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Research Article

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Prognostic Model of Stage II/III Colon Cancer Constructed using Gene Expression Subtypes and *KRAS* Mutation Status

Kengo Gotoh[1#], Eiji Shinto[2#], Yuichiro Yoshida[1*], Hideki Ueno[2], Yoshiki Kajiwara[2], Masato Yamadera[2], Ken Nagata[2], Hitoshi Tsuda[3], Junji Yamamoto[2] and Kazuo Hase[2]

Abstract

Objectives: Cancer subtypes classified according to DNA microarray data predict prognosis with high accuracy. Here we constructed a new colon cancer (CC) subtype classification based on only of genes with known biological functions with the aim of establishing a new prognostic model for clinical use.

Methods: We performed an expression correlation analysis using data for 73 primary CC cases in the public dataset (learning set), focusing on genes located on the long arms of chromosomes 18 and 20 and stromal-related genes. We determined the representation of each gene in the modules with closely correlated expression levels in the same module. Mutations in *KRAS, BRAF* and *TP53* were assessed using direct sequencing. Microsatellite instability (MSI) was analyzed using the Bethesda reference panel.

Results: We constructed a discriminant model with a view to classifying CC into three subtypes ("stromal", "chromosomal instability [CIN]-like", "MSI-like") based on the expression levels of 55 genes of the Learning set. When we applied this predictor to microarray data from other patients with stage II/III colon cancer (n=258, test set), we discovered a significant difference in disease-free survival between the stromal subtype and the other subtypes (p=1.25e-03). Accordingly, we created an integrated prognostic model for classifying the patients into high- and low-risk groups according to the expression levels of the 55 genes and *KRAS* mutations (p=1.56e-06). Analysis of independent specimens from patients with stage II/III colon cancer who underwent radical resection (n=59, validation set) confirmed the prognostic value of our model (p=4.75e-02).

Conclusion: The model produced a biologically discriminatory classifier that associated MSI status with the risk of recurrence that may be clinically applicable to the selection of patients with Stage II/III CC for adjuvant therapy.

Keywords

Colon cancer; Prognosis; DNA microarray; Chromosomal instability; Microsatellite instability; Stroma

Introduction

Colorectal cancer (CRC) is the second and third most common cancer of women and men, with nearly 1.4 million new cases diagnosed worldwide in 2012 [1]. In the same year, there were 693,933 CRC-related deaths (8.5% of all cancer deaths) [1]. The postoperative recurrence rate of Stage II CRC is 10%-20% [2]. Postoperative adjuvant chemotherapy is recommended for patients with Stage II CRC with high-risk pathological factors [3]. Nevertheless, risk factors have not been determined with precision, and this recommendation is not justified through randomized control trials. Large-scale studies of patients with stage III CRC show that postoperative adjuvant chemotherapy effectively prolongs survival [4]. However, prognosis is better for patients with Stage IIIA compared with Stage IIB/IIC disease [2]. Therefore, precise prediction of prognosis is urgently required for patients with Stage III CRC as well. CRC subtypes classified according to DNA microarray data predict prognosis with high accuracy [5-9]. However, there is little consistency among reports regarding the genes selected for subtype classification. This diversity is explained by the employment of different methodologies. For example, some researchers determine the expression levels of genes with known functions (candidate-gene approach), while others select genes through unbiased microarray analysis (data-driven approach) [10]. The former strategy risks missing potentially important but poorly annotated genes, while the latter is associated with a serious risk of over fitting to the training set.

Most colon cancers (CCs) can be separated into at least two types, the Chromosomal instability (CIN) type or the Microsatellite instability (MSI) type [11]. The prognosis of MSI-type patients with CC is more favorable compared with that of those with CIN-type [12-15]. Chromosomal abnormalities in CC frequently occur on the long arms of chromosomes 18 and 20 [16,17]. Therefore, we hypothesized that we could distinguish CIN-type from MSI-type by analyzing the expression levels of genes located in these chromosomal regions. Otherwise, the rate of the MSI-type would be higher than the rates of the other subtypes. Moreover, convincing evidence reveals an association between the number of infiltrating stroma cells in tumor tissue and the prognosis of CC [5,18-20]. We therefore reasoned that the analysis of expression levels of stroma-related genes might aid in the definition of cancer subtypes.

Here we developed a new model of classifying CC subtypes using a methodology that combines candidate-gene and data-driven approaches to overcome the disadvantages of each. The method involves analysis of the unique combination of genes (referred to as modules) located on 18q and 20q as well as those expressed by the tumor stroma. First, we extracted these gene modules through correlating gene expression levels and selected representative genes from each module. Using a 55 gene-expression signature, we subtyped CCs into CIN-like, MSI-like, and stromal subtypes and assessed their prognostic significance. Moreover, we evaluated the relationship between common gene mutations in CC and clinical outcomes of patients with each CC subtype. This allowed us to establish a new prognostic model based on the assessment of a novel combination of the three gene modules and *KRAS* mutations. The clinical significance of the proposed prognostic model was tested using an independent

***Corresponding author:** Yuichiro Yoshida, Central Research Laboratories, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe 651-2271, Japan, Tel: 81-78-991-2367; Fax: 81-78-992-7065; E-mail: Yoshida.Yuichiro@sysmex.co.jp

#These authors contributed equally to this work.

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public dataset, and its clinical utility was demonstrated using an in-house dataset and clinical data.

Materials and Methods

Public microarray datasets

We used two public datasets to construct the model. Gene expression data from the H. Lee Moffitt Cancer Center [21], which is part of the GSE14333 (learning set; n=73, Dukes stages A, B and C CC, without adjuvant chemotherapy), were used for gene cluster discovery. Gene expression data from The French Cartes d'Identite´ des Tumeurs (CIT) program, which is a part of GSE39582 [9] (test set; n=258, Stages II and III CC, without adjuvant chemotherapy), were used to construct our prognostic model. The datasets are available from the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo).

Tissue samples

We collected tumor tissue specimens from patients with Stage II/III CC who underwent curative surgery at the National Defense Medical College (validation set; n=60, without adjuvant chemotherapy, 2002-2003) according to a protocol approved by the Institutional Review Board. Archived primary tumor and adjacent non-neoplastic colon tissues were collected during surgery. Frozen sections with a tumor component >60% were prepared from each specimen and subjected to DNA microarray and gene mutation analyses. Patients and tumor characteristics of the three datasets are presented in Table 1.

Construction of a molecular subtype predictor using a learning set

To discover functional gene modules, we performed co-expression analysis using R software. We filtered out low intensity probes (average signal intensity<350) and low deviation probes (CV<0.18) from all probes in GSE14333 microarray data. Then we calculated Pearson's correlation coefficient of gene expression between the remaining 10,449 genes, and we extracted gene modules from gene clusters. After we identified three gene modules representing the long arms of chromosomes 18 and 20 and stromal-related genes, representative genes were selected according to the significant associations of their expression levels within each module as well as to information about their biological functions. We were limited to 73 samples because of our relatively narrow selection criteria.

Accordingly, we next constructed a subtype predictor with reference to Hu et al. [22]. First, we performed hierarchical clustering of the learning dataset using the expression profiles. We next averaged the gene expression profiles (centroid) for samples assigned to each subtype using the hierarchical clustering dendrogram as a guide. New

Table 1: Patient characteristics.

Characteristics		Learning set (n=73)	Test set (n=258)	Validation set (n=59)	P value
Age at diagnosis	Mean, years (SD)	68.9(12.0)	70.9(12.8)	69.4(8.3)	0.308
	<40, n (%)	1(1.4%)	6(2.3%)	0(0%)	
	40-49, n (%)	2(2.7%)	13(5.0%)	1(1.7%)	
	50-59, n (%)	11(15.1%)	22(8.5%)	7(11.9%)	
	60-69, n (%)	22(30.1%)	61(23.6%)	20(33.9%)	
	>70, n (%)	37(50.7%)	156(60.5%)	31(52.5%)	
Sex, n (%)	Male	37(50.7%)	147(57.0%)	32(54.2%)	6.00e-03
	Female	36(49.3%)	111(43.0%)	27(45.8%)	
Stage, n (%)	I	21(28.8%)	0 (0%)	0 (0%)	2.42E-22
	II	37(50.7%)	203(78.7%)	43(72.9%)	
	III	15(20.5%)	55(21.3%)	16(27.1%)	
Location, n (%)	Right	35(47.9%)	118(45.7%)	25(42.4%)	0.814
	Left	38(52.1%)	140(54.3%)	34(57.6%)	
Follow up, n (%)	Mean	4.02(0.08-11.9)	4.41(0-16.8)	5.92(0.05-11.5)	2.00e-03
Relapse, n (%)	+	11(15.1%)	62(24.0%)	6(10.2%)	0.027
	-	62(84.9%)	196(76.0%)	53(89.8%)	
KRAS mutant, n (%)	+	-	86(34.5%)	23(39.0%)	0.624
	-	-	163(65.5%)	36(61.0%)	
	N/A	73	9	0	
BRAF mutant, n (%)	+	-	33(13.8%)	2(3.4%)	0.046
	-	-	206(86.2%)	57(96.6%)	
	N/A	73	19	0	
TP53 mutant, n (%)	+	-	79(49.7%)	35(59.3%)	0.266
	-	-	80(50.3%)	24(40.7%)	
	N/A	73	99	0	
MSI, n (%)	+	-	47(20.7%)	5(8.6%)	0.053
	-	-	180(79.3%)	53(91.4%)	
	N/A	73	31	1	
Recurrence, n (%)	+	11(15.1%)	62(24.0%)	6(10.2%)	0.272
	-	62(84.9%)	196(76.0%)	53(89.8%)	
5-year survival rate, %		83.9%	72.5%	89.5%	-

*__Note:__ P values for characteristics, except for follow-up, were obtained using the Chi-squared test. A p value for follow-up was using a *t* test. N/A, not available; SD, standard deviation

samples were assigned to the nearest subtype/centroid as determined using the Spearman correlation. This predictor was then used to classify tumors into subtypes within the test and validation datasets.

Gene expression analysis

Total RNA and DNA were extracted using an RNeasy Mini Kit and a QIAamp DNA Mini Kit (Qiagen Valencia, CA). The RNA quality of samples was then assessed using a 2100 Bio analyzer (Agilent Technologies, Palo Alto, CA). The preparations were of optimal quality for oligonucleotide microarray analysis, as shown by the integrity of the 18S and 28S ribosomal RNA bands. Gene expression data were generated using an Affymetrix Human Genome U133 Plus 2.0 Gene Chip Array (Affymetrix, Santa Clara, CA). Each dataset was normalized using the Microarray Suite (MAS) 5.0 in the Expression console (Affymetrix). Data are available from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE 92921).

Gene mutation and MSI analysis

The mutations in exon 2 and 3 of *KRAS* [23], exon 15 of *BRAF* [24] and exons 5 to 9 of *TP53* [25] were assessed using direct sequencing as previously described. MSI was analyzed using a panel of five microsatellite loci of the Bethesda reference panel [26]. MSI-high tumors were classified as deficient in DNA mismatch repair (dMMR), and MSI-low and microsatellite-stable (MSS) tumors as proficient MMR (pMMR).

Survival analysis

We used Kaplan-Meier analysis and the log-rank test to estimate prognosis. Recurrence-free survival (RFS) was defined as the time from surgery until primary tumor recurrence. $P<0.05$ indicates statistical significance, and all statistical tests were two-tailed. All statistical analyses were conducted using the statistical software EZR [27].

Results

Identification of three CC subtypes and development of a subtype predictor

We performed an expression correlation analysis using data for 73 primary CC cases in GSE14333 (learning set, Dukes Stage A/B/C CC). We identified several gene clusters, which included gene groups located on chromosomes 18q, 20q, and 13q as well as the gene groups associated with the immune response, cell proliferation, and stromal related genes (Figure 1).

Among these gene groups, we focused on genes located on the long arms of chromosomes 18 and 20 and stromal-related genes. We determined the representation of each gene in the modules with closely correlated expression levels in the same module. Finally, we extracted 55 genes according to their representation among the three gene groups and information about their biological functions (Table 2).

Unsupervised clustering using the 55 genes revealed that patients with CC (learning set) could be classified into the subtypes as follows: Subtype 1 (n=32; 43.2%), characterized by high expression of stromal genes; Subtype 2 (N=24; 33.8%), associated with the 18q-lower/20q-higher expression than average; and Subtype 3 (n=17; 23.0%), associated with the 18q-higher/20q-lower expression than average (Figure 2).

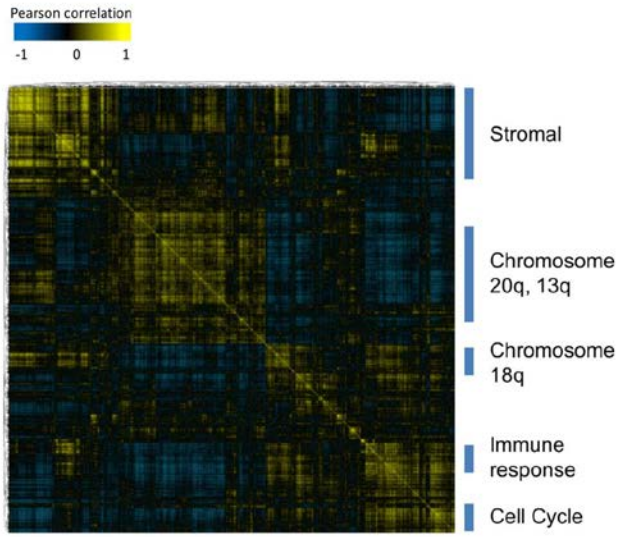

Figure 1: Correlation heat map. The heat map shows pairwise Pearson correlation coefficients between genes. The Pearson correlation coefficients from low to high are indicated in blue to yellow, respectively. The bars on the right side of heat map indicate gene clusters.

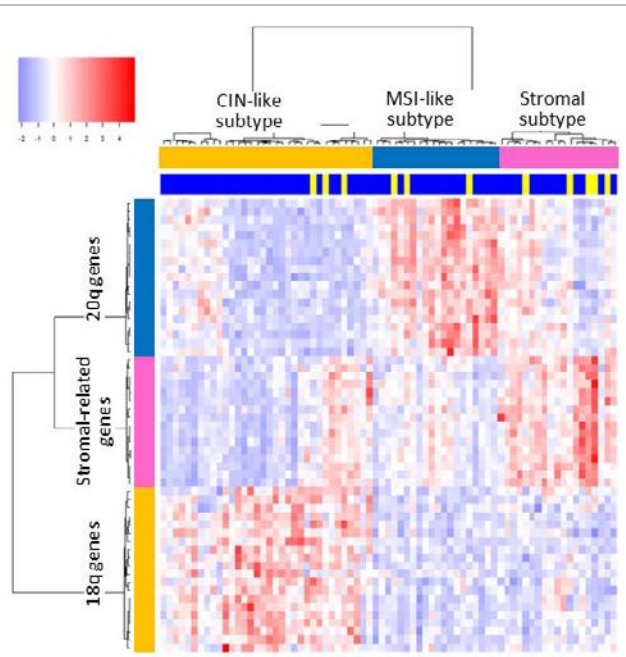

Figure 2: Heat map of unsupervised hierarchal clustering. Unsupervised hierarchical clustering of the learning dataset comprising 55 genes expressed at detectable levels in all samples. Each column represents a sample and each row a gene. Gene expression above and below the median is depicted in red and blue, respectively. The blue and yellow squares in the second upper bar indicate patients with no recurrence and recurrence, respectively.

Subtype 1 was named the 'stromal subtype' because of the high expression of stroma-related genes.

Subtype 2 was named the 'CIN-like subtype', because CIN cancers frequently express the 18q-low/20q-high pattern [16,17]. Subtype 3 was named the 'MSI-like subtype', because we suspect that MSI cases are infrequently categorized as Subtype 2. Thus, Subtype 3 is enriched with MSI cases. The 5-year disease free survival (DFS) rates in the

Table 2: Reference genes.

Affymetrix Probe Set ID	Representative Public ID	UniGene ID	Gene Symbol	Gene Title	Chromosomal Location
200027_at	NM_004539	Hs.465224	*NARS*	asparaginyl-tRNA synthetase	chr18q21.31
201588_at	NM_004786	Hs.114412	*TXNL1*	thioredoxin-like 1	chr18q21.31
202484_s_at	AF072242	Hs.25674	*MBD2*	methyl-CpG binding domain protein 2	chr18q21
202836_s_at	NM_006701	Hs.465498	*TXNL4A*	thioredoxin-like 4A	chr18q23
209366_x_at	M22865	Hs.465413	*CYB5A*	cytochrome b5 type A (microsomal)	chr18q23
217752_s_at	NM_018235	Hs.149185	*CNDP2*	CNDP dipeptidase 2 (metallopeptidase M20 family)	chr18q22.3
218171_at	AF195514	Hs.126550	*VPS4B*	vacuolar protein sorting 4 homolog B (S. cerevisiae)	chr18q21.33
218208_at	NM_025078	Hs.288284	*PQLC1*	PQ loop repeat containing 1	chr18q23
219419_at	NM_024805	Hs.593610	*RBFA*	ribosome binding factor A (putative)	chr18q23
220180_at	NM_025214	Hs.120790	*CCDC68*	coiled-coil domain containing 68	chr18q21
223180_s_at	BC000892	Hs.532835	*C18orf55*	chromosome 18 open reading frame 55	chr18q22.3
223299_at	AF212233	Hs.45107	*SEC11C*	SEC11 homolog C (S. cerevisiae)	chr18q21.32
225301_s_at	AI991160	Hs.720076	*MYO5B*	myosin VB	chr18q21
225407_at	N37023	Hs.551713	*MBP*	myelin basic protein	chr18q23
226924_at	AI016355	Hs.61508	*LOC400657*	hypothetical LOC400657	chr18q22.3
227072_at	BG167480	Hs.654809	*RTTN*	Rotatin	chr18q22.2
227542_at	AU157543	Hs.44439	*SOCS6*	suppressor of cytokine signaling 6	chr18q22.2
227768_at	AB051490	Hs.536490	*ZNF407*	zinc finger protein 407	chr18q23
232594_at	AK001829	Hs.191582	*HSBP1L1*	heat shock factor binding protein 1-like 1	chr18q23
238846_at	AW026379	Hs.204044	*TNFRSF11A*	tumor necrosis factor receptor superfamily, member 11a, NFKB activator	chr18q22.1
202716_at	NM_002827	Hs.417549	*PTPN1*	protein tyrosine phosphatase, non-receptor type 1	chr20q13.1-q13.2
202925_s_at	NM_002657	Hs.154104	*PLAGL2*	pleomorphic adenoma gene-like 2	chr20q11.21
203943_at	NM_004798	Hs.369670	*KIF3B*	kinesin family member 3B	chr20q11.21
204554_at	AL109928	Hs.42215	*PPP1R3D*	protein phosphatase 1, regulatory (inhibitor) subunit 3D	chr20q13.3
204849_at	NM_006602	Hs.126248	*TCFL5*	transcription factor-like 5 (basic helix-loop-helix)	chr20q13.3-qter
206918_s_at	NM_003915	Hs.246413	*CPNE1*	copine I	chr20q11.22
207941_s_at	NM_004902	Hs.282901	*RBM39*	RNA binding motif protein 39	chr20q11.22
208979_at	AF128458	Hs.368971	*NCOA6*	nuclear receptor coactivator 6	chr20q11
209422_at	AL109965	Hs.517044	*PHF20*	PHD finger protein 20	chr20q11.22-q11.23
212237_at	N64780	Hs.374043	*ASXL1*	additional sex combs like 1 (Drosophila)	chr20q11.1
212349_at	AL045513	Hs.472409	*POFUT1*	protein O-fucosyltransferase 1	chr20q11
213090_s_at	AI744029	Hs.18857	*TAF4*	TAF4 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 135kDa	chr20q13.33
218325_s_at	NM_022105	Hs.517172	*DIDO1*	death inducer-obliterator 1	chr20q13.33
224611_s_at	AL118506	Hs.164419	*DNAJC5*	DnaJ (Hsp40) homolog, subfamily C, member 5	chr20q13.33
224815_at	AA148301	Hs.408427	*COMMD7*	COMM domain containing 7	chr20q11.21
225224_at	AL034550	Hs.516978	*C20orf112*	chromosome 20 open reading frame 112	chr20q11.1-q11.23
225313_at	AI627538	Hs.504920	*C20orf177*	chromosome 20 open reading frame 177	chr20q13.2-q13.33
225498_at	AV713673	Hs.472471	*CHMP4B*	chromatin modifying protein 4B	chr20q11.22
225903_at	AL118520	Hs.253319	*PIGU*	phosphatidylinositol glycan anchor biosynthesis, class U	chr20q11.22
1555630_a_at	AF327350	Hs.301853	*RAB34*	RAB34, member RAS oncogene family	chr17q11.2
201069_at	NM_004530	Hs.513617	*MMP2*	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	chr16q13-q21
201426_s_at	AI922599	Hs.455493	*VIM*	Vimentin	chr10p13
201893_x_at	AF138300	Hs.156316	*DCN*	Decorin	chr12q21.33
202351_at	AI093579	Hs.436873	*ITGAV*	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	chr2q31-q32
202450_s_at	NM_000396	Hs.632466	*CTSK*	cathepsin K	chr1q21
202686_s_at	NM_021913	Hs.590970	*AXL*	AXL receptor tyrosine kinase	chr19q13.1
203729_at	NM_001425	Hs.9999	*EMP3*	epithelial membrane protein 3	chr19q13.3
208747_s_at	M18767	Hs.458355	*C1S*	complement component 1, s subcomponent	chr12p13
212067_s_at	AL573058	Hs.524224	*C1R*	complement component 1, r subcomponent	chr12p13
213001_at	AF007150	Hs.653262	*ANGPTL2*	angiopoietin-like 2	chr9q34
218469_at	NM_013372	Hs.40098	*GREM1*	gremlin 1	chr15q13.3
226001_at	AK002174	Hs.272251	*KLHL5*	kelch-like 5 (Drosophila)	chr4p14
226051_at	BF973568	Hs.55940	*SELM*	selenoprotein M	chr22q12.2
229067_at	BF977829	Hs.523529	*SRGAP2P1*	SLIT-ROBO Rho GTPases activating protein 2 pseudo gene 1	chr1p11.2
57715_at	W72694	Hs.241545	*CALHM2*	calcium homeostasis modulator 2	chr10pter-q26.12

CIN-like, MSI-like, and stromal subtypes were 88.2%, 86.4%, and 72.8%, respectively. We found no statistically significant difference, though patients with the stromal subtype tended to have a higher risk of recurrence compared with the other subtypes (Figure 3 and Table 3). For general use, we developed a subtype classifier '55-gene centroid predictor' to divide CCs into the three subtypes, according to a previous report [22].

Prognostic significance of the subtype model and its biological importance for the test set

We applied the subtype predictor to an independent public data set of 258 stage II/III CC samples (GSE39582). The CIN-like, MSI-like, and stromal subtypes accounted for 39.9% (103 specimens), 39.5% (102 specimens), and 20.5% (53 specimens), respectively. The 5-year RFS of patients with the CIN-like, MSI-like, and stromal subtype cancers were 72.2%, 81.2%, and 56.0%, respectively (Figure 4). The log-rank test revealed that there was a significantly higher incidence of recurrent events in the stromal subtype (stromal subtype vs. other subtypes, p=3.62e−03).

The relationships between each subtype and factor in the test dataset are summarized in Table 4. The results supported our assumption that CIN was significantly enriched in CIN-like subtype cases (p=3.86e-15), MSI was significantly enriched in MSI-like subtype cases (p=2.20e-16), and CpG island methylated phenotype (CIMP) was significantly enriched in MSI-like subtype cases (p=2.38e-13). There was a high incidence of *BRAF* mutations

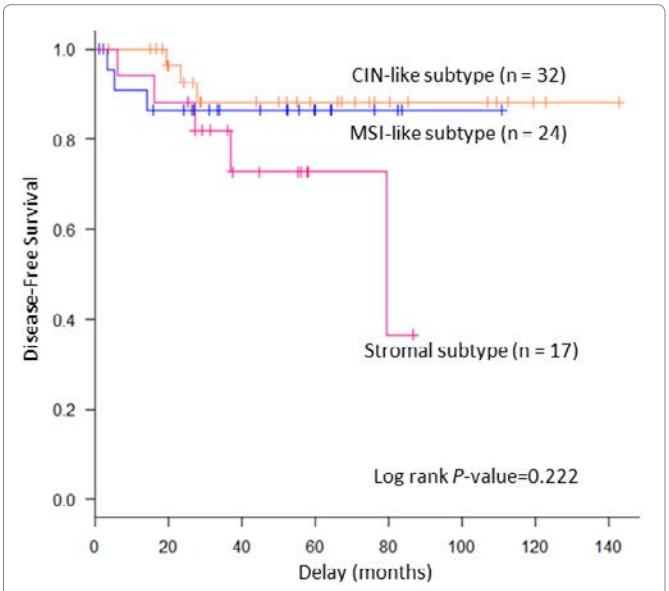

Figure 3: Survival of subtypes in the learning dataset. Kaplan–Meier analysis of disease-free survival times of patients in each subtype. The orange, blue and pink lines indicate the CIN-like, MSI-like and Stromal subtypes, respectively. Censored data are marked at each line. P values were calculated using the log-rank test.

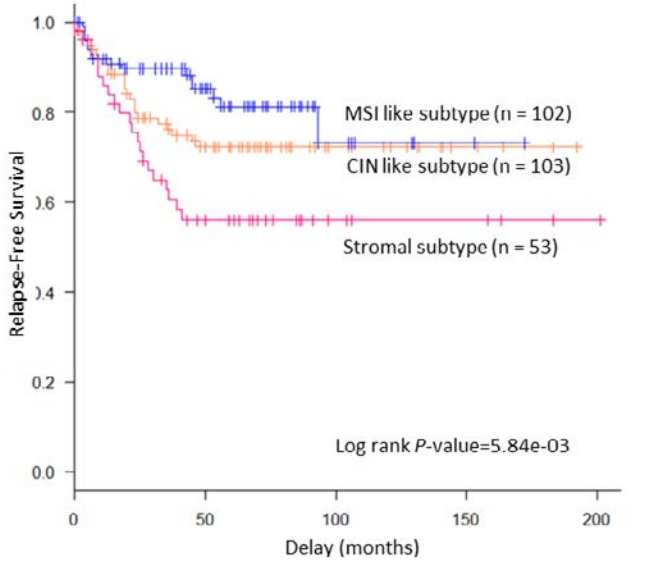

Figure 4: Survival curves corresponding to each subtypes in the test dataset. Kaplan–Meier analysis of RFS of patients in each subtype. The orange, blue and pink lines indicate CIN-like, MSI-like and Stromal subtypes, respectively. Censored data are marked at each line. P values were calculated using the log-rank test.

Table 3: Correlation of subtypes with clinical parameters of the learning set.

Characteristic		CIN-like subtype (n=32, 43.8%)	MSI-like subtype (n=24, 32.9%)	Stromal subtype (n=17, 23.3%)	P value
Age at diagnosis	Mean, Year (SD)	72.9(10.5)	67.2(12.7)	63.8(11.9%)	0.2856
	<40, n (%)	0(0%)	0(0%)	1(5.9%)	
	40-49, n (%)	0(0%)	2(8.3%)	0(0%)	
	50-59, n (%)	4(12.5%)	4(16.7%)	3(17.6%)	
	60-69, n (%)	8(25.0%)	8(33.3%)	6(35.3%)	
	>70, n (%)	20(62.5%)	10(41.7%)	7(41.2%)	
Sex, n (%)	Male	18(56.3%)	11(45.8%)	8(47.1%)	0.724
	Female	14(43.8%)	13(54.2%)	9(52.9%)	
Stage at diagnosis, n (%)	Dukes A	13(40.6%)	5(20.8%)	3(17.6%)	0.315
	Dukes B	13(40.6%)	15(62.5%)	9(52.9%)	
	Dukes C	6(18.8%)	4(16.7%)	5(29.4%)	
Location, n (%)	Right	12(37.5%)	16(66.7%)	7(41.2%)	9.19e-02
	Left	20(62.5%)	8(33.3%)	10(58.8%)	
Recurrence, n (%)	+	3(9.4%)	3(12.5%)	5(29.4%)	0.226
	-	29(90.6%)	21(87.5%)	12(70.6%)	
5-year DFS,%		88.2%	86.4%	72.8%	0.222

*Note: P values for characteristics, except for 5-year DFS, were determined using the Chi-squared test. A p value for 5-year DFS was obtained using the log-rank test. SD, standard deviation

Table 4: Correlation of subtypes with clinical parameters in the test set.

Characteristic		CIN-like subtype (n=103, 39.9%)	MSI-like subtype (n=102, 39.5%)	Stromal subtype (n=53, 20.5%)	P value
Age at diagnosis	Mean, Year (SD)	70.5(11.9)	71.3(14.0)	71(12.3)	0.198
	<40, n (%)	1(1.0%)	4(3.9%)	1(1.9%)	
	40-49, n (%)	4(3.9%)	7(6.9%)	2(3.8%)	
	50-59, n (%)	13(12.6%)	3(2.9%)	6(11.3%)	
	60-69, n (%)	25(24.3%)	21(20.6%)	15(28.3%)	
	>70, n (%)	60(58.3%)	67(65.7%)	29(54.7%)	
Sex, n (%)	Male	63(61.2%)	56(54.9%)	28(52.8%)	0.525
	Female	40(38.8%)	46(45.1%)	25(47.2%)	
Stage at diagnosis, n (%)	II	87(84.5%)	79(77.5%)	37(69.8%)	0.096
	III	48(46.6%)	23(22.5%)	16(30.2%)	
Location, n (%)	distal	80(77.7%)	30(29.4%)	30(56.6%)	3.35e-11
	proximal	23(22.3%)	72(70.6%)	23(43.4%)	
MSI status, n (%)	MSI	0(0%)	47(51.6%)	0(0%)	<2.2e-16
	MSS	93(100%)	44(48.4%)	43(100%)	
	N/A	10	11	10	
CIN status, n (%)	+	82(95.3%)	39(41.9%)	37(84.1%)	3.86e-15
	-	4(4.7%)	54(58.1%)	7(15.9%)	
	N/A	17	9	9	
CIMP status, n (%)	+	2(2.2%)	45(47.4%)	5(11.4%)	2.38e-13
	-	89(97.8%)	50(52.6%)	39(88.6%)	
	N/A	12	7	9	
KRAS mutation status, n (%)	+	24(24.7%)	36(36.0%)	26(50%)	7.79e-03
	-	73(75.3%)	64(64.0%)	26(50%)	
	N/A	6	2	1	
BRAF mutation status, n (%)	+	0(0%)	31(32.0%)	2(4.2%)	1.22e-10
	-	94(100%)	66(68.0%)	46(95.8%)	
	N/A	9	5	5	
TP53 mutation status, n (%)	+	47(67.1%)	13(22.4%)	19(61.3%)	1.09e-06
	-	23(32.9%)	45(77.6%)	12(38.7%)	
	N/A	33	44	22	
Recurrence, n (%)	+	25(24.3%)	16(15.7%)	21(39.6%)	4.19e-03
	-	78(75.7%)	86(84.3%)	32(60.4%)	
5-year RFS, %		72.2%	81.2%	56.0%	5.84e-03

***Note:** P values for characteristics, except for 5-year RFS, were obtained using the Chi-squared test. A p value for 5-year RFS was obtained using the log-rank test. N/A, not available; SD, standard deviation

(p=1.22e-10) in MSI-like subtype cases and a low incidence of *TP53* mutations (p=1.09e-06).

Modification of the subtype model incorporating gene mutation status

When we evaluated the prognostic values of common gene mutations in each subtype, we found that *KRAS* mutations represented a significant risk factor for recurrence (p=2.74e-04) in the CIN-like subtype cases (Figure 5), while *BRAF* and *TP53* mutations did not have significant prognostic value. Accordingly, we created a prognostic model that classified patients with CC into the good or poor prognosis group according to the molecular subtype and *KRAS* mutation status (Figure 6). Our prognostic model classified patients with stage II/III CC into two groups with significantly different risks of recurrence (p=1.56e-06) in the test set GSE39582 (Figure 7).

The results of these analyses are summarized in Figure 6. The relative risks of the subtypes associated with 5-year RFS are as follows: MSI-like, 81.2%; CIN-like, wild-type KRAS, 78.9%; CIN-like, mutant KRAS, 46.6%; and stromal, 56.0%.

Validation of the prognostic model with in-house data set

We next validated the prognostic model with the newly acquired microarray datasets of tissue specimens from 59 patients treated at the National Defense Medical College with resected stage II/III CC who had not received postoperative adjuvant chemotherapy (in-house validation dataset). Patients were classified into the CIN-like (32.2%, 19 specimens), MSI-like (32.2%, 19 specimens), and stromal (35.6%, 21 specimens) subtypes using the 55-gene centroid predictor. Finally, we applied our prognostic model to the independent dataset and demonstrated that it identified high- and low-risk patient groups (p=0.0475) (Figure 8 and Table 5).

Discussion

To develop a commercially available prognostic test for patients with CC that offers unique advantages over existing assays [28-34], we focused on selecting genes with known biological functions that reside on specific chromosomal loci as well as those that are expressed by the tumor stroma known to influence tumor phenotypes (i.e. “rational” vs. “shotgun” approach”). Here we describe such a test that importantly does not infringe on patents for others designed to predict prognosis of patients with CC (Our model described in this

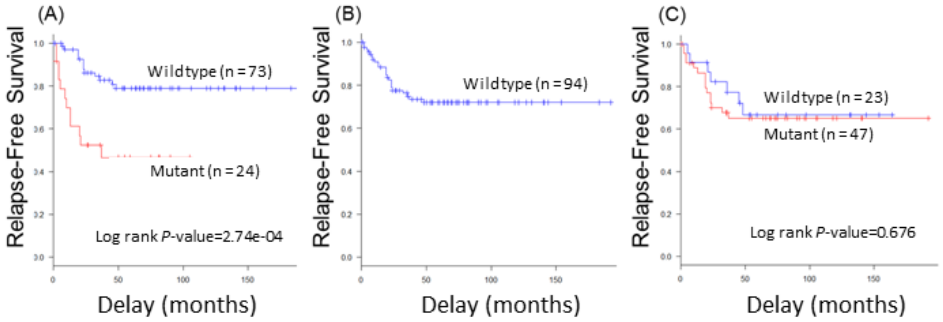

Figure 5: Prognostic values of common gene mutations in the CIN subtype. Kaplan–Meyer analysis of RFS of CIN-like subtype patients in the test dataset compared with patients with wild-type (blue line) or mutated (red line) *KRAS* (A), *BRAF* (B), or *TP53* (C). Censored data are marked at each line.

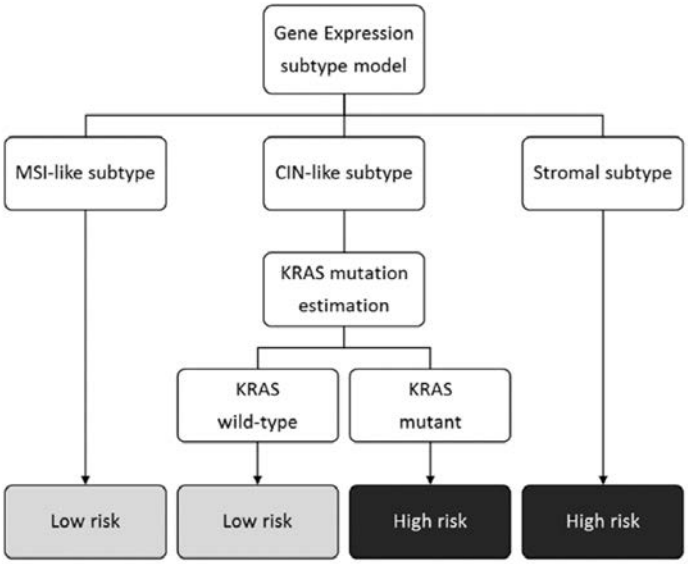

Figure 6: Flow chart of the prognosis model.

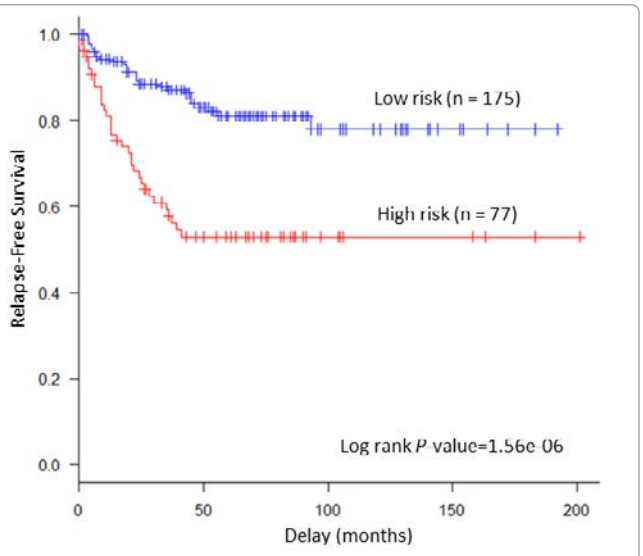

Figure 7: Kaplan-Meier analysis of RFS of patients in the test data predicted with a high versus low risk of recurrence. The blue and red lines indicate low-risk and high-risk patients, respectively. Censored data are marked at each line. *P* values were calculated using the log-rank test.

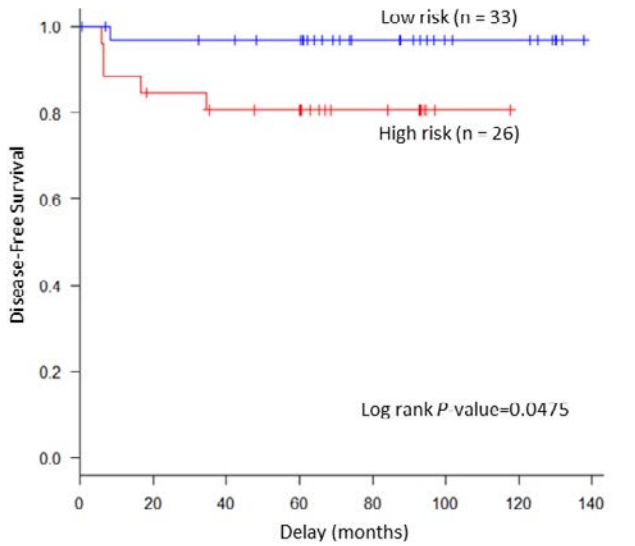

Figure 8: Kaplan-Meier analysis of RFS of patients in the test data predicted with a high versus low risk of recurrence. The blue and red lines indicate low-risk and high-risk patients, respectively. Censored data are marked at each line. *P* values were calculated using the log-rank test.

Table 5: Correlation of subtypes with clinical parameters of the validation set.

Variable		CIN-like subtype (n=19, 32.2%)	MSI-like subtype (n=19, 32.2%)	Stromal subtype (n=21, 35.6%)	P value
Age at diagnosis	Mean, Year (SD)	68.7 (8.3)	71.3 (9.0)	68.3 (7.8)	0.220
	<40, n (%)	0 (0%)	0 (0%)	0 (0%)	
	40-49, n (%)	0 (0%)	1 (5.3%)	0 (0%)	
	50-59, n (%)	3 (15.8%)	1 (5.3%)	3 (14.3%)	
	60-69, n (%)	8 (42.1%)	3 (15.8%)	9 (42.9%)	
	>70, n (%)	8 (42.1%)	14 (73.7%)	9 (42.9%)	
Sex, n (%)	Male	9 (47.4%)	12 (63.2%)	11 (52.4%)	0.648
	Female	10 (52.6%)	7 (36.8%)	10 (47.6%)	
Stage at diagnosis, n (%)	II	12 (63.2%)	16 (84.2%)	15 (71.4%)	0.356
	III	7 (36.8%)	3 (15.8%)	6 (28.6%)	
Location, n (%)	distal	7 (36.8%)	10 (52.6%)	8 (38.1%)	0.545
	proximal	12 (63.2%)	9 (47.4%)	13 (61.9%)	
MSI status, n (%)	dMMR	0 (0%)	5 (27.8%)	0 (0%)	1.87e-03
	pMMR	19 (100%)	13 (72.2%)	21 (100%)	
	N/A	0	1	0	
KRAS mutation status, n (%)	+	5 (26.3%)	12 (63.2%)	6 (28.6%)	0.0472
	-	14 (73.7%)	7 (36.8%)	15 (71.4%)	
	N/A	0	0	0	
BRAF mutation status, n (%)	+	0 (0%)	2 (10.5%)	0 (0%)	0.200
	-	19 (100%)	17 (89.5%)	21 (100%)	
	N/A	0	0	0	
TP53 mutation status, n (%)	+	14 (73.7%)	7 (36.8%)	14 (66.7%)	0.0561
	-	5 (26.3%)	12 (63.2%)	7 (33.3)	
	N/A	0	0	0	
Recurrence, n (%)	+	1 (5.3%)	1 (5.3%)	4 (19.0%)	0.346
	-	18 (94.7%)	18 (94.7%)	17 (81.0%)	
5-year DFS,%		94.4%	94.4%	80.7%	0.267

***Note:** P values for characteristics, except for 5-year DFS, were obtained using the Chi-squared test. A p value for 5-year DFS was obtained using the log-rank test. N/A, not available; SD, standard deviation

paper is patent pending). In particular, selection of differentially expressed genes using bioinformatics without considering function may diminish the test's specificity and sensitivity to levels that might disqualify it for routine clinical use. Specifically, we estimated the performance of the prognostic model using 4,818 genes in three gene clusters (Stromal/Chromosome 20q, 13q/Chromosome 18q (Figure 1 and Table 6). The prognostic performance of this model was diminished in the test and validation sets. This result indicates that our gene selection methods effectively help to construct a highly reproducible discriminative model comprising multiple genes.

For this purpose, here we performed unsupervised clustering of gene expression in CC samples, focusing on genes located on the long arms of chromosomes 18 and 20 as well as stroma-related genes. We selected the gene modules "Chromosomal 18q Loss", "Chromosomal 20q Amplification," and "Stromal" to construct a classifier comprising 55 genes. This strategy enabled us to categorize CCs into the subtypes "Stromal," "CIN-like," and "MSI-like." Next, we discovered that *KRAS* mutations were associated with shorter RFS of patients with the CIN-subtype but not those with the MSI- and Stromal-types. We incorporated these findings to construct a new prognostic model that incorporated the combination of subtype classifications and *KRAS* mutations.

Adjuvant chemotherapy is frequently considered as the treatment option of choice for high-risk patients with stage II CC. The National Comprehensive Cancer Network [3] and the European Society for Medical Oncology [35] recommend considering adjuvant treatment for patients with stage II CC if specific features are present as follows:

Table 6: Comparison of 5-year survival rates of subtypes using 55 and 4,818 genes.

		Learning set		Test set		Validation set	
		n	5 year DFS	n	5 year RFS	n	5 year DFS
55 genes	CIN	32	88.2%	103	72.2%	19	94.4%
	MSI	24	86.4%	102	81.2%	19	94.4%
	Stromal	17	72.8%	53	56.0%	21	80.7%
	P value	0.222		**5.84e-03**		0.267	
	High risk	-	-	77	52.9%	26	80.6%
	Low risk	-	-	181	81.0%	33	96.8%
	P value			**1.56e-06**		**4.75e-02**	
4,818 genes	CIN	35	82.9%	110	68.7%	20	90.0%
	MSI	27	96.0%	100	80.8%	33	87.3%
	Stromal	11	56.0%	48	63.3%	6	100.0%
	P value	**3.23e-03**		**2.00e-02**		0.666	
	High risk	-	-	80	57.6%	9	100.0%
	Low risk	-	-	171	78.7%	50	87.5%
	P value			**7.64e-05**		0.276	

***Note:** P alludes for characteristics, except for 5-year DFS and 5-year RFS, were obtained using the Chi-squared test. P values for 5-year DFS and 5-year RFS were obtained using the log-rank test.

clinical obstruction, perforation, T4 tumor, suboptimal lymph node sampling (<12 lymph nodes), presence of lymphovascular or perineural invasion, or poor differentiation. Kumar et al. [36] determined the effectiveness of adjuvant chemotherapy for high-risk patients with these features. Further, ≥ 76% of Stage II patients are categorized as high-risk [36], although the recurrence rate of Stage II patients ranges from 10% to 20% [2].

Patients with stage III CC are generally advised to undergo adjuvant chemotherapy; however, we doubt that patients with Stage IIIA CC require this type of therapy, because their prognosis is better compared with those with Stage IIB/IIC. Further investigations are therefore necessary to develop a robust standard to select candidate adjuvant chemotherapeutic strategies according to cancer stage and subtyping performed using gene-expression profiles.

The RFS of low-risk patients with Stage II/III CC judged using our prognostic model was 81.0% in the test set and 96.8% in the validation set, indicating that adjuvant therapy is not required for such patients. Thus, we used our model to assign 77% and 50% of the test and validation sets, respectively, to the low-risk category. These frequencies are significantly higher compared with the proportion of patients classified as low-risk [36]. Thus, the developed model was designed to identify CC patients with good and poor prognosis, if used clinically, would spare numerous patients from unnecessary and potentially harmful adjuvant therapy and reduce medical costs.

MSI status is a key feature of CC, and evidence [37,38] indicates that the benefits of adjuvant chemotherapy differ between patients with MSI and MSS. In the present study, all MSI cancers were included in MSI-like subtype, not only in the test set, but in the validation set as well. Consequently, all CIN-like and stromal subtypes exhibited the MSS phenotype, indicating that additional screening for MSI screening of patients with CIN-like and stromal subtype cancers is not required. The 55-gene centroid predictor was significant for predicting prognosis and was biologically significant by its ability to identify MSI subtypes.

Gene expression profiling is increasingly utilized for cancer diagnosis, prognosis, and treatment decisions. The Mamma Print assay [39-41], introduced by Agendia in 2007, and was the first FDA-approved molecular test that profiles gene expression levels to predict the risk of early-stage breast cancer to guide treatment decisions. The OncotypeDX Colon Cancer Assay manufactured by Genomic Health improves prediction of recurrence risk for patients with stage II CC [28-30,32,33,36]. The ColoPrint, produced by Agendia, significantly improves prognostic accuracy in predicting the survival of patients with stages II and III colorectal cancer [31,34]. Subsequently, several clinical trials [28-30] validated the performance of these two classifiers. However, the OncotypeDX Colon Cancer Assay categorized 26%–31% of these patients as “intermediate risk” [33]. This high incidence of “intermediate risk” patients complicates clinical decision-making. Moreover, these assays required an MSI test to improve their quality, because a good prognosis was predicted for patients with MSI-type cancer regardless of the results of the OncotypeDX Colon Cancer Assay. Thus, our prognostic model has a distinct advantage, because it divides patients with CC into two risk groups without performing the MSI test.

Although these findings seem promising, the present study has some limitations. First, our models were structured using data acquired from frozen specimens. For practical use, the quality of our model must be confirmed using formalin-fixed paraffin-embedded specimens. Second, we were unable to obtain statistically significant data for Stage II or Stage III patients because few patient samples were available. We believe that a larger study will aid in determining the potential of the subtype classifier and the prognostic model described here.

Conclusion

We developed a new prognostic model for patients with CC based on analysis of the expression of 55 genes and *KRAS* mutations status. This novel model was developed by analyzing a combination of 18q, 20q, and stromal gene modules. The model produced a biologically discriminatory classifier that associated MSI status with the risk of recurrence. Finally, we demonstrated the prognostic significance of the proposed model that may be clinically applicable for the selection of patients with Stage II/III CC for adjuvant therapy.

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Author Affiliations

Top

[1]*Central Research Laboratories, Sysmex Corporation, Kobe, Japan*

[2]*Surgery, National Defense Medical College, Saitama, Japan*

[3]*Pathology, National Defense Medical College, Saitama, Japan*