The role of molecular markers in the adjuvant treatment of colorectal cancer

Wendy L. Allen and Patrick G. Johnston

Drug Resistance Group, Centre for Cancer Research and Cell Biology, Queen' s University Belfast, University Floor, Belfast City Hospital, Lisburn Road, Belfast, BT9 7AB, Northern Ireland, United Kingdom

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the Western world. Approximately 75% of patients with colorectal cancer present with localised disease, however, despite curative surgery, around 40% of patients still experience disease relapse leading to morbidity and eventual mortality. The use of adjuvant chemotherapy eliminates microscopic disease, with the hope of preventing recurrent disease.

The most active drug in colorectal cancer, the anti-metabolite 5-fluorouracil (5-FU), was developed more than 40 years ago. 5-FU exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA (Fig. 1). In the last 5 years the median survival for patients with metastatic colorectal cancer has nearly doubled from 12 months to 22 months and new agents in late phase clinical trails may soon extend this survival benefit further. In the metastatic disease setting, single-agent 5-FU produced response rates of only 10–20% [1]. However, combination of 5-FU with new classes of drugs such as oxaliplatin and irinotecan, has significantly improved response rates to the 40–50% range in patients with metastatic colorectal cancer [2,3].

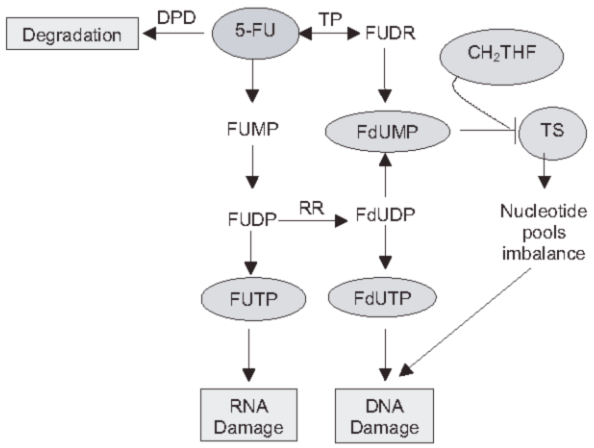

Fig. 1. Mechanism of action of 5-FU. 5-FU: 5-fluorouracil; CH2THF: 5,10-methylene-tetrahydrofolate; DPD: dihydropyrimidine dehydrogenase; FdUDP: 5′-fluoro-2′-deoxyuridine-5′-diphosphate; FdUMP: 5′-fluoro-2′-deoxyuridine-5′-monophosphate; FUDR: 2′-deoxy-5′-fluorouridine; FUMP: 5′-fluorouridine-5′-monophosphate; FUTP: 5′-fluorouridine-5′-triphosphate; RR: ribonucleotide reductase; TP: thymidine phosphorylase; TS: thymidylate synthase.

In patients with resectable stage III colorectal cancer, adjuvant therapy has been demonstrated to improve disease-free survival (DFS) and overall survival (OS) by 35% and 22%, respectively. However, the role of adjuvant therapy in stage II colorectal cancer remains controversial. The 5-year survival for patients with stage II colorectal cancer is 75%, demonstrating that the majority of patients are cured by surgery alone. A recent meta-analysis of prospective randomised clinical trials of adjuvant therapy has not shown a significant survival benefit in these patients. Approximately, 40% of these patients will develop recurrent disease within their lifetime; hence there is a need to identify which of these patients would benefit form adjuvant therapy. The identification of patients at high risk of relapse has traditionally depended on pathological features. However, these have been too inaccurate to provide detailed predictions of patient outcome. Recent advances in our understanding of the molecular biology of colorectal cancer have lead to the identification of other potential prognostic and predictive factors (Table 1).

Current molecular markers for 5-FU-based therapy

Microsatellite instability (MSI)

Genetic instability has been recognised as a central element in the genesis of malignant lesions, resulting in clonal evolution of genetic events acquired in the

Table 1
Currently studied molecular markers for 5-FU-based therapy[a]

Marker	Results	Reference(s)
TS	High TS correlates with poor survival	[4–7]
	No prognostic role for TS	[8]
DPD	Low DPD levels correlate with increased survival	[9,10]
	No prognostic role for DPD	[11]
p53	*p53* over-expression correlates with poor survival	[12–16]
	p53 displays no independent prognostic role	[17,4,18–20]
DCC	*DCC*+ tumours are associated with increased overall survival and disease-free recurrence	[21]
	LOH displays no prognostic role in colorectal cancer	[17]
MSI	MSI-H correlates with increased survival	[22,23]
	MSI+ displays no prognostic significance	[24]
K-ras	*K-ras* mutations are associated with an increased risk of death	[25–28]
	K-ras mutations display no prognostic significance	[29–31]

[a] 5-FU: 5-fluorouracil; DPD: dihydropyrimidine dehydrogenase; TS: thymidylate synthase; DCC: deleted in colon cancer; MSI: microsatellite instability; LOH: loss of heterozygosity.

course of tumour progression [32]. MSI is common to many forms of cancer and is found in half to two-thirds of sporadic colon cancers. MSI is caused by mutations in the mismatch repair (MMR) genes such as *hMSH2*, *hMLH1* and *hMSH6*, which results in failure of the DNA mismatch repair system to correct errors that occur during replication [33]. Inactivation of mismatch repair genes causes replication errors, termed microsatellite instability (MSI), or the mutator phenotype. MSI is defined as variations in the numbers of repetitive di-, tri- and tetra-nucleotide repeats (called microsatellites) found in DNA. The DNA base-base mismatch repair system (MMR genes) have been shown to account for microsatellite instability in nearly all cases of hereditary non-polyposis colorectal cancer (HNPCC)-associated replication error (RER) cancer [34].

The MMR genes (*hMLH1*, *hMSH2* and *hMSH6*) are responsible for HNPCC and patients with HNPCC inherit a single defective allele of a MMR gene and require an additional somatic mutation to inactivate the relevant gene. DNA mismatch repair is found in 10–15% of all colorectal cancers and accounts for greater than 90% of HNPCC [34]. The majority of sporadic MSI tumours are due to transcriptional silencing of the *hMLH1* gene, which is caused by promoter methylation [35]. The cluster of MMR genes acts as a unit and when mutations occur in any of these genes the mutation rate increases 100–1000-fold.

Various studies have investigated the prognostic role of MSI in stage II colorectal cancer (Table 2). The studies have confirmed a consistent and independent association between MSI-high (MSI-H) phenotype and improved survival in patients with stage II and stage III colorectal cancer. These studies have noted that the 5-year survival rate for all patients with MSI-H tumours was significantly better than that of patients with microsatellite stable (MSS) tumours (76% versus 54%) [22]. Furthermore, Lim and colleagues demonstrated that patients with MSI tumours exhibited better recurrence-free survival (RFS) compared with those with MSS tumours, and the use of adjuvant chemotherapy did not benefit these patients [36]. A conflicting result was confirmed by Wang and colleagues, in which 396 stage II colorectal cancer patients were assessed for MSI. They found that 23% of proximal tumours were MSI+ and demonstrated that MSI was not a prognostic indicator in this cohort of stage II colorectal cancer patients [24].

In vitro studies suggest that MSI influences cell survival after 5-FU treatment. Meyers and colleagues demonstrated that restoration of *hMLH1* activity in MMR-deficient HCT116 cells increased their sensitivity to 5-FU [42]. However, *in vivo* studies have shown the MSI-positive phenotype has been associated with increased survival in patients with stage III colorectal cancer who receive adjuvant 5-FU-based therapy. Furthermore, several other studies have demonstrated that there is a survival advantage and a less aggressive phenotype in patients who have a high frequency of MSI who receive adjuvant 5-FU-based chemotherapy. It is believed that tumours with MSI+ phenotype appear to be more chemosensitive [43]. Two independent studies have demonstrated that there is no survival advantage for MSI-H patients who are treated with adjuvant 5-FU, compared with no adjuvant

Table 2
Studies that have investigated the prognostic role of MSI in stage II and III colorectal cancer[a]

Observation	Reference(s)
70% of colorectal cancer have lost a portion of 17p or 18q or both	Kern et al. 1989 [37]
LOH at the *p53* allele mediated conversion to the highest grade of dysphasia	Miyaki et al. 2002 [38]
Loss of 18q correlated with poorer prognosis in stage II and III colorectal cancer, while retention of both 18q alleles resulted in 93% 5-year survival compared with 54% in patients who have lost one allele.	Lanza et al. 1998 [39]
Retention of both 18q alleles result in a more favourable outcome after adjuvant 5-FU-based therapy.	Watanabe et al. 2001 [17]
LOH at 18q had a prognostic role and was associated with worse prognosis in stage II and III colorectal cancer.	Font et al. 2001 [25]; Shibata et al. 1996 [40]; Jen et al. 1994 [41]

[a] 5-FU: 5-fluorouracil; MSI: microsatellite instability; LOH: loss of heterozygosity.

therapy. However, 5-FU-based adjuvant chemotherapy benefited those patients who were MSS or low-frequency MSI [44,45]. The discrepancy between the *in vitro* and *in vivo* findings may be due to other intrinsic biological differences between MSI-positive and MSI-negative tumours. For example, most MSI-positive tumours have wild-type *p53*, and most MSI-negative tumours are mutant for *p53*. The major limitation to the analysis is the low frequency of this phenomenon, as it usually only occurs at a rate of 17%, which makes it difficult to quantify whether there is a survival advantage.

MSI (replication error; RER) is common to many sporadic forms of cancer and is found in half to two-thirds of colon cancers [36]. There also appears to be a link between MSI and transforming growth factor-beta (TGFβ) RII mutation. A recent study demonstrated that 61% of stage III colorectal cancer patients with MSI-high tumours also had a TGFβ RII mutation [17]. This study also demonstrated that patients who had MSI-high tumours and TGFβ RII mutations had a 5-year survival of 74% following adjuvant 5-FU therapy compared with 46% in patients with MSI-high tumours without TGFβ RII mutations [17].

TGFβ is a multifunctional polypeptide that regulates a number of cellular processes including growth, differentiation, deposition of the extracellular matrix (ECM), immunosuppression, embryogenesis, repair of soft and hard tissue and regulation of haematopoiesis [46–49]. TGFβ exerts its effects through binding to specific cell surface proteins. These receptors have been termed type I (RI), type II (RII) and type III (RIII) [50–52]. Type RI and RII are glycoproteins, while type RIII is a proteoglycan [53]. It has been demonstrated that RI and RII are serine/threonine kinases, which are indispensable for TGFβ signalling [54,55]. Furthermore, cells that lose the ability to express or respond to TGFβ, are more likely to exhibit uncontrolled growth and over time become tumourigenic. RII, but not RI can individually bind TGFβ. Binding of RII to TGFβ induces the assembly of the RII–RI heterodimer and transphosphorylation by RII of RI [55–57].

Furthermore, a study has demonstrated that RII is inactivated by BAT-RII frameshift mutation in 90% of RER colon cancers [58]. TGFβ RII is a downstream mutation target resulting in the disruption of growth regulation of HNPCC in both cell lines and tissues [59]. It has also been observed that re-expression of RII in HCT116 colon cancer cells which are resistant to TGFβ leads to a reversal of tumourigenicity both *in vitro* and *in vivo* [60,61]. This suggests that TGFβ plays a significant role in the suppression of malignancy and mutations that inactivate RII are actively selected for in colon cancer and do not randomly accumulate [54,58]. Taken together the data suggests that the TGFβ pathway provides important targets for therapeutic intervention as well as for chemoprevention strategies.

Loss of heterozygosity (LOH)

Studies on allelic losses in patients with familial adenomatous polyposis (FAP) and non-familial adenomatous polyposis (non-FAP) established that 5q LOH occurred in a substantial proportion of adenomas, whereas 17p and 18q LOH were a later event [62,63]. This served as the model for colorectal carcinogenesis proposed by Fearon and Vogelstein [64]. The emphasis of the model was on the accumulation of mutations rather than the order in which they arise. It is interesting to note that no allelic losses have been observed in normal colonic epithelium surrounding colorectal neoplasm [65,66]. Consistent allelic loss

Table 3
Studies that have investigated the prognostic role of LOH in stage II and III colorectal cancer[a]

Observation	Reference(s)
MSI-H phenotype correlates with improved survival in stage II and III colorectal cancer patients. The 5-year survival for MSI-H tumours was significantly better than that of patients with MSS (76% versus 54%, respectively).	Gryfe et al. 2000 [8]
MSI tumours exhibited better recurrence-free survival compared with those with MSS tumours. Furthermore, the use of adjuvant therapy did not benefit MSI patients.	Lim et al. 2004 [36]
MSI was not shown to be a prognostic indicator in stage II colorectal cancer patients.	Wang et al. 2003 [24]
MSI-H has been associated with increased survival and a less aggressive phenotype in patients with stage III colorectal cancer who receive adjuvant 5-FU-based therapy.	Elsaleh et al. 2001 [43]
Studies have demonstrated that there is no survival advantage for MSI-H patients who receive adjuvant 5-FU, compared with no adjuvant therapy. However, 5-FU-based adjuvant therapy benefited those patients who were MSS or low frequency MSI.	Carethers et al. 2004 [44]; Ribic et al. 2003 [45]
There is a link between MSI and TGF-b RII mutation, 61% of stage III colorectal cancer patients with MSI-H tumours also had TGF-b RII mutation. Patients who had TGF-b RII mutation and MSI-H phenotype had a 5-year survival of 74% following adjuvant 5-FU therapy compared with 46% in patients with MSI-H tumours without TGF-b RII mutation.	Watanabe et al. 2001 [17]

[a] 5-FU: 5-fluorouracil; MSI: microsatellite instability; MSS: microsatellite stable; LOH: loss of heterozygosity.

of a specific chromosomal region in a particular cancer type is generally taken as evidence for the location of a putative tumour suppressor gene in that region. Many studies have now demonstrated that loss of 5q (adenomatous polyposis coli (*APC*) gene) occurs at the transition phase of normal to benign adenoma, whereas loss of 17p (*p53* gene) or 18q (deleted in colon cancer (*DCC*) gene) occurs at a later stage transition of adenoma to carcinoma [65]. However, additional genetic lesions may participate in the growth of adenomas, including point mutations in other genes such as the *K-ras* gene [67,68]. This allelic picture has demonstrated an essential role for these genes in tumour progression.

It has been reported that 70% of colorectal cancers have lost a portion of chromosome 17p or 18q or both [37]. The prognostic role of LOH has been investigated in several studies (Table 3). The 17p chromosome contains *p53*, which is an important tumour suppressor, and is mutated in 40–60% of colorectal cancers. Miyaki and colleagues reported that LOH at the *p53* allele mediated conversion to the highest grade of dysplasia [38]. It has also been demonstrated that loss of 18q is associated with a poorer prognosis in stage II and III tumours [69]. Furthermore, retention of both 18q alleles in patients with stage II colorectal cancer results in a 93% 5-year survival compared with 54% in patients who have lost one allele [69]. Watanabe and colleagues showed that patients with stage III colorectal cancer who retained both 18q alleles had a more favourable outcome after adjuvant 5-FU-based chemotherapy [17]. The 18q chromosome segment contains three candidate tumour suppressor genes, *DCC*, *smad 2* and *smad 4* [70]. Smad proteins are transcription factors involved in the TGFβ pathway [71]. They are involved in signalling from the TGFβ receptor and have been shown to regulate transcription of target genes including *c-myc* [72,73]. In addition, 50% of colorectal cancers have lost a portion of chromosome 8p and re-introduction of 8p into colon cancer cells has been shown to decrease tumourigenicity and invasiveness. Moreover, loss of 8p is associated with a worse prognosis. The identity and function of the genes contained within this region of 8p have yet to be identified [74].

p53 tumour suppressor gene

p53 has been described as the universal sensor of genotoxic stress [75]. *p53* status has been studied as a prognostic factor, and more recently as a predictor of response to cancer chemotherapy. Two methods have been employed to assess *p53* status; DNA analysis, to detect a variety of mutations, and immunohistochemistry, to detect abnormal nuclear accumulation of the *p53* protein. Immunohistochemically detected *p53* over-expression has been used as a surrogate marker for *p53* mutation; however, this assumption is not always correct. Many genetic changes do not

result in *p53* over-expression, and positive immunohistochemical analysis of *p53* may occur in the absence of *p53* mutation. A number of studies have demonstrated that *p53* over-expression correlated with poor survival when measured by immunohistochemistry in stage II disease [12–14]. However, a number of other studies assessed *p53* by both immunohistochemistry and polymerase chain reaction-single conformation polymorphism (PCR-SSCP) and found that *p53* did not display any independent prognostic role in early stage colorectal cancer [4,18,19,76].

A recent study by Tang and colleagues [77] found that *p53* mutation was associated with a poorer prognosis in stage II and III colorectal cancer patients who received surgery alone, whereas *p53* was not a prognostic factor among those patients who had received 5-FU-based adjuvant chemotherapy. Yet, Ahnen and colleagues [15] found that patients with stage III colorectal cancer whose tumours over-expressed *p53* did not derive significant survival benefit from adjuvant 5-FU-based treatment, whereas those without *p53* over-expression did. These conflicting findings may be due to the techniques used as well as the various antibodies used to detect *p53*. Due to these conflicting results the use of *p53* as a predictive marker for 5-FU remains controversial.

Deleted in colon cancer (DCC)

DCC encodes a transmembrane protein with high homology to cell adhesion molecules. A decrease in *DCC* expression may lead to altered adhesion and contribute to enhanced tumour growth and metastatic spread of colorectal cancer [78]. It has been demonstrated that *DCC* abnormalities occur in around 40–50% of all patients with colorectal cancer. *DCC* status is currently measured by molecular genetic assays or immunohistochemistry. *DCC* staining has been reported to be an ' all or nothing' event, and the immunohistochemical assessment would normally be scored as the presence of any cytoplasmic reactivity or negative staining.

A number of studies have investigated the prognostic role of *DCC* in early stage colorectal cancer (stage II and III) (Table 1). *DCC* was measured either by immunohistochemistry or molecular analysis using 3–10 microsatellite markers. The studies found that LOH had a prognostic role in stage II and III colorectal cancer and was associated with worse prognosis [25, 13,41,39]. One study by Watanabe and colleagues found that LOH did not have a prognostic role in stage II colorectal cancer; however, this group of 121 patients had received adjuvant chemotherapy [17]. All of the above investigations were retrospective and a consistent and significant prognostic role for *DCC* was found in 4/5 studies. A study by Gal and colleagues investigated the prognostic role of *DCC* in early stage colorectal cancer with and without adjuvant chemotherapy. *DCC* status was measured by immunohistochemistry from paraffin-embedded tissue and the primary endpoint of the study was RFS. They showed that OS and RFS were higher in *DCC*+ (83%) tumours than *DCC*– (54%). Furthermore, at 5 years, all *DCC*+ patients who received adjuvant chemotherapy were alive with no evidence of disease. However, only 54% of patients who did not receive adjuvant treatment were alive at 5 years ($P=0.0001$) [21].

Thymidylate synthase

A number of *in vitro* and *in vivo* studies have demonstrated that TS is a prognostic and predictive marker in fluoropyrimidine-based chemotherapy (Table 1). The primary mechanism of resistance to fluoropyrimidines is an increase in TS expression [79]. Furthermore, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical studies have demonstrated that patients with low tumoural TS expression have higher response rates to 5-FU than those with higher levels of TS expression [80,81,5]. Several *in vitro* and *in vivo* studies have demonstrated that treatment of cancer cells with 5-FU acutely up-regulates TS synthesis [5,82,83]. The molecular basis for this acute up-regulation appears to be the inhibition of an auto-regulatory feedback loop, in which ligand-free TS binds to, and inhibits, its own translation. However, when FdUMP stably binds TS, it is no longer able to bind to its mRNA, resulting in increased TS protein expression [82]. The role of acute TS induction in 5-FU resistance was investigated in MDA435 breast cancer cells in which expression of a TS trans-gene was controlled by a tetracycline-regulated promoter. This study demonstrated that inducible expression of TS increased the IC50 dose of 5-FU by ~3-fold, suggesting that acute induction of TS is a factor in determining sensitivity to 5-FU [84]. There is, however, a subset of patients who have low TS expression who do not respond to 5-FU treatment. These non-responders may have other mechanisms of resistance, such as high levels of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase. This highlights the need for and importance of measuring multiple markers of resistance to fluoropyrimidines.

TS gene polymorphisms also have the potential to predict clinical outcome and toxicity; therefore it may be an important factor to consider when

deciding on patient treatment. The TS gene promoter is polymorphic and usually has two (TSER*2) or three (TSER*3) 28-base pair tandem repeat sequences [85]. These tandem repeats may affect transcriptional and/or translational efficiency of the TS gene. It has been demonstrated that TSER*3/TSER*3 homozygous patients are less likely to respond to 5-FU than TSER*2/TSER*2 homozygous, or TSER*2/TSER*3 heterozygous patients [86]. This may be due to the fact that TS promoters with the TSER*3 sequence have been reported to generate ~3-fold higher mRNA than those with the TSER*2 sequence [87], and therefore patients with this genotype may express higher levels of TS and be less responsive to 5-FU. The identification of the polymorphism provides a means of selecting patients who are likely to respond to 5-FU-based chemotherapy and also identifies patients who will experience increased toxicity. Recently a six base-pair polymorphic deletion in the 3′ untranslated region (UTR) of the TS gene has been identified; however, it is unclear at present whether this polymorphic change influences TS gene expression or mRNA stability.

Several clinical studies have investigated the role of TS as a predictive and prognostic marker in stage II and III colorectal cancer. A number of these studies have demonstrated that high levels of TS, as measured by immunohistochemistry (IHC), predict for worse survival in stage II and III patients [5–7]. Allegra and colleagues carried out a further study of patients with stage II and III colorectal cancer who were treated with either surgery alone or surgery and adjuvant 5-FU. They stained for three markers, *p53*, TS and *Ki67*, by immunohistochemistry (IHC). They found that both high TS and positive *p53* were associated with a worse outcome. They also demonstrated that high *Ki67* correlated with improved outcome when compared with low *Ki67*. However, they did not identify any interaction with treatment for any of the markers tested [4]. An intergroup combined analysis was carried out to assess the prognostic significance of TS expression in stage II and III colorectal cancer and further evaluate the role of TS as a predictor of response to adjuvant 5-FU-based therapy. The results established that TS was a prognostic marker for OS in stage III colorectal cancer, however, TS was not a predictor of response in the adjuvant setting [88].

Dihydropyrimidine dehydrogenase (DPD)

Dihydropyrimidine dehydrogenase (DPD) catalyses the rate-limiting step in the catabolism of fluoropyrimidines. More than 80% of 5-FU administered is degraded in the liver by DPD. Thus, DPD limits the bioavailability of 5-FU [89]. DPD is very active in the liver, but is also found in other tissues. Of note DPD levels are higher in normal mucosa as compared with levels in tumour tissue [90]. DPD has variable activity in human tumours, and tumoural DPD has been reported to be an important determinant of response to 5-FU both *in vitro* [91] and *in vivo* in the metastatic setting [92]. The differences in variation among patients who receive 5-FU must be due to genetic differences in the activity of the DPD gene. Patients deficient in DPD experience profound systemic toxicity when treated with 5-FU, which may prove fatal [93], as a result of its decreased catabolism of 5-FU resulting in higher systemic levels of 5-FU in patients.

The DPD gene is also polymorphic and studies have identified a common G → A point mutation in the invariant GT splice donor site flanking exon 14 (IVS14 + 1G>A), which results in the loss of exon 14 and a truncated protein product. The mutated DPD activity is severely compromised, especially in individuals homozygous for this mutation where the enzyme activity is virtually absent. Patients who are homozygous for this mutation are at higher risk from toxicity [93–95].

Several studies have investigated the association of DPD levels with RFS in patients with stage II and III colorectal cancer (Table 1). An initial study by Kornmann and colleagues examined whether there was an association between DPD and TS mRNA levels and disease-free recurrence in patients receiving adjuvant 5-FU-based chemotherapy. They measured DPD and TS mRNA levels by RT-PCR in a retrospective analysis of the primary tumour. They demonstrated that patients with a recurrence who had low TS had a median RFS of 18 months and those with high TS had a median RFS of 11 months. However, they found no significant difference in median RFS in patients with low and high DPD mRNA levels [11]. They carried out a further analysis on paraffin-embedded primary samples from 309 patients treated with adjuvant 5-FU. They found that in patients receiving 5-FU-based adjuvant therapy, those with high TS survived longer than those with low TS and in each subgroup those with low DPD survived longer than those with high DPD levels as measured by real-time PCR [9]. Tsuji and colleagues [10] also analysed DPD enzymatic activity to evaluate the relationship between DPD expression and enzymatic activity. They analysed 53/89 tumours for DPD activity and found that DPD expression significantly correlated with DPD enzymatic activity in that small group. Furthermore, they demonstrated that patients with low DPD expression had longer

disease-free recurrence than those with high levels of DPD according to univariate analysis ($P=0.026$). Further multivariate analysis showed that low DPD expression was significantly and independently associated with better survival [10]. Tsuji et al. carried out a further study to analyse the prognostic significance of tumour DPD expression in curatively resected colorectal cancer patients who received adjuvant 5-FU or not. They found that high DPD expression in the surgery alone group was associated with a better survival ($P=0.02$), while high tumour DPD expression in the adjuvant chemotherapy group was associated with poor survival ($P=0.03$). These results would suggest that estimating tumour DPD expression will provide useful information and help assess which treatment, if any, stage II and III colorectal cancer patients should be given after curative surgery [90].

K-ras

K-ras controls growth and differentiation by transduction of extracellular signals. *K-ras* belongs to the *RAS* family (*K-ras*, *H-ras* and *N-ras*) of cellular proto-oncogenes. It has been established that approximately 30% of colorectal cancer have a mutation in the *K-ras* gene. *K-ras* abnormalities have been identified in codons 12, 13, 31 and 61, with more than 80% of cases in codons 12 and 13. Studies have shown that, in stage I and II colorectal cancer, *K-ras* mutations are positively associated with recurrence and poorer long-term survival [96]. These studies noted that mutations within codons 12 and 13 were linked to increased risk of nodal metastasis [97].

In 1998 the K-ras in Colorectal Cancer Collaborative Group (RASCAL) study was initiated to assess the association between *K-ras* mutation and patient outcome and tumour characteristics. The study concluded that the glycine to valine mutation on codon 12 (10% of the study) was an independent factor for increased risk of death [26]. The second RASCAL study allowed further subgroup analysis in patients with stage II and III colorectal cancer (8.6% of the study), which demonstrated that the valine mutation on codon 12 lost its prognostic role in this particular subgroup of patients [29]. The conclusions from these studies suggest that not only does the mutation lead to cancer progression, but also may lead to a tumour with more aggressive biological behaviour.

New technologies

The advent of high throughput methodologies, such as microarray-based gene expression profiling, proteomic profiling, comparative genomic hybridisation (CGH) analysis and the newly developed metabolomics enables tumour samples to be profiled on a global scale. This has major implications for the diagnostic capability and prognostic classification of tumours, where we can ultimately predict response of each individual tumour to chemotherapy. Whereas microarray expression profiling of colorectal cancer has been performed, no comparable protein analysis has been reported. It would be of great interest to identify a group of consistently changing proteins whose functions may reveal insight into critical events in disease progression and which may hold value as potential therapeutic targets [98].

CGH allows the entire genome to be scanned, in a single step, for copy number aberrations in chromosomal material [99]. CGH identifies specific chromosomal regions that are consistently gained or lost at a high frequency within colorectal cancer and has demonstrated an increase in the genetic grade of a tumour with disease progression [100,101]. Rooney and colleagues carried out CGH analysis in 29 stage III colorectal cancer samples to assess any genomic aberrations and the overall level of chromosome instability. Also, eight colorectal cancer cell lines were used to evaluate their usefulness as model systems for colorectal cancer genomics. They found a high level of variation between both the number and type of genetic aberration detected in the 29 colorectal cancer samples and found that almost every chromosomal arm was detected as changed in at least one genome that was assessed. They were unable to demonstrate an association between any specific chromosomal aberration and patient survival; however, they did find a link between the number of aberrations and survival, with greater than two aberrations resulting in a better survival benefit [102].

The most frequently used genome wide approach is DNA microarray profiling, this has been utilised to either diagnose/stage cancer or to predict outcome, e.g. recurrence. Mariadason and colleagues carried out gene expression profiling on 30 colorectal cancer cell lines and correlated this with 5-FU sensitivity using three different assays of response. They were able to identify panels of genes that correlated with 5-FU sensitivity and in addition used leave-one-out cross-validation to demonstrate that these genes were predictive for 5-FU response. They noted that this gene set had a greater power to predict response than four ' classical' determinants of 5-FU response: TS, TP53 and MMR status. In a similar study they repeated the correlation analysis for sensitivity to irinotecan and this generated a second gene set that showed great

predictive power for sensitivity to irinotecan [103]. Furthermore, the group re-analysed the same microarray data in order to predict response to oxaliplatin and demonstrated that the oxaliplatin marker set again, showed great predictive power [104].

Several groups have used gene expression profiling for prognosis in several different tumour types including, leukaemia [105], breast [106], early stage lung adenocarcinoma [107], mesothelioma [108] and inflammatory breast cancer [109]. A number of expression studies in colorectal cancer have been carried out [110,111]. A study by Bertucci and colleagues used 50 cancerous and non-cancerous tissues and demonstrated that the gene expression profile was able to separate stage IV from stage I–III disease in an unsupervised manner using global hierarchical clustering. They further demonstrated that the segregations were improved using supervised clustering. From their clustering they were able to predict the likelihood of metastasis, and suggested that this group of patients may benefit from a more aggressive treatment regime [111].

Motoori and colleagues carried out a study to establish whether they could predict recurrence in patients with advanced gastric cancer after curative resection. They carried out gene expression profiling on 60 advanced gastric patients using a high throughput quantitative RT-PCR technique. From their ' training set' of 40 samples, they identified 29 genes, which could distinguish between good and poor response. They used the remaining 20 samples as a test set and demonstrated that the genes had a predictive accuracy of 75%. They further found that the predictive accuracy was 81.8% in patients with early stage disease, 88.89% in patients who had a small tumour and 84.6% in patients with less developed lymph node metastasis [N (0,1)] [112]. The predictive accuracy could possibly be increased in this study if the number of samples in the ' training set' was increased further.

An interesting study was carried out by Clarke and colleagues [113], who compared the gene expression profiles pre- and during treatment in patients who had rectal cancer. The patients were receiving 5-FU in combination with mitomycin C and fractionated radiation. The investigators wished to assess whether it was possible to use microarray profiling to detect altered gene expression in solid tumours in response to treatment. They found that altered gene expression profiles were apparent in all patients after treatment, with a major cluster of genes involved in synthesis and metabolism down-regulated after treatment. They also found that there was down-regulation in genes involved in RNA and protein synthesis and processing and in cellular metabolism. Importantly, one-third of the genes were found to be positively regulated by *c-Myc*. They also noted that *c-Myc* was down-regulated following treatment. This is an interesting paper as it highlights the possibilities of microarray profiling and demonstrates that it is possible to detect global gene expression changes within solid tumour tissue in response to ongoing treatment [113].

Finally, Wang and colleagues [114] also used gene expression profiling to identify markers for stage II colorectal cancer. The study contained 74 patients with stage II colorectal cancer. They used two supervised class prediction approaches to select markers from the 17,616 informative genes from the microarray. Firstly, the patients were divided equally into a training set and a test set. The training set was used to select markers and build a prognostic signature, while the test set was used independently to validate the training set. This approach yielded 60 genes from 38 patients. Secondly, the patients were divided into one of two groups based on unsupervised clustering results. From that each subgroup was further divided into a training set and a test set and again were analysed to select markers. This approach yielded 23 markers from the training set, which were then analysed in the test set. The investigators then compared the predictive power of the 23-gene set and the 60-gene set and found that only the 23-gene set was predictive. The 23-gene set was then further validated in 36 independent patients and demonstrated an overall accuracy of 78% [114]. This study highlights the power of predictive marker testing but also highlights the need to carefully select the correct analysis for the purpose of the test.

A major limitation to the advancement of microarray technology as a tool for identification of predictive marker sets is the availability of fresh frozen tumour samples, and the absence of a large enough study group powered to allow the identification of a highly significant predictive marker set. Furthermore, gene expression profiling may fail to provide information regarding the functionality or likelihood of induction of critical drug response determining genes. As a result there may be a need to combine gene expression profiling with proteomic profiling to obtain a better vision of the mechanism involved in drug response/ resistance [115].

Conclusion

To date there have been significant limitations to the studies carried out on individual predictive and

prognostic markers, such as the choice of cut-off used, the statistical methods employed to assess importance of the genes and the size of the study population. There have been several discrete molecular markers identified to date that have demonstrated clinical efficacy, LOH, MSI, TGFβ RII mutation and TS. Various studies have revealed that loss of 18q is associated with poorer prognosis in patients with stage II and III colorectal cancer, while its retention is linked with increased 5-year survival and a more favourable outcome after adjuvant 5-FU therapy [17,39]. Additional studies have confirmed a consistent and independent correlation between MSI-H phenotype and increased survival in patients with stage II and III colorectal cancer [23,8]. Furthermore, there appears to be a link between MSI and TGFβ RII mutation, since patients with both MSI-H and TGFβ RII mutation had an increased survival rate compared with patients who were MSI-H without TGFβ RII mutation [17]. Finally, studies have established that high levels of TS predict for inferior survival in patients with stage II and III colorectal cancer [5–7].

In the future molecular profiling of tumours may identify individuals more likely to benefit from adjuvant therapy and tailor individual treatment in the future. The area of pharmacogenomics will ultimately lead to a more rationalised, molecular approach to cancer treatment and will undoubtedly make a huge contribution in the field of oncology. The next step in the field of pharmacogenomics will be to develop clinical trials that will assess prospectively the benefits of profiling a patient' s particular tumour, which should translate into improvements in both overall response and toxicity. If the goal of pharmacogenomics is realised, a new era of individualised treatment will become a reality, with a better overall response rate, less toxicity and enhanced survival benefit for patients with colorectal cancer.

Conflict of interest statement

None declared.

References

1 Johnston PG, Kaye S. Capecitabine: a novel agent for the treatment of solid tumors. *Anticancer Drugs* 2001, **12**, 639–646.

2 Giacchetti S, Perpoint B, Zidani R, *et al.* Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2000, **18**, 136–147.

3 Douillard JY, Cunningham D, Roth AD, *et al.* Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 2000, **355**, 1041–1047.

4 Allegra CJ, Paik S, Colangelo LH, *et al.* Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. *J Clin Oncol* 2003, **21**, 241–250.

5 Lenz HJ, Danenberg KD, Leichman CG, *et al.* p53 and thymidylate synthase expression in untreated stage II colon cancer: associations with recurrence, survival, and site. *Clin Cancer Res* 1998, **4**, 1227–1234.

6 Johnston PG, Fisher ER, Rockette HE, *et al.* The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994, **12**, 2640–2647.

7 Edler D, Kressner U, Ragnhammar P, *et al.* Immunohistochemically detected thymidylate synthase in colorectal cancer: an independent prognostic factor of survival. *Clin Cancer Res* 2000, **6**, 488–492.

8 Allegra CJ, Parr AL, Wold LE, *et al.* Investigation of the prognostic and predictive value of thymidylate synthase, p53, and Ki-67 in patients with locally advanced colon cancer. *J Clin Oncol* 2002, **20**, 1735–1743.

9 Kornmann M, Schwabe W, Sander S, *et al.* Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression levels: predictors for survival in colorectal cancer patients receiving adjuvant 5-fluorouracil. *Clin Cancer Res* 2003, **9**, 4116–4124.

10 Tsuji T, Sawai T, Takeshita H, Nakagoe T, *et al.* Tumor dihydropyrimidine dehydrogenase expression is a useful marker in adjuvant therapy with oral fluoropyrimidines after curative resection of colorectal cancer. *Cancer Chemother Pharmacol* 2004, **54**, 531–536.

11 Kornmann M, Link KH, Galuba I, *et al.* Association of time to recurrence with thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression in stage II and III colorectal cancer. *J Gastrointest Surg* 2002, **6**, 331–337.

12 Buglioni S, D' Agnano I, Vasselli S, *et al.* p53 nuclear accumulation and multiploidy are adverse prognostic factors in surgically resected stage II colorectal cancers independent of fluorouracil-based adjuvant therapy. *Am J Clin Pathol* 2001, **116**, 360–368.

13 Gervaz P, Bouzourene H, Cerottini JP, *et al.* Dukes B colorectal cancer: distinct genetic categories and clinical outcome based on proximal or distal tumor location. *Dis Colon Rectum* 2001, **44**, 364–372; discussion 372–363.

14 Bouzourene H, Gervaz P, Cerottini JP, *et al.* p53 and Ki-ras as prognostic factors for Dukes' stage B colorectal cancer.*Eur J Cancer* 2000, **36**, 1008–1015.

15 Ahnen DJ, Feigl P, Quan G, *et al.* Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. *Cancer Res* 1998, **58**, 1149–1158.

16 Liang JT, Huang KC, Cheng YM, *et al.* P53 overexpression predicts poor chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV colorectal cancers after palliative bowel resection. *Int J Cancer* 2002, **97**, 451–457.

17 Watanabe T, Wu TT, Catalano PJ, *et al.* Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med* 2001, **344**, 1196–1206.

18 Giatromanolaki A, Stathopoulos GP, Tsiobanou E, *et al.* Combined role of tumor angiogenesis, bcl-2, and p53 expression in the prognosis of patients with colorectal carcinoma. *Cancer* 1999, **86**, 1421–1430.
19 Soong R, Grieu F, Robbins P, *et al.* p53 alterations are associated with improved prognosis in distal colonic carcinomas. *Clin Cancer Res* 1997, **3**, 1405–1411.
20 Paradiso A, Simone G, Petroni S, *et al.* Thymidilate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. *Br J Cancer* 2000, **82**, 560–567.
21 Gal R, Sadikov E, Sulkes J, Klein B, Koren R. Deleted in colorectal cancer protein expression as a possible predictor of response to adjuvant chemotherapy in colorectal cancer patients. *Dis Colon Rectum* 2004, **47**, 1216–1224.
22 Gryfe R, Kim H, Hsieh ET, Aronson MD, *et al.* Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000, **342**, 69–77.
23 Lim SB, Jeong SY, Lee MR, *et al.* Prognostic significance of microsatellite instability in sporadic colorectal cancer. *Int J Colorectal Dis* 2004, **19**, 533–537.
24 Wang C, van Rijnsoever M, Grieu F, *et al.* Prognostic significance of microsatellite instability and Ki-ras mutation type in stage II colorectal cancer. *Oncology* 2003, **64**, 259–265.
25 Font A, Abad A, Monzo M, *et al.* Prognostic value of K-ras mutations and allelic imbalance on chromosome 18q in patients with resected colorectal cancer. *Dis Colon Rectum* 2001, **44**, 549–557.
26 Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter ' RASCAL' study. *J Natl Cancer Inst* 1998, **90**, 675–684.
27 Bazan V, Migliavacca M, Zanna I, *et al.* Specific codon 13 K-ras mutations are predictive of clinical outcome in colorectal cancer patients, whereas codon 12 K-ras mutations are associated with mucinous histotype. *Ann Oncol* 2002, **13**, 1438–1446.
28 Esteller M, Gonzalez S, Risques RA, *et al.* K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. *J Clin Oncol* 2001, **19**, 299–304.
29 Andreyev HJ, Norman AR, Cunningham D, *et al.* Kirsten ras mutations in patients with colorectal cancer: the ' RASCAL II' study. *Br J Cancer* 2001, **85**, 692–696.
30 Bleeker WA, Hayes VM, Karrenbeld A, *et al.* Impact of KRAS and TP53 mutations on survival in patients with left- and right-sided Dukes' C colon cancer. *Am J Gastroenterol* 2000, **95**, 2953–2957.
31 Morrin M, Kelly M, Barrett N, Delaney P. Mutations of Ki-ras and p53 genes in colorectal cancer and their prognostic significance. *Gut* 1994, **35**, 1627–1631.
32 Remvikos Y, Muleris M, Salmon RJ, Dutrillaux B. Colorectal carcinogenesis: from chromosomal evolution pathways to molecular pathogenesis. *Cancer Genet Cytogenet* 1997, **93**, 63–73.
33 Chai SM, Zeps N, Shearwood AM, *et al.* Screening for defective DNA mismatch repair in stage II and III colorectal cancer patients. *Clin Gastroenterol Hepatol* 2004, **2**, 1017–1025.
34 Liu B, Parsons R, Papadopoulos N, *et al.* Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat Med* 1996, **2**, 169–174.
35 Jacob S, Praz F. DNA mismatch repair defects: role in colorectal carcinogenesis. *Biochimie* 2002, **84**, 27–47.
36 Lim SB, Jeong SY, Lee MR, Ku JL, *et al.* Prognostic significance of microsatellite instability in sporadic colorectal cancer. *Int J Colorectal Dis* 2004, **19**, 533–537.
37 Kern SE, Fearon ER, Tersmette KW, *et al.* Clinical and pathological associations with allelic loss in colorectal carcinoma [corrected]. *JAMA* 1989, **261**, 3099–3103.
38 Miyaki M, Iijima T, Yasuno M, *et al.* High incidence of protein-truncating mutations of the p53 gene in liver metastases of colorectal carcinomas. *Oncogene* 2002, **21**, 6689–6693.
39 Lanza G, Matteuzzi M, Gafa R, *et al.* Chromosome 18q allelic loss and prognosis in stage II and III colon cancer. *Int J Cancer* 1998, **79**, 390–395.
40 Shibata D, Reale MA, Lavin P, *et al.* The DCC protein and prognosis in colorectal cancer. *N Engl J Med* 1996, **335**, 1727–1732.
41 Jen J, Kim H, Piantadosi S, *et al.* Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N Engl J Med* 1994, **331**, 213–221.
42 Meyers M, Wagner MW, Hwang HS, Kinsella TJ, Boothman DA. Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. *Cancer Res* 2001, **61**, 5193–5201.
43 Elsaleh H, Shannon B, Iacopetta B. Microsatellite instability as a molecular marker for very good survival in colorectal cancer patients receiving adjuvant chemotherapy. *Gastroenterology* 2001, **120**, 1309–1310.
44 Carethers JM, Smith EJ, Behling CA, *et al.* Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. *Gastroenterology* 2004, **126**, 394–401.
45 Ribic CM, Sargent DJ, Moore MJ, *et al.* Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 2003, **349**, 247–257.
46 Roberts AB, Flanders KC, Heine UI, *et al.* Transforming growth factor-beta: multifunctional regulator of differentiation and development. *Philos Trans R Soc Lond B Biol Sci* 1990, **327**, 145–154.
47 Massague J, Cheifetz S, Boyd FT, Andres JL. TGF-beta receptors and TGF-beta binding proteoglycans: recent progress in identifying their functional properties. *Ann NY Acad Sci* 1990, **593**, 59–72.
48 Shull MM, Ormsby I, Kier AB, *et al.* Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992, **359**, 693–699.
49 Kulkarni AB, Huh CG, Becker D, *et al.* Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993, **90**, 770–774.
50 Moustakas A, Lin HY, Henis YI, *et al.* The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J Biol Chem* 1993, **268**, 22215–22218.
51 Lin HY, Lodish HF. Receptors for the TGF-beta superfamily: multiple polypeptides and serine/threonine kinases. *Trends Cell Biol* 1993, **3**, 14–19.
52 Boyd FT, Massague J. Transforming growth factor-beta inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. *J Biol Chem* 1989, **264**, 2272–2278.
53 Lopez-Casillas F, Wrana JL, Massague J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993, **73**, 1435–1444.

54 Lin JK, Chou CK. In vitro apoptosis in the human hepatoma cell line induced by transforming growth factor beta 1. *Cancer Res* 1992, **52**, 385–388.

55 Wrana JL, Tran H, Attisano L, Arora K, Childs SR, Massague J, O' Connor MB. Two distinct transmembrane serine/threonine kinases from Drosophila melanogaster form an activin receptor complex. *Mol Cell Biol* 1994, **14**, 944–950.

56 Wrana JL, Attisano L, Carcamo J, *et al.* TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 1992, **71**, 1003–1014.

57 Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-beta receptor. *Nature* 1994, **370**, 341–347.

58 Parsons R, Myeroff LL, Liu B, *et al.* Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* 1995, **55**, 5548–5550.

59 Markowitz S, Wang J, Myeroff L, *et al.* Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995, **268**, 1336–1338.

60 Wang J, Sun L, Myeroff L, *et al.* Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. *J Biol Chem* 1995, **270**, 22044–22049.

61 Geiser AG, Burmester JK, Webbink R, Roberts AB, Sporn MB. Inhibition of growth by transforming growth factor-beta following fusion of two nonresponsive human carcinoma cell lines. Implication of the type II receptor in growth inhibitory responses. *J Biol Chem* 1992, 267, 2588–2593.

62 Vogelstein B, Fearon ER, Hamilton SR, *et al.* Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988, **319**, 525–532.

63 Powell SM, Zilz N, Beazer-Barclay Y, *et al.* APC mutations occur early during colorectal tumorigenesis. *Nature* 1992, **359**, 235–237.

64 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990, **61**, 759–767.

65 Boland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP. Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. *Nat Med* 1995, **1**, 902–909.

66 Herbergs J, Hopman AH, De Bruine AP, Ramaekers FC, Arends JW. In situ hybridization and flow cytometric analysis of colorectal tumours suggests two routes of tumourigenesis characterized by gain of chromosome 7 or loss of chromosomes 17 and 18. *J Pathol* 1996, **179**, 243–247.

67 Yamagata S, Muto T, Uchida Y, *et al.* Polypoid growth and K-ras codon 12 mutation in colorectal cancer. *Cancer* 1995, **75**, 953–957.

68 Isogaki J, Shinmura K, Yin W, *et al.* Microsatellite instability and K-ras mutations in gastric adenomas, with reference to associated gastric cancers. *Cancer Detect Prev* 1999, **23**, 204–214.

69 Lanza G, Gafa R, Santini A, *et al.* Prognostic significance of DNA ploidy in patients with stage II and stage III colon carcinoma: a prospective flow cytometric study. *Cancer* 1998, **82**, 49–59.

70 Papadimitrakopoulou VA, Oh Y, El-Naggar A, *et al.* Presence of multiple incontiguous deleted regions at the long arm of chromosome 18 in head and neck cancer. *Clin Cancer Res* 1998, **4**, 539–544.

71 Ijichi H, Ikenoue T, Kato N, *et al.* Systematic analysis of the TGF-beta-Smad signaling pathway in gastrointestinal cancer cells. *Biochem Biophys Res Commun* 2001, **289**, 350–357.

72 Frederick JP, Liberati NT, Waddell DS, Shi Y, Wang XF. Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. *Mol Cell Biol* 2004, **24**, 2546–2559.

73 Yagi K, Furuhashi M, Aoki H, *et al.* c-myc is a downstream target of the Smad pathway. *J Biol Chem* 2002, **277**, 854–861.

74 Paredes-Zaglul A, Kang JJ, Essig YP, *et al.* Analysis of colorectal cancer by comparative genomic hybridization: evidence for induction of the metastatic phenotype by loss of tumor suppressor genes. *Clin Cancer Res* 1998, **4**, 879–886.

75 Liu ZG, Baskaran R, Lea-Chou ET, *et al.* Three distinct signalling responses by murine fibroblasts to genotoxic stress. *Nature* 1996, **384**, 273–276.

76 Bhatavdekar JM, Patel DD, Chikhlikar PR, *et al.* Molecular markers are predictors of recurrence and survival in patients with Dukes B and Dukes C colorectal adenocarcinoma. *Dis Colon Rectum* 2001, **44**, 523–533.

77 Tang R, Wang JY, Fan CW, *et al.* p53 is an independent pre-treatment markers for long-term survival in stage II and III colorectal cancers: an analysis of interaction between genetic markers and fluorouracil-based adjuvant therapy. *Cancer Lett* 2004, **210**, 101–109.

78 Gotley DC, Reeder JA, Fawcett J, *et al.* The deleted in colon cancer (DCC) gene is consistently expressed in colorectal cancers and metastases. Oncogene 1996, **13**, 787–795.

79 Johnston PG, Drake JC, Trepel J, Allegra CJ. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. *Cancer Res* 1992, **52**, 4306–4312.

80 Johnston PG, Lenz HJ, Leichman CG, *et al.* Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995, **55**, 1407–1412.

81 Edler D, Blomgren H, Allegra CJ, *et al.* Immunohistochemical determination of thymidylate synthase in colorectal cancer – methodological studies. *Eur J Cancer* 1997, **33**, 2278–2281.

82 Chu E, Koeller DM, Johnston PG, Zinn S, Allegra CJ. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. *Mol Pharmacol* 1993, **43**, 527–533.

83 Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, **7**, 890–899.

84 Longley DB, Ferguson PR, Boyer J, *et al.* Characterization of a thymidylate synthase (TS)-inducible cell line: a model system for studying sensitivity to TS- and non-TS-targeted chemotherapies. *Clin Cancer Res* 2001, **7**, 3533–3539.

85 Marsh S, McKay JA, Cassidy J, McLeod HL. Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. *Int J Oncol* 2001, **19**, 383–386.

86 Marsh S, McLeod HL. Thymidylate synthase pharmacogenetics in colorectal cancer. *Clin Colorectal Cancer* 2001, **1**, 175–178; discussion 179–181.

87 Kawakami K, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res* 1999, **19**, 3249–3252.

88 Johnston PG, Benson AB, 3rd, Catalano PJ, *et al. The Clinical Significance Of Thymidylate Synthase (TS) Expression In Primary Colorectal Cancer: An Intergroup Combined Analysis.* ASCO Annual Meeting, 2005.
89 Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* 1989, **16**, 215–237.
90 Tsuji T, Sawai T, Takeshita H, *et al.* Tumor dihydropyrimidine dehydrogenase in stage II and III colorectal cancer: low level expression is a beneficial marker in oral-adjuvant chemotherapy, but is also a predictor for poor prognosis in patients treated with curative surgery alone. *Cancer Lett* 2004, **204**, 97–104.
91 Takebe N, Zhao SC, Ural AU, *et al.* Retroviral transduction of human dihydropyrimidine dehydrogenase cDNA confers resistance to 5-fluorouracil in murine hematopoietic progenitor cells and human CD34+-enriched peripheral blood progenitor cells. *Cancer Gene Ther* 2001, **8**, 966–973.
92 Salonga D, Danenberg KD, Johnson M, *et al.* Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000, **6**, 1322–1327.
93 Johnston SJ, Ridge SA, Cassidy J, McLeod HL. Regulation of dihydropyrimidine dehydrogenase in colorectal cancer. *Clin Cancer Res* 1999, **5**, 2566–2570.
94 Wei X, McLeod HL, McMurrough J, Gonzalez FJ, Fernandez-Salguero P. Molecular basis of the human dihydropyrimidine dehydrogenase deficiency and 5-fluorouracil toxicity. *J Clin Invest* 1996, **98**, 610–615.
95 van Kuilenburg AB, Haasjes J, Meinsma R, *et al.* Lethal outcome of a patient with a complete dihydropyrimidine dehydrogenase (DPD) deficiency after administration of 5-fluorouracil: frequency of the common IVS14+1G>A mutation causing DPD deficiency. *Clin Cancer Res* 2001, **7**, 1149–1153.
96 Benhattar J, Losi L, Chaubert P, Givel JC, Costa J. Prognostic significance of K-ras mutations in colorectal carcinoma. *Gastroenterology* 1993, **104**, 1044–1048.
97 Lee JC, Wang ST, Lai MD, Lin YJ, Yang HB. K-ras gene mutation is a useful predictor of the survival of early stage colorectal cancers. *Anticancer Res* 1996, **16**, 3839–3844.
98 Friedman DB, Hill S, Keller JW, *et al.* Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics* 2004, **4**, 793–811.
99 Kallioniemi A, Kallioniemi OP, Sudar D, *et al.* Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992, **258**, 818–821.
100 Reid LH, Crider-Miller SJ, West A, *et al.* Genomic organization of the human p57KIP2 gene and its analysis in the G401 Wilms' tumor assay. *Cancer Res* 1996, **56**, 1214–1218.
101 Al-Mulla F, Keith WN, Pickford IR, Going JJ, Birnie GD. Comparative genomic hybridization analysis of primary colorectal carcinomas and their synchronous metastases. *Genes Chromosomes Cancer* 1999, **24**, 306–314.
102 Rooney PH, Boonsong A, McKay JA, *et al.* Colorectal cancer genomics: evidence for multiple genotypes which influence survival. *Br J Cancer* 2001, **85**, 1492–1498.
103 Mariadason, JM, Arango, D, Shi, Q, *et al.* Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. *Cancer Res* 2003, **63**, 8791–8812.
104 Arango D, Wilson AJ, Shi Q, *et al.* Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. *Br J Cancer* 2004, **91**(11), 1931–1946.
105 Golub TR, Slonim DK, Tamayo P, *et al.* Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999, **286**, 531–537.
106 van de Vijver MJ, He YD, van' t Veer LJ, *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002, **347**, 1999–2009.
107 Beer DG, Kardia SL, Huang CC, *et al.* Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002, **8**, 816–824.
108 Gordon GJ, Jensen RV, Hsiao LL, *et al.* Using gene expression ratios to predict outcome among patients with mesothelioma. *J Natl Cancer Inst* 2003, **95**, 598–605.
109 Bertucci F, Finetti P, Rougemont J, *et al.* Gene expression profiling for molecular characterization of inflammatory breast cancer and prediction of response to chemotherapy. *Cancer Res* 2004, **64**, 8558–8565.
110 Alon U, Barkai N, Notterman DA, *et al.* Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc Natl Acad Sci USA* 1999, **96**, 6745–6750.
111 Bertucci F, Salas S, Eysteries S, *et al.* Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. *Oncogene* 2004, **23**, 1377–1391.
112 Motoori M, Takemasa I, Yano M, *et al.* Prediction of recurrence in advanced gastric cancer patients after curative resection by gene expression profiling. *Int J Cancer* 2005, **114**(6), 963–968.
113 Clarke PA, George ML, Easdale S, *et al.* Molecular pharmacology of cancer therapy in human colorectal cancer by gene expression profiling. *Cancer Res* 2003, **63**, 6855–6863.
114 Wang Y, Jatkoe T, Zhang Y, *et al.* Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. *J Clin Oncol* 2004, **22**, 1564–1571.
115 Mariadason JM, Arango D, Augenlicht LH. Customizing chemotherapy for colon cancer: the potential of gene expression profiling. *Drug Resist Updat* 2004, **7**, 209–218.