

## Colorectal Carcinogenic Pathways and Chemotherapeutic Responsiveness : A Review

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### ABSTRACT

Colorectal cancer is one of the most common malignancies encountered in the developed world and its incidence has noted to be rising in the developing world as well. The considerable morbidity and mortality associated with this condition has driven research into elucidating the underlying molecular mechanisms in the hope that new therapeutic targets would be identified. This research has revealed the presence of distinct molecular pathways that culminate in malignant transformation of colorectal tissue. Mutations in several other pathways, most commonly involved in intracellular signalling have also been shown to act in conjunction with the distinct carcinogenic pathways and thereby predisposing to the development of cancer. These discoveries are beginning to have clinical applications as the identification of responders and non responders to particular chemotherapeutic agents is now possible to a certain extent via various molecular markers. In this paper, we briefly review the different carcinogenic pathways along with mutations in certain molecular pathways that aid the progression of malignant transformation. We also go on to discuss in detail, the different pharmacogenomic factors that influence a patient's response to various chemotherapeutic agents. Such knowledge not only would result in lower costs but would also minimise the exposure of patients to any undesirable side effects. These advances represent a step towards making personalised medicine a reality.

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## Introduction

Colorectal cancer is one of the most common malignancies encountered in the western world and is now the third most common cause of cancer related mortality<sup>1</sup>. Indeed, such statistics are not limited to the western world as similar observations have been made in Asia where the incidence of colorectal cancer is rising. The growing size of the problem has necessitated the importance of continued research into this field that would not only lead to an enhanced understanding but also impact on clinical practice<sup>2,3</sup>.

Research into the molecular mechanisms has led to advances in therapies targeted at various molecular pathways<sup>4</sup>. Apart from unravelling the complexities of carcinogenesis, it has also enabled clinicians to predict response to chemotherapy. The translation of this research into clinical practice means that we are closer to developing individualised therapies for patients. This is also of considerable relevance as targeted individualised therapies would mean that the patients are less likely to suffer from any adverse side effects of treatment.

In this review, a brief survey of the various molecular pathological mechanisms underlying colorectal carcinogenesis is presented. In the context of this approach, the response of patients to the various therapies is described. A brief description of the novel approach of molecular pathological epidemiology is also presented.

## Methods

A pubmed search was performed for relevant literature using the terms colorectal cancer, chemotherapy, molecular biology colorectal cancer, monoclonal antibodies, colorectal cancer genetics, pharmacogenetics of colorectal cancer. The bibliographies of the retrieved papers were also searched for articles of relevance.

## The Adenoma-Carcinoma Sequence and Molecular Pathways

Histological observations indicated that colorectal malignancies develop via a worsening degree of dysplasia of normal colonic mucosa<sup>5</sup>. Fearon &

Vogelstein proposed the adenoma-Carcinoma model of carcinogenesis which has undergone various modifications as precise molecular Details have been elucidated<sup>6</sup>. Colorectal cancer has been found to be a heterogeneous disease with four main aetiological pathways - the Chromosomal Instability pathway (CIN), CpG Island Methylator Phenotype (CIMP) pathway, Microsatellite Instability (MSI) pathway and the Serrated pathway<sup>7,8,9,10</sup>. A very brief description of these pathways and several other molecular mechanisms is described in the following paragraphs and also lay the foundation for a better understanding of the molecular pharmacology of the various chemotherapeutic agents.

The CIN pathway is identified by aneuploidy and structurally altered chromosomes and is associated with deletions in chromosome 5, 18q or 17q<sup>11</sup>. Loss of heterozygosity (LOH) results in deletions of the SMAD group of genes and also of the Deleted in Colorectal Cancer (DCC) gene. SMAD2 and SMAD4 are known to play a role in the TGF- $\beta$  signalling pathway<sup>12</sup>. Other mutations that are commonly found in this pathway are mutations in the APC gene and in the KRAS gene<sup>13</sup>. APC is a tumour suppressor gene and mutations in this gene have been found early on in the development of sporadic colorectal cancers<sup>14</sup>. KRAS plays a crucial role in the numerous intracellular signalling pathways and is reflected in the fact that KRAS mutations are found in a variety of cancers<sup>15,16,17</sup>. The downstream mediators of the KRAS pathway include the mitogen-activated protein kinase kinase (MAPKK) and mitogen-activated protein kinase (MAPK), both of which have roles in cell division<sup>18</sup>. Mutations in codons 12 and 13 in exon 1 and codon 61 in exon 2 lead to a decrease in gtpase activity resulting in a constitutively active K-RAS protein which predisposes to the development and malignant progression of polyps<sup>19</sup>.

The CIMP pathway results from the silencing of tumour suppressor genes by the hypermethylation of CpG islands within the promoter regions of these genes with a concomitant global DNA hypomethylation<sup>20,21</sup>. The exact mechanism underlying this process is yet to be fully understood<sup>22</sup>. The degree of CIMP is determined from the number of markers positive for CIMP from a predetermined set of genes. Despite the fact that CIMP is the second most common aetiological

pathway for colorectal cancer, there is a lack of standardisation of the panel of markers used to determine the CIMP status<sup>23</sup>.

Defects in the DNA mismatch repair (MMR) system result in the accumulation of mutations in repeat sequences known as microsatellites and are the first steps along the MSI pathway<sup>24</sup>. Germline mutations in the MMR system result in Hereditary Non Polyposis Colorectal Cancer (HNPCC)<sup>25,26</sup>. Alternatively, silencing of one of the genes involved in the MMR system can occur by hypermethylation of the promoter of one or more of the constituent genes. This appears to be the mechanism that underlies sporadic MSI tumours. More specifically, a high degree of MLH1 promoter hypermethylation and consequent silencing of this gene has been noted in sporadic MSI tumours<sup>27</sup>.

The recently recognised serrated pathway describes the progression of traditional serrated adenomas (TSA) and sessile serrated adenomas (SSA) to adenocarcinomas<sup>28, 29</sup>. TSA and SSA along with true hyperplastic polyps were previously classified as hyperplastic polyps and were believed to have no malignant potential. Increasing amounts of research do seem to suggest that TSA and SSA progress to adenocarcinomas via distinct pathways as most TSA have a lower degree of MSI (MSI-L) than SSA (MSI-H)<sup>30</sup>. Also, around 80% of TSA are associated with KRAS mutations whereas BRAF mutations are more common in SSA. Interestingly KRAS and BRAF mutations appear to almost mutually exclusive<sup>31</sup>.

### Mutations in Specific Pathways

There are various other mutations, particularly in signalling pathways that can also predispose to malignant transformation and act in conjunction with the above mentioned pathways. These are usually pathways involved in cellular proliferation or apoptosis and the mutation can result in a constitutive activation of the pathway which favours proliferation. Many of the genetic abnormalities result in overlapping dysregulation of molecular pathways and are not mutually exclusive<sup>32, 33, 34</sup>.

The diverse biological roles of the EGFR pathway are reflected in the different downstream

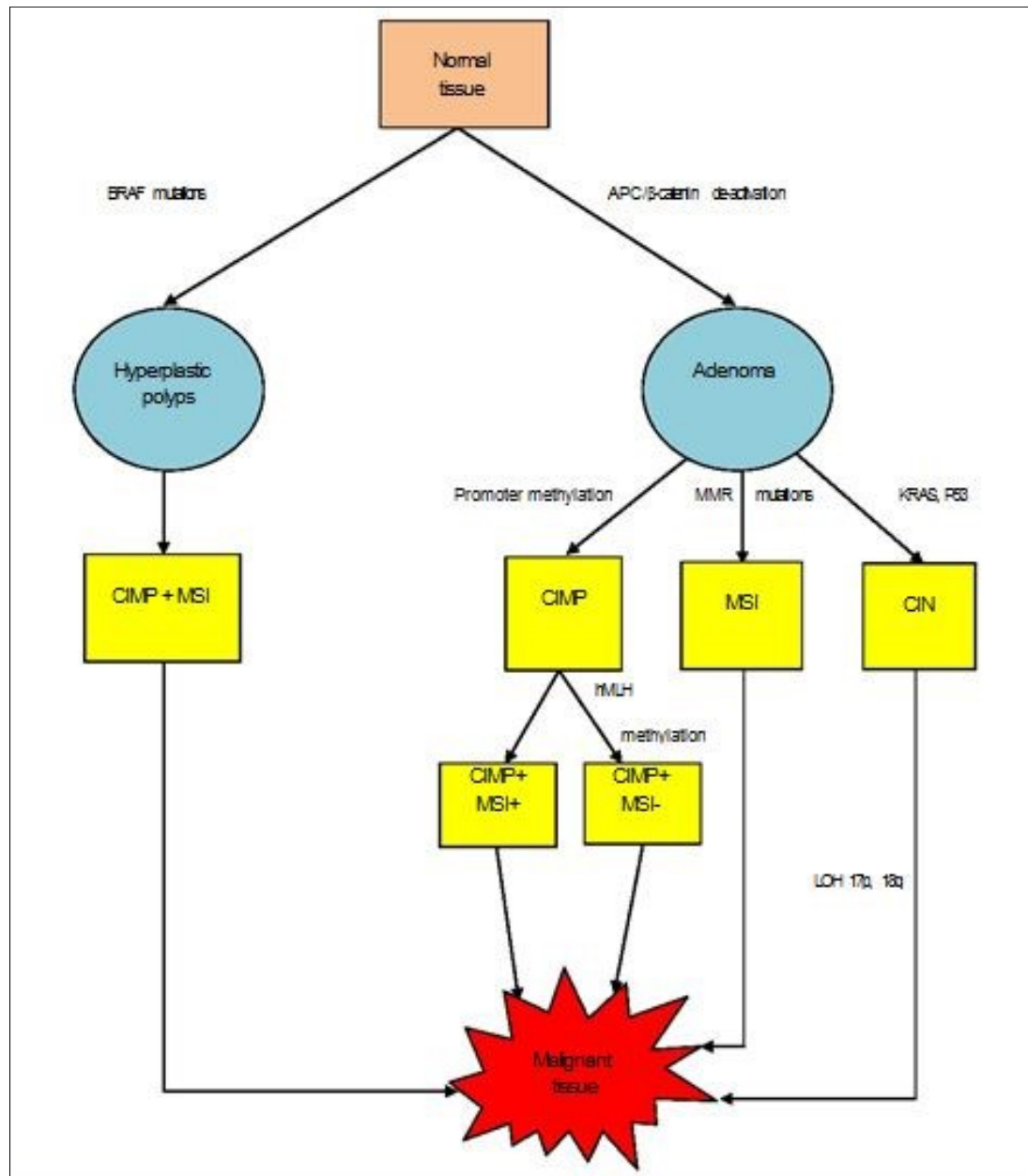
pathways it can activate. Pathways known to be activated by EGFR ligand binding are the RAS-RAF-MAPK pathway, PI3K pathway and the protein serine/threonine kinase Akt pathway<sup>35, 36, 37</sup>. Increased expression of EGFR has been associated with advanced stage and even metastasis and has been noted in almost 70% of advanced stage colorectal cancers<sup>38</sup>. Expression of EGFR is highest deep within the tumour and correlates with the invasiveness of the tumour<sup>39</sup>.

Components of the TGF- $\beta$  pathway also act on the RAS-RAF-MAPK pathway, PI3K/Akt pathway overlapping with the EGFR pathway and therefore these mutations tend to have a synergistic effect<sup>40</sup>. Mutations in the TGF- $\beta$  receptors can also result in aberrant activation of the pathway and indeed mutations in TGFBR2 have been detected in 30% of all colorectal cancers<sup>41</sup>. The existence of microsatellite regions in the TGFBR2 receptor gene means that these mutations tend to occur with higher frequency in MSI positive tumours<sup>42</sup>.

The role of p53 in a variety of carcinogenic pathways has been reported and it has been noted in almost 50% of colorectal cancers worldwide<sup>43</sup>. P53 is a major constituent of cell cycle regulatory pathways and therefore deleterious mutations would make it very likely to predispose to malignancy. P53 is regulated by various mechanisms such as phosphorylation, methylation and acetylation and disruption of all these intracellular mechanisms would lead to aberrant p53 function, however the most common mutation appears to be a missense mutation that interferes with its ability to bind to specific cognate sequences<sup>44</sup>.

### CHEMOTHERAPEUTIC RESPONSIVENESS

The considerable amount of research conducted into the molecular aetiology of colorectal cancer has led to the development of therapies that have increased survival rates among patients<sup>45</sup>. Despite these improvements, a clear understanding of which patients would respond to and benefit from these therapies is still lacking. Research is ongoing in terms of identifying predictive molecular markers that would help identify patients who would benefit from such therapies rather than surgery alone.



**Figure 1:** A schematic depiction of the different colorectal carcinogenic pathways with the main predisposing mutations. APC-adenomatous polyposis coli gene, MMR-mismatch repair system, CIMP-CpG island methylator phenotype, MSI-microsatellite instability, CIN-chromosomal instability, LOH-loss of heterozygosity.

### 5-Fluorouracil & Capecitabine

5-Fluorouracil (5-FU) which is administered intravenously has been used as first line chemotherapy in the adjuvant setting for colorectal cancer for decades. Capecitabine, which is an oral fluoropyrimidine is a prodrug which gets converted to a 5-FU following enzymatic conversion<sup>46</sup>. 5-FU is preferentially incorporated by cancer cells via the same pathway as uracil, and is converted to thymidine for DNA production<sup>47</sup>. An alternative metabolic route is via the

inhibition of thymidylate synthase (TS)<sup>48</sup>. Most of the 5-FU, however is catabolised by dihydropyrimidine dehydrogenase (DPD) in the liver. The conversion of capecitabine to 5-FU is via the action of hepatic carboxyl-esterases and cytidine deaminase and subsequently by thymidine phosphorylase (TP) and uridine phosphorylase (UP)<sup>49</sup>.

Various studies have been done that have looked at how the levels of the various enzymes involved in the metabolism of the fluoropyrimidines

relate to response rates. However, many of these studies have given conflicting results due to the difference in methodology and patient population. Ciaparrone et al demonstrated a correlation between a low level of DPD determined by immunohistochemistry (IHC) and RT-PCR with prolonged overall survival and disease free survival whereas no such correlation was seen in a study by Westra et al<sup>50, 51</sup>.

UP is a key enzyme in the conversion of 5-FU to its active metabolite and therefore it would be expected that a high level of UP would correlate with greater effectiveness. This has been demonstrated in vitro by Mader et al<sup>52</sup>. TP also plays a similar role as UP and together they constitute the rate limiting step of the conversion of capecitabine to its active metabolite<sup>53</sup>. TP also has angiogenic properties and therefore promotes angiogenesis in tumours. Contradictory results have been obtained from studies looking at the role of TP in relation to clinical outcome which may be due to the dual roles played by TP in enhancing 5-FU activity and angiogenesis<sup>54, 55</sup>.

TS which is a target of fluoropyrimidines is necessary for DNA synthesis and repair and therefore low levels of TS can lead to the accumulation of DNA damage. However, despite malignant cells being more proliferative than non malignant cells, a lack of TS also results in a lack of DNA synthesis. This implies that TS deficient tumours tend to be less proliferative. While in vitro studies demonstrate a positive correlation between TS levels and 5-FU responsiveness, in vivo studies are inconclusive<sup>56, 57, 58</sup>. This is primarily due to the heterogeneity of methodology used in various studies. Defects in the MMR system result in an increased tolerance to 5-FU. This is most likely because DNA damage does not trigger cell cycle arrest or death when the MMR system is defective<sup>59</sup>. Therefore, knowledge of the underlying carcinogenic pathway can help in determining the effectiveness of 5-FU.

### Irinotecan

Irinotecan mediates its action via the inhibition of topoisomerase 1 (topo-1). Topo-1 plays an important role in DNA replication by relaxing the supercoiled DNA helix by the introduction of single stranded breaks<sup>60</sup>. Around 43-51% of colorectal cancers

express increased levels of topo-1. The association of irinotecan with topo-1 results in a stable complex which induces double stranded breaks in the replication fork during DNA synthesis. This in turn serves as an apoptotic signal resulting in cell death<sup>61</sup>. Following transport to the liver, irinotecan is metabolised by two carboxyesterases (CES1 and CES2) to its active metabolite<sup>62</sup>.

In vitro studies have demonstrated an increased responsiveness to irinotecan in cell lines with higher topo-1 activity. A study by Braun et al showed that in patients expressing higher levels of topo-1 determined by IHC, a major overall survival benefit was seen with the use of irinotecan or oxaliplatin compared to patients on 5-FU<sup>63</sup>. Further studies are required to definitively establish the role of topo-1 in determining irinotecan sensitivity.

Both CES1 and CES2 are expressed in hepatocytes and malignant colon cells<sup>64</sup>. Since they play a major role in the production of the active metabolite, it would be expected that higher levels of these enzymes in tumour tissue would correlate with an improved outcome. An in vitro study by Sanghani et al have shown that CES2 levels in colon cancer cell lines are indeed associated with a greater ability to result in the active metabolite of irinotecan<sup>65</sup>. However, larger in vivo studies are lacking and are therefore required to definitively demonstrate a positive correlation.

### Oxaliplatin

Oxaliplatin is a third generation platinum compound which is notable for its anti-tumour activity in colorectal cancers and its synergistic action with other chemotherapeutic agents such as irinotecan and 5-FU<sup>66</sup>. Although the main mechanism by which oxaliplatin mediates its cytotoxic effects is via the formation of DNA adducts, it also combines non enzymatically with glutathione, methionine and cysteine. The formation of DNA adducts act as apoptotic triggers and result in cell death<sup>67</sup>.

Intracellular levels of oxaliplatin are determined by the relative rates of uptake and efflux. Various uptake and efflux transporters have been identified such as organic cation transporters (OCT), copper efflux transporters and P-type ATPases ATP7A and ATP7B. These transporters may

play a role in determining the sensitivity to oxaliplatin<sup>68, 69</sup>.

The MMR system, despite its role in DNA repair, does not appear to play much of a role in determining the response to oxaliplatin. Rather, a different pathway known as the Nucleotide Excision Repair (NER) pathway is what is involved in the excision and repair of DNA-platinum adducts<sup>70</sup>. The NER pathway consists of the Xeroderma Pigmentosum group of genes (XP-A to G), ERCC1, RPA, RAD23A and RAD23B. The products of these genes work in conjunction with each other and recognise distortions in the DNA helix and subsequently excise the DNA lesion along with a few nucleotides either side of it. The gap is then filled in by a polymerase enzyme using the unbroken strand as a template<sup>71, 72</sup>.

On a theoretical basis alone, one would expect a high sensitivity to oxaliplatin if there is a deficiency in the NER pathway. Studies have been done looking at the levels of ERCC1 and oxaliplatin responsiveness which indeed do suggest this relation<sup>73, 74</sup>. A low ERCC1 gene expression has been associated with a better overall survival in patients with late stage colorectal cancer treated with oxaliplatin based regimens<sup>75</sup>. However, in a phase III trial, ERCC1 expression levels did not have any prognostic value in patients treated with capecitabine and oxaliplatin<sup>76</sup>. The contradictory results suggest the need for further research in the use of ERCC1 levels as a prognostic indicator.

## Monoclonal Antibodies

Monoclonal antibodies target specific molecules in specific carcinogenic pathways. The issue of predictive biomarkers is particularly important when it comes to monoclonal antibody therapies as these are very expensive and used only in advanced or metastatic cancer. The two pathways targeted by monoclonal antibody therapies in clinical use are the vascular endothelial growth factor (VEGF) pathway and the EGF pathway.

VEGF, of which there are types A to E, is a potent pro-angiogenic factor and its importance is highlighted by the fact that neoangiogenesis is required for the survival and metastasis of all solid tumours beyond a certain size<sup>77, 78</sup>. VEGF binds to specific

receptors which results in receptor dimerisation and subsequent activation of intracellular signalling pathways which also inhibit apoptosis<sup>79, 80</sup>. In addition to their role in angiogenesis, VEGF expressed on the surface of colorectal tumours also promotes the degradation of the extracellular matrix and vascular permeability which are both characteristic of advanced disease and poor prognosis<sup>81</sup>.

Bevacizumab, which is a monoclonal antibody targeted against VEGF-A has been shown to be more effective when used in conjunction with another cytotoxic agent and its use has been approved in the United States as first line treatment of metastatic colorectal cancer. The improvement noted when it is used in combination has been hypothesised to be due to the destruction of the peripheral vasculature of the tumour resulting in the remaining vasculature becoming more organised. This would lead to an improved delivery of the cytotoxic agent used in combination<sup>82</sup>.

Larger studies need to be performed to identify and validate predictive biomarkers for bevacizumab. In a study of 40 patients with metastatic cancer, Ronzoni et al demonstrated a significant correlation between the levels of total and resting circulating endothelial cells (tcec, rcec) and the antitumor efficacy of bevacizumab<sup>83</sup>. They therefore suggest that the tcec and rcec levels can be used as non invasive predictive biomarkers. A larger study by Simkens et al consisting of 473 patients failed to demonstrate any such correlation<sup>84</sup>. Although the reason for this discrepancy may be due to the different techniques and a lack of standardisation, further studies would be required to conclusively determine the clinical use of circulating endothelial cell levels.

Cetuximab and panitumab are two different monoclonal antibodies targeted at the EGF receptor (EGFR). These agents have been demonstrated to be effective either as part of a combination therapy regimen or as single agents. The observation that these agents are only effective against a minority of patients with metastatic disease highlighted the need for predictive biomarkers<sup>85</sup>. Large randomised studies have definitively established KRAS mutations as a predictor of poor response<sup>86</sup>. Prior to these results, such a correlation had already been indicated in several

smaller studies<sup>87, 88</sup>. The biological mechanism for this is evident as KRAS is a component of the EGF pathway. The common mutations that occur in the KRAS gene that are predictive of a poor response occur in codons 12 and 13<sup>89</sup>. In the United States, candidates for anti-EGFR therapy undergo KRAS mutations in codons 12 and 13 and are commenced on the therapy only if they are found to be negative.

Despite the biological rationale, only a small proportion of patients with an unmutated KRAS gene respond to anti-EGFR therapy indicating that there could be other predictive biomarkers<sup>90</sup>. Recent research suggests that mutations in codons 61 and 146 are also indicative of a poor response<sup>91</sup>. The analysis of BRAF mutations has also attracted attention as potential biomarkers. BRAF is the immediate downstream mediator of KRAS and the V600E mutation occurs in the BRAF gene mutually exclusive of mutations in KRAS<sup>31</sup>. Current research, although limited, seems to suggest that V600E mutations in BRAF imply a poor response to anti-EGFR therapy<sup>92</sup>. Further large scale studies are required to definitively establish its clinical use. However, there is an increasing usage of BRAF mutation testing in wild type KRAS patients as a means of further stratifying their response to anti-EGFR therapy.

The PI3K pathway is also activated by EGF and therefore its role as a potential predictive marker is the subject of much research. Several small studies have associated mutations in this pathway with a resistance to anti-EGFR therapy, however since these mutations can coexist with BRAF or KRAS mutations, its importance is unclear<sup>93</sup>. It is likely that PI3K mutations and loss of PTEN protein expression along with KRAS/BRAF mutations and potentially other markers in the future would form a 'set of molecular markers' with which a patient's response to anti-EGFR therapy would be able to be predicted with great accuracy. Research into the EGFR gene amplification points to a possible role in predicting response, however these studies lack standardisation and have been fraught with technical challenges. At present, it does not appear to be clinically useful<sup>94</sup>.

### The Role of Inflammation

Chronic inflammation is known to be associated

with a predilection towards malignant transformation and is evidenced in the higher incidence of colorectal cancer in patients with inflammatory bowel disease (IBD)<sup>95</sup>. The underlying mechanisms although not completely elucidated, appear to involve an aberrant host immune response to intraluminal bacteria in the presence of predisposing genetic alterations. This process involves the complex interplay of various factors such as cyclo-oxygenase 1 and 2 (COX1, COX2), NF- $\kappa$ B, TNF- $\alpha$  and toll like receptors (TLR)<sup>96</sup>. COX2 converts arachidonic acid to prostaglandins which is then acted upon by specific prostaglandin synthases to yield at least five structurally related molecules one of which, called PGE<sub>2</sub>, plays a pivotal role in carcinogenesis<sup>97</sup>.

COX has also been demonstrated to promote angiogenesis by activating angiogenic factors such as vascular endothelial growth factor (VEGF) via the action of PGE<sub>2</sub>. In fact, clinical studies have demonstrated reduced mortality from colorectal cancer with aspirin use and this benefit seems to be stronger with prolonged use. The benefit seems to be limited to cases of sporadic cancer only and not colorectal cancers of a hereditary aetiology such as those with FAP or Lynch syndrome<sup>98, 99</sup>.

Recent research by Liao et al has demonstrated a better prognosis for colorectal cancer with aspirin use in patients with mutations in PIK3CA<sup>100</sup>. This research is noteworthy as it identifies and suggests the use of somatic PIK3CA mutations as a biomarker to predict the clinical response of patients to aspirin therapy. A subsequent systematic review and meta analysis by Paleari et al of published studies suggested similar results although they do acknowledge that the low number of studies addressing this issue does mean that it is not yet possible to draw definitive conclusions and therefore further studies are warranted<sup>101</sup>.

### The Molecular Pathological Epidemiology Approach

The new field of molecular pathological epidemiology is leading to a paradigm shift that not only applies to colorectal cancer but also to

various other malignant and benign pathologies.

The fundamental premise of this approach is the unique disease principle which posits that each patient's pathology results from the interaction of heterogeneous biological and environmental factors that include genetic mutations, inter cellular communication, microbial presence and exposures derived from the patient's lifestyle and environment<sup>102</sup>.

This is an interdisciplinary field and draws on subjects such as molecular biology, epidemiology, statistics and bio informatics. The driving force behind the natural evolution of this discipline has been the desire to develop personalised medicine. One of the noteworthy successes of this approach in colorectal cancer had been the identification of PIK3CA mutations as a potential biomarker to determine a patient's response to aspirin<sup>103</sup>. More recently, attempts have been made to expand the field to incorporate disciplines from the social sciences such as economics, psychology and sociology<sup>104</sup>.

Despite the logical appeal to this approach, there are challenges ahead in the form of social, economic and healthcare disparities. How such disparities can be effectively incorporated into a single theory to produce a workable model should remain the focus of researchers as such a theory would then be best suited to address not only diseases such as colorectal cancer but also other pathologies that would in due course become widespread around the world.

## Conclusion

The last few decades have led to a considerable understanding of the underlying molecular processes of malignant transformation in colorectal tissue. Our understanding has come a long way from the initial adenoma-carcinoma model and it is possible that in the future, the details of new carcinogenic pathways would be elucidated. All this would aid towards the development of personalised medicine and new therapeutic modalities as more molecular targets are identified. The identification of responders to specific therapies would not only result in lower costs, but would also be able to minimise the exposure of the

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