Predictive Biomarkers and Personalized Medicine

Novel Chemosensitive Single-Nucleotide Polymorphism Markers to Targeted Regimens in Metastatic Colorectal Cancer

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Introduction

More than 50% of patients with colorectal cancer (CRC) exhibit recurrence or metastasis, commonly designated as metastatic CRC, regardless of curative operations (1). Numerous treatment regimens for such patients have been developed to achieve a significant improvement in the response rate and survival time. Among them, 2 biologically targeted agents, bevacizumab (Avastin; Genentech Inc.) and cetuximab (Erbitux; ImClone Systems Inc.), have been successfully combined with irinotecan and oxaliplatin regimens (2, 3). Bevacizumab is a humanized monoclonal antibody targeting the proangiogenic VEGF-A. Cetuximab is a chimeric monoclonal antibody that binds to and subsequently blocks the activity of epidermal growth factor receptor (EGFR). Targeted chemotherapy clearly benefits a significant proportion of patients with metastatic CRC whereas other patients gain very little or even no benefit. In addition, health economics have limited the ordinary use of targeted regimens. Therefore, efficient predictive biomarkers are urgently required to enhance the responsiveness and cost-effectiveness of such therapies.

Although identification of predictive biomarkers to targeted regimens has been attempted using biomolecular and clinical approaches, few efficient markers are available for targeted regimens. KRAS mutations, which are present in 35% to 45% of CRC patients, have emerged as an important predictive marker of resistance to panitumumab or cetuximab treatment (4). Other molecular changes encompassing EGFR effector pathways and cognate EGFR ligands [i.e., PI3K (phosphoinositide 3-kinase)/PTEN/AKT, RAS/RAF, AREG (amphiregulin), and EREG (epiregulin)] have been investigated to find chemo-sensitive markers to cetuximab (4–6). To date, few biomarker studies have clinically
Materials and Methods

Screening procedures and subjects
A 3-step process of genome-wide SNP screening, clinical association analysis, and biological utility assessment was used for chemosensitive SNP discovery. For the first step, 118 patients with sporadic CRC who had previously been tested by in vitro chemosensitivity assay were consecutively recruited for GWAS analysis at Asan Medical Center (Seoul, Korea; Table 1). For Hardy–Weinberg equilibrium (HWE) and clinical association analyses, a separate cohort of 460 healthy controls and 98 evaluable patients with targeted chemotherapy who were a different cohort from the first step were also included for genotyping the selected SNPs during GWAS screening, using their genomic DNA. Genomic DNA was extracted from buffy coats preserved in the liquid nitrogen, using a nucleic lysis buffer (Promega). Healthy controls recruited from the Health Evaluation Center of Asan Medical Center came from the general Korean population and reported no known family history of cancer. All patients provided written informed consent. The study protocol was approved by the Institutional Review Board for Human Genetic and Genomic Research (registration no.: 2009–0091) in accordance with the Declaration of Helsinki.

Initial genome-wide SNP screening
The chemosensitivity of tumor tissue from the initial cohort of 118 tumor tissues from 118 patients was assessed using the in vitro chemosensitivity assay. The assessment was performed in a manner similar to a previous histoculture drug response assay (Supplementary Fig. 1), using MTT as a quantitative endpoint to assess the total tumor cell viability (12, 13). Targeted agents were freshly solved into PBS with other chemotherapy agents and all drugs were concurrently treated with patients’ cancer cells. The cutoff concentrations of the established drugs used to distinguish in vitro sensitivity from resistance were set at 50 μg/mL for 5-fluorouracil (5-FU), 2 μg/mL for leucovorin, 5 mmol/L for capecitabine, 20 μg/mL for irinotecan, 40 μg/mL for oxaliplatin, and 10 μg/mL each for bevacizumab and cetuximab. Triplicates of the 4 regimens were cultured and examined on a 24-well plate at the same time. The inhibition rate (IR) of tumor growth was calculated using the following equation: IR (%) = (1 – mean absorbance of treated wells per gram of tumor/mean absorbance of control wells per gram of tumor) × 100. The sample quantity was determined as the approximate number needed to establish an 85% chance of detecting the IR difference, with a success rate of 25% between the responsive and nonresponsive groups. Genomic DNAs from peripheral blood samples of these patients were genotyped using the Affymetrix Genome-Wide Human SNP Nsp/Sty Assay Kit and SNP Array 6.0 according to the manufacturer’s protocol. From 863,309 autosomal SNPs, a total of 651,262 SNPs were selected for association with the efficacy of bevacizumab and cetuximab regimens. A dichotomous measure was used, with an IR cutoff value for a
positive response of 30% or more, similar to the RECIST (Response Evaluation Criteria in Solid Tumors) criteria (14). The following criteria were used: (i) SNP call rate of 95% or more, (ii) minor allele frequency (MAF) of 1% or more, and (iii) HWE with \( P < 0.001 \). A liberal \( P \) value (< 0.001) was used for the initial candidate region screening, due to the relatively small sample size. After quality control filtering, the overall genotype call rate for 118 individuals was 99.7%. The ancestral allele for the respective SNP was designated as a reference allele based on the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/snp). The raw data were deposited in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GEO21228.

### Chemotherapy using targeted regimens and evaluations

Eligibility criteria included histologically verified colorectal adenocarcinoma, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, and an age of 75 years or younger. A total of 98 patients underwent metastatic chemotherapy using bevacizumab (69 patients) or cetuximab (67 patients) regimens, including 38 patients with crossover treatment (bevacizumab to cetuximab regimens in 22 patients and cetuximab to bevacizumab regimens in 16 patients). Chemotherapy regimens were administered in accordance with the NCCN Clinical Practice Guidelines in Oncology (www.nccn.org). Intravenous bevacizumab (7.5 mg/kg) or cetuximab (400 mg/m\(^2\) initial dose and then 250 mg/m\(^2\) ) were administered every week or on day 1 with FOLFOX and FOLFIRI regimens, respectively. The mean number of chemotherapy cycles was 9 (range, 2–23) for bevacizumab and 14 (range, 2–90) for cetuximab. Patients who underwent metastatic chemotherapy received follow-up care every 6 to 8 weeks (mean, 13.3 months; range, 1–39 months). The tumor response was assessed by objective response and the disease control rate calculated according to the intent-to-treat analysis using RECIST criteria (14). As our main purpose was to evaluate the chemosensitivity of targeted regimens in the limited cohort; the primary endpoint was response rates, accompanying survival rates as supplementary one. Accordingly, the best response with the time to this response was measured as primary endpoint in patients with crossover treatment. Adverse events were evaluated on the basis of NCI Common Toxicity Criteria (ver. 3.0). Clinical responses were associated with genotyping results under a codominant inheritance model.

### Genotyping of initially screened SNPs and KRAS mutation analysis

The genotypes of initially screened SNPs identified by the initial genome-wide SNP screening were assayed by pyrosequencing using genomic DNA samples of the 98 patients with targeted chemotherapy and 460 healthy controls for

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### Table 1. Demographic and biological features of patients in the initial screening and clinical association analysis for chemosensitive SNP markers

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>No. of patients</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial screening</strong> (( n = 118 )$$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>67/51</td>
<td>37/32</td>
</tr>
<tr>
<td>Age, mean ± SD, y</td>
<td>57 ± 10</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>Stage, ( I/II/III/IV $$</td>
<td>11/41/42/24</td>
<td>0/2/19/48</td>
</tr>
<tr>
<td>Tumor characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location, right/left/rectum</td>
<td>42/34/42</td>
<td>18/22/29</td>
</tr>
<tr>
<td>Infiltrative growth</td>
<td>31</td>
<td>22</td>
</tr>
<tr>
<td>Poorly differentiated or mucinous</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>Lymphovascular invasion+</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>Defective mismatch repair(d $$</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>KRAS codons 12–13 mutation+</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Nuclear p53 expression &gt;10%</td>
<td>69</td>
<td>41</td>
</tr>
</tbody>
</table>

**NOTE:** Bold font, \( P < 0.05 \).

\( a \)Comparison of initial screening and clinical association by Pearson’s \( \chi^2 \) test or unpaired \( t \) test.

\( b \)Including 38 patients receiving crossover treatment.

\( c \)Cancer staging according to the American Joint Committee on Cancer (6th ed., 2001). The 98 patients in the clinical association were all metastatic or recurrent CRC patients regardless of their initial stages.

\( d \)High frequency of MSI or 10% or less MLH1/MSH2 expression by immunohistochemistry.
Biological utility assay

The candidate SNPs by clinical association analysis were assessed using a biological utility assay that included mutagenesis and transfection, cell viability, and caspase-3 activity measurements. ANXA11 cDNA [Korea Research Institute of Bioscience & Biotechnology (KRIIBB, Daejeon, Korea)] was amplified by PCR and subcloned into a Myc/His-tagged pcDNA3 vector. LIFR, LINS1, DFN831 (KRIIBB), ISX (ImaGenes), and cDNAs were amplified and subcloned into an HA-tagged pcDNA3 vector. The substitution allele of each clone was generated using a site-directed mutagenesis kit (Intron Biotechnology). Each mutagenesis was then confirmed by DNA sequencing analysis. Among the 12 CRC cell lines, 4 (RKO, HCT116, HCT15, and SW480; obtained from American Type Culture Collection) were preferentially chosen for their short doubling time and because they carried at least one chemosensitive allele to the targeted regimens. Transient transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. Stably expressing cells were generated by G418 selection for 10 days. At least 2 clones bearing the reference or substitution alleles were established for each cell line. The clones were confirmed by Western blotting, using either an anti-Myc or anti-HA antibody (Santa Cruz Biotechnology). Cytotoxicity was assayed by measuring cellular MTT redox activity. Caspase-3 activity was additionally measured for clones showing a significant viability difference between the reference and substitution alleles by Western blotting, using an anti-caspase-3 antibody (Cell Signaling Technology).

Statistical analysis

Association of an SNP with in vitro chemosensitivity was examined by linear regression, using PLINK (version 1.06). WGAViewer was used to create a Manhattan plot showing the $-\log_{10}(P)$ calculated from the drug efficacy association analysis (16). Genotype and allele frequencies were compared in terms of drug responses and case–control associations by cross-table analysis, using Fisher’s exact test with 2-sided verification. The viability outcomes between clones with the reference and substitution alleles were compared using Student’s $t$ test. Cytotoxicity was also verified with potential confounders by multivariate analysis, using logistic regression. Overall and progression-free survival (OS and PFS) rates were compared and verified using the Kaplan–Meier method with a log-rank test and Cox’s regression model, respectively. Multiple comparisons in the current study were adjusted using the Bonferroni correction by the number of genes tested for each regimen. Statistical significance was defined as $P < 0.05$. All calculations were performed using SPSS software (ver. 18; SPSS Inc.).

Results

Initial genome-wide screening of chemosensitive SNPs

Among a total of 651,262 SNPs, the top SNPs with $P < 10^{-3}$ were listed for the 6 regimens: the 2,232 and 1,931 SNPs were highly associated with efficacy of bevacizumab and cetuximab regimens, respectively, by examining each regimen on 118 tumor samples (Supplementary Fig. 2). To select primary candidate SNPs, we focused on nonsynonymous SNPs (nsSNP) that were strongly linked to the associated SNPs ($r^2 > 0.8$) in the Japanese HapMap population (WGAViewer) with an MAF > 15% in the Japanese and Han Chinese populations (www.hapmap.org). Thirty-four nsSNPs were identified as being potentially associated with

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Gene</th>
<th>Chromosome</th>
<th>SNP ID</th>
<th>Alleles</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab + FOLFIRI</td>
<td>UTRN</td>
<td>6</td>
<td>rs1534443</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>ANXA11</td>
<td>10</td>
<td>rs1049650</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>TNC</td>
<td>9</td>
<td>rs13321</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>LINS1</td>
<td>15</td>
<td>rs1124722</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>SEMA4B</td>
<td>15</td>
<td>rs4932005</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>DFN831</td>
<td>9</td>
<td>rs2274159</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>ISX</td>
<td>22</td>
<td>rs361863</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>LIFR</td>
<td>5</td>
<td>rs3729740</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>MDM1</td>
<td>12</td>
<td>rs2306393</td>
<td>T</td>
<td>C</td>
</tr>
</tbody>
</table>

Abbreviations: FL, 5-FU/leucovorin; FOLFIRI, FL + irinotecan; FOLFOX, FL + oxaliplatin.
Table 3. Genotype frequencies of relevant candidate SNPs associated with overall responses\(^a\) in patients treated with bevacizumab and cetuximab regimens\(^b\)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>Genotype(^c)</th>
<th>Bevacizumab regimen</th>
<th>Cetuximab regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Responders/all patients (%)</td>
<td>OR</td>
</tr>
<tr>
<td>UTRN</td>
<td>rs1534443</td>
<td>CC</td>
<td>7/26 (26.9)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT + TT</td>
<td>13/43 (30.2)</td>
<td>1.176</td>
</tr>
<tr>
<td>TNC</td>
<td>rs13321</td>
<td>CC</td>
<td>5/24 (20.8)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CG + GG</td>
<td>15/45 (33.3)</td>
<td>1.9</td>
</tr>
<tr>
<td>ANXA11</td>
<td>rs1049550</td>
<td>CC + CT</td>
<td>9/32 (28.1)</td>
<td>1</td>
</tr>
<tr>
<td>LNS1</td>
<td>rs11247226</td>
<td>TT</td>
<td>11/37 (29.7)</td>
<td>1.081</td>
</tr>
<tr>
<td>SEMA4B</td>
<td>rs4932305</td>
<td>AA</td>
<td>14/46 (30.4)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG + GG</td>
<td>6/23 (26.1)</td>
<td>0.807</td>
</tr>
</tbody>
</table>

NOTE: Bold font, \(P < 0.05\).
\(^a\)Tumor response was assessed using RECIST criteria (14). Objective (OR = CR + PR) and disease control (DCR = CR + PR + SD) responses. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
\(^b\)Including bevacizumab or cetuximab with FOLFIRI (5-FU/leucovorin + irinotecan) and FOLFOX (5-FU/leucovorin + oxaliplatin).
\(^c\)All genotypes were compared between patients with 2 reference alleles and patients with 2 substitution alleles in addition to patients with 1 reference allele with 1 substitution allele (dominant model), except for ANXA11 rs1049550 (recessive model), using Fisher’s exact test.
\(^d\)The \(P\) values using 2-sided verification were shown as unadjusted (without parenthesis) and adjusted (parenthesis) values using Bonferroni corrections.
the efficacy of the 4 regimens (Supplementary Table 2). For the practical purpose, we further narrow down to 10 SNPs, excluding 13 SNPs with significant cross-sensitivity to the FOLFOX and FOLFIRI regimens, 7 SNPs with no expression in CRC or referring to functionally undetermined genes, and 4 SNPs located in the same linkage disequilibrium (LD) block. Because 1 SNP (MTR rs1805087) exhibiting a significant departure from HWE (DHW) in control populations was additionally excluded (Supplementary Table 3), 9 candidate SNPs were finally subjected to clinical and biological validations (Table 2). The mean difference of \textit{in vitro} chemosensitivity was 16.9\% (range, 7.6\%–26.5\%) between the 2 alleles of the 9 candidate SNPs (Supplementary Table 4). Among the 118 tumor samples of initial screening, the \textit{in vitro} chemosensitivities of bevacizumab or cetuximab regimen were not associated with sex, age, tumor stage, and microsatellite instability (MSI; $P = 0.06–0.716$). However, 5 SNPs (i.e., LINS1 rs11247226, SEMA4B rs4932305, DFNB31 rs2274159, and 15x rs361863) were significantly associated with their specific chemosensitivities on a multivariate analysis, using these potential confounders ($P = 0.005–0.047$).

**Identification of chemosensitive SNPs to targeted regimens**

An objective response was achieved in 27.5\% and 25.4\% of bevacizumab- and cetuximab-treated patients, respectively. A disease control response was achieved in 63.8\% and 62.7\% of bevacizumab- and cetuximab-treated patients, respectively (Supplementary Table 5). The genotypes of the 9 chemosensitive SNPs in 118 patients with initial genome-wide screening did not differ with those in 98 patients with targeted therapy ($P = 0.127–0.484$). Among these 9 chemosensitive SNPs, specific genotypes of 3 SNPs (ANXA11 rs1049550, LINS1 rs11247226, and DFNB31 rs2274159) were associated with objective or disease control responses (Table 3). The SNP LIFR rs3729740 tended toward an association.

For the bevacizumab regimens, patients carrying homozygous substitution alleles (TT) of ANXA11 rs1049550...
exhibited greater disease control rates than those with homozygous or heterozygous reference alleles (CC and CT; \( P = 0.044 \)). Patients carrying heterozygous or homozygous reference alleles (AG and GG) of LIN51 rs11247226 exhibited greater disease control rates than those with homozygous reference alleles (AA; \( P = 0.033 \)). For the cetuximab regimens, patients carrying homozygous reference alleles (GG) of DFNB31 rs2274159 or LIFR rs3729740 exhibited greater or a tendency toward greater response than those with heterozygous or homozygous substitution alleles (GA and AA; \( P = 0.025 \) and 0.07, respectively). However, these 4 SNPs did not exhibit a significance adjusted by the number of SNPs tested for each regimen.

KRAS mutations at codons 12 and 13 were identified in 22 of 66 samples (33.3%), and acneform rash occurred in 41 of 67 patients (61.2%) who received cetuximab regimens. Wild-type KRAS codons 12 and 13 (\( P = 0.008 \)) and acneform rash (\( P = 0.006 \)) were associated with enhanced disease control responses to cetuximab regimens. The KRAS mutation was not related to specific genotypes of DFNB31 rs2274159 (\( P = 0.825 \)) or LIFR rs3729740 (\( P = 0.079 \)). Among these 98 patients with targeted therapy, overall responses were not associated with sex, age, tumor stage, and MSI (\( P = 0.24 – 0.759 \)). However, the 2 SNPs ANXA11 rs1049550 and DFNB31 rs2274159 were significantly associated with chemosensitivities to bevacizumab regimen and cetuximab regimen, respectively, on a multivariate analysis, using these potential confounders (\( P = 0.015 \) and 0.017, respectively).

### Identification of chemosensitive SNPs related to survival outcome and adverse events

In patients who received cetuximab regimens, patients carrying the homozygous reference alleles of ISX rs361863 (CC) seem to exhibit longer PFS periods than those carrying heterozygous or homozygous substitution alleles (CT and TT; PFS, mean ± SEM: 8.5 ± 1.2 months

![Figure 2. Reference allele (C) of the SNP ISX rs361863 (C allele; C1 and C2 clones) is more sensitive to FRC-induced cytotoxicity than the substitution allele (T allele; T1 and T2 clones). Stable expression of ISX respective allele was confirmed by Western blot, using an anti-HA antibody (A and D). RKO and HCT116 cell clones stably expressing the reference or substitution allele (a and d, respectively) were incubated with FRC (50 \( \mu \)g/mL of 5-FU, 10 \( \mu \)g/mL of leucovorin, 50 \( \mu \)g/mL of irinotecan, and 50 \( \mu \)g/mL of cetuximab) for 24 hours. Drug response activity was determined by cell viability assay (MTT assay; B and E). Caspase-3 activation in the presence or absence of FRC was analyzed by Western blot (C and F). Actin was used as a loading control. Values are means ± SEM of quadruplicates. \( P \) values (Student’s t test) are displayed over the bars. Bold font, \( P < 0.05 \).]
vs. 4.4 ± 0.5 months, \( P = 0.046 \) (unadjusted); 0.23 (adjusted). The specific genotype of ISX rs361863 was not correlated with mutations of KRAS codons 12 and 13 \( (P = 0.567) \).

Grade IV hematologic toxicities occurred in 8.7% and 4.5% of patients receiving bevacizumab and cetuximab regimens, respectively (Supplementary Table 6). However, these adverse events were not associated with specific genotypes of the 5 chemosensitive SNPs chosen by the overall response or survival outcome.

**In vitro cytotoxicity of transfected CRC cells**

Cytotoxicity analysis with MIT showed that RKO and HCT116 cells expressing the reference allele \((G)\) of LIFR rs3729740 were more sensitive to FXC (FOLFOX + cetuximab)-induced cell death than those expressing the substitution allele \((A; P ≤ 0.001; \text{Fig. 1B and E})\). Cells expressing the reference allele \((C)\) of ISX rs361863 were more sensitive to FRC (FOLFIRI + cetuximab)-induced cell death than those expressing the substitution allele \((T; P ≤ 0.001–0.024; \text{Fig. 2B and E})\). Caspase-3 was more activated in CRC cells expressing the reference alleles of LIFR rs3729740 or ISX rs361863 than in those expressing the substitution alleles to FXC and FRC regimens, respectively \((P ≤ 0.001–0.004; \text{Figs. 1 and 2C and F})\). Enhanced chemosensitivity of the reference allele \((G)\) of the other FRC-chemoresponsive SNP, DFNB31 rs22274159, was variably identified in some clones of CRC cells (Supplementary Fig. 3A). Otherwise, RKO cells expressing the substitution allele \((T)\) of ANXA11 rs1049559 were more sensitive to FRB (FOLFIRI + bevacizumab)-induced cell death than those expressing the reference allele \((C; P < 0.001–0.026; \text{Supplementary Fig. 3B})\). Differential cytotoxicity to FXB was not identified for the LINS1 rs11247226 alleles (Supplementary Fig. 3C).

A summarized schema, comprising the 3-step process of initial screening, clinical endpoint, and *in vitro* findings, is shown in Figure 3.

![Figure 3](https://example.com/figure3.png)

**Discussion**

It is vital to identify biomarkers that can predict which cancer patients would most benefit from a specific chemotherapeutic regimen (e.g., with maximal efficacy and minimal toxicity). To date, most biomarkers focus on drug metabolism and downstream processes. For example, polymorphisms of dihydropyrimidine dehydrogenase (DPYD) and thymidylate synthase (TYMS) genes are reportedly correlated with increased toxicity and reduced response rate, respectively, to 5-FU-based chemotherapy (17). The *UGT1A1* +28 polymorphism was also identified as the major predictive pharmacogenetic marker for severe hematologic toxicity of neutropenia to irinotecan. With the recently available knowledge of concrete human genetic variations, HapMap LCLs have been introduced in pharmacogenomic studies to identify genetic loci responsible for drug response and toxicity (8). One study used Epstein–Barr virus (EBV)-transformed LCLs to perform GWA experiments designed to identify genetic variations correlated with variations in gemcitabine and AraC IC_{50} values (18). One limitation of LCL use is the possibility that EBV transformation might influence drug sensitivity and expression profiles because transfected genes either are not expressed in these cell lines or are downregulated after transformation (18). In this study, we propose a surrogate SNP discovery scheme that uses a 3-step process of genome-wide SNP screening, clinical association analysis, and biological utility assessment. This method may reduce the huge sample number, cost, and time associated with GWAS.

In the first step of this study, genome-wide chemosensitive SNPs were filtered in association with individual tumor cell apoptosis to a specific regimen, using an *in vitro* chemosensitivity assay. Despite its inherent limitations (e.g., reproducibility), the MIT-based tissue culture assay is a valuable tool for predicting individual chemosensitivity to clinical responses (19). We chose nsSNPs located in strong LD in Japanese populations. The set of associated variants might include those with particularly strong biological credentials for the nsSNP in a compelling biological candidate (20). Otherwise, alterations in splicing efficiencies due to a single base substitution have rarely been observed (21). The MAF was determined in the oriental populations including Japanese and Han Chinese. For a combined population of various races without a sufficient number in each population, unique genetic findings in one population may be masked by the noise produced by the lack of a genetic effect in another (10). One SNP exhibiting significant DHW in the control populations was excluded in the current study. Most SNPs showing extreme DHW in controls can be safely discarded (20). However, overenthusiastic use of HWE as a quality criterion can be counterproductive, given that a modest disequilibrium can signify a true association (22).

In the second step, pharmacokinetic and pharmacodynamic parameters that were somewhat disregarded in the first step were evaluated as candidate SNPs by clinical...
association analysis. False discoveries, possibly occurring from multiple tests during GWA, could be diminished by replication in the other independent subjects. An MAF of more than 15% provided the least sample number (~70 patients) for clinical association analysis to establish an 85% chance for allele discrimination. In most circumstances, particularly when the total GWA sample size has financial or operational constraints, efforts to enrich case selection are likely to improve power, obviating selection of familial cases or extreme individuals (20).

Finally, type I errors, such as from the limited sample size during the first and second steps, were further eliminated by use of a biological utility assay with cloned cells expressing specific alleles of candidate SNPs. Cell lines can be used as model systems for cellular pharmacologic effects such as drug-induced cytotoxicity or apoptosis, biochemical effects, enzymatic reactions (8). In the current study, at least 2 clones from different cell lines were used for each regimen, assuming tumor cell heterogeneity in individual patients and clonal variation. Because targeted chemotherapy is administered to patients who are resistant to traditional regimens, a chemosensitive marker is required to exclude completely unresponsive subjects. Accordingly, the 4 CRC cell lines in our experiments were chosen because each had at least 1 chemosensitive allele of the 5 candidate SNPs to the respective regimen, minimizing negative responsiveness to the examined allele. The MTT-based cell viability assay was verified using caspase-dependent apoptosis. The main mechanism of cell death leads to a cascade of events that ultimately converge in the activation of an effector enzyme, caspase-3 (23).

Overexpression of EGFR is reported in 60% to 80% of patients with metastatic CRC (2). The binding of EGF or TGF-α to EGFR initiates a cascade of intracellular signaling that stimulates tumor cell-cycle progression and proliferation, activating 2 major downstream pathways [RAS/RAF/MEK (MAP/ERK kinase)/ERK (extracellular signal regulated kinase) and PI3K/Pten/AKT; refs. 2, 4]). Cetuximab, which selectively binds to EGFR, inhibits EGFR-mediated intracellular signaling. It increases the response to both oxaliplatin- and irinotecan-based regimens and thereby plays a useful role in addition to conversion chemotherapy (3). Patients and CRC cells carrying reference alleles of LIFR rs3729740 and ISX rs361863 seemed to display improved disease control responses and PFS rates to EXC and FRC, respectively. According to the cell viability and caspase-3 activation assays, the RKO and HCT116 CRC cells transfected with each reference allele were consistently chemosensitive. Leukemia inhibitory factor receptor (LIFR) is an integral component of the glycoprotein 130–LIFR signaling complex that participates in signal transduction by IL-6 cytokine family members (24). Various carcinoma cells constitutively express LIF and receptor (LIFRb and gp130) mRNAs (25). A direct link between the PI3K/AKT–FOXO and the LIFR/STAT3 pathways suggests LIFR loss of function may act in concert with Ras/RAF/MAPK signaling by using LIFR as a tumor suppressor in glioblastoma (26). In the clinical setting, deregulation of either PI3K or PI3K impairs the response to cetuximab-based treatment in patients with CRC (4). Thus, the association of LIFR expression and cetuximab chemosensitivity may be constitutive according to the EGFR-PTEN and LIFR link. As yet, the influence of intestine-specific homeodomain transcription factor ISX on various anticancer regimens has not been investigated. Recently, ISX paralleled by intestinal SR-B1 and BCMO1 expression was reported to act as a retinoic acid–sensitive gatekeeper that controls vitamin A production (27). KRAS mutations that predominantly involve activating mutations at codons 12 and 13 were identified in one third of our patients, which is in the mid range (20%–46%) of previous studies of CRCs (4, 28). As in the current study, wild-type tumor KRAS and skin toxicity are consistently linked to the efficacy of anti-EGFR treatments, including cetuximab and panitumumab (1, 4, 28, 29). Because the LIFR rs3729740 and ISX rs361863 genotypes did not correlate with KRAS codons 12 and 13 mutations, these two SNPs can be separately informative in addition to the KRAS mutation in predicting cetuximab chemoresponsiveness.

Unfortunately, no efficient chemotherapy marker has been identified for bevacizumab regimens. Circulating VEGF and endothelial cell levels have been evaluated as surrogate markers of angiogenic activity (3), but clinical trials could not reliably confirm the association. In the current study, patients carrying substitution alleles of ANXA11 rs1049550 showed greater disease control rates, respectively, to bevacizumab regimens than those carrying reference alleles. Although our cell viability assay on allele-specific transfected cells did not show significantly reduced apoptosis in a quarter of clones, the overall chemosensitivity to bevacizumab regimens cannot be ignored when considered together with clinical association results on a multivariate analysis. Annexin A11 (ANXA11) has been assigned to a spectrum of regulatory functions in calcium signaling, cell division, vesicle trafficking, and apoptosis (30). The mSNP rs1049550 of ANXA11 leads to an amino acid change (R230C, basic arginine to a polar cysteine) of the first conserved annexin domain that is responsible for intracellular Ca2+-dependent trafficking.

To our knowledge, this is the first study to identify SNP markers chemosensitive to targeted regimens in a 3-step process of genome-wide analysis. The 2 novel markers of the current study LIFR rs3729740 and possibly ISX rs361863 will hopefully improve the prediction of metastatic CRC patient sensitivity to cetuximab regimens. However, these chemosensitive SNPs reported must be further validated in large cohorts. Furthermore, the regulatory pathway associated with potentially surrogate SNPs remains to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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