Table 1. Primer sequences used in this study

Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]
PCR (Ex-taq)			
5′-Flanking (for −1.9 k to −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA	20289134–20289443
5′-Flanking (for −1.7 k to −950)	ATGAGGTGGTATCTAACTGTGG	AAATGTTTTCTGTAGGGACGGG	20289392–20290182
1st PCR (Z-taq)			
5′-Flanking (for −1.2 k) to exon 6	ATACTGCATGGGTGGTTATG	AACCTGCCTCCAAATTTTTC	20289942–20303347
Exons 7 to 11	GGAGAATCACTTTGAAGCCG	CTAGCAAGTGTGAGGGGTGT	20304874–20314079
Exons 12 to 19	TCTGTGAATGTGGCAAAACT	GGATCTACCAAGAATTTAGC	20315189–20328004
Exons 20 to 25	GATGAGCATTTTCAATTTAC	TCAGTTCACCCAGCACTTAT	20338211–20344941
Exons 26 to 32	GAGCAAGACCTTGTCTCATA	CCATGGATGAATCTCAGATA	20349821–20360334
2nd PCR (Ex-taq)			
5′-Flanking (for −880 to −130)	GGAAGATCGCTTGAACCCAT	TCATCCCAACCATTTAATCG	20290245–20290994
Exon 1	TTGTTGGCCAGCTCTGTTG	TTCTGGTTCTTGTTGGTGAC	20290810–20291254
Exon 2	GGGTAAGGCTGGATATGGAT	CTGGCTCTACCTGAGACAAT	20292767–20293194
Exon 3	CACCGGAAACCATTCTGTTC	TTTGCCTCACTATGGATCCC	20300442–20300773
Exon 4	GCCAGATTAGTCACGACAGT	CCAAAGGAAGTCTACATGGCC	20301708–20302134
Exon 5	CAGGTAAGGAAAAAAAGAGTGG	CCTTGTCATAAAATGGTCTG	20301966–20302418
Exon 6	TATGCCAGAAAATCTGATTA	AGGTGGAACATGAGCTTGAGT	20302499–20303070
Exon 7	GGTGGAGATAGCCTCTGACC	TGCACTGAGAAGTATGAAGTGC	20305320–20305728
Exon 8	CCTGTACAGAGAAGGCCACG	TGCGGTCTTCATGAACACAA	20307385–20307816
Exon 9	GGCTTTGGACAATTCTGGTC	TCCACCCATTGTCTGTGAAC	20308539–20309038
Exon 10	AGGCAAGAAGTCACAGTGCC	TTGCCCAAACTCCCATTAAG	20312158–20312650
Exon 11	ACAGTCAGGCAAGGGCTATG	GACAGGAGGACATGAAACAA	20313420–20313873
Exon 12	GATTTCTATTCCCCACATTT	GAGCTGGGGGTATGGTACAA	20315554–20315983
Exon 13	GTGACCTTGGAGAAGATATT	CTCTTGAAAGTTTACCAGCA	20316189–20316623
Exon 14	TTGCTCAAGGACTGAAATAG	CCTGCTTATCCTCAGAAGAG	20318223–20318732
Exon 15	GGTCTCATGGTCTCATTCTA	GGGTTTATCCTGCACTAGTA	20319650–20320025
Exon 16	AGAAGCACTTTGGGGTCTTGTA	GCTGAAATGGGAAGGAGAATC	20321144–20321581
Exon 17	GCTGAAAAACGATAGTCCAA	TCAACTAGATTACCCCTGTGT	20325354–20325863
Exons 18 and 19	TCACAGGGTGACAAGCAAC	TTGAATCTCTGGGTAGTTTG	20326820–20327678
Exon 20	GAAACCAGCAAGATCAGAGGA	TCACTCAGCTGGCATCAAAG	20338493–20338929
Exon 21	TGACTGTGACATCTGCTTGC	GGACAGAGGACATATTGCTCC	20338927–20339248
Exons 22 and 23	GCATTGTATTTCAGCATTGT	ACAGTGTTGTCTAGGGGGAC	20339701–20340506
Exon 24	GAACACACAGAATCCAACAGA	TCACTTCAGCTTCAGACAGT	20342562–20343001
Exon 25	TCTCATTGGTCTCCTCCTCG	AATTTCACACCACTAGCCAT	20344186–20344672
Exon 26	GAGGCATTGCCTAAGAGTGC	AAAGATGGAGCCAGGGTTTG	20350122–20350523
Exons 27 and 28	GGCAAGGATTGTCTTTCTTA	CGACAGCTGCGGTAAGTCTG	20351928–20352954
Exon 29	AGAGATGGAGTAGCCAGTCAC	CAGCCACAAATGCATATTACC	20353790–20354262
Exon 30	GAAGCTCAACCACAAACCAG	GCTCGACCAGTTTTCAAGAG	20355106–20355610
Exon 31	GCAAGGTACAGCTAGTTGAA	GCGTGATGTAAAATTTTGGC	20358730–20359248
Exon 32	GCTGTGGCTCATTGATTTTC	AAGGTGATAAAACAGAAATG	20359651–20360213
Sequencing			
5′-Flanking (for −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA[b]	
(for −1.7 k to −1.3 k)	GGTATCTAACTGTGGTTTTG	GAAGGAAAGGAGTCAAAGGAAC	
(for −1.5 k to −950)	TCCCACACTGAATGCTGCCTTT	TAGGGACGGGGTCTCACTAT	
(for −880 to −400)	GGAAGATCGCTTGAACCCAT[b]	ATGTGCAGTTTCGCTTCTG	
(for −570 to −130)	CATATAGGCTCACACTGGAT	TCATCCCAACCATTTAATCG[b]	
Exon 1	TGGTTCCTTTTATGTATGGC	GTTCTTGTTGGTGACCACCC	
Exon 2	AAAGCAGTGGGATGTGCTG	TGTCTCTACTGTGCACCAAGG	
Exon 3	CACCGGAAACCATTCTGTTC[b]	TTTGCCTCACTATGGATCCC[b]	
Exon 4	CCTCCTTTCTTCCCATGTTC	CTCAACTTGATGCCATTTAC	
Exon 5	TGGGGCAACCTCTAACTCATA	TGAGACCCAGACATCTTAAA	
Exon 6	TTAGGGTCTCCAAATAAACA	ACTTTCAGAGGAGTGAGAGAGT	
Exon 7	GGTGGAGATAGCCTCTGACC[b]	TGCACTGAGAAGTATGAAGTGC[b]	
Exon 8	CCTGTACAGAGAAGGCCACG[b]	CACAATGCTGTAAGGTTAAG	
Exon 9	GGCTTTGGACAATTCTGGTC[b]	TCCACCCATTGTCTGTGAAC[b]	
Exon 10	GTGCCTTGGAGAAGCTGTGT	TTGCCCAAACTCCCATTAAG[b]	
Exon 11	TCACTGGGCACCTCAAGTTC	GGAATCCATCACCTCTACCA	
Exon 12	ACATTTTGGGGACTATATCT	ATGCCAGCTAGTCTATCAAA	
Exon 13	GGAGGCTGGATGATCCTTAAG	CTCTTGAAAGTTTACCAGCA[b]	
Exon 14	CATCTGTCTATGGTGGGATA	ATAGGCTCAAGACAAATCTC	
Exon 15	GATTTCATTCACCTCCTGTT	CATTTCCCCATGCATTCTAT	
Exon 16	CCAATCTTGAGGGGAAATCT	TCCAAGACCTCACCTACTAGC	

Table 1. continued

Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]
Exon 17	GTGGAATAACTACAAGCACG	TCAACTAGATTACCCCTGTGT[b]	
Exon 18	GGTGACAAGCAACAAAACTA	CCACCATCTTCCCTGTCTTA	
Exon 19	GATGCTCATGTAGGAAAACA	TTTACCATTCCACCCATGGC	
Exon 20	GGCTTCTCTCTCCTTGTTCA	CAAAGAAACAAAGGAAGAGC	
Exon 21	TGACTGTGACATCTGCTTGC[b]	GGACAGAGGACATATTGCTCC[b]	
Exon 22	GCATTGTATTTCAGCATTGT[b]	GATATTTGATGCATGGACGA	
Exon 23	GAATCTGTCTGGACCCTGTA	GTCTAGGGGGACATAATAAT	
Exon 24	ACACACAGAATCCAACAGAT	TCAACATATGACTAAATGGC	
Exon 25	GGAGCCTCTCATCATTCTGC	TTTCACACCACTAGCCATGC	
Exon 26	CCGATCAAGTCAAACCCTCT	TTTGAACCTCAGTCTTCTTT	
Exon 27	TTTCCTTACTCCCTTGTAGA	AAACTTTAGGGACCCATTAT	
Exon 28	CTGCTACCCTTCTCCTGTTC	CCTTCCCTCTGATACTGTGT	
Exon 29	TACCTCCTGTGACTGTGAAT	CAGCCACAAATGCATATTACC[b]	
Exon 30	GCCAGTCCTATCCACCATCT	AACACGAGGAACACGAGGAG	
Exon 31	GATCTGGAACATGAAAATGG	TTTTGGCCAGATTACTTGAC	
Exon 32	GCTCATTGATTTTCACTGCT	AAGGCAAAGGAATAATTATCG	

[a]The reference sequence is NT_030059.12.
[b]The same primer that was used for the 2nd PCR.

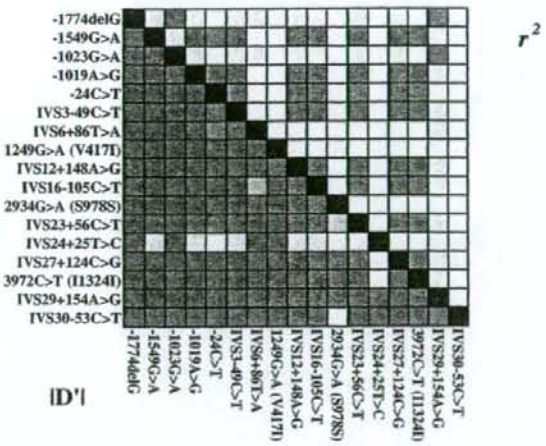

Fig. 1. Linkage disequilibrium (LD) analysis of *ABCC2*
Pairwise LD (r^2 values and |D′|) of polymorphisms detected in no less than 3% of allele frequencies is shown as a 10-graded blue color.

(**Table 2**). All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$). Novel variations consisted of 5 non-synonymous and 4 synonymous variations in the coding region, 22 in the intronic regions, 3 in the 5′-flanking region, 1 in the 3′- flanking region, and 1 in the 3′-UTR. The novel non-synonymous variations were 1177C>T (Arg393Trp), 1202A>G (Tyr401Cys), 2358C>A (Asp786Glu), 2801G>A (Arg934Gln), and 3320T>G (Leu1107Arg), and their frequencies were 0.002. No statistically significant differences were found in the allele frequencies of all variations between 177 cancer patients and 59 healthy subjects ($P > 0.05$, Fisher's exact test), although a larger number of subjects would be needed to conclude.

The frequency of the known common SNP −24C>T (0.173) was comparable to those reported in Asians (0.17–0.25)[8,12,20] and Caucasians (0.15–0.23)[9,10,14,15,21]. The allele frequency of another common SNP, 3972C>T (Ile1324Ile) (0.216), was also comparable to those in Asians (0.22–0.30)[8,12,20] but lower than those in Caucasians (0.32–0.37)[9,10,14,15,21]. The other major variations in the 5′-flanking region, −1774delG and −1549G>A, were found at frequencies of 0.343 and 0.203, respectively, and these values were similar to those obtained in Koreans (0.34 and 0.21, respectively).[8] However, the relatively frequent SNPs 1446C>G (Thr482Thr) (allele frequency=0.125), IVS15-28C>A (0.333) and IVS28+16G>A (0.167) in Caucasians[17] were not detected in our study.

The LD profile of the *ABCC* variations (no less than 3% allele frequency) is shown in **Figure 1**. As assessed by r^2 values, close linkages were observed among −1774delG, −1023G>A and IVS29+154A>G, and among −1549G>A, −1019A>G, −24C>T, IVS3-49C>T, IVS12+148A>G, IVS15+169T>C, IVS16-105C>T, IVA23+56C>T, IVS27+124C>G, and 3972C>T (Ile1324Ile). It must be noted that complete linkage was observed between −1549G>A and −1019A>G in our population. In |D′| values, strong LD was also observed almost throughout the region analyzed. Overall, since close associations between the variations were observed throughout the entire *ABCC2* gene, the region sequenced was analyzed as a single LD block for the haplotype inference.

The *ABCC2* haplotype structures were analyzed using 61 detected genetic variations and a total of 64 haplotypes were identified/inferred. **Figure 2** summarizes the haplotypes and their grouping. Our nomenclature system is based on the recommendation of Nebert.[22] Haplotypes without

Table 2. Summary of *ABCC2* variations detected in this study

SNP ID			Position					
This Study	dbSNP (NCBI)	JSNP	Reference	Location	NT_030059.12	From the translational initiation site or from the end of the nearest exon	Nucleotide change	Amino acid change	Frequency (total = 472)
MPJ6_AC 2082			8	5′-Flanking	**20289354**	−1774	acttatcttgttG/_ttttttttttt		0.343
MPJ6_AC 2078[a]				5′-Flanking	**20289538**	−1590	tttaatttgttaG/Atgtatgtttgct		0.002
MPJ6_AC 2079			8, 10, 17	5′-Flanking	**20289579**	−1549	tccttatagtatG/Attgtggatatta		0.203
MPJ6_AC 2080			9, 17	5′-Flanking	**20290105**	−1023	tgggaggccaagG/Acagaaggattgt		0.343
MPJ6_AC 2081			10, 17	5′-Flanking	**20290109**	−1019	aggccaaggcagA/Gaggattgttgaa		0.203
MPJ6_AC 2028[a]				5′-Flanking	**20290395**	−733	acagtttctagcG/Tactgatgccacc		0.004
MPJ6_AC 2029				5′-Flanking	**20290395**	−733	acagtttctagcG/Aactgatgccacc		0.002
MPJ6_AC 2030[a]				5′-Flanking	**20290715**	−413	ttgcagcagaagC/Tgaaactgcacat		0.002
MPJ6_AC 2003		**ssj0000371**	9, 12, 15–18, 20, 26	Exon 1	**20291104**	−24	tagaagagtcttcG/Tgttccagacgca		0.174
MPJ6_AC 2004			18	Exon 1	**20291105**	−23	agaagagtcttcG/Attccagacgcag		0.006
MPJ6_AC 2031		**ssj0000386**	17, 26	Intron 3	**20301785**	IVS3 −49	ctccccctcagtcC/Ttcggttagtggc		0.203
MPJ6_AC 2032[a]				Intron 6	**20302837**	IVS6 +86	tattttattattT/Attttttgagat		0.076
MPJ6_AC 2033[a]				Exon 7	**20305479**	732	caagtttgaaacG/Acacatgaagaga	Thr244Thr	0.002
MPJ6_AC 2066[a]				Intron 7	**20307421**	IVS7 −69	tcacaggctgacC/Gaccctggagctg		0.002
MPJ6_AC 2067[a]				Intron 7	**20307423**	IVS7 −67	acaggctgaccaC/Accctggagctgct		0.002
MPJ6_AC 2035[a]				Exon 9	**20308814**	1177	ggtgtaaaagtaC/Tggacagctatca	Arg393Trp	0.002
MPJ6_AC 2068[a]				Exon 9	**20308839**	1202	tggcttctgtatA/Gtaagaaggtaag	Tyr401Cys	0.002
MPJ6_AC 2036[a]				Intron 9	**20308859**	IVS9 +13	gtaagcagaataC/Tggcaggtatcac		0.002
MPJ6_AC 2037[a]				Exon 10	**20312319**	1227	gaccctatccaaC/Tttggccaggaag	Asn409Asn	0.002
MPJ6_AC 2009		**ssj0000388**	17, 18, 20, 23–26	Exon 10	**20312341**	1249	aaggagtacaccG/Attggagaaacag	Val417Ile	0.097
MPJ6_AC 2010			18	Exon 10	**20312549**	1457	ccaagagtaagaC/Tcattcaggtaaa	Thr486Ile	0.019
MPJ6_AC 2069[a]				Intron 11	**20315600**	IVS11 −67	taaaacatgggtG/Agatcagatacac		0.002
MPJ6_AC 2038		**ssj0000390**	26	Intron 12	**20315952**	IVS12 +148	ccgccccatgccA/Gcttttcctcctt		0.210
MPJ6_AC 2039[a]				Intron 13	**20318344**	IVS13 −73	tcatggactaacG/Aacaaagtcaaaa		0.002
MPJ6_AC 2070[a]				Intron 14	**20318515**	IVS14 +14	taaataaatttgG/Taagttgcttccc		0.002
MPJ6_AC 2040[a]				Intron 14	**20318521**	IVS14 +20	aatttggaagtt(del/ins)[b]cagcaaactga		0.002
MPJ6_AC 2071[a]				Intron 14	**20318594**	IVS14 +93	agcaaactgagaG/Tagagtgtggaga		0.002
MPJ6_AC 2041[a]				Intron 14	**20319757**	IVS14 −62	cggagagagacaC/Tgtgagggcagac		0.002
MPJ6_AC 2042[a]				Intron 14	**20319758**	IVS14 −61	ggagagagacacG/Atgagggcagaca		0.006
MPJ6_AC 2043		**ssj0000393**	26	Intron 15	**20320054**	IVS15 +169	aaagcaaaggttT/Ctcagccccttcc		0.210
MPJ6_AC 2044[a]				Intron 15	**20321170**	IVS15 −131	gtcttgtatatcC/Gaaggcaaatttt		0.004
MPJ6_AC 2045[a]				Intron 16	**20325422**	IVS16 −169	ttgagtcctgagA/Tgtggaataacta		0.004
MPJ6_AC 2046		**ssj0000396**	17	Intron 16	**20325486**	IVS16 −105	tgcacagttattC/Taaatttaagctc		0.214
MPJ6_AC 2072[a]				Exon 18	**20327159**	2358	tcttctagatgaC/Acccctgtctgca	Asp786Glu	0.002
MPJ6_AC 2012			18, 20, 24	Exon 18	**20327167**	2366	atgaccccctgtC/Ttgcagtggatgc	Ser789Phe	0.008
MPJ6_AC 2074[a]				Intron 19	**20327555**	IVS19 +3	gaagccacaggtA/Gtgtaagaaggat		0.002
MPJ6_AC 2047				Intron 19	**20327645**	IVS19 +93	agtatccagtgaA/Tctagatttggaa		0.002
MPJ6_AC 2048				Intron 20	**20338745**	IVS20 +29	gctggcagcccttC/Agtcagctctata		0.002
MPJ6_AC 2049[a]				Exon 21	**20339052**	2801	ccttgaaaactcG/Agaatgtgaatag	Arg934Gln	0.002
MPJ6_AC 2015		**ssj0000398**	8, 18, 26	Exon 22	**20339944**	2934	aggattgtttccG/Aatattcttcatc	Ser978Ser	0.040
MPJ6_AC 2050[a]				Exon 22	**20340061**	3051	cgactatccagcA/Gtctcagagggac	Ala1017Ala	0.002
MPJ6_AC 2051[a]				Exon 23	**20340337**	3181	cacaagcaactgC/Ttgaacaatatcc	Leu1061Leu	0.002
MPJ6_AC 2052		**ssj0000399**	17, 26	Intron 23	**20340470**	IVS23 +56	ggatcttttctgaC/Tagggaggaatta		0.222
MPJ6_AC 2074[a]				Exon 24	**20342724**	3320	ttacatgcttccT/Gggggataatcag	Leu1107Arg	0.002
MPJ6_AC 2053				Intron 24	**20342843**	IVS24 +25	atggctaagtcaT/Ccctccttcctc		0.030
MPJ6_AC 2075[a]				Intron 24	**20342880**	IVS24 +62	agcccagcctctT/Ctcctgagaatct		0.002
MPJ6_AC 2054				Intron 24	**20342926**	IVS24 +108	cactcactcctcC/Tcctcagcagctt		0.023
MPJ6_AC 2055[a]				Intron 24	**20344318**	IVS24 −56	agaaaggaggaaG/Aatggtggatgcc		0.002
MPJ6_AC 2056[a]				Intron 26	**20352061**	IVS26 −21	atgatgattttcA/Ggtcttctggttt		0.002
MPJ6_AC 2057[a]				Intron 27	**20352227**	IVS27 +44	ggcaaaaacaacA/Gtgcaactccttc		0.008
MPJ6_AC 2058		**ssj0000404**	17, 26	Intron 27	**20352307**	IVS27 +124	aaagttttctttC/Gctctaactcaaa		0.222
MPJ6_AC 2076			26	Exon 28	**20352688**	3927	ccaagtgcggtaC/Tcgacctgagctg	Tyr1309Tyr	0.002
MPJ6_AC 2022		**ssj0000407**	8, 12, 13, 17, 18, 20, 26	Exon 28	**20352733**	3972	cacttgtgacatC/Tggtagcatggag	Ile1324Ile	0.216
MPJ6_AC 2059[a]				Intron 28	**20352920**	IVS28 +172	agggaaggatagC/Tagccagggatca		0.004
MPJ6_AC 2060[a]				Intron 29	**20354201**	IVS29 +136	cttgagctagttC/Tcctaggatggac		0.002
MPJ6_AC 2061		**ssj0000408**	26	Intron 29	**20354219**	IVS29 +154	gatggacacgtcA/Gtttccagaactt		0.367
MPJ6_AC 2062		**IMS-JST090926**	17	Intron 29	**20355209**	IVS29 −35	cttttctggcatG/Aagccccaacagc		0.015
MPJ6_AC 2063[a]				Intron 30	**20358793**	IVS30 −92	gggggttttgaA/Gagtctgatctgg		0.008
MPJ6_AC 2064		**IMS-JST185750**		Intron 30	**20358832**	IVS30 −53	ccccctgccctgC/Tgtcttttcttgg		0.051
MPJ6_AC 2077[a]				3′-UTR	**20359975**	*61	taattttatttttT/Gtataaaatacag		0.002
MPJ6_AC 2065[a]				3′-Flanking	**20360190**	*193+83	ttattccttttgcC/Gtttcatttctgt		0.00288

[a]Novel genetic variation

[b]delGCTTCCCAAACTTATTCGCAGTACTGGTGCCAGAATTTTGATAATACAAGAGCTTAGTAG/insTATTTACCT

[c]Numbered from the termination codon.

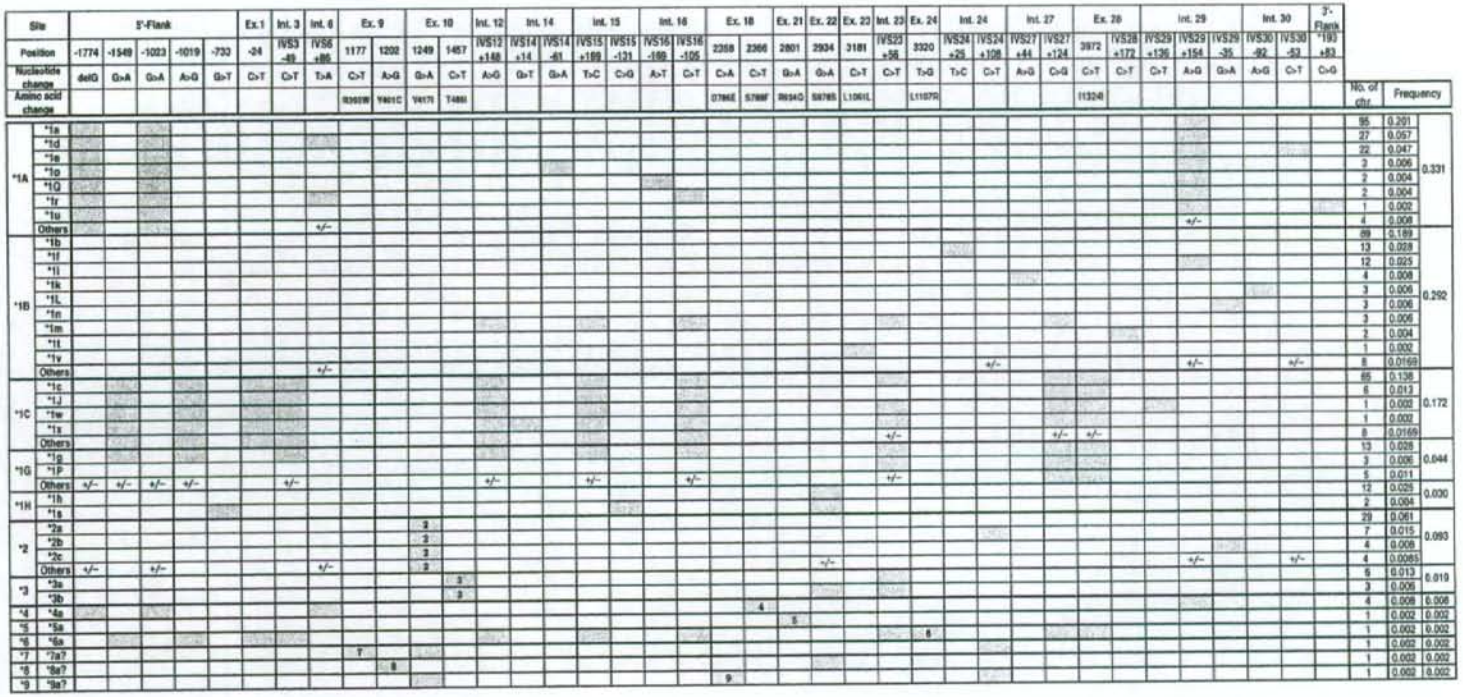

Fig. 2. *ABCC2* **haplotypes in 236 Japanese subjects**

The *1 groups (without nonsynonymous substitutions) were classified into *1A (harboring −1774delG), *1C (harboring −24C>T), *1G [harboring 3972C>T (Ile1324Ile) without −24C>T], *1H [harboring 2934G>A (Ser978Ser)] and *1B [without the common variations]. Marker SNPs for *2 to *9 are indicated by numbers. Rare and ambiguous haplotypes (n = 1) are shown with "?" or grouped into "others".

any amino acid substitution were assigned as the *1 group and named with small alphabetical letters in descending frequency order (*1a to *1x). Haplotypes with nonsynonymous variations were assigned from *2 to *9 groups, and their subtypes were named with small alphabetical letters. The haplotypes (*7a to *9a) were inferred in only one patient and described with "?" due to their ambiguity. Also, ambiguous rare haplotypes in the *1 and *2 groups were classified as "Others" in **Figure 2**. The *1 haplotypes were further classified into the *1A, *1B, *1C, *1G and *1H groups (capital alphabetical letters of the most frequent haplotypes were used) according to the common tagging SNPs, such as −1774delG, −24C>T, 3972C>T (Ile1324Ile), and 2937G>A (Ser978Ser).

The most frequent *1 group, *1A, harbors the common SNPs −1774delG and −1023G>A in the 5′-flanking region and mostly IVS29+154A>G, and the frequency of *1A (0.331) is almost the same as that in healthy Koreans (0.323) reported by Choi *et al.*[8] They have shown that −1774delG reduced promoter activity both at the basal level and after induction by chenodeoxycolic acid (CDCA), a component of bile acids, and that the haplotype bearing −1774delG is associated with chemical-induced hepatitis (cholestatis and mixed types).[8] Therefore, it is possible that *1A can affect the pharmacokinetics or pharmacodynamics of MRP2-transported drugs.

The *1B group haplotypes (0.292 frequency) harbor no or any intronic or synonymous variations the functions of which are unknown. The functional significance of variations in the *1B group, including the most frequent SNP IVS24+25T>C, needs further confirmation.

The third group *1C (0.172 frequency) harbors the known common SNPs −1549G>A, −1019A>G, −24C>T, IVS3−49C>T, and 3972C>T (Ile1324Ile), except for one rare ambiguous haplotype lacking 3972C>T (Ile1324Ile). The *1C haplotypes also harbor IVS12+148A>G, IVS15+169T>C and IVS16−105C>T. The haplotypes bearing −1549G>A, −24C>T and 3972C>T (Ile1324Ile) are commonly found in Korean populations (frequency 0.14–0.25)[8] and Caucasians (0.14–0.17).[10,14,21] The functional importance of the tagging SNP in the *1C group, −24C>T, has been reported by several researchers; *e.g.*, reduced promoter activity,[8,11] reduced mRNA expression in the kidney,[11] association with chemical-induced hepatitis (hepatocellular type),[8] and influence on irinotecan-pharmacokinetics and pharmacodynamics.[12,16] For other SNPs in the *1C group, functional alterations *in vitro* have not been shown; no change in promoter activity by −1549G>A, no influence of IVS3−49C>T on splicing, and no change induced by 3972C>T (Ile1324Ile) on MRP2 expression or transporter activity.[8] Although −24C>T caused reduced promoter activity in the absence of the bile acid CDCA,[8,11], enhanced promoter activity of −24C>T under induction by CDCA has been demonstrated.[8] Therefore the function of this SNP might depend on cholestatic status.

Our data demonstrated that −1019A>G was closely associated with the other *1C SNPs (complete linkage with −1549G>A). The close linkage between −1019A>G and −1549G>A was also observed in Caucasians, but their linkages with −24C>T and 3972C>T were relatively weak.[14] In contrast, another study on Caucasians reported that −1019A>G was exclusive to −1549G>A, −24C>T and 3972C>T.[10] Although the reasons for these discrepancies are not clear, some ethnic differences might exist in the 5′-flanking region.

The *1G group harbors 3972C>T (Ile1324Ile) but not −24C>T. Caucasians have haplotypes bearing 3972C>T (Ile1324Ile) without −24C>T at frequencies of 0.15–0.20.[10,21] In contrast, the frequency of the corresponding haplotype group in our study (*1G) was much lower (0.044). Although no *in vitro* effect of 3972C>T (Ile1324Ile) was shown,[8] its *in vivo* association with increased area under the concentration-time curve of irinotecan and its metabolites was reported in Caucasians.[13]

The *1H group (*1h and *1s) harbors a synonymous substitution of 2934G>A (Ser978Ser) (0.03 frequency). No influence of 2934G>A(Ser978Ser)on MRP2 expression or transport activity has been shown.[8]

As for haplotypes with nonsynonymous substitutions, eight haplotype groups (*2 to *9) were identified. The *2 [including 1249G>A (Val417Ile)] was the most frequent among them, and its frequency (0.093) was similar to those for Asians (0.10–0.13)[8,12,20] and slightly lower than those for Caucasians (0.13–0.22).[9,10,14,15,21] The haplotype frequencies of *3 [harboring 1457C>T (Thr486Ile)] and *4 [2366C>T (Ser789Phe)] were 0.019 and 0.008. Other rare haplotypes with novel nonsynonymous variation, *5 [2801G>A (Arg934Gln)], *6 [3320T>G (Leu1107Arg)], *7 [1177C>T (Arg393Trp)], *8 [1202A>G (Tyr401Cys)], and *9 [2358C>A (Asp786Glu)] were found each in only one subject as heterozygote at a 0.002 frequency. No functional significance of the marker SNP [1249G>A (Val417Ile)] of *2 has been shown *in vitro*,[8,23] but its *in vivo* associations with lower MRP2 expression in the placenta[24] and chemical-induced renal toxicity[25] have been reported. The variation 2366C>T (Ser789Phe) (*4) has been shown to cause reduced MRP2 expression and alter localization *in vitro*,[23] but clinical data are limited. Functional changes in *3 [1457C>T (Thr486Ile)] and *5 to *9 (novel nonsynonymous variations) are currently unknown. Possible effects of these amino acid substitutions were speculated using PolyPhen analysis (http://genetics.bwh.harvard.edu/pph); its prediction is based on the analysis of substitution site [e.g., a substitution in transmenbrane domain is assessed by the predicted hydrophobic and transmembrane (PHAT) matrix score], likelihood of the substitution assessed by the position-specific independent count (PSIC) profile scores, and protein 3D structures. This analysis predicted a possible functional change of Leu1107Arg (*6) due to substitution in

the transmembrane region (PHAT matrix element difference = −6), and probable functional effects of Arg393Trp (*7) (PSIC score difference = 3.053), Tyr401Cys (*8) (3.382) and Asp786Glu (*9) (2.277), but no functional effects of *3 (1.446) and *5 (0.326).

In conclusion, the current study provided detailed information on *ABCC2* variations and haplotype structures in Japanese and also suggested a large ethnic difference in the frequencies of 3972C>T(Ile1324Ile) and 1446C>G (Thr482Thr) and their related haplotypes between Asians and Caucasians. This information would be useful for studies investigating the clinical significance of *ABCC2* alleles and haplotypes.

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Phase I/II study of oxaliplatin with oral S-1 as first-line therapy for patients with metastatic colorectal cancer

Y Yamada*[,1], M Tahara[2], T Miya[3], T Satoh[4], K Shirao[1], Y Shimada[1], A Ohtsu[2], Y Sasaki[3] and Y Tanigawara[5]

[1]Gastrointestinal Oncology Division, National Cancer Center Hospital, Tokyo, Japan; [2]Gastrointestinal Oncology and Endoscopy Division, National Cancer Center Hospital East, Chiba, Japan; [3]Department of Medical Oncology, Saitama International Medical Center-Comprehensive Cancer Center, Saitama Medical University, Saitama, Japan; [4]Department of Medical Oncology, Kinki University School of Medicine, Osaka, Japan; [5]Department of Pharmacy, Keio University, Tokyo, Japan

Two phase II studies of S-1 monotherapy have shown promising response rates (RR) of 35–40% with good tolerability in patients with untreated metastatic colorectal cancer. To investigate the usefulness of S-1 plus oxaliplatin (SOX) as an alternative to infusional 5-fluorouracil/leucovorin plus oxaliplatin, the recommended dose (RD) of SOX was determined, and its safety and preliminary efficacy were evaluated in a phase I/II study. Oxaliplatin was administered at a dose of 100 mg m^{-2} (level 1) or 130 mg m^{-2} (level 2) on day 1, and S-1 (80–120) was given twice daily for 2 weeks followed by a 1-week rest. This schedule was repeated every 3 weeks. Level 2 was determined to be the RD. For the 28 patients who received the RD, the median treatment course was 6.5 cycles (2–14), RR of 50% (1 CR and 13 PR; 95% CI 31–69%), with a median progression-free survival of 196 days. Survival rate (1 year) was 79%. Peripheral neuropathy was observed in all patients but with no functional disorders. Major grade 3 or 4 adverse reactions at the RD were neutropaenia (14%), thrombocytopaenia (28%), and diarrhoea (3%). SOX regimen is effective and easily manageable without central vein access.

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Oral fluoropyrimidine derivatives have been developed to circumvent the problems associated with continuous infusion of 5-fluorouracil (5-FU). S-1 is an effective derivative that combines tegafur with two modulators of 5-FU metabolism, 5-chloro-2,4-dihydroxypyridine (CDHP), a reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate in a molar ratio of 1:0.4:1 (Kato *et al*, 2001). Tegafur, an oral prodrug of 5-FU, is gradually converted to 5-FU and rapidly metabolised by DPD in the liver. The maximum concentration (C_{max}) and area under the concentration–time curve (AUC) of 5-FU in plasma during S-1 treatment have been found to be higher than the steady-state concentration and AUC of 5-FU in plasma during protracted intravenous infusion of 5-FU at a dose of 250 mg m^{-2} day^{-1} (Yamada *et al*, 2003).

Potassium oxonate is an orotate phosphoribosyl transferase inhibitor that is distributed primarily to the gastrointestinal tract. This component of S-1 decreases incorporation of 5-fluorouridine triphosphate into RNA in the gastrointestinal mucosa and reduces the incidence of diarrhoea. F-β-alanine (FBAL) is the main metabolite of 5-FU. F-β-alanine and fluorocitrate are thought to cause the neurotoxic and cardiotoxic effects of 5-FU by inhibiting the tricarboxylic acid cycle (Okeda *et al*, 1990; Robben *et al*, 1993; Diasio 1998). The CDHP component of S-1 inhibits DPD, the rate-limiting enzyme in the catabolic pathway of 5-FU. Consequently, the plasma FBAL concentration after oral administration of S-1 is significantly lower than that after continuous infusion of 5-FU (Yamada *et al*, 2003). Therefore, S-1 may decrease the incidence of neurotoxicity and cardiotoxicity. The response rate of S-1 monotherapy has been found to be 35–40% for patients with metastatic colorectal cancer (Ohtsu *et al*, 2000; Shirao *et al*, 2004), with grade 3 or 4 neutropaenia observed in 5–13%, thrombocytopaenia in 0–8%, diarrhoea in 2–3%, and grade 1 hand–foot syndrome (HFS) in 5%.

Oxaliplatin is a third-generation platinum compound with less toxicity and improved convenience. The regimen of infusional 5-FU and leucovorin (LV) with oxaliplatin is the standard for first- and second-line chemotherapy in patients with metastatic colorectal cancer (de Gramont *et al*, 2000; Rothenberg *et al*, 2003; Goldberg *et al*, 2004). However, infusional 5-FU with LV has the disadvantages of increased inconvenience, cost, and morbidity related to the use of a portable infusion pump and a central venous catheter. Therefore, oral fluoropyrimidine monotherapy has been commonly used in Japan.

The primary objectives of this phase I/II study were to determine the maximum tolerated dose (MTD) of S-1 plus oxaliplatin (SOX). In the phase II study, the toxicity and antitumour activity of SOX were evaluated at the recommended dose (RD).

MATERIALS AND METHODS

Patient selection

Patients with histologically confirmed colorectal cancer who had measurable metastatic disease were eligible for the study. Patients

*Correspondence: Dr Y Yamada, Gastrointestinal Oncology Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; E-mail: yayamada@ncc.go.jp
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with prior chemotherapy and radiotherapy for metastatic disease were not permitted. Patients who had received adjuvant oral fluorouracil-based therapy other than S-1 were eligible if they had remained free of disease for at least 6 months after the completion of such therapy. Other eligibility criteria included an age between 20 and 74 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; adequate baseline bone marrow function (white blood cell count more than the lower limit of normal at each hospital and less than 12 000 μl^{-1}, neutrophil count more than 2000 μl^{-1}, and a platelet count more than 100 000 μl^{-1}), hepatic function (serum total bilirubin (T.Bil) level 1.5 times the upper limit of normal or less, and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) 2.5 times the upper limit of normal or less), and renal function (serum creatinine level the upper limit of normal or less); and a life expectancy of at least 90 days. All patients gave written informed consent. Patients were excluded if they had symptomatic brain metastasis, pre-existing watery diarrhoea, or concomitant non-malignant disease, such as cardiac, pulmonary, renal, or hepatic disease, or uncontrolled infection. This study was approved by the institutional review board of each centre. Before enrolment, all patients underwent a physical examination (including documentation of measurable disease), a complete blood cell count (CBC) with differential count, serum chemical analysis, electrocardiography, and computed tomographic (CT) scanning or magnetic resonance imaging (MRI).

Toxicity and response criteria

Toxicity was assessed according to the Common Terminology Criteria for Adverse Events, Version 3.0 (CTCAE v3.0) (Therasse *et al*, 2000). Neurotoxicity was assessed according to the following specific neurotoxicity grading scale: grade 1, dysesthesia or paresthesia that completely regressed within 6 days; grade 2, dysesthesia or paresthesia persisting for 7 days or longer; and grade 3, dysesthesia or paresthesia causing functional impairment. During the study, all patients were evaluated weekly for signs and symptoms of toxicity. Complete blood cell counts, including differential count, liver function tests, measurement of urea nitrogen, creatinine, and electrolyte levels, and urinalysis were performed weekly. The response of measurable and assessable disease sites was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) (Shimoyama, 1999). Tumour dimensions were assessed by CT scanning or MRI every month to confirm response, and after RECIST efficacy was confirmed, every 2 months subsequently.

Treatment plan

Oxaliplatin was administered as a 2-h infusion every 3 weeks. The duration of the infusion could be extended to 6 h in patients who had pharyngolaryngeal dysesthesia during infusion. S-1 was available in capsule forms containing 20 or 25 mg of tegafur. Patients received S-1 orally twice daily from the evening of day 1 to the morning of day 15 at a dose of 40 mg (BSA $< 1.25\,m^2$), 50 mg ($\geqslant 1.25 - < 1.50\,m^2$), or 60 mg ($\geqslant 1.50\,m^2$) followed by a 7-day rest period in the 3-weekly schedule. All patients received premedication with a 5-hydroxytryptamine-3-receptor antagonist with or without dexamethasone, given as a 30 min drip infusion before chemotherapy. Treatment was routinely given on an outpatient basis. Subsequent treatment was withheld until the neutrophil and platelet counts were greater than 1500 and 75 000 μl^{-1}, respectively, AST or ALT less than 150 IU l^{-1}, T.Bil less than 1.5 times the upper limit of normal, creatinine less than the upper limit of normal, and diarrhoea, stomatitis, and HFS had resolved to grade 0 or 1. Treatment was repeated until the onset of disease progression or severe toxicity. When the administration of oxaliplatin was discontinued due to oxaliplatin-induced neuropathy, S-1 was also discontinued.

Dose-escalation schedule

The dose of S-1 was fixed and oxaliplatin was examined at doses of 100 mg m^{-2} (level 1) and 130 mg m^{-2} (level 2). A minimum of three patients were studied per dose level. Dose-limiting toxicity (DLT) was defined as any of the following findings during cycle 1: (i) a neutrophil count of less than 500 μl^{-1} for more than 4 days, (ii) a platelet count of less than 50 000 μl^{-1}, (iii) diarrhoea of grade 3 or more that occurred despite adequate supportive therapy, (iv) grade 3 or 4 non-haematologic toxicity, excluding nausea, vomiting, anorexia, and electrolyte imbalance, or (v) a treatment delay longer than 1 week due to drug-related toxicity in the phase I portion. If DLT occurred in one of the first three patients assigned to a given dose level, three additional patients were assigned to the same dose level. The MTD was defined as the dose that induced DLT during cycle 1 in 50% or more of the subjects. The RD was defined as one dose level below the MTD. If the MTD was not achieved, even at level 2, it was regarded as the RD.

The dose was modified for each patient based on haematologic or non-haematologic toxicity. If DLT occurred, the dose of oxaliplatin in the subsequent course was reduced to 75% of the initial dose and that of S-1 was reduced by one dose level: from 80 to 50, 100 to 80, and 120 to 100. S-1 intake was interrupted mid-cycle if there was a neutrophil count less than 1000 μl^{-1}, a platelet count less than 75 000 μl^{-1}, diarrhoea, stomatitis, or HFS occurred at grade 1 or more, AST or ALT more than 150 IU l^{-1}, T.Bil more than 1.5 times the upper limit of normal, or creatinine more than the upper limit of normal. The treatment in the subsequent cycle could be resumed if these adverse events resolved within 3 weeks after the last S-1 treatment. If peripheral neuropathy persisted between courses, the next treatment cycle was started at 75% of the previous dose of oxaliplatin. In a case with pharyngolaryngeal dysesthesia, the duration of the oxaliplatin infusion was prolonged from 2 to 6 h. Recombinant granulocyte colony-stimulating factor was subcutaneously injected if patients had grade 4 neutropaenia or grade 3 febrile neutropaenia, but prophylactic use was not allowed.

Statistical analysis

The sample size was calculated to be at least 28 patients on the assumption of the null hypothesis of overall response rate of $\leqslant 30\%$ *vs* the alternative hypothesis of overall response rate of $> 60\%$, power 80%, and α 2.5% (one-sided). The efficacy was analysed by the full analysis set. The primary end point was overall response rate as determined by an External Review Board. The 95% CI for response rate was calculated. Twenty-eight evaluable patients were required. Progression-free survival (PFS) and overall survival were analysed by the Kaplan–Meier method. Safety was analysed in all patients who received at least one dose of study medications. Clinical cutoff date for the study analysis was 31 May 2007.

RESULTS

Patient characteristics

A total of 32 patients, 23 men and 9 women, were recruited into this study between March 2005 and June 2006. The median age was 57 years. Four patients had received adjuvant oral fluorouracil-based therapy. Out of 32 patients, 31 received at least one cycle of the study treatment. The demographic data, sites of metastatic tumour, and prior adjuvant therapies are summarised in Table 1. Among the nine patients entered into the phase I study, six patients were treated at the RD. Twenty-three patients entered into the phase II study. However, one patient was excluded from the analysis of efficacy due to symptoms of brain metastasis suspected

to have existed before enrolment. All 32 patients were evaluated for toxicity and 28 patients for efficacy.

Dose-escalation findings

The first three patients were enrolled at dose level 1 (oxaliplatin 100 mg m^{-2}, S-1 80–120 mg day^{-1}). No DLTs were observed, and six patients were enrolled at dose level 2 (oxaliplatin 130 mg m^{-2}, S-1 80–120 mg day^{-1}). At level 1, one patient had grade 3 thrombocytopaenia. At level 2, one patient had grade 3 neutropaenia and one patient had grade 4 thrombocytopaenia. The RD was determined to be 130 mg m^{-2} of oxaliplatin in combination with the Japanese standard daily dose of S-1.

Table 1 Patients characteristics

Characteristic	Level 1, $n=3$ L-OHP (100 mg m^{-2}) No. of patients (%)	Level 2, $n=29$ L-OHP (130 mg m^{-2}) No. of patients (%)
Age (years)		
Median	57	57
Range	47–60	34–71
Sex		
Male	3 (100)	20 (69)
Female	0	9 (31)
ECOG performance status		
0	3 (100)	26 (90)
1	0	3 (10)
Primary tumour		
Colon	2 (67)	18 (62)
Rectum	1 (33)	11 (38)
Metastatic site		
Liver only	1 (33)	10 (35)
Lung	0	3 (10)
Liver and other lesions	1 (33)	10 (35)
Others	1 (33)	6 (21)
No. of metastatic sites		
1	1 (33)	15 (52)
⩾2	2 (67)	14 (48)
Previous treatment		
Resection	2 (67)	25 (86)
Adjuvant 5-FU	0	4 (14)

ECOG = Eastern Cooperative Oncology Group; L-OHP = oxaliplatin.

Safety assessment

After identification of tolerability at level 2 (130 mg m^{-2}) of oxaliplatin, 29 other patients received the RD at 130 mg m^{-2}, including the phase I part patients, to further evaluate the tolerability and toxicity of the study regimen. The median number of administered cycles was 6.5 (range: 2–14), and the total number of cycles for the 29 patients was 180. Oxaliplatin could be administered at the RD without dose reduction in 57% of 28 patients. At the RD, grade 3 neutropaenia was observed in four patients (14%), and grade 3 and 4 thrombocytopaenia in seven patients (24%) and one patient (3%), respectively. The median relative dose intensity was 82.8% for oxaliplatin and 74.6% for S-1 at level 2. The causes of treatment discontinuation at the RD were PD in 13 patients (36%), delayed recovery from toxicity such as neutropaenia, thrombocytopaenia, and slight hyperbilirubinaemia in 8 patients, discretion of the investigator in 2 patients, allergic reaction in 1 patient, and symptomatic deterioration in 1 patient. The treatment was discontinued due to prolonged thrombocytopaenia in eight patients after a median of seven cycles (range: 3–8). No treatment-related death was observed.

Sensory neuropathy occurred in all patients. However, no functional impairment was observed in this study. The most common non-haematologic toxicities were anorexia, nausea, and diarrhoea. One patient had grade 3 diarrhoea at the RD. Another mild adverse event related to treatment was injection site reactions (45%). One patient had severe allergic reactions such as skin rash and fever, which are typical platinum-related reactions during the sixth cycle (Table 2).

Response to therapy

The objective tumour response was determined by the External Review Board. One of the 28 patients given the RD at level 2 had CR and 13 patients had PR, yielding a response rate of 50% (95% CI: 30.6–69.4%). In the 28 patients studied, the median PFS was

Table 2 Toxicity

	Level 1, L-OHP (100 mg m^{-2}), $n=3$			Level 2, L-OHP (130 mg m^{-2}), $n=29$		
	All (%)	G3 (%)	G4 (%)	All (%)	G3 (%)	G4 (%)
Non-haematologic						
Nausea	1 (33)	0	0	21 (72)	0	0
Vomiting	0	0	0	7 (24)	0	0
Diarrhoea	1 (33)	0	0	17 (59)	1 (3)	0
Fatigue	1 (33)	0	0	25 (86)	0	0
Anorexia	2 (67)	0	0	26 (90)	0	0
Rush	3 (100)	0	0	13 (45)	0	0
Pigmentation disorder	1 (33)	0	0	22 (76)	0	0
Hand–foot syndrome	0	0	0	0	0	0
Peripheral neuropathy	3 (100)	0	0	29 (100)	0	0
Allergic reaction	0	0	0	0	1 (3)	0
Haematologic						
Neutropaenia	2 (67)	0	0	18 (62)	4 (14)	0
Leukopaenia	2 (67)	0	0	20 (69)	0	0
Thrombocytopaenia	3 (100)	1 (33)	0	27 (93)	7 (24)	1 (3)
Anaemia	1 (33)	0	0	18 (62)	1 (3)	0

L-OHP = oxaliplatin.

Table 3 Response rate

	No. of patients	CR	PR	SD	PD	Response rate (%)
Level 1						
L-OHP (100 mg m^{-2})	3	0	2	1	0	67 (CI: 9.4–99.2)
Level 2						
L-OHP (130 mg m^{-2})	28	1	13	9	5	50 (CI: 30.6–69.4)

CI = confidence interval; CR = complete response; L-OHP = oxaliplatin; PD = progressive disease; PR = partial response; SD = stable disease.

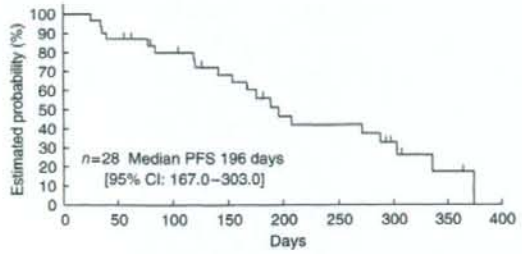

Figure 1 Progression-free survival.

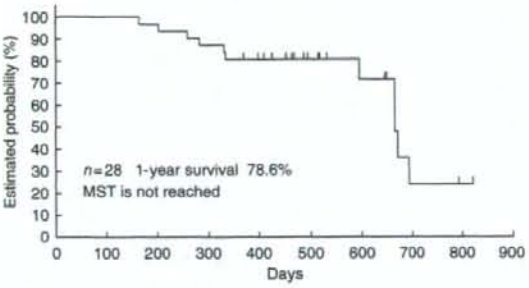

Figure 2 Overall survival.

196 days (95% CI: 167–303). The median overall survival time was not reached when 1 year passed since the last patient enrolment, namely 18 patients were alive and 10 patients were dead, and the 1-year survival rate was 78.6% by the Kaplan–Meier method (Table 3) (Figures 1 and 2).

DISCUSSION

Our results suggest that SOX regimen is safe and effective as a first-line treatment for metastatic colorectal cancer. The RD was determined to be 130 mg m^{-2} of oxaliplatin on day 1 with 40–60 mg of S-1 twice daily from the evening of day 1 to the morning of day 15, followed by a 7-day rest period in a 3-weekly schedule. This result indicates that both oxaliplatin and S-1 could be administered at doses similar to those recommended for monotherapy for each drug. SOX regimen has demonstrated promising efficacy with a response rate of 50%, median PFS of 196 days, and a 1-year survival rate of 78.6%. Efficacy of this combination is superior to that reported for monotherapy by each drug (Diaz-Rubio *et al*, 1998; Ohtsu *et al*, 2000; Shirao *et al*, 2004; Boku *et al*, 2007). No DLTs were observed during the first cycle at levels 1 and 2. At the RD (level 2), the toxicity profile was acceptable. The frequent non-haematologic toxicities were anorexia, nausea, and diarrhoea. Most cases of gastrointestinal toxicity were grade 1 or 2, and good oral intake was maintained. There was no grade 3 neurotoxicity observed. Although the incidence of grade 3 or 4 thrombocytopaenia seems to be higher with SOX compared with the reported result of FOLFOX4 (Diaz-Rubio *et al*, 1998; Shirao *et al*, 2004), it was well managed by adequate dose modification of oxaliplatin and S-1 in subsequent cycles (Goldberg *et al*, 2004). Since the severity of thrombocytopaenia is dependent on the dose of oxaliplatin, FOLFOX7 with oxaliplatin at a dose of 130 mg m^{-2} caused 9% of grade 3 thrombocytopaenia (Maindrault-Gœbel *et al*, 2001; Tournigand *et al*, 2006).

The median time to first dose reduction was five cycles (range: 2–7) due to any reason in 16 of the 28 patients at the RD, and 4.5 cycles (range: 3–5) due to grade 3 or 4 thrombocytopaenia in 6 of the 28 patients. Therapy was delayed in 22 of the 28 patients and 40 of 209 cycles, commonly due to neutropaenia, thrombocytopaenia, and sensory peripheral neuropathy. SOX requires only one clinic visit per 3-week cycle for a 2-h infusion of oxaliplatin. This convenience constitutes a marked advantage over regimens combining infused 5-FU/LV by ambulatory pump and oxaliplatin in terms of the impact on the daily lives of patients. In addition, very busy hospitals may have logistic issues providing pumps to all patients; therefore, oral S-1 offers an advantage over infusional 5-FU in respects of convenience and practicability.

The combination regimens of other oral fluoropyrimidine, capecitabine, and oxaliplatin have been reported in other phase II and III studies. Cassidy *et al* (2004) reported the results of a phase II study of oxaliplatin plus capecitabine (XELOX) as a first-line therapy in patients with colorectal cancer (Díaz-Rubio *et al*, 2002). Oxaliplatin (130 mg m^{-2}) was administered on day 1 and capecitabine (2000 mg m^{-2} day^{-1}) for 14 days with a 1-week rest, every 3 weeks. The response rate, median TTP, and MST were 55%, 7.7 months, and 19.5 months, respectively. Grade 3 or 4 neutropaenia according to NCI-CTC developed in 7% of patients by XELOX, and grade 3 or 4 diarrhoea developed in 16%.

The efficacy and safety of XELOX were also compared with that of 5-FU/LV plus oxaliplatin regimens (FUOX) in several phase III studies. The efficacy of XELOX was statistically not inferior to that of the FUOX regimen: median TTP 8.9 *vs* 9.5 months ($P=0.153$), and MST 18.1 *vs* 20.8 months ($P=0.145$) (Díaz-Rubio *et al*, 2007). Grade 3 or 4 diarrhoea was observed in 14% with both XELOX and FUOX regimens, respectively, and grade 3 or 4 HFS in 2% with XELOX. The efficacy of XELOX was also statistically not inferior to that of FOLFOX4: median TTP 8.0 *vs* 8.5 months, and MST 18.8 *vs* 17.7 months (Cassidy *et al*, 2007). Grade 3 or 4 diarrhoea was observed in 20% with XELOX and 11% with FOLFOX4, and grade 3 HFS in 6% with XELOX and 1% with FOLFOX4. Other schedules of oxaliplatin and capecitabine (CAPOX: 70 mg m^{-2} oxaliplatin on days 1 and 8 and 2000 mg m^{-2} day^{-1} capecitabine for 14 days with a 1-week rest) were compared with FUFOX. CAPOX was slightly inferior to FUFOX in TTP: median TTP 7.1 *vs* 8.0 months ($P=0.117$), and MST 16.8 *vs* 18.8 months ($P=0.26$) (Porschen *et al*, 2007). Both regimens were generally well tolerated, although grade 2 or 3 HFS occurred more often with CAPOX (10 *vs* 4%) ($P=0.028$). In summary, the results of these phase III studies show that the efficacy of XELOX or CAPOX was not inferior to or was slightly inferior to that of infusional 5-FU/LV plus oxaliplatin regimens. Although HFS is more commonly observed in capecitabine-combined regimens, capecitabine is expected to replace infusional 5-FU/LV.

Our limited experience of SOX regimen suggests that tri-weekly treatment with oxaliplatin and S-1 may be comparable to that of XELOX or CAPOX. The response rate of SOX was 50%, suggesting that it is worth while comparing the efficacy of SOX with that of XELOX in the phase III study. Grade 3 or 4 thrombocytopaenia was observed in 28% of patients, and this incidence seems to be higher than that reported by FOLFOX4 (de Gramont *et al*, 2000).

Grade 3 or 4 thrombocytopaenia with oxaliplatin monotherapy was reported in 12% of patients (Boku *et al*, 2007), and that with S-1 monotherapy in 0–8% of patients in previous phase II studies (Ohtsu *et al*, 2000; Shirao *et al*, 2004). The most commonly observed grade 3 or 4 toxicity after SOX therapy was cumulative prolonged thrombocytopaenia in this phase I/II trial, which is a well-known toxicity of oxaliplatin. The protocol therapy was discontinued due to prolonged thrombocytopaenia in seven patients after a median of seven cycles (range: 3–8). In cases where sudden and severe thrombocytopaenia is observed, type II allergic reaction to oxaliplatin should be considered and definitive withdrawal is strongly suggested (Maindrault-Gœbel *et al*, 2001). A phase I study of XELOX with 130 mg m^{-2} of oxaliplatin tri-weekly has also shown a relatively higher incidence of grade 3 thrombocytopaenia in 22% (Díaz-Rubio *et al*, 2002), but only 4% during phase II with weekly assessment of CBC (Cassidy *et al*, 2004). Thrombocytopaenia of SOX should be evaluated in the future phase II or III studies with a larger number of patients.

In conclusion, SOX holds promise of being a safe and effective treatment for metastatic colorectal cancer. Further evaluation is expected to examine whether SOX can be a substitute for FOLFOX.

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Clinical Significance of Insulin-Like Growth Factor Type 1 Receptor and Epidermal Growth Factor Receptor in Patients with Advanced Gastric Cancer

Junichi Matsubara[a] Yasuhide Yamada[a] Takako E. Nakajima[a] Ken Kato[a]
Tetsuya Hamaguchi[a] Kuniaki Shirao[a] Yasuhiro Shimada[a] Tadakazu Shimoda[b]

Divisions of [a]Gastrointestinal Oncology and [b]Clinical Laboratory, National Cancer Center Hospital, Tokyo, Japan

Key Words
Advanced gastric cancer · EGFR · Gastric cancer prognosticators · HER2 · IGF-1R

Abstract
Objective: To better understand the clinical implications of insulin-like growth factor type 1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and HER2 expressions in gastric cancer (GC). ***Methods:*** The study group comprised 86 patients who received first-line chemotherapy for advanced GC at the National Cancer Center Hospital. Using laser-captured microdissection and a real-time RT-PCR assay, we quantitatively evaluated mRNA levels of IGF-1R, EGFR and HER2 in paraffin-embedded cancer specimens of surgically removed primary tumors. ***Results:*** In univariate analysis of the study group as a whole, patients with low expression of both IGF-1R and EGFR (n = 13) had a significantly longer overall survival than the other patients (n = 51; median, 24.6 vs. 12.8 months; log-rank p = 0.013). Multivariate survival analysis demonstrated that high EGFR expression [hazard ratio, HR: 2.94 (95% confidence interval, CI: 1.40–6.17), p = 0.004] and poor performance status [HR: 1.96 (95% CI: 1.12–3.42), p = 0.018] were significant predictors of poor survival. In patients given first-line S-1 monotherapy (n = 29), low IGF-1R (p = 0.002) and low EGFR (p = 0.035) gene expression correlated with a better response, without a significant prolongation of survival. ***Conclusion:*** Our data warrant further investigations on the strategy of co-targeting IGF-1R and EGFR in GC.

Introduction

Globally, gastric cancer (GC) is the second most common cause of cancer-related death. Even though the incidence of GC is declining, approximately 930,000 cases are newly diagnosed each year [1]. Despite the identification and development of several new classes of anticancer agents, GC remains an aggressive malignancy, with a median survival of 7–10 months in patients with metastatic or unresectable disease. However, recent advances in molecularly targeted cancer therapeutics have led to some early success, and the insulin-like growth factor (IGF) signaling axis has newly emerged as an important target for cancer therapy [2, 3].

IGF type 1 receptor (IGF-1R) is a cell membrane receptor that is activated by its ligands, IGF-1 and IGF-2. IGF-1R participates in cell proliferation, differentiation and prevention of apoptosis [2, 4]. Since IGF-1R is also involved in malignant transformation [4], development of

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Yasuhide Yamada, MD, PhD
Gastrointestinal Oncology Division
National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku
Tokyo 1040045 (Japan)
Tel. +81 3 3542 2511, Fax +81 3 3542 3815, E-Mail yayamada@ncc.go.jp

IGF-1R-directed cancer therapy has been initiated. IGF-1R is frequently overexpressed in human cancers, and the association between IGF-1R expression and outcome has been assessed for breast cancer and other solid tumors [5, 6]. However, IGF-1R expression in GC remains poorly understood.

Previous studies have indicated that IGF-1R can interact with epidermal growth factor receptor (EGFR) to augment the malignant behavior of tumors [7]. EGFR and its homologues HER2 (also known as erbB-2) are members of the *erbB* gene family. These receptors encode for transmembrane receptor-type tyrosine kinases, for which therapeutic approaches do exist. They play a crucial role in tumor cell proliferation, resistance to apoptotic stimuli, adhesion, migration and differentiation, as well as participate in tumor angiogenesis [8]. EGF and transforming growth factor-α activate EGFR and HER2, which form either homodimers or heterodimers, and initiate intracellular signaling cascades. EGFR and IGF-1R regulate overlapping downstream signaling pathways and EGFR might also regulate the IGF-1R signaling pathway through IGF-binding protein-3 [9]. HER2 is the preferred co-receptor for the formation of dimers with EGFR, HER3 or HER4; the heterodimers consisting of HER2 and these other receptors have a greater capacity for translating mitogenic signals than the homodimers and act synergistically to promote cellular transformation [10]. In many cancers, the expression of these receptors may be related to patient survival [11]. Expression of EGFR or HER2 has been evaluated using several methods in GC. However, whether the expressions of EGFR and HER2 are significant predictors of survival in patients with GC remains controversial.

This study was designed to further delineate the clinical implications of IGF-1R, EGFR and HER2 mRNA expression in GC. We tested the hypothesis that the clinical outcome of patients with advanced GC, such as rate of objective response to first-line chemotherapy and overall survival, is related to the pretreatment intratumor mRNA levels of these three biomarkers. We also analyzed relationships between the study variables and clinicopathological characteristics.

Patients and Methods

Patient Eligibility

Patients with a diagnosis of histologically proven advanced GC were eligible for the study. Inclusion criteria were as follows: unresectable, locally advanced or metastatic disease; no prior chemotherapy and no prior adjuvant/neoadjuvant chemotherapy; specimens of primary gastric adenocarcinomas were obtained before the start of chemotherapy by surgical resection at the National Cancer Center Hospital (Tokyo, Japan); first-line chemotherapy was administered at the National Cancer Center Hospital between July 1997 and June 2004; radiographically measurable disease, and written informed consent. Measurable disease was assessed by a standardized CT examination every 2 months. Response was evaluated according to the standard guidelines of the International Union against Cancer as complete response (CR), partial response (PR), no change (NC) or progressive disease (PD) [12]. Tumor response and survival times as of December 2006 were confirmed in all patients. Written informed consent was obtained before treatment and evaluation of tumor samples. This study was approved by the Institutional Review Board of the National Cancer Center Hospital.

Chemotherapy

The following first-line chemotherapy regimens were administered to the patients in our study: S-1 monotherapy (n = 29), cisplatin plus irinotecan (n = 29), 5-FU monotherapy (n = 23) and other regimens (5-FU plus methotrexate, n = 2; paclitaxel, n = 2, and uracil/ftorafur, n = 1). For S-1 monotherapy, patients received S-1 (40 mg/m^2 twice daily) on days 1–28 of a 42-day cycle. Treatment with cisplatin plus irinotecan consisted of cisplatin (80 mg/m^2) on day 1 and irinotecan (70 mg/m^2) on days 1 and 15 of a 28-day cycle. For 5-FU monotherapy, patients received 5-FU (800 mg/m^2/day) as a continuous infusion on days 1–5 of a 28-day cycle.

Laboratory Methods

Ten-micrometer-thick sections obtained from identified areas with the highest tumor cell concentration were mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with hematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (American MasterTech Scientific, Lodi, Calif., USA). The sections of interest were selectively isolated by laser-captured microdissection (PALM Microsystem; Leica, Wetzlar, Germany), according to standard procedures [13]. The dissected tissue particles were transferred to a reaction tube containing 400 µl of RNA lysis buffer.

The samples were homogenized and heated at 92°C for 30 min. Fifty microliters of 2 M sodium acetate were added at pH 4.0, followed by 600 µl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were placed on ice for 15 min and then centrifuged at 13,000 rpm for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was carefully removed. Glycogen (10 µl) and 300–400 µl of isopropanol were added. The tubes were chilled at –20°C for 30–45 min to precipitate the RNA. The samples were washed in 500 µl of 75% ethanol and air-dried for 15 min. The pellet was resuspended in 50 µl of 5 mM Tris. Finally, cDNA was prepared as described by Lord et al. [14].

Quantification of three genes of interest and an internal reference gene (β-actin) was done with a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence detection System, TaqMan®, Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) using the standard curve method. The PCR reaction mixture consisted of 1,200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of $MgCl_2$ and 1× TaqMan buffer A containing

Table 1. Primer and probe sequences for quantitative RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan® Probe (5′–3′)
IGF-1R	TGGAGTGCTGTATGCCTCTG	CACCTCCCACTCATCAGGA	CCCGGAGTACTTCAGCGCTGCTG
EGFR	TGCGTCTCTTGCCGGAAT	GGCTCACCCTCCAGAAGGTT	ACGCATTCCCTGCCTCGGCTG
HER2	CTGAACTGGTGTATGCAGATTGC	TTCCGAGCGGCCAAGTC	TGTGTACGAGCCGCACATCCTCCA
β-Actin	GAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	ACCACCACGGCCGAGCGG

a reference dye. The final volume of the reaction mixture was 20 µl (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. The primers and probes used are listed in table 1. Gene expression values (relative mRNA levels) are expressed as ratios [differences between threshold cycle (Ct) values] between the gene of interest and an internal reference gene (β-actin).

For each gene, we established a usable Ct range for the data and documented the precision of the measurements within the usable range. For maximum accuracy, we demonstrated that the slopes of the plots of ΔCt versus log pg RNA for target genes and the housekeeping gene (β-actin) were parallel. Each replicate Ct data point is the average of Ct values obtained in three PCR reactions. To compare the results of two different TaqMan plates with each other, the same standardized samples are analyzed on every plate.

Statistical Analysis

We examined the objective tumor response to chemotherapy and overall survival. The objective response rate was calculated as the ratio of (CR + PR)/(CR + PR + NC + PD). Overall survival was calculated as the period from the start of first-line chemotherapy until death from any cause. If patients were lost to follow-up, data were censored at the date of the last evaluation.

To assess associations of gene expression levels with tumor response and overall survival, the expression levels of each gene were categorized into low and high values at optimal cutoff points. The maximal χ^2 method [15–17] was used to determine which gene expression (optimal cutoff point) best segregated patients into poor- and good-outcome subgroups (in terms of likelihood of response and survival). To determine the corrected p values based on the maximal χ^2 analysis, 2,000 bootstrap-like simulations were used in univariate analyses to estimate the distribution of the maximal χ^2 statistics under the null hypothesis of no association. Variables for multivariate analysis were selected by the stepwise method, using a significance level of $p < 0.050$ for entering into or remaining in the model. The estimates of hazard ratios (HRs) with 95% confidence intervals (CIs) based on a Cox proportional hazard model were used to provide quantitative summaries of the gene expression data. The probability of overall survival was calculated with the Kaplan-Meier method, and differences between curves were evaluated with the log-rank test.

Correlations of three biomarker expressions with each other were examined by Spearman's rank correlation test. The Wilcoxon test was used to compare the mRNA expression levels of each growth factor receptor between two different histological types of tumors. All reported p values are two-sided, and the level of statistical significance was set at $p < 0.050$. All analyses were performed using the statistical software package R, version 2.4.1, and the SAS statistical package, version 9.1.3 (SAS Institute, Cary, N.C., USA).

Results

Eight hundred ninety-nine patients received first-line chemotherapy for advanced GC at our hospital between July 1997 and June 2004. A total of 86 of these patients were eligible for the present study. The demographic characteristics of the patients at the start of first-line chemotherapy are shown in table 2. There were 69 (80%) men and 17 (20%) women, with a median age of 64 years. At the time of analysis, 78 (91%) patients had died and 8 (9%) patients were alive. No patient was lost to follow up.

The chemotherapy regimens received by the patients and the response rates are also shown in table 2. The rates of response to first-line chemotherapy in our study were comparable to those reported previously [18, 19].

Gene Expression Levels of IGF-1R, EGFR and HER2, Clinical Characteristics, and Overall Survival since the Start of First-line Chemotherapy in All Patients

The gene expression levels of the three biomarkers were quantifiable in 86.0–93.0% of the 86 tumors (table 2) and their cutoff values for overall survival analyses were determined using the maximal χ^2 method. The median overall survival was 13.6 months in our study. In univariate analyses, overall survival in the study group as a whole correlated with the expression level of EGFR alone (table 3). Since the results of univariate survival analysis for HER2 expression showed a trend in an opposite direction to the results for IGF-1R and EGFR, we performed a combined analysis of IGF-1R and EGFR expressions. Patients with low mRNA expression of both IGF-1R and EGFR (n = 13) had significantly longer overall survival than the other patients (n = 51; median overall survival,

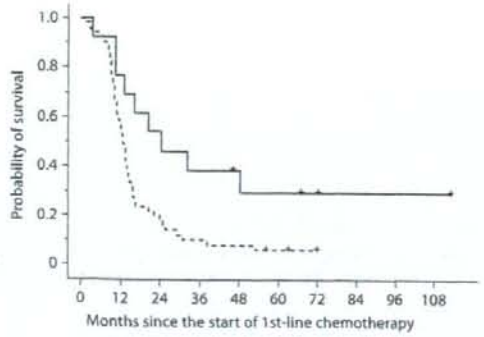

Fig. 1. Kaplan-Meier plot of overall survival for all patients according to the mRNA expression levels of IGF-1R and EGFR: —— = Low IGF-1R and low EGFR expression (n = 13; median survival: 24.6 months); ------ = other (n = 51; median survival: 12.8 months); log-rank test p = 0.013.

24.6 vs. 12.8 months; log-rank p = 0.013; fig. 1). Multivariate analysis with a Cox proportional hazard model demonstrated that high EGFR expression [HR: 2.94 (95% CI: 1.40–6.17)] and poor performance status [HR: 1.96 (95% CI:1.12–3.42)] were significant predictors of shorter survival (table 3).

Gene Expression Levels of IGF-1R, EGFR and HER2, Tumor Response, and Overall Survival in Patients Treated with S-1 Monotherapy or Cisplatin plus Irinotecan as First-Line Chemotherapy

To better understand the relationship between the mRNA levels of the three biomarkers and treatment outcomes with each chemotherapy regimen, we performed subgroup analysis. Gene expression cutoff values that best segregated patients into poor- and good-response subgroups were defined by the same methods as those used for the analyses of overall survival. In patients given first-line S-1 monotherapy, low IGF-1R gene expression (objective response rate: 57 vs. 0% in patients with low vs. high IGF-1R, respectively; p = 0.002) and low EGFR gene expression (objective response rate: 71 vs. 26% in patients with low vs. high EGFR, respectively, p = 0.035) correlated with a better response (table 4). However, the expression level of none of the three biomarkers correlated with overall survival (table 4).

In patients treated with first-line cisplatin plus irinotecan, expressions of the three biomarkers did not show any correlation with response (table 4). However, the expression level of EGFR correlated with overall survival (table 4). At an EGFR cutoff value of 1.39×10^{-3}, low EGFR gene expression was associated with a trend toward a better response (60 vs. 25% in patients with low vs. high EGFR, p = 0.100), and the median overall survival was as long as 20.6 months in the low EGFR expression group, compared with 14.3 months in the high EGFR expression group (log-rank p = 0.042). Expression levels of the other two genes did not correlate with overall survival (table 4).

Other first-line regimens were not examined because the number of patients who responded to treatment was too small.

Table 2. Patient characteristics

Characteristics	Patients (n = 86)	
	n	%
Sex		
Male	69	80
Female	17	20
Age, years		
Median	64	
Range	39–84	
ECOG performance status		
0	42	48
1	41	48
2	3	3
Metastatic site		
Lymph nodes	43	49
Liver	26	30
Peritoneum	22	26
Lung	4	5
Other	4	5
Histological type		
Intestinal	38	44
Diffuse	48	56
First-line chemotherapy regimen		
S-1	29	38[1]
Cisplatin + irinotecan	29	36[1]
5-FU	23	4[1]
Other	5	20[1]
Gene expression levels[2]		
IGF-1R [2.03 (0.16–14.98)]	75	87.2
EGFR [3.66 (0.35–57.78)]	74	86.0
HER2 [0.19 (0.01–2.39)]	80	93.0

ECOG = Eastern cooperative oncology group.
[1] Response rates are shown (in percentages).
[2] Medians (in brackets) and ranges (in parentheses) of mRNA expression levels are given relative to the internal reference gene β-actin ($\times 10^{-3}$).

Table 3. Univariate analysis and Cox regression multivariate analysis of overall survival in all patients included in this study: correlation with mRNA expression levels and clinical data

Factors	Cutoff	Patients	MST months	Univariate analysis		Multivariate analysis*	
				HR (95% CI)	p	HR (95% CI)	p
EGFR	$\leq 0.77 \times 10^{-3}$	16	24.6	1	**0.001**	1	**0.004**
	$>0.77 \times 10^{-3}$	58	13.5	1.49 (1.10–2.13)		2.94 (1.40–6.17)	
PS	continuous variable	86	–	–	0.055	1	**0.018**
				1.48 (0.99–2.19)		1.96 (1.12–3.42)	
IGF-1R	$\leq 3.28 \times 10^{-3}$	65	13.5	1	0.055		
	$>3.28 \times 10^{-3}$	10	10.4	1.44 (0.99–1.98)			
HER2	$\leq 0.179 \times 10^{-3}$	65	13.3	1	0.064		
	$>0.179 \times 10^{-3}$	15	22.0	0.76 (0.55–1.02)			
Tissue type	intestinal	38	16.0	1	0.089		
	diffuse	48	12.6	1.21 (0.97–1.53)			

PS = Performance status. Factors are listed in ascending order of p values, and cutoff points were determined by the maximal χ^2 method.

* Factors were selected by the stepwise method from among all five factors used for univariate analysis. The level of significance was set at $p < 0.050$. Significant values are shown in bold.

Table 4. Correlation of gene expression levels with tumor response and overall survival in patients with advanced GC according to first-line chemotherapy

	S-1 monotherapy (n = 29)						Cisplatin + irinotecan (n = 29)					
	cutoff $\times 10^{-3}$	patients	RR %	p	MST months	p log rank	cutoff $\times 10^{-3}$	patients	RR %	p	MST months	p log rank
IGF-1R	≤1.88	14	57	**0.002**	13.5	0.306	≤0.64	10	30	0.134	15.5	0.255
	>1.88	11	0		10.4		>0.64	15	60		14.6	
EGFR	≤0.76	7	71	**0.035**	24.6	0.197	≤1.39	15	60	0.100	20.6	**0.042**
	>0.76	19	26		13.2		>1.39	8	25		14.3	
HER2	≤0.026	6	67	0.054	13.5	0.741	≤0.021	7	71	0.126	12.7	0.304
	>0.026	19	26		13.2		>0.021	21	38		17.9	

RR = Objective response rate. The cutoff point was determined by the maximal χ^2 method. The level of significance was set at $p < 0.050$. Significant values are shown in bold.

Expressions of IGF-1R, EGFR and HER2, and Histological Tumor Types

There was no correlation between the mRNA expression levels of IGF-1R and EGFR (Spearman's rank correlation coefficient: r = 0.180), IGF-1R and HER2 (r = −0.088), and EGFR and HER2 (r = −0.087). Intestinal tumors had lower IGF-1R (p = 0.021, Wilcoxon test; fig. 2a) and higher HER2 ($p < 0.001$, fig. 2c) expression levels than diffuse tumors.

Discussion

Since reports that the IGF system participates in cancer progression, angiogenesis, metastasis and resistance to apoptosis, IGF-1R has received considerable attention as a potential target for cancer therapy [3, 20, 21]. Two phase I/II clinical trials of receptor-specific blocking of monoclonal antibodies are now underway [22, 23], as are many phase I and preclinical trials of other monoclonal antibodies and small-molecule tyrosine kinase inhibitors. In GC, evidence supporting an association of IGF-1R mRNA

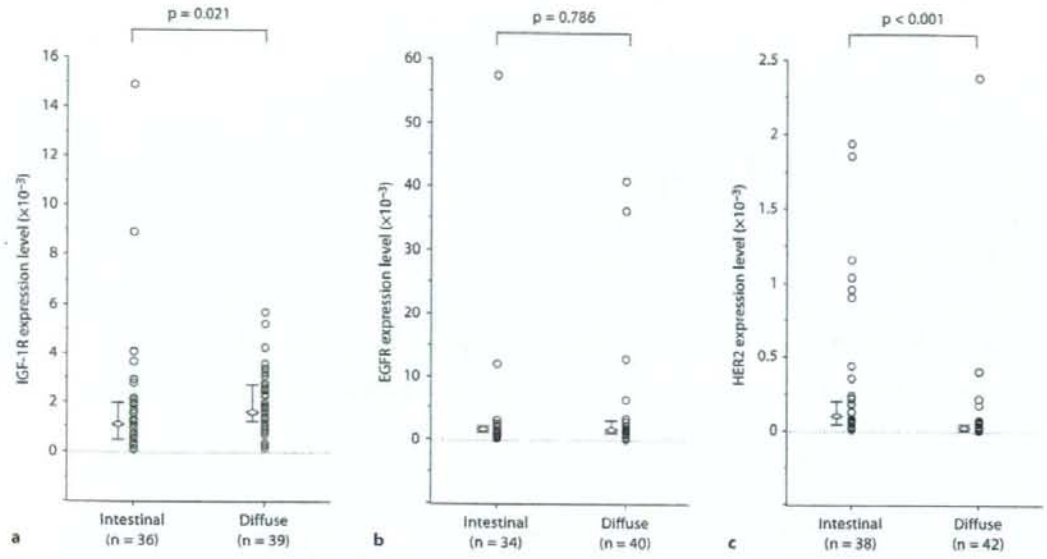

Fig. 2. Expression levels of IGF-1R (**a**), EGFR (**b**) and HER2 (**c**) in intestinal and diffuse tumors. Every result is plotted, and median values (diamonds) and interquartile ranges from 25 to 75% (bars) are also shown. Correlations of IGF-1R, EGFR and HER2 expressions with histological tumor types were tested by the Wilcoxon test. **a** IGF-1R, $p = 0.021$. **b** EGFR, $p = 0.786$. **c** HER2, $p < 0.001$.

expression with survival and clinicopathological characteristics remains scant so far. Our study showed that patients with low IGF-1R expression had slightly but not significantly longer survival. Our findings suggest that anti-IGF-1R strategies with specific monoclonal antibodies or tyrosine kinase inhibitors, as well as anti-EGFR strategies, may prove valuable in patients with GC.

Targeting EGFR has been a successful approach to treat colorectal cancer [24, 25]. In GC, the results of phase II clinical trials of cetuximab (anti-EGFR monoclonal antibody) and lapatinib (a dual tyrosine kinase inhibitor of EGFR and HER2) were reported in 2007 [26, 27]. Our data suggest that anti-EGFR strategies may be beneficial in patients with GC and contribute to improved outcomes. Since EGFR antagonists will be approved for the clinical treatment of GC in the near future, there is an urgent need for a better understanding of the clinical significance of EGFR expression at the molecular level.

Cross-talk between IGF-1R and EGFR has been implicated in the development of tumor cell resistance to treatment with EGFR inhibitors [28, 29]. Recently, a combination of IGF-1R-targeted therapy and anti-EGFR strategies has been shown to synergistically enhance antitumor activity in vitro [30, 31]. Our study showed that overall survival in patients with low expression of both IGF-1R and EGFR in primary GC was significantly longer than in the other patients. Although we did not use anti-IGF-1R and anti-EGFR compounds in the present study, our results suggest that the co-targeting strategy of anti-IGF-1R and anti-EGFR may be beneficial in GC, potentially leading to improved survival.

In contrast to IGF-1R and EGFR expression, high HER2 expression was associated with a longer overall survival than low HER2 expression (table 3). This finding was probably related to the facts that intestinal-type tumors had higher HER2 expression levels than diffuse-type tumors (fig. 2c) and that patients with intestinal-type tumors had longer overall survival than those with the diffuse type (table 3), as is typically the case.

Patients with low expression of IGF-1R or EGFR had higher response rates to first-line S-1 monotherapy (table 4). Given that survival signals originating at IGF-1R and EGFR limit the efficacy of cytotoxic agents designed to induce apoptosis, treatments targeting IGF-1R and EGFR might augment the efficacy of such agents [32]. However, the small sample size of our study precludes drawing any firm conclusions.

mRNA expression of IGF-1R, EGFR, and HER2 could not be measured in approximately 10% of the patients (table 2). Response and survival were similar between patients with complete data and patients with missing data (data not shown). We excluded those patients with missing data from some analyses in the present study because there would be little effect of missing data on the overall results of our study.

In conclusion, our study provides evidence that low mRNA expression of both IGF-1R and EGFR in primary GC correlates with significantly longer overall survival, whereas high EGFR expression in GC specimens and poor performance status are significant predictors of poorer survival in patients with advanced GC. Although our results may provide a specific rationale for combining anti-IGF-1R and anti-EGFR strategies for the antineoplastic treatment of GC, our small sample size precludes drawing any firm conclusions. The potential therapeutic benefits of simultaneously co-targeting such receptors in patients with GC should be critically evaluated, especially in prospective studies.

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