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REVIEW

Irinotecan therapy and molecular targets in colorectal cancer: A systemic review

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Abstract

Irinotecan is the second line chemotherapy for advanced stage colorectal cancer (CRC) after failure of first line chemotherapy with oxaliplatin and 5-fluorouracil. The aim of this review is to analyse the data on irinotecan as second line chemotherapy for advanced CRC and the potential roles of the molecular markers, p53 and vascular endothelial growth factor (VEGF) in the management of advanced CRC. Thus, the English literature from 1980 to 2008 concerning irinotecan, p53, VEGF and CRC was reviewed. On review, Phase Ⅱ and Ⅲ clinical trials showed that irinotecan improves pain-free survival, quality of life, 1-year survival, progression-free survival and overall survival in advanced CRC. p53 and VEGF were expressed in CRC and had a predictive power of aggressive clinical behaviour in CRC. Irinotecan sensitizes p53 wild type, mutant and null cells to Fas-mediated cell apoptosis in CRC cells. Wild type p53 cells were more sensitive to irinotecan than mutated p53. Irinotecan has an anti-VEGF effect inhibiting endothelial cell proliferation, increasing apoptosis and reducing microvascular density which is only limited by irinotecan toxicity levels. To conclude, irinotecan improves the patient's quality of life and the survival rates of patients with advanced CRC. p53 and VEGF status of the patients' tumour is likely to affect the responsiveness of CRC to irinotecan. It is recommended that studies of the expression of these molecular markers in relation to chemo-responsiveness of irinotecan should be carried out for better management of patients with advanced CRC.

Key words: Colorectal cancer; Irinotecan; Molecular; p53; Vascular endothelial growth factor

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common types of cancer in Western countries[1]. Five-year survival in CRC patients is related to diagnostic staging of CRC[2]. Due to the late onset of symptoms, the majority of cases are diagnosed in Duke's stages C or D. Most patients with CRC undergo surgical resection and then commence adjuvant chemotherapy, with the exception of stage A and some stage B cancers where side effects outweigh potential benefits. In patients who develop recurrent or metastatic CRC, oxaliplatin and 5-fluorouracil (5-FU) combined are the most extensively used first line treatment, with a response in approximately half of patients[3]. If the cancer progresses after this treatment, irinotecan is commenced. This review was undertaken to study the impact of irinotecan in the treatment of advanced CRC and to identify advanced CRC molecular markers that predict response to irinotecan. The aims are to influence patient selection and to recommend

evidence-based treatment options for patients with advanced CRC and to reduce patient morbidity and mortality.

DATA COLLECTOIN

The English literature between 1980 and 2008 on the treatment of advanced CRC with irinotecan was reviewed; the effects of irinotecan on CRC, p53, vascular endothelial growth factor (VEGF) and CRC in Australia. Studies not including irinotecan as the variable were excluded, as were those without CRC. The studies were statistically significant when $P < 0.05$. Only original full text publications were reviewed. The selection criteria included adequate follow-up, sample numbers, second line irinotecan therapy and clinicopathological features: grading and staging of the CRCs, histological subtype, nodal and metastatic status, threshold cut off values and complete reference lists. The blinded status and number of experimental observers were noted. The review was limited by suboptimal cohort sizes as it focuses on recently emerging research. Whilst the methodology and statistical analysis varied between studies, their designs were similar.

LITERATURE OVERVIEW

Irinotecan therapy

Irinotecan is activated by hydrolysis to SN-38, an inhibitor of topoisomerase I . This is then inactivated by glucuronidation by uridine diphosphate glucoronosyltransferase 1A1[4]. The inhibition of topoisomerase I by the active metabolite SN-38 eventually leads to inhibition of both DNA replication and transcription. Unlike hepatocytes, other cells in the body have no way of detoxifying SN-38 through glucoronidation, thus contributing to its high cytotoxicity. The most frequent irinotecan toxicities are severe diarrhoea and suppression of the immune system (neutropenia). Other side effects include nausea, hyperbilirubinaemia, fatigue, emesis, fever, weight loss, alopecia, oedema, dyspnoea and thromboembolism.

A Phase Ⅱ study on irinotecan therapy was performed by Cunningham and colleagues in 289 patients with advanced CRC[5]. It was shown that the survival rates were much better in 189 patients who received chemotherapy than the 90 patients who received supportive care only (36.2% *vs* 13.8%). In addition, both pain-free survival and quality of life were higher with irinotecan 2nd line chemotherapy. Also, irinotecan increased survival of patients without World Health Organisation (WHO) performance status deterioration and without weight loss exceeding 5%.

A Phase Ⅲ study by Rougier and colleagues concluded that irinotecan produced better 1-year survival of 45% and median survival of 10.8 mo than the comparison groups treated with three different regimes of infused FU, where a survival rate of 32% and a median survival of 8.5 mo were recorded[6]. These were both well designed randomised non-blinded studies with large cohorts both in excess of 265 patients. The patients were assessed every 3 wk with imaging and CEA levels to detect progression of disease. All the patients had metastatic CRC and had received treatment within the preceding 6 mo with 5-FU.

Clinicopathological features were a major confounding factor, however, the results of Cunningham *et al*[5] and Rougier *et al*[6] remained significant after the WHO baseline performance status of the patients was analysed with multivariate analysis. These studies support the use of second line irinotecan monotherapy to increase pain-free survival, quality of life, 1-year survival, progression-free survival and overall survival in metastatic CRC, both in comparison to best supportive care and to 5-FU.

Rothenberg and colleagues conducted a Phase Ⅱ trial, involving 166 patients with metastases involving either liver, lung, lymph nodes or other soft tissue, treated with second line irinotecan monotherapy[7]. They found a significant response to irinotecan with 10.8% of patients achieving complete or partial response and 40.4% with stabilised disease. The median progression-free survival was 3.9 mo with a median survival of 9.9 mo. Unlike the above studies which examined the effect of clinicopathological variants through multivariate analysis, Rothenberg *et al*[7] directly examined the effect of symptomatic disease on overall survival. They found that asymptomatic patients had a longer overall survival than those patients with symptoms prior to irinotecan therapy. However, on further analysis the 1-year survival was 42.4%, with no significant effect from clinicopathological factors or baseline variables. There were several limitations in this study. Most importantly there was no control or comparison group. Despite the tumors being assessed every 12 wk, the assessment criteria states the tumors must decrease or remain stable for 4 wk or more to be classified as responding to therapy. Therefore the tumors needed to respond for 12 or more weeks. Such a long time lapse between monitoring affects progression-free results, leading to an underestimation of true progression-free survival. Also, the study was not blinded. However, the authors overcame this by the use of two teams assessing the imaging and CEA results, one of which involved radiologists and oncologists independent of the study.

These three major studies show statistically that clinicopathological features have no effect on overall survival, and that the presence of symptomatic disease is not an independent predictor for outcome in a multivariate analysis. Overall, irinotecan is an effective second line chemotherapeutic agent for CRC. In Phase Ⅱ and Ⅲ clinical trials, it has been shown to increase 1-year survival, quality of life and symptom control despite a high side effect profile.

Molecular targeted therapy

Molecular targeted therapy is a "drug or therapeutic strategy with a focused mechanism specifically acting on a well-defined target or biological pathway that, when inactivated, causes the regression or destruction of cancer". The advantages of molecular targeted therapy include (1) providing evidence-based treatment options

for patients, (2) treating patients most likely to respond to the therapy (3) sparing patients with poor response profiles from further harm in terms of side effects, (4) minimising side effects (5) streamlining healthcare resources and finally (6) to promote further research into molecular targeted therapy.

This review focused on two common genes that are commonly investigated in cancer, namely VEGF and p53. Being a second line chemotherapeutic agent, it is important to research the effects of irinotecan on such mutations as these antigens are key determinants in the survival of advanced CRCs. By researching irinotecan it is hoped that results will show different molecular targeted pathways by which irinotecan can potentially inhibit CRCs so that patients with the most responsive marker profile may be treated with a molecular targeted drug that acts on different sites rather than just one site.

p53 in CRC

p53 is a tumour suppressor gene[8]. Alterations in p53 are the most common genetic changes noted in human cancer. p53 senses DNA damage in the G1 stage of the cell cycle and either prevents cell cycle progression until DNA is repaired or induces apoptosis. p53 acts as a central mediator of the cellular response to stressful stimuli. The growth-suppressive function of p53 is lost with mutation and this occurs commonly in human cancer. In addition to suppressing cancer development and progression, wild-type p53 further confers chemo-sensitivity and radio-sensitivity in tumour cells.

Our recent study, involving 188 patients, showed that p53 is over-expressed in 63% of Australian patients with CRC[9]. The survival of the CRC patients was related to staging and p53 protein nuclear expression in the tumors. In our other study, p53 was also noted to predict poorer survival in a subset of CRC patients with mucinous adenocarcinomas[10].

Bosari *et al*[11] conducted a 5-year retrospective study on p53 immunostaining in 206 CRC specimens from patients with no neoadjuvant therapy or history of other malignancies and with clear resection margins. 65 specimens stained positive for nuclear p53 and 99 for cytoplasmic p53 accumulation. Cytoplasmic p53 accumulation correlated with reduced overall survival and reduced disease-free survival. In addition, cytoplasmic accumulation of p53 was a significant prognostic factor for poorer overall survival and disease-free survival in left sided CRC. The study could have been strengthened by the identification of the type of p53, rather than relying on past research that indicates cytoplasmic accumulation of p53 is usually WTp53. WTp53 and Mp53 respond differently in CRC and induce cell death *via* different mechanisms.

Flamini and colleagues studied 96 CRC patients, of which 47% where p53 positive[12]. The patients were studied for 3 years and treated exclusively by surgical resection of the CRC and metastases. Compared to other stages, Duke's D CRC had increased cytoplasmic p53 expression, whilst nuclear p53 was over expressed in Duke's B CRC. This study had unique findings and is unlikely to ever be repeated due to the ethical restrictions of not treating patients with either adjuvant or neoadjuvant therapy.

Diez and colleagues studied 174 patients and concluded that p53 positivity in the primary CRC increased the risk of recurrence only after the first year of follow-up[13]. Lanza and colleagues studied 204 CRCs by IHC, of which 60.4% where p53 positive[14]. The study concluded that there was no statistical significance with regard to age, gender, tumour site, tumour stage or grade of differentiation. Of the 141 patients with TNM Ⅰ-Ⅲ disease who underwent curative resection, positive p53 staining was associated with poorer overall survival.

Adrover and colleagues randomly selected 111 patients with sporadic CRCs TNM stages Ⅰ-Ⅳ, and quantitatively measured the cytoplasmic WTp53 and Mp53 in both the cancerous and non-neoplastic tissue of all patients using immunoassay with the p53 antibodies Ab1801 and DO1 as markers[15]. High p53 expression was defined as having ⩾ 2.7 ng/mg cytosolic protein. TNM stage Ⅲ and high p53 expression correlated with increased disease-free survival. In multivariate analysis, p53 expression is related to a survival advantage in stage Ⅲ CRCs. These results significantly contradict previous studies. However, Adrover *et al*[15] were the first to identify the difference in normal p53, wild type and mutant p53. The definition of high levels of p53 is debatable as the p53 expression in adjacent non-neoplastic tissue has not been evaluated.

To conclude, p53 expression is important in the prognosis of CRC. The identification of WTp53 and Mp53 is controversial due to the half life of WTp53. Based on the larger studies, the overexpression of p53, especially cytoplasmic p53, is a crucial target for molecular targeted chemotherapy.

Vascular endothelial growth factor in CRC

The VEGF family of genes are key regulators of angiogenesis[16]. VEGF expression correlated with clinical and pathological parameters in cancers[17-19]. For instance, we have shown that strong immunohistochemical VEGF expression levels tended also to have higher serum VEGF level than those with low expression levels. In addition, elevated serum VEGF levels are strongly correlated to the recurrence of thyroid cancer and the presence of lymph node metastases. VEGF expression was noted in the non-cancerous tissue adjacent to the cancer indicating that genetic changes may occur before the morphological appearance of cancer.

Colorectal mucosa contains all the subtypes of the VEGF family A-D[20]. VEGF mRNA is expressed in higher levels in human CRC cells compared to adjacent normal tissue[21]. VEGF mRNA expression rises most between CRC stages Tis to T1.

A meta-analysis involving 27 studies of VEGF expression and overall survival of CRC observed a 1.65 times poorer survival in those with higher ratios of VEGF in cancer tissue[22]. Kondo *et al*[23] found VEGF mRNA and protein only in CRCs (15 of 26 studied) compared to no expression in adenomas. They

suggested that mutant p53 induced VEGF expression, which coincides with the progression from adenoma to carcinoma in CRC.

Of the 31 human CRC cell lines examined with IHC, Kuramochi *et al*[24] concluded there was no significant difference in median VEGF mRNA levels of expression in the primary CRC in patients with or without hepatic metastases. However, the level of VEGF expression is significantly less in the hepatic metastatic tissue compared to the adjacent non-cancerous hepatic tissue. Patients with more than one site of metastasis expressed higher levels of mRNA VEGF compared to those with one metastasis site[22].

Comparing CRC (T) to the adjacent colorectal mucosa (N), Hanrahan *et al*[25] concluded that VEGF-A and VEGF-B play a significant role in the early development of CRC from adenomas, whilst VEGF-C is expressed at higher levels in metastatic CRC. They concluded that VEGF A and C mRNA levels were correlated to tumour grade and tumour size, but not significantly related to the staging of CRC.

Ottaiano and colleagues concluded from their study of 71 patients that CXCL12 stimulates ICAM-1 and VEGF expression and clonogenic growth of CRC cells, which all lead to metastases, and that over expression of VEGF was an independent predictor of early metastases in CRC patients[26]. Ishigami *et al*[27] further concluded that over expression of VEGF mRNA also correlated with poor overall survival.

Saad and colleagues showed that VEGF expression correlated with the presence of angiolymphatic invasion, lymph node metastasis and the depth of invasion[28]. They found no significant correlation between VEGF expression and tumour grade and development of liver metastases.

Molecular markers and irinotecan

p53: Yu *et al*[29] profiled the 24 genes associated with the mechanism of action of irinotecan in 52 CRC specimens from humans with no previous neoadjuvant therapy. Irinotecan is converted to SN-38 which acts on topoisomerase-1 then subsequently *via* a cascade effect on TNFSF6 to FDXR and then p53 which finally induces cell apoptosis. Through *in vitro* gene profiling and cluster analysis, Yu *et al*[29] found that the pathway leading to p53 was expressed at higher levels in tumourous tissues (T) compared to adjacent normal tissue (N), however, none as high as p53. Whilst the level of p53 RNA expression was not identical for each specimen, the general trend remained. The study showed that irinotecan is more effective in inducing apoptosis in CRC when the p53 T:N is high, despite the type of mutation in p53.

In-vitro studies concluded that irinotecan sensitizes p53 wild type, mutant and null cells to Fas-mediated cell apoptosis in CRC cells[30]. Irinotecan caused a significant rise in Fas mRNA in WTp53 cells[31]. However, irinotecan also caused a small increase in Fas mRNA in p53 mutant and null cells. This indicates that not only does p53 have a major role in Fas cell surface expression, but that irinotecan also increases Fas cell surface expression independent of p53. Irinotecan induces signal transducer and activator of transcription 1 (STAT1) phosphorylation in the p53-null cell lines and increases the expression of genes involved in cell surface trafficking of Fas, despite STAT1 not being identified in the promoter of Fas. Absence of STAT-1 decreases Fas expression.

McDermott and colleagues found a significant increase in STAT-1 Ser727 phosphorylation in irinotecan treated null cells[32]. The experiment was repeated with the addition of STAT1 small interfering RNA which caused a down regulation of STAT1 expression in the WTp53 and null cell lines. Subsequent treatment with irinotecan produced no change in the level of Fas mRNA, however, down regulation of STAT1 resulted in a significant decrease in Fas cell surface expression in the p53-null cell line. They proposed that STAT1 silencing was incomplete or that STAT-1 independent pathways regulate Fas ligand expression in response to irinotecan

p53 wild type cells were also more sensitive to irinotecan treatment compared to mutant p53 at low dose and high dose treatment[33]. No significant response to irinotecan treatment was recorded in the mutant p53 cell lines As irinotecan is known to have a higher response in both cytoplasmic accumulation of p53 and of WTp53, this study further suggests that WTp53 accumulates in the CRC cell cytoplasm and that WTp53 in CRC is a positive predictive factor for response to irinotecan.

Irinotecan was added to cells from a single Duke's B CRC with mutant p53 and its sub-clone transfected with wide type p53. The cells were synchronised to G0/G1 stage of the cell cycle by starvation. Following the addition of irinotecan, the wild type p53 cells were arrested in S phase whilst the mutated p53 cells continued to progress through the cell cycle, indicating a lack of functionality of p53. The functional response of irinotecan in the wide type p53 line and not the mutant p53 was observed during *in-vivo* studies of nu/nu mice with xenografted human CRC, whereby there was increased apoptosis and decreased proliferation.

Overall, irinotecan reduces progression in WTp53 CRCs, and plays a less significant role in inhibiting mutant and null p53 CRCs *via* the STAT1 pathway. Having identified p53 as the key component of the apoptotic pathway and the significance of the T:N p53 ratio in predicting a positive response to irinotecan, it is necessary that these findings are implemented in practice. Thus, further research should be conducted using *in-vivo* research methods on a large scale to confirm the clinical significance of such results.

Vascular endothelial growth factor: In CRCs, high levels of EGFR correlated with response to irinotecan and progression-free survival[34]. However, this did not prove that VEGF expression is correlated with response to irinotecan. The study had a small sample size, all were treated with irinotecan, 5-FU and leucovorin, and no control groups treated with single agent therapy. Whilst there was an adequate follow up period of 23 mo, there were several variables.

Koizumi and colleagues developed NK012, a SN-38 (irinotecan metabolite) releasing nanodevice and tested its efficacy in xenografted CRCs in mice[35]. A comparison of tumour size reduction with NK012 (doses 15 and 30 mg/kg per day) *vs* irinotecan (66.7 mg/kg per day) showed a significant reduction in tumour size compared to the irinotecan treatment. Compared to irinotecan, NK012 was more cytotoxic with potent anti-tumour activity, thought to be secondary to the enhanced and prolonged distribution of free SN-38 in the tumour. This showed that irinotecan and its active metabolite has an anti-VEGF effect. Thus, it can be proposed that these activities should be reflected in VEGF positive CRCs if adequate levels of SN-38 are produced, which is only limited by the irinotecan dose side effects.

Bocci and colleagues compared the effects of irinotecan metronomic monotherapy against irinotecan combined with semaxinib on xenografted CRCs in mice and in *in-vivo* CRC cell lines[36]. They analysed *in-vitro* proliferation of cells, apoptosis and thrombospondin-1 (TSP-1)/VEGF expression and concluded that in the cells treated with SN-38 monotherapy, there was inhibited endothelial cell proliferation alone, and that irinotecan worked synergistically with semaxinib to increase apoptosis and increase expression and secretion of TSP-1 and to reduce microvascular density[35].

In *in-vitro* studies, SN-38 increased TSP-1 expression and in *in-vivo* studies, SN-38 reduced tumour and microvessel growth[37]. Higher levels of TSP-1, an anti-angiogenic factor are linked with low levels of VEGF[37]. TSP-1 inhibits angiogenesis through the inhibition of matrix metalloproteinase-9 (MMP9). TSP-1 null mice have both increased levels of MMP9, whilst over expression of TSP-1 in mammary tissue reduced the levels of active MMP9. The levels of MMP9 correlated with the level of expression of VEGF binding to VEGFR-2[38]. Gupta *et al*[39] concluded that TSP-1 inhibits VEGF mobilisation from the extracellular matrix by inhibiting active MMP9 and that TSP-1 also has a direct role in inhibiting VEGF activity.

Irinotecan increases TSP-1 expression and secretion and TSP-1 reduces VEGF expression and activity[36]. CRCs expressing high levels of VEGF respond more significantly to irinotecan as the stimulus for angiogenesis and thus subsequent growth of the tumour is reduced.

CONCLUSION

Increased expression of p53, especially cytoplasmic WTp53 is associated with poor overall survival, decreased disease-free survival, increased relapse and metastases. However, cytoplasmic accumulation of wild type p53 is a positive predictive factor for irinotecan response. Irinotecan induces apoptosis in both mutant and null p53 CRCs to a lesser extent *via* the STAT1 pathway.

Increased expression of VEGF is correlated with multiple metastases, tissue invasion and lymph node invasion. Whilst there is evidence that irinotecan increases TSP-1 expression which in turn reduces VEGF expression and the angiogenic growth of tumors, there is no conclusive evidence linking irinotecan with VEGF expression and response. Irinotecan has the potential for multiple effects within CRC cells which may reduce the number of prescribed drugs and side effects for patients.

As a second line therapy, irinotecan improves patient quality of life, 1-year survival and progression-free survival. It would be beneficial to all patients to determine the molecular profiles of CRC most likely to respond to irinotecan and to spare those unlikely to respond from unnecessary side effects.

A standardised criteria needs to be developed to compare marker expression in normal colorectal mucosa to that of cancerous mucosa in order to define overexpression of markers. Further research would also benefit from a standard CRC staging system so that studies can be compared directly, as well as 12 mo follow-up periods for irinotecan treatment. Areas of potential research focus include: (1) large studies on the effects of irinotecan with regard to VEGF and p53 expressions in CRC and (2) determine whether the expression of molecular markers can predict response to irinotecan.

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