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TOPIC HIGHLIGHT

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Recent applications of chemosensitivity tests for colorectal cancer treatment

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Abstract

The evaluation of therapeutic efficacy is necessary to predict the outcome of patients with metastatic colorectal cancer (CRC). In these patients, there is a critical need for predictive chemosensitivity assays and biomarkers to optimize efficacy and minimize toxicity. The introduction of targeted agents has improved the progression-free survival and overall survival of patients with metastatic disease. However, approximately 50% of patients do not show a positive response to chemotherapy and the selection of patients likely to respond to a specific regimen remains challenging. Cell culture-based chemosensitivity tests use autologous viable tumor cells to evaluate susceptibility to specific agents *in vitro* and predict their direct effects. Adenosine triphosphate-based assays and methyl thiazolyl-diphenyl-tetrazolium bromide-based assays are used widely as sensitivity tests because of their short assay period, technical simplicity, and the requirement of small amount of specimen. Among protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status predicts the response to epidermal growth factor receptor-targeted therapy in CRC patients. The validation of predictive and prognostic markers enables the selection of therapeutic regimens with optimal efficacy and minimal toxicity for each patient, which has been termed personalized treatment. This review summarizes currently available predictive and prognostic chemosensitivity tests for metastatic CRC.

Key words: Colorectal adenocarcinomas; Colorectal cancer; Chemotherapy; *In vitro* assays; Molecular targeted therapy; Individualized therapy

Core tip: This review summarizes currently available predictive and prognostic chemosensitivity tests and biomarkers in terms of cell culture, protein, and gene. Cell culture-based chemosensitivity tests are used widely in clinical practice because of their short assay period, technical simplicity, and the requirement of small amount of specimen. Among protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status predicts the response to epidermal growth factor receptor-targeted therapy in colorectal cancer patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common can-

cer and the fourth most frequent cause of cancer death worldwide[1]. CRC develops as a consequence of accumulated genetic and epigenetic alterations that result in the loss of tumor suppressor genes and activation of oncogenes.

The response to chemotherapy varies among patients, with objective tumor response rates to standard chemotherapy regimens of 30%-40% in patients with metastatic CRC. Therefore, a reliable method to determine the sensitivity or resistance of tumors to specific chemotherapy agents would be useful in clinical practice. For this purpose, cell culture-based chemosensitivity tests have been investigated for more than 30 years; however, their use is limited by technical issues, a low success rate for primary culture, length of time required, and poor correlation with clinical response. To overcome these obstacles, gene- and protein-based chemosensitivity tests have been investigated, and certain gene alterations have been identified that are predictive of clinical drug response.

In the present review, we discuss recent advances in cell culture-based chemosensitivity tests and the identification of genomic alterations as biomarkers for the design of efficient chemotherapy regimens for CRC patients.

CELL CULTURE-BASED CHEMOSENSITIVITY TESTS

In cell culture-based chemosensitivity tests, autologous viable tumor cells are evaluated to determine the susceptibility of that tumor to specific agents *in vitro* and to predict the response to therapy. Although cell culture-based chemosensitivity tests have been investigated extensively, they are not widely used because of technical problems, a low success rate for primary culture, length of time required, and poor correlation with clinical response[2]. In 2004, the American Society of Clinical Oncology (ASCO) stated that the use of *in vitro* drug response assays to select chemotherapeutic agents for individual patients is not recommended outside of the clinical trial setting[3]. In a 2011 update, no changes were made to the original ASCO guidelines because of insufficient evidence to support the use of these assays in clinical practice[4]. Several *in vitro* chemosensitivity and drug resistance assays have been developed, including the human tumor cloning assay, differential staining toxicity, adenosine triphosphate (ATP)-based and methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) assays, histoculture drug response assay (HDRA), and extreme drug response assay (EDRA)[3,5]. Among these assays, ATP-based and MTT assays are commonly used as simple sensitivity tests. The advantages of these assays are a short assay period, technical simplicity and the requirement of a relatively small amount of specimen[6,7]. Table 1 describes cell culture-based *in vitro* assays that have been recently used in clinical trials of human solid cancers.

ATP-based assay

The ATP-based assay is a sensitive cytometric assay that evaluates tumor cell viability by measuring the intracellular ATP levels of drug-exposed cells and untreated controls[8]. This test has several advantages over other cell-based assays, including higher sensitivity for predicting cell viability, accurate distinction between cancer cells and normal cells and the requirement of a small number of cells[9]. The ATP-based chemotherapy response assay (ATP-CRA) is an improved method in which the proliferation of normal cells in tumor tissues can be inhibited through the use of ultralow attachment culture plates; this assay does not require large amounts of specimen and has a relatively short test turnaround time[10]. Several preclinical and clinical studies have shown the feasibility and good treatment outcomes of ATP-CRA-guided chemotherapy in ovarian, breast, stomach, and lung cancer[11-14].

Differences in the chemosensitivity of CRC patients to several anticancer drugs, including 5-fluorouracil (5-FU), oxaliplatin, and irinotecan have been investigated in preclinical studies[15,16]. The only clinical study was reported by Hur *et al*[6] who showed that ATP-CRA could improve treatment response and resectability in initially unresectable colorectal liver metastasis. In their study, the authors showed that the ATP-CRA guided chemotherapy group showed better treatment response (48.4% *vs* 21.9%, $P = 0.027$) and a higher rate of resectability of hepatic lesions (35.5% *vs* 12.5%, $P = 0.032$). However, multi-institutional randomized controlled trials are needed to validate the use of ATP-CRA for individualized chemotherapy in CRCs.

MTT assay and histoculture drug response assay

The MTT assay is a high throughput (96-well plates) method for the quantification of viable cells without the need for elaborate cell counting. It is commonly used to determine the cytotoxicity of drugs at different concentrations. The principle of the MTT assay is that mitochondrial activity remains constant in viable cells, and therefore an increase or decrease in the number of viable cells is correlated with changes in mitochondrial activity. The mitochondrial activity of cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilized. The absorbance of the resulting solution, which indicates cell viability, is quantified by measuring the optical density (OD) at 540 and 720 nm using a plate reader. Presently, clinical correlation studies using the MTT assay have been reported for breast and stomach cancers[17,18].

Hoffman *et al*[19] developed the HDRA and applied it to the three-dimensional culture of tumor tissue fragments using a collagen gel matrix and an MTT end point[20]. Several conventional drug sensitivity tests use isolated tumor cells obtained after enzymatic digestion. By contrast, the HDRA technique uses cancer tissue fragments in which cells maintain their native architecture and can grow in three dimensions. This enables the maintenance of intercellular contacts and interactions with stromal cells. The HDRA thus enables assessment of the sensitivity of tumor cells to anticancer drugs un-

Table 1 Overview of the cell culture-based chemosensitivity tests

Name	Studied tumor type	Description
MTT[17,18]	Breast and stomach	The MTT assay measures mitochondrial activity and is most often used to detect loss of cell survival/cell viability in response to a drug or toxin. Tumor cell suspensions are cultured with various chemotherapy agents for 3-4 d and then exposed to the MTT reagent; because it reduces intracellularly to a blue dye, the intensity of uptake yields an estimate of the number of viable cells to determine drug sensitivity
HDRA[5,21,22]	Stomach, breast, ovary, and colon	The HDRA uses cancer tissue fragments and three-dimensional cell culture, in which intercellular contacts and interactions with stromal cells are maintained. Tumor specimens are cut into 1-mm^3 pieces and put on a gelatin sponge infiltrated with culture medium containing a test drug. After incubation for 3-7 d, cell viability is assessed using the MTT assay
ATP[6,11-14]	Ovary, breast, stomach, and colon	The quantification of intracellular concentrations of ATP as a measure of cell survival has gained wide acceptance for the evaluation of the medium and long-term cytotoxic effects of drugs (2-3 d). The assay is based on the bioluminescent detection of cellular ATP and is extremely sensitive, allowing the measurement of ATP levels in a single adherent or non-adherent mammalian cell
EDRA[26,31]	Ovary, breast, lung, and colon	After 3-5 d of culture, tumor cells obtained from fresh biopsy specimens are labeled with tritiated thymidine. The level of uptake is tracked after exposure to chemotherapy drug concentrations that approximate the peak level achieved clinically. Extreme resistance is identified when thymidine incorporation is inhibited in the presence of the drug by less than one standard deviation of the median cell inhibition measured for several hundred reference tumor samples

MTT: Methyl thiazolyl-diphenyl-tetrazolium bromide; HDRA: Histoculture drug response assay; ATP: Adenosine triphosphate bioluminescence; EDRA: Extreme drug resistance assay.

der conditions that mimic those of the *in vivo* environment[21]. The correlation rate of the HDRA to clinical response was reported to range from 74% to 92.1% in several studies of head and neck, gastric, and colorectal cancers[5,22,23].

Recently, our group compared chemosensitivity assessed using the HDRA with the clinical response to different treatment regimens in patients with advanced CRC[7]. HDRAs were performed to assess the effect of seven combinations of anticancer drugs, including 5-FU with leucovorin (FL), FL with oxaliplatin (FOLFOX) and with irinotecan (FOLFIRI), and their combinations with bevacizumab and cetuximab. The results of 324 HDRAs showed that tumor inhibition rates were higher for FOLFOX (34.2%-39.2%) than for FOLFIRI (24.2%-32.7%, $P < 0.001$). Evaluation of 86 chemotherapeutic regimens showed that the correlation rate of HDRA to the clinical response to chemotherapy was 66.3% (57/86), with sensitivity and specificity values of 72.7% (40/55) and 54.7% (17/31), respectively. Despite variations in accuracy, HDRA might be a feasible and useful technique to predict chemosensitivity in individual patients. Similar to the ATP-CRA, further randomized multi-institutional studies are necessary to support the routine clinical application of the HDRA.

Extreme drug response assay

The EDRA was developed by Kern *et al*[24] as an exclusion test to identify drugs unlikely to elicit a response. According to the Bayesian theory, any laboratory assays will be accurate only when the assays are extremely (> 98%) specific for drug resistance, concurrently with high overall response rates. The EDRA measures inhibition of DNA synthesis by calculating the rate of proliferating tumor cells plated in agar medium using a thymidine incorporation methodology. The percent inhibition of cellular thymidine incorporation (PCI) comparing the quadruplicate negative and duplicate positive controls is calculated for each drug using a liquid scintillation counter. Tumor specimens are classified as exhibiting extreme drug resistant (EDR) to an agent when the PCI result is more than one standard deviation below the median PCI for examining drug[25]. Kern *et al*[24] reviewed 450 correlations between EDRA results and clinical response over an 8-year period and identified EDR with > 99% specificity. The EDRA has been used to identify patients with therapeutic failure and relapse in various types of tumors including ovary, breast, and lung cancers[26-29].

In CRC, Fan *et al*[30] analyzed the outcomes of EDRAs in 102 CRC patients treated with 5-FU single chemotherapy using cell viability and ATP assays. In the clinical correlation of 25 Dukes' D patients with EDRA, the sensitivity and specificity of the assay were 100% and 95%, respectively. Recently, Mechetner *et al*[31] analyzed the results of EDRA performed in 4854 CRC specimens and showed that primary and metastatic tumors showing EDR to FL had up to 58% cross-resistance to a variety of chemotherapy agents, with the lowest percentages for oxaliplatin (11% and 8%, respectively) and irinotecan (16% and 14%, respectively). Approximately 20% of tumors showed EDR to either FOLFOX or FOLFIRI. They concluded that the results of the EDRA obtained at initial diagnosis may be useful for the selection of therapeutic regimens for metastatic disease.

GENE-AND PROTEIN-BASED CHEMOSENSITIVITY TESTS

Molecular markers of fluoropyrimidines

Thymidylate synthase: The gene expressions of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and thymidine phosphorylase (TP) play a key role in 5-FU resistance. TS, an essential enzyme for DNA synthesis, is the target of 5-FU. Despite controversial results[32,33], many studies and meta-analyses have shown that the downregulation of intra-tumoral TS protein

Table 2 Protein and gene-based chemosensitivity tests in colorectal cancer

Marker	Target chemotherapy drug	Function	Change	Consequence
TS[34,35]	5-FU	Essential enzyme for DNA synthesis	TS expression ↓	[1]Chemotherapy response ↑
DPD[33,35]	5-FU	Degradation of 5-FU	DPD expression ↓	[1]Chemotherapy response ↑
TP[39]	5-FU	Activation of 5-FU (from 5′-DFUR to 5-FU)	Stromal TP expression ↑	[1]Chemotherapy response ↑
UGT1A1[49]	Irinotecan	Degradation of the active metabolite of irinotecan (SN-38)	Polymorphism of UGT1A (UGT1A1*28)	Irinotecan toxicity↑
ERCC1[54]	Oxaliplatin	Excision nuclease that repairs platinum-induced DNA adducts	ERCC1 expression ↓	[1]Chemotherapy response ↑
KRAS[65-69]	Anti-EGFR	Proto-oncogene in the EGFR signaling pathway	Mutation of the *KRAS* gene	Chemotherapy response↓
NRAS[72]	Anti-EGFR	Proto-oncogene in the EGFR signaling pathway	Mutation of the *NRAS* gene	Chemotherapy response↓
BRAF[74-77]	Anti-EGFR	Signaling gene acting downstream of KRAS	Mutation of the *BRAF* gene (V600E)	Chemotherapy response↓

[1]Chemotherapy responses of these markers are generally inconsistent without strong evidences. TS: Thymidylate synthase; 5-FU: 5-fluoropyrimidine; DPD: Dihydropyrimidine dehydrogenase; TP: Thymidine phosphorylase; 5′-DFUR: 5′-Deoxy-5-fluorouridine; UGT1A1: Uridine diphosphate glucuronosyltransferase 1A1; ERCC1: Excision repair cross-complementation group 1; anti-EGFR: Anti-epidermal growth factor receptor (cetuximab or panitumumab).

and mRNA expression is a strong prognostic marker for the response to 5-FU based chemotherapy regimens in CRC[34,35] (Table 2).

DPD: DPD catalyzes the first and rate-limiting step of the pyrimidine catabolic pathway. DPD is also responsible for the degradation of 5-FU and influences the antitumor and adverse effects of 5-FU. High intratumoral DPD activity markedly decreases the cytotoxic effect of 5-FU. Despite its low incidence, DPD deficiency is associated with severe adverse effects after 5-FU-based chemotherapy and can result in death mainly from infectious disease due to neutropenia[36]. DPD protein and mRNA expression is a strong prognostic marker of the response to 5-FU based chemotherapy regimens in CRC[33,35].

TP: TP is a key enzyme involved in the synthesis and degradation of pyrimidine nucleotides. The antiapoptotic and angiogenic effects of TP are closely related to the growth and metastasis of CRC. In addition, TP is a key enzyme in the activation pathway of the 5-FU prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) to 5-FU[37]. The expression of TP in CRC has a dual function. High expression of TP is associated with poor prognosis in patients with CRC, as indicated by increased infiltration, growth, and tumor metastasis. However, the upregulation of TP expression in CRC tissues improves the curative effect of 5-FU, which is important in the treatment of CRC. Therefore, the up- and down-regulation of TP expression in tissues plays an important role in the emergence and development of tumors and may affect prognostic and therapeutic indices. Despite conflicting results regarding the association between TP expression and prognosis[37-39], TP serves as an indicator of angiogenic potential and plays an important role in cancer chemotherapy as a target for antiangiogenic agents and as an activating enzyme of 5-FU prodrugs[40].

Single-nucleotide polymorphisms: Genome-wide single nucleotide polymorphism (SNP) analysis may represent a promising approach for the identification of new predictive biomarkers for clinical application. The chemosensitive SNP markers GPC5 rs553717 (AA), SSTR4 rs2567608 (AA) and EPHA7 rs2278107 (TT) were identified through a three step process consisting of *in vitro* screening, identification, and validation[41]. These candidate markers are significantly correlated with recurrence or chemoresponsiveness in patients receiving fluoropyrimidine-based adjuvant chemotherapy. Recently, our group identified two chemosensitive SNP markers for chemoradiation (CRT) therapy in patients with low lying rectal cancer. Two candidate markers, CORO2A rs1985859 and FAM101A rs7955740, may be of value for the prediction of radiosensitivity to preoperative CRT, although further validation is needed in large cohorts[42].

Molecular markers of irinotecan

Uridine diphosphate glucuronosyltransferase 1A1: Irinotecan (CPT-11) is an inhibitor of DNA topoisomerase I that is widely used in the treatment of CRC. Irinotecan is metabolized to its active metabolite, SN-38, which is 1000 times more active than the unmodified drug. The major route of SN-38 elimination is *via* glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), an essential enzyme involved in the complex metabolism of irinotecan. UGT1A1*28 is a common allele with seven TA repeats in the promoter of UGT1A1 compared with the wild-type allele (UGT1A1*1) with six repeats. A seven-repeat allele is associated with decreased transcription and expression of UGT1A1 and reduced enzymatic activity, which lead to higher or more prolonged exposure to SN-38. Investigation of the variant UGT1A1*28 showed that the homozygous variant allele is associated with a significantly increased risk for myelosuppression and gastrointestinal toxicities in patients treated with irinotecan[43]. The frequency of UGT1A1*28 is very low in Asians compared with that in Caucasian[44,45]. Another polymorphism, UGT1A1*6, characterized by replacing single nucleotide in exon1 of UGT1A1, has been also considered to be related with reduced SN-38 glucuronidation activity and bears a higher allele frequency in Asians than Caucasians[45]. A recent

meta-analysis of 11 studies revealed that UGT1A1*6 polymorphisms is also potential biomarkers predicting irinotecan-induced severe toxicity in Asians in addition to UGT1A1*28[46]. Studies investigating the efficacy of irinotecan in CRC patients bearing different UGT1A1*28 genotypes have yielded conflicting results that are difficult to interpret because of small sample sizes and the associated poor statistical power[47]. Overall, UGT1A1 genotypes predict severe neutropenia and diarrhea, but not treatment efficacy[48,49].

Molecular markers of oxaliplatin

Excision repair cross-complementation group 1: Oxaliplatin is a platinum analogue that differs from cisplatin by the presence of a diaminocyclohexane ligand in its chemical structure. Excision repair cross-complementation group 1 (ERCC1) is an excision nuclease within the nucleotide excision repair (NER) pathway that plays a major role in the repair of platinum-induced DNA adducts. Overexpression of ERCC1 has been reported in cisplatin-resistant cancer cell lines[50]. Downregulation of ERCC1 expression in tumor tissues is associated with favorable overall survival in advanced CRC patients treated with oxaliplatin-based chemotherapy[51]. However, clinical correlations between ERCC1 polymorphisms and poor oncologic outcomes have been reported[52,53]. In a recent study, immunohistochemical analysis showed a correlation between negative expression of the ERCC1 protein and favorable overall survival and low recurrence rates[54]. However, the precise role of ERCC1 expression needs to be validated in further studies.

Molecular markers of EGFR-targeted treatment

The monoclonal antibodies cetuximab and panitumumab, which target the epidermal growth factor receptor (EGFR), have expanded the range of treatment options for metastatic CRC[55]. EGFR and downstream signaling pathways are activated by several mechanisms, including overexpression of the receptor, overexpression of the ligand, activating mutation of the receptor, or inactivation of tumor suppressor genes. EGFR ligands, the receptor itself, and the downstream signaling molecules, such as KRAS, NRAS, BRAF, PIK3CA, and its suppressor PTEN, have all been examined as potential effectors of resistance to EGFR-targeted therapy[56]. The mutation status of signaling molecules downstream of the EGFR target may predict the clinical response to EGFR-targeted therapies.

EGFR ligands: EGFR ligands, such as amphiregulin and epiregulin, may stimulate EGFR through an autocrine or paracrine loop with positive feedback[57]. Amphiregulin and epiregulin are coregulated by binding to the same receptor. Accordingly, similar prognostic or predictive effects would be expected. Despite inconsistent results[58], many studies reported that increased expression of genes encoding amphiregulin and epiregulin strongly associated with increased therapeutic benefit from cetuximab in metastatic CRC patients with KRAS wild-type[59-62]. Although previous data have shown similar results for amphiregulin and epiregulin, epiregulin is recently favored as a better predictor[58,63]. Further researches confirming the usefulness of these candidate markers are needed.

KRAS: Activating mutations in the KRAS oncogene, located on the short arm of chromosome 12, are commonly associated with progression from a benign adenoma to a dysplastic adenocarcinoma and occur in 30%-40% of CRCs[64]. The value of KRAS as a predictive biomarker for anti-EGFR therapy has been demonstrated, as mutations of this gene result in the activation of the EGFR pathway. In 2006, Lièvre *et al*[65] showed that whereas all patients who responded to cetuximab presented with wild-type KRAS, 68% of non-responders showed mutations in this gene. Phase Ⅲ CRYSTAL and phase Ⅱ OPUS trials showed the benefit of cetuximab in metastatic CRC patients treated with FOLFIRI and FOLFOX, respectively[66,67]. These findings were confirmed in many other studies[68,69]. Currently, the presence of the wild-type form of KRAS is considered a positive predictive marker of response to EGFR inhibitor therapy. KRAS mutations are associated with lack of treatment response and a reduction in median progression-free survival (PFS) in patients treated with cetuximab/panitumumab alone or in combination with chemotherapy. The results of several clinical trials have led to the recommendation of KRAS mutational screening of codons 12 and 13 in patients with metastatic CRC[70].

NRAS: NRAS mutations, which occur in a smaller percentage (approximately 5%) of patients than KRAS mutations, arise at a later stage in the development of CRC and suppress apoptosis[71]. A recent PRIME trial showed that extended RAS mutations including NRAS exons 2, 3, or 4 were associated with inferior PFS and overall survival after panitumumab-FOLFOX4 treatment[72]. Therefore, NRAS mutational screening should be considered in terms of its low incidence and time-cost benefits.

BRAF: An activating mutation (V600E) of the KRAS downstream signaling protein BRAF is present in 3%-12% of CRC patients[73]. BRAF mutations are mutually exclusive of KRAS mutations in CRC[68]. The negative prognostic value of BRAF mutations in KRAS wild-type patients treated with anti-EGFR therapy was demonstrated in several studies[74-77]. A recent pooled analysis of the CRYSTAL and OPUS trials confirmed that BRAF mutation is not a predictive marker for response of cetuximab in combination chemotherapy but shows as a negative prognostic marker[78]. Although evidence is still insufficient to demonstrate an actual association of BRAF mutations with non-responsiveness to anti-EGFR therapy, BRAF genetic screening is recommended by National Comprehensive Cancer Network in patients with KRAS wild-type before anti-EGFR therapy.

PI3K/PTEN: Another major downstream signaling pathway activated by EGFR in addition to the KRAS-BRAF-MAPK pathway is the PI3K/PTEN/AKT signaling pathway. PIK3CA can be dysregulated by activating mutations in the PIK3CA p110 subunit or through inactivation of the tumor suppressor phosphatase and tensin homologue (PTEN) phosphatase. PIK3CA and PTEN mutations can coexist with KRAS and BRAF mutations[79,80]. The clinical impact of PTEN protein expression and PIK3CA mutations remains controversial. Sartore-Bianchi *et al*[81] showed that PIK3CA mutations and PTEN loss in CRCs are significantly associated with lack of response to panitumumab or cetuximab treatment. However, Prenen *et al*[82] reported no strong rationale for using PIK3CA mutations as a single marker for sensitivity to cetuximab in chemotherapy-refractory metastatic CRC. A recent randomized controlled trial also showed that neither PIK3CA mutation status nor PTEN expression are prognostic or predictive of response to cetuximab[83]. Further studies are needed to confirm the usefulness of these candidate markers.

SNP: Patients carrying the GG genotype at DFNB31 rs2274159 or LIFR rs3729740 are more sensitive to cetuximab-containing regimens than those carrying at least one A allele[84]. Cell lines transfected with the G allele at LIFR rs3729740 and the C allele at ISX rs361863 showed higher sensitivity to cetuximab-containing regimens than those with the A and T alleles. Recently, a clinical association study conducted by our group showed that patients homozygous for the wild-type alleles (GG) of LIFR rs3729740 exhibited a 1.9 times greater overall response rate and 1.4 mo longer PFS than those homozygous or heterozygous for the mutant allele[85].

Molecular markers for VEGF targeted treatment

Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1, VEGFR-2, and VEGFR-3 are intimately involved in cell migration and proliferation and promote endothelial cell survival and protection against endothelial cell apoptosis and senescence[86]. Bevacizumab is a recombinant humanized monoclonal IgG1 antibody against VEGF-A that decreases the availability of free circulating VEGF-A, preventing receptor activation. Hurwitz *et al*[87] showed that bevacizumab significantly improved overall survival in patients with metastatic CRC[87]. Although certain candidate markers have been identified, no efficient chemotherapy marker for bevacizumab-based regimens has been established.

Plasma VEGF-A: The measurement of concentrations of circulating protein is an attractive biomarker strategy, as blood is easily accessible, the assays are inexpensive, and the proteins may be readily and quantitatively measured by automated methods[88]. Plasma VEGF levels have been proposed to reflect VEGF-dependent tumor angiogenesis, and might predict benefit from bevacizumab[89]. Although increased plasma VEGF-A levels are well established as indicators of poor prognosis[90], data regarding the predictive effect of baseline VEGF-A levels have largely been inconsistent[91]. A recent study demonstrated that an early increase of plasma VEGF-A level after the initial decrease is a potential predictive marker of a poor response and reactive resistance to bevacizumab[92]. The predictive value of VEGF-A to bevacizumab will be evaluated in the phase Ⅲ MERiDiAN trial (opening in 2012).

Neuropilin-1: Neuropilin-1 (NRP1) is a VEGF co-receptor that enhances VEGF binding to VEGFR-2, VEGFR-2 phosphorylation, and VEGF-induced signaling and migration[93]. Preclinical data suggest that NRP1 is a valid anticancer target which has roles in both the proliferation of tumor cells and pathological angiogenesis[94,95]. In gastric and breast cancers, tumor NRP1 expression was identified as a potential predictor of bevacizumab efficacy[96,97]. Despite insufficient data for CRC, tumor NRP1 expression appears as a promising biomarker for anti-angiogenic therapy.

SNP: Several SNP markers have been identified as markers of chemosensitivity to bevacizumab therapy. Koutras *et al*[98] reported that the VEGF-1154 GG genotype was a significant adverse prognostic factor for overall survival in patients with metastatic CRC receiving irinotecan-based chemotherapy plus bevacizumab. Recently, Loupakis *et al*[99] showed that VEGFR-2 rs12505758 C-variants were associated with shorter PFS [HR = 1.36 (1.05-1.75), $P = 0.015$] compared to T/T variants. Our group found that patients carrying the TT genotype at ANXA11 rs1049550 or at least one G allele at LINS1 rs11247226 were more sensitive to bevacizumab therapy than those carrying at least one C allele or the AA genotype[84]. In a recent clinical association study, we showed that the TT genotype at ANXA11 rs1049550 was correlated with increased sensitivity to bevacizumab[85]. These data indicate that SNP analysis may represent a promising approach for the identification of novel predictive biomarkers for clinical application.

CD133: CD133, a surface protein widely used for the isolation of colon cancer stem cells, is associated with tumor angiogenesis and recurrence. Pohl *et al*[100] showed that patients with high gene expression levels of CD113 (> 7.76) showed a significantly greater tumor response (RR = 86%) than patients with low expression levels (≤ 7.76, RR = 38%, adjusted $P = 0.003$), independent of the expression of VEGF or its receptor. Combined analyses of two CD113 polymorphisms (rs2286455 and rs3130) showed a significant association with PFS (18.5 mo *vs* 9.8 mo, $P = 0.004$) in multivariate analysis as an independent prognostic factor for PFS (adjusted $P = 0.002$)[100].

CONCLUSION

Although cell culture-based chemosensitivity tests have

been investigated extensively, consistent results have not been achieved mainly because of technical problems and variable clinical correlations. Certain *in vitro* sensitivity tests, including ATP- and MTT-based assays, have recently been used in clinical practice, although validation in large and well-controlled cohorts is necessary. Further development of these chemosensitivity assays may enable the accurate prediction of sensitivity or resistance to chemotherapy drugs. Regarding protein- and gene-based chemosensitivity assays, assessment of KRAS mutation status to predict the efficacy of antibodies targeting EGFR in patients with metastatic CRC is an important step. In addition, gene expression-based panels aimed at determining the risk of relapse in elderly and marginal patients who are more sensitive to chemotherapy may represent a valuable clinical tool[101]. Although many potential biomarkers have recently been reported, few have emerged as clinically useful, mainly because of limited reproducibility, technical faults, and their assessment in small and heterogeneous cohorts. The two types of chemosensitivity tests, *in vitro* assays and molecular markers, could be used in combination for an accurate prediction of the clinical response to chemotherapy. The development of novel cell-based assays and genomic technologies could usher in an era of personalized molecular medicine in which patients will be accurately stratified based on their specific molecular profile.

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