* mutations; likewise, there is a negative correlation between *BRAF* V600E mutation and alcohol intake (Nishihara et al., 2013; Chen et al., 2014). While *BRAF* mutated carcinomas are linked with anorexia and weight loss (Sorbye et al., 2015), carcinomas with wild type *BRAF* are associated with increased weight, body fat percentage, hip, waist and body mass index (Brändstedt et al., 2014).

Primary carcinomas with mutated *BRAF* are often noted in the proximal colon with highest prevalence in the right colon (Phipps et al., 2012; Ito et al., 2014; El-Deiry et al., 2015; Haley et al., 2015; Sorbye et al., 2015). Yamauchi et al. hypothesise that this is due to the changes in molecular features that occur along the bowel subsites due to the change in bowel content constantly interacting with host cells and causing cellular molecular

Table 1. Ethnical frequencies of *BRAF* mutation in colorectal carcinoma.

Ethnicity	Prevalence of *BRAF* mutation in colorectal cancers	References
Italian	9.5%	Simi et al., 2008
Greece	7.2%	Saridaki et al., 2011
Spanish	6.25% to 13%	Borrás et al., 2011; Herreros-Villanueva et al., 2011
Netherlands	21.8%	Siraj et al., 2014
Chinese	7%	Li et al., 2011
Korean	3.3%	Kwon et al., 2011
Thai	1.1%	Hsieh et al., 2012
Israel	5%	Siraj et al., 2014
Saudi Arabia	2.5%	Siraj et al., 2014
Ashkenazi Jews	5.8%	Siraj et al., 2014

changes (Yamauchi et al., 2012). The authors also noted that caecal carcinomas have a lower prevalence of *BRAF* mutation than other sites in the right colon. This may be due to the unique molecular features of caecal cancers. In addition, in mouse models where a loss of retinoblastoma protein Rb1 in the gastrointestinal tract promotes tumour formation, loss of Rb1 is specific to caecal cancers (Yamauchi et al., 2012). Rectal carcinomas seldom harbour *BRAF* mutation (Burnett-Hartman et al., 2013; Sekal et al., 2015). In contrast, wild type *BRAF* and microsatellite stable carcinomas often occur in distal portion of the large intestine (Bond et al., 2014).

Association of *BRAF* mutation with pathology and prognosis

BRAF mutation is associated with mucinous histology and signet ring cell histology in colorectal carcinoma (El-Deiry et al., 2015). In signet ring cell colorectal carcinomas, *BRAF* V600E mutations adversely affect survival of patients with microsatellite stable carcinomas, but not in high-level microsatellite instable carcinomas (Kakar et al., 2012).

Compared to wild type *BRAF*, the presence of *BRAF* V600E mutation is a poor prognostic factor and is associated with a shorter progression-free survival and shorter overall survival in patients with colorectal carcinoma (Yokota, 2012). In addition to this, *BRAF* mutations are associated with advanced cancer stages, aggressive biological behaviour and poorly differentiated carcinomas (Sylvester et al., 2011; Kim, 2014).

Another feature correlated with poor patient survival in colorectal carcinomas is the expression of SOX2, a gene which encodes for a transcription factor and is a member of the SRY-related HMG-box (SOX) gene family. SOX2 plays essential roles in cell fate determination, thereby regulating developmental processes. Expression of SOX2 is correlated with down-regulation of CDX2 expression, which in colorectal cancers has been linked to CpG island methylator phenotype-high, microsatellite instability, and *BRAF* mutated tumours (Lundberg et al., 2016). This gene was expressed in 11% of colorectal tumours in a study undertaken by Lundberg et al and is significantly associated with *BRAF* V600E mutants. Lundberg et al. have shown in a cell line study that SOX2 expression is partly regulated by *BRAF* signalling, and an increased SOX2 expression may promote colorectal cancer metastasis and mediate poor prognosis in patients with colorectal carcinoma (Lundberg et al., 2014).

BRAF mutations were more predominant in patients having colorectal carcinoma with metastatic spread to at least one local lymph node (Hernowo et al., 2014) and were associated with advanced T stage (T4) carcinoma (Nam et al., 2016). Furthermore, *BRAF* mutated colorectal carcinomas frequently demonstrated adverse histologic features such as lymphatic invasion, higher mean number of lymph node metastases, perineural invasion, and high tumour budding (El Deiry et al., 2015). Mutated *BRAF* colorectal carcinomas are often associated with multiple metastatic sites and are more likely to develop peritoneal, liver and distant nodal metastases when compared with wild type *BRAF* carcinomas (Karagkounis et al., 2013; Saridaki et al., 2013; Sorbye et al., 2015). In metastatic disease, the mutation is associated with inferior overall survival of patients with colorectal carcinoma; this is particularly evident in the patients treated with chemotherapy (Tie et al., 2011). In addition, distant lymph node metastases are uncommon at time of diagnosis but are usually the first site of relapse (Phipps et al., 2013; Russo et al., 2014).

BRAF mutation and genetic syndromes

BRAF mutations are commonly found in patients with a history of polyps and bear a strong correlation between patients with serrated polyposis syndrome but a negative correlation with familial syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC)/ Lynch syndrome (Boparai et al., 2011; Buchanan et al., 2013; Russo et al., 2014). HNPCC is a familial autosomal dominant cancer predisposition syndrome accounting for 2-5% of colorectal carcinomas. Patients with HNPCC inherit a germline mutation in one of the mismatch repair genes.

BRAF mutations are important for serrated polyp carcinogenesis and correlated with polyposis comprising multiple serrated adenomas and polyps. Gaiser et al. speculated that serrated polyps act as an anti-apoptotic stimulus, resulting in serrated morphology (Gaiser et al., 2012). On the other hand, *BRAF* mutations are not common in colorectal conventional adenomas. The mutations occur in 2.83% of flat adenomas and 2.15% of polypoid adenomas (Voorham et al., 2012). In addition, mutated *BRAF* is low in tubulovillous adenoma with serrated features (Tsai et al., 2014).

BRAF mutant carcinomas that are CpG island methylator phenotype low and harbor microsatellite stability are linked to impaired survival of patients with colorectal carcinoma (Eklöf et al., 2013; Etienne-Grimaldi et al., 2014). *BRAF* mutation is a negative prognosis factor in microsatellite stable colorectal carcinoma; this is not clearly observed in microsatellite unstable colorectal carcinoma, mainly due to the low incidence of these molecular alterations (Sanz-Garcia et al., 2017). Nevertheless, the presence of a *BRAF* mutation in microsatellite stable colorectal carcinomas is a warning of a poorer prognosis in patients with colorectal carcinoma (Pakneshan et al., 2013). In addition, *BRAF* mutated tumours are partially resistant to conventional chemotherapy (Sanz-Garcia et al., 2017).

In clinical settings, immunohistochemical studies of protein markers MSH2, MSH6, MLH1, and PMS2 are performed to detect microsatellite instability (Rosty et al., 2013; van den Broek et al., 2015). Sporadic colorectal carcinomas with microsatellite instability result from methylation of mismatch repair genes that

have negative staining of two (or one) of these four markers in carcinoma cells in the presence of positive staining in adjacent benign tissues. The loss of expression of these proteins occurs in pairs. MSH2 and MSH6 is one pair, MLH1 and PMS2 is the other. Often, two of the four proteins are lost in HNPCC.

Negative expression of MLH1 and PMS2 is the most common finding in microsatellite instability. It may denote HNPCC or sporadic microsatellite instability. Sporadic microsatellite instability could be due to methylation of the protein and is *BRAF* mutation positive. It is easier to detect *BRAF* mutation than methylation status of mismatch repair genes. Thus, *BRAF* mutation in colorectal cancers is the standard protocol in patients with negative expression of MLH1 and PMS2 to identify sporadic microsatellite unstable colorectal cancer in public laboratories in Australia (Pakneshan et al., 2013). If *BRAF* mutation is negative, colorectal carcinomas with negative expression of MLH1 and PMS2 are highly likely to be HNPCC.

Methods of detection

In clinical settings, apart from detecting *BRAF* mutation in the settings of HNPCC, *BRAF* mutation could often be detected together with RAS mutation when considering target therapy for metastatic colorectal carcinoma.

There are multiple methods of detecting mutated *BRAF*, each with their own benefits and disadvantages. The traditional approach in clinical practice is to test for *BRAF* mutation by Sanger sequencing. In some instances, traditional techniques of Sanger sequencing are suboptimal for detection of somatic mutations in metastatic and treated colorectal cancers as they can miss mutant cases if tissue specimens contain a percentage of tumour cells less than 50% (Arcila et al., 2011; Roma et al., 2016). In addition, Sanger sequencing has up until recently been a costly method of detection.

Several polymerase chain reaction (PCR) based methods have been developed to test *BRAF* mutation. For instance, Negru and colleagues described high resolution melting, a technique with 99% concordance with Sanger sequencing (Negru et al., 2014). The combination of co-amplification at lower denaturation temperature PCR with high resolution melting permits the correct identification of less represented mutations in colorectal cancers (Mancini et al., 2010). These methods are less expensive than sequencing. However, the application of these methods in clinical laboratories require expertise and the sensitivity is inferior to that of sequencing.

Testing of circulating tumour cells obtained from peripheral blood may allow non-invasive evaluation of the status of a patient's cancer. Genotyping of circulating tumour cells with high sensitivity and specificity could be through circulating tumour cells enrichment coupled with application of optimized PCR-based assay, or by direct detection from plasma with high mutant allelic frequencies (Mohamed et al., 2017; Sefrioui et al., 2017). However, the technique is only effective for cancers that have circulating tumour DNA. In addition, sophisticated preparation and techniques are required, and thus the technique may not be practical in the current settings of many diagnostic laboratories.

Immunohistochemistry is a simple, rapid and inexpensive method for detecting *BRAF* mutations. In melanoma, the detection of *BRAF* mutation is by positive staining of anti-*BRAF* antibody using immunohistochemical stain. Only melanomas that are negative by immunohistochemistry need confirmation by mutation analysis. However, currently, this method is not accredited for use in other cancers. In colorectal cancer, Kuan and colleagues proposed that immunohistochemistry could be a useful surrogate for the detection of the *BRAF* V600E mutation although weak staining must be evaluated by *BRAF* PCR analysis or sequencing (Capper et al., 2013; Day et al., 2014; Kuan et al., 2014). On the other hand, Szymonek and colleagues recently found lack of concordance between immunohistochemistry and Sanger sequencing/real time PCR in the detection of *BRAF* V600E (Szymonek et al., 2017) in a large series of colorectal carcinoma. Thus, although immunohistochemistry is the most simple and economic method to detect *BRAF* mutation in colorectal cancer, further studies and protocols need to be established for it to be for clinical use.

It is now common to use next generation sequencing to detect *BRAF* mutation in colorectal carcinoma. Next generation sequencing demonstrates a high analytic sensitivity, broad reportable range of mutation spectrum and capacity for quantitative measurement of mutant allele frequencies. Simultaneous detection of concomitant mutations is possible with this technique (Haley et al., 2015; Hempelmann et al., 2015). Methods of targeted sequencing and whole genome sequencing have been trialled and confirmed to be the most suitable for clinical diagnostic applications of primary and metastatic colorectal carcinomas (Brannon et al., 2014). Currently, the next generation sequencing test costs roughly $230 American dollars more than laboratory developed allele specific *BRAF* V600E polymerase chain reaction (Zhu et al., 2018). In 2016, Patel and colleagues in United Kingdom did a cost analysis of standard Sanger sequencing versus next generation sequencing on two genes (Patel, et al., 2016). According to them, the mean cost per sample with next generation sequencing was 119 pounds whereas that of Sanger sequencing of each of two genes were 178 pounds and 79 pounds respectively, generating a cost saving of 59 pounds for one gene and a surplus of 40 pounds for the others. As the cost of the next generation sequencing has decreased in recent years, it is now the method of choice in many clinical units.

Potential applications of anti-BRAF therapies

BRAF protein is a key player in growth and

survival in several signalling pathways such as MEK-ERK, NF-kB, P13/AKT pathways (Rahman et al., 2013, 2015) (Fig. 1). BRAF kinase inhibitors are widely used to treat metastatic melanoma. BRAF inhibitors are categorized into two types; type I inhibitors which bind with the protein kinase in its active conformation (including Vemurafenib, Dabrafenib, LGX818, PLX4720, SB-590885, GDC-0879) and Type II inhibitors (including sorafenib, regorafenib, XL281, RAF265, ARQ736) which bind with the protein kinase in its inactive conformation (Rahman et al., 2014a). Development of resistance to BRAF kinase inhibitors (intrinsic and acquired) could be the reason for failure of this target therapy for cancer (Rahman et al., 2014b). In colorectal carcinomas, single therapy with BRAF inhibitor appears to be ineffective with a response rate of approximately 5% (Barras 2015). Compared to melanoma, colon carcinoma demonstrates higher levels of PI3K/AKT pathway activation, a mechanism of both innate and acquired resistance to BRAF inhibitors in *BRAF* V600E mutated cancers (Coffee et al., 2013).

BRAF inhibition decreases cell viability in *BRAF* mutated colorectal carcinoma and in vitro studies have shown that BRAF inhibition decreases MAPK signalling and cell viability. However, isolated BRAF inhibition does not induce apoptosis; the PI3K/mTOR pathway sustains signalling even after BRAF inhibition and provides resistance to BRAF inhibition. (Coffee et al., 2013). *In vivo* BRAF blockade in a genetically engineered mouse model for microsatellite stable *BRAF* V600E mutated colon carcinoma resulted in inhibition of MAPK, sustained PI3K/mTOR signalling and did not induce apoptosis. Nonetheless, the use of concomitant BRAF and PI3K/mTOR blockade enhanced the effects of in vivo BRAF inhibition via the induction of in vivo tumour regression, resulting in a significant decrease in proliferation and induction of apoptosis, supporting the adoption of combinatorial approaches (Coffee et al., 2013).

Multiple proliferation and survival signalling pathway are simultaneously active in *BRAF* mutated carcinoma (Rahman et al., 2015) and suppression of one pathway is not enough for complete suppression of cancer growth (Rahman et al., 2016). Combinatorial approaches have been shown to be beneficial in improving outcomes in patients with *BRAF* mutations. For instance, combined treatment of PLX4720 (type I BRAF inhibitor) with PI3K inhibitors causes synergistic growth inhibition in *BRAF* mutant colorectal cancer cells with both primary and secondary resistance. *In vivo*, the addition of PLX4720 to AKT inhibitors AKT demonstrated more tumour growth inhibition than PLX4720 alone. In vitro, clones with acquired resistance to PLX4720 exhibit PI3K/AKT activation with EGFR or KRAS amplification (Mao et al., 2012).

Selective pan RAF inhibitors like LY3009120 have shown be superior in inhibiting proliferating and tumour growth in *BRAF* mutations when compared to previously investigated selective *BRAF* inhibition in preclinical models of human colorectal carcinoma. Vakana et al showed that LY3009120 was efficacious against mutated *BRAF* colorectal cancer in xenograft models (Vakana et al., 2016). After application of LY3009120, anti-

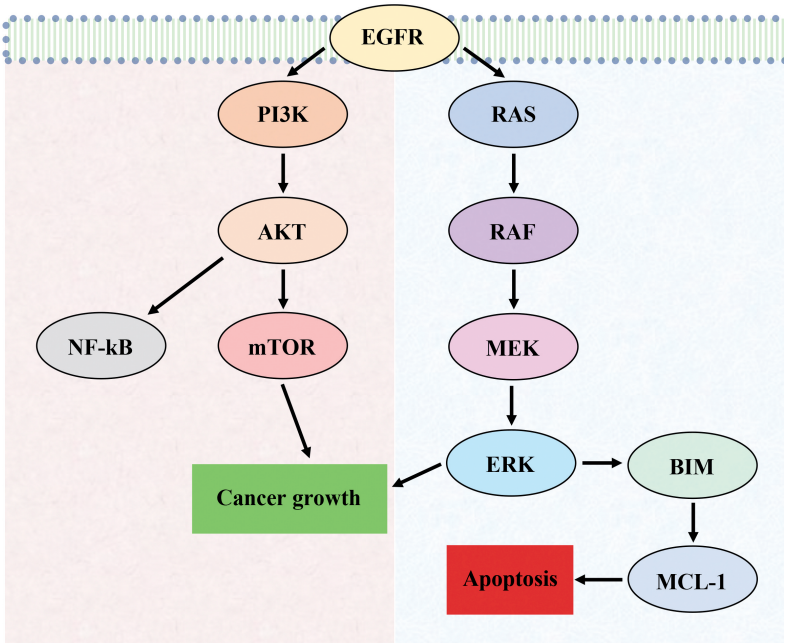

Fig. 1. The molecular pathways in which BRAF protein is a key player in growth and survival. The proteins in these pathways are molecular targets for the drugs in trails for colorectal cancer.

proliferative effects were exhibited in mutated *BRAF* colon carcinoma cells associated with G1 cell cycle arrest and resulted in isolated induction of cell death (Vakana et al., 2016).

Anti-BRAF therapies are associated with a range of cutaneous disorders. The most important side effect is squamous cell carcinoma. The other cutaneous manifestations include keratoacanthoma, Grover's disease, seborrheic keratosis, epidermal cysts, acneiform eruptions, hair loss and changes in hair structure, keratosispilaris-like reactions, and photosensitivity (Anforth et al., 2013; Pakneshan et al., 2013).

BRAF also controls MEK and ERK. MEK inhibitors cause upregulation of BIM (a proapoptotic protein that functions as a prosurvival molecule in cancer) and in turn promotes cancer apoptosis in colon cancer cell lines (Wickenden et al., 2008).

Other strategies of treatment proposed include the use of cobimetinib (MEK inhibitor) and a MCL-1 antagonist (apoptosis regulator) as a therapeutic strategy against mutant *BRAF* V600E to reverse apoptosis resistance caused by MEK/ERK activation and MCL-1. MCL-1 is a protein which promotes cell survival by interfering with the release of cytochrome c from mitochondria (Kawakami et al., 2016). It is a protein affected by the expression of ERK. *In vitro* and *in vivo* studies on colon carcinoma showed a direct link between oncogenic transformation and aberrant expression of immunosuppressive glycans from association between *BRAF* V600E mutation and high expression of MGL-binding ligands (Lenos et al., 2015).

There are multiple clinical trials targeting *BRAF* V600E mutation in colorectal carcinoma (https://clinicaltrials.gov/). However, there is only one trial with published results in the literature. The trial could not identify a meaningful clinical benefit with single agent targeting the *BRAF* V600E mutation in colorectal carcinoma (Kopetz, et al., 2015). In addition, there are seven clinical trials currently active using different BRAF inhibitors (retrieved from https://clinicaltrials.gov/ in November 2018)

Conclusion

In conclusion, *BRAF* mutation is important in the pathogenesis of colorectal carcinoma. The understanding of *BRAF* mutation allows for personalized treatment of colorectal cancers. Further research on the detection methods of *BRAF* mutation to make them of relevance in clinical practice is essential. In addition, further clinical trials on combination target therapy against BRAF mutated carcinoma will help to improve the management of the patients with colorectal cancer.

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