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Differential gene expression signatures between colorectal cancers with and without KRAS mutations: Crosstalk between the KRAS pathway and other signalling pathways

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ABSTRACT

Purpose: KRAS mutation is an important predictive marker in determining resistance to anti-Epidermal Growth Factor Receptor (EGFR) antibody therapies. In order to clarify whether not only KRAS related signalling pathways but also other signalling pathways are altered in patients with colorectal cancers (CRCs) with KRAS mutations, we examined the differences in the gene expression signatures between CRCs with and without KRAS mutation.

Patients and methods: One-hundred and thirteen patients who underwent a surgical resection of a primary CRC were examined. KRAS mutational status was determined using the Peptide Nucleic Acid (PNA)-clamp real-time polymerase chain reaction (PCR) TaqMan assay. Gene expression profiles were compared between CRCs with and without KRAS mutation using the Human Genome GeneChip array U133.

Results: Among 113 CRCs, KRAS mutations were present in 35 tumours (31%). We identified 30 genes (probes) that were differentially expressed between CRCs with and without KRAS mutation (False Discovery Rate (FDR), $p < 0.01$), by which we were able to predict the KRAS status with an accuracy of 90.3%. Thirty discriminating genes included TC21, paired-like homeodomain 1 (PITX1), Sprouty-2, dickkopf homologue 4 (DKK-4), SET and MYND domain containing 3 (SMYD3), mitogen-activated protein kinase kinase kinase 14 (MAP3K14) and c-mer Proto-oncogene tyrosine kinase (MerTK). These genes were related to not only KRAS related signalling pathway but also to other signalling pathways, such as the Wnt-signalling pathway, the NF-kappa B activation pathway and the TGF-beta signalling pathway.

Conclusions: KRAS mutant CRCs exhibited a distinct gene expression signature different from wild-type KRAS CRCs. Using human CRC samples, we were able to show that there is crosstalk between the KRAS-mediated pathway and other signalling pathways. These results are necessary to be taken into account in establishing chemotherapeutic strategies for patients with anti-EGFR-refractory KRAS mutant CRCs.

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1. Introduction

Cetuximab and panitumumab are anti-Epidermal Growth Factor Receptor (EGFR) antibody agents used in the treatment of patients with metastatic colorectal cancer (CRC). EGFR is a member of the transmembrane tyrosine kinase receptor family and activates the RAS–RAF–MAP kinase pathway, thereby controlling cell growth, differentiation and proliferation.[1,2] EGFR activation contributes to malignant transformation and metastatic dissemination, whereas the blockade of EGFR disrupts the downstream signalling pathways. Because cetuximab and panitumumab are monoclonal antibodies directed against EGFR, KRAS mutations in the RAS–RAF–MAP kinase pathway have been reported to be an important predictive marker in determining tumour resistance to anti-EGFR antibody therapies.[3–8] Studies demonstrated that the benefits of anti-EGFR antibody treatment are restricted to patients with wild-type KRAS disease; therefore, other chemotherapeutic agents are recommended for the treatment of CRC patients with KRAS mutations.[9,10]

On the other hand, studies have shown that multiple molecular signalling pathways initiating from KRAS are interconnected since the mutation of genes in one pathway may influence the activity of kinases in the other pathways.[11] Therefore, not only KRAS related signalling pathways such as the RAS/PI3K/PTEN/Akt pathways, but also other signalling pathways have been shown to be altered in CRCs with KRAS mutations.[11,12] Because of these molecular interactions, the effects of the activation of KRAS may result in complex functional effects, including changes in cellular proliferation, apoptosis, drug resistance and prognosis.[11,13] KRAS has been reported to be a predictive marker for response to anti-EGFR therapy and as a prognostic factor for CRC patients.[13] This suggests that patients with mutant KRAS-expressing CRCs may harbour multiple genetic alterations that may determine the prognosis for CRC patients.

To date, few studies have examined the difference in gene expression between cancer cell lines with and without KRAS mutations. Three *in vitro* studies characterised the different gene signatures between wild-type and mutant KRAS cells using a microarray analysis.[14–16] By using recombinant adenoviruses to express the oncogenic activities of Ras in human primary mammary epithelial cells, Bild et al. examined the activation and deregulation of Ras pathway via DNA microarray. The authors identified 348 genes whose expression significantly differed according to the Ras status.[14] Monticone et al. also demonstrated that cells with a KRAS mutation exhibit a different gene expression signature compared to wild-type KRAS cells by a microarray analysis.[15] The authors also demonstrated that KRAS mutant cells dysregulated several genes associated with cell cycle, apoptosis and nitrogen metabolism, indicating reduced survival and proliferation compared to wild-type KRAS cells. Li et al. described that KRAS mutant transfected Caco cells have a different gene expression compared to wild-type Caco cells via microarray.[16] These *in vitro* studies clearly revealed that cells with mutated KRAS have changes in various signalling pathways that reflect the activation status of the KRAS pathway. However, few studies have examined the differences in gene expression signatures between human CRC samples with wild-type or mutated KRAS status.

The aim of the present study was to clarify the differences in the gene expression signature between CRCs with or without KRAS mutation. Identification of a characteristic signature of mutant KRAS CRCs may help to determine the chemotherapeutic response to other chemotherapeutic agents for the individualised selection of therapies and regimens.

2. Materials and methods

2.1. Patients and samples

One hundred and thirteen patients who underwent a surgical resection of the primary CRC were included in this study. The patient and tumour characteristics are shown in Table 1.

2.2. DNA and RNA isolation, microarray expression profiling and validation

Genomic DNA was extracted from frozen samples as described previously using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA).[17] Frozen samples were taken from surgically-resected specimens, snap-frozen in liquid nitrogen and stored at –80 °C. Parallel tumour specimens were formalin-fixed and paraffin-embedded for the histological studies. Samples were used for DNA extraction once microscopic examination verified that specimens contained at least 70% tumour cells.[17,18] The gene expression profiles were determined with the Affymetrix HG-U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA).[17] The present study was approved by the local ethics committee, and all patients provided their informed consent in regard to data handling.

2.3. KRAS mutational analyses

2.3.1. Primer, probe and PNA design

We designed primers to KRAS using the Primer Express Software programme, version 3 (Applied Biosystems, Foster City, CA). We generated an amplicon of 157 bp (KRAS). The chosen primers were subjected to a Basic Local Alignment Search Tool (BLAST) N, BLAST X, and BLAST P database searches to identify any sequence similarities. The chosen reporter fluorophores for TaqMan MGB probes were 6-carboxyfluorescein (FAM) for KRAS. The Peptide Nucleic Acid (PNA) clamp was designed to hybridise to the wild-type (wtDNA) KRAS allele surrounding codons 12 and 13. The primers, probes and PNA-clamp were as follows: KRAS, KRAS-F (forward) 5′-ATCGTCAA GGCACTCTTGCCTAC-3′, KRAS-R (reverse) 5′-GTA CTGGTGGAG TATTTGATAGTG-3′, KRAS-MGB-probe 5′-FAM-A CCTTATGTG TGACATGTT-3′, and KRAS–PNA clamp H_2N-TAC GCCAC CAGCTCC-CO-N_2H.

2.3.2. Real-time TaqMan PNA clamp PCR

The KRAS mutation status of DNA extracted from patient samples was determined using the PNA-clamp real-time PCR TaqMan assay. PCR amplification was performed in a total volume of 10 μl, containing 2 μl DNA (20 ng), 5 μl 2×

Table 1 – Clinical and pathologic patient characteristics.

Characteristics	KRAS mutation (–) (n = 78)		KRAS mutation (+) (n = 35)		Total (n = 113)		p-Value*
	No. of patients	%	No. of patients	%	No. of patients	%	
Age (years)							
Median	65		68		66		0.21
Range	32–87		26–83		26–87		
Gender							
Male	55	71	21	60	76	67	0.27
Female	23	29	14	40	37	33	
Tumour location							
Proximal colon	27	35	11	31	38	34	0.68
Distal colon	29	37	16	46	45	39	
Rectum	22	28	8	23	30	27	
Differentiation							
Well	45	58	15	43	60	523	0.31
Moderate	25	32	14	40	39	35	
Others	8	10	6	17	14	12	
pN							
pN0	56	71	19	54	75	66	0.10
pN1	16	21	9	26	25	22	
pN2	6	8	7	20	13	12	
pT							
pT1–2	18	23	6	17	24	21	0.65
pT3	39	50	17	49	56	50	
pT4	21	27	12	34	33	29	

* P value: KRAS mutation (–) versus KRAS mutation (+).

TaqMan Gene Expression master mix (ABI), 160 nM of each primer, 500 nM of TaqMan probe and 800 nM PNA (Panagene, Daejeon, Korea). The parallel non-PNA-clamp reaction was performed without any PNAs. PCR was performed in MicroAmp optical 384-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were performed with an ABI prism 7900 sequence detection system (Applied Biosystems). The PCR conditions were 95 °C for 5 min, followed by three-step cycling: 40 cycles of 95 °C for 30 s, 70 °C for 30 s, and 60 °C for 1 min. In each experiment, PCR reagents without template were run in parallel as the no-template control. The fluorescence data were analysed with the ABI Prism 7900 software programme, and amplification plots produced in the PCR reaction were also analysed. PNA hybridisation securely inhibits the annealing of the partially overlapping reverse primer and inhibits the amplification of wtDNA at the KRAS allele. The PNA/DNA hybrid is unstable due to base pair mismatches, and, therefore, does not inhibit Taq polymerase from extending the reverse primer on the mutated tumour DNA. The threshold cycle (C_t value) was automatically calculated from the PCR amplification plots, in which the fluorescence was plotted against the number of cycles. All possible combinations of primers and sets of probes were tested with the controls of previously-sequenced DNAs from CRC tissues.

2.4. Microarray data analysis and class prediction

The expression analyses were carried out using the GeneSpring GX software program, versions 7.3 and 11 (Agilent Technologies, Santa Clara, CA). The gene expression data, when classified as either flag-P or flag-M in more than 10% of all samples, were loaded into the software programme and were normalised in one of two ways: 'per-chip normalisation' and 'per-gene normalisation'. For 'per chip normalisation', all expression data on a chip were normalised to the 75th percentile of all values on that chip. For 'per gene normalisation', the data for a given gene were normalised to the median expression level of that gene across all samples.

Expression profiles were compared between CRC with and without KRAS mutation using a statistical multiple test procedure based on the Benjamini and Hochberg false discovery rate.[19] Two-dimensional hierarchical clustering was then applied to the log-transformed data with average-linkage clustering and standard correlation as the similarity metric for the discriminating genes that were differentially expressed between CRCs with and without KRAS mutation. Variations in the multigene expression between CRCs with and without KRAS mutations were also compared by a principal component analysis (PCA). We thereafter carried out supervised class prediction using the k-nearest-neighbour method and a leave-one-out cross-validation with the discriminating genes.[20]

2.5. Comparison with the GEO dataset (GSE3151 and GSE16125)

Bild et al. identified 348 genes whose expression differed significantly depending on the RAS mutational status.[14] The gene expression data of the 348 genes were analysed with

Table 2 – A list of 30 probes that were differentially expressed between colorectal cancers with and without KRAS mutation.

Probe ID	UniGene ID	Gene symbol	Gene name	Absolute fold change	Regulation in KRAS mutant colorectal cancer	*p*-Value	Corrected *p*-value
201968_s_at	Hs.1869	PGM1	Phosphoglucomutase 1	1.47	Down	2.30E-05	0.0033
204011_at	Hs.18676	SPRY2	Sprouty homologue 2 (Drosophila)	1.41	Up	2.21E-05	0.0033
204820_s_at	Hs.376046	BTN3A2	Butyrophilin, subfamily 3, member A2	1.43	Down	5.85E-05	0.0060
205192_at	Hs.404183	MAP3K14	Mitogen-activated protein kinase kinase kinase 14	1.32	Down	2.30E-05	0.0033
206028_s_at	Hs.306178	MerTK	c-mer Proto-oncogene tyrosine kinase	1.45	Down	1.30E-05	0.0027
206619_at	Hs.159311	DKK-4	Dickkopf homologue 4 (*Xenopus laevis*)	5.44	Up	1.26E-07	0.0002
208502_s_at	Hs.84136	PITX1	Paired-like homeodomain 1	1.62	Up	9.40E-05	0.0089
208608_s_at	Hs.46701	SNTB1	Syntrophin, beta 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	1.81	Up	3.61E-05	0.0048
208916_at	Hs.631582	SLC1A5	Solute carrier family 1 (neutral amino acid transporter), member 5	1.53	Up	2.18E-05	0.0033
209846_s_at	Hs.376046	BTN3A2	Butyrophilin, subfamily 3, member A2	1.58	Down	4.48E-05	0.0052
211518_s_at	Hs.68879	BMP4	Bone morphogenetic protein 4	3.06	Up	2.04E-06	0.0011
212589_at	Hs.502004	RRAS2	Related RAS viral (r-ras) oncogene homologue 2	1.43	Down	1.58E-05	0.0029
212590_at	Hs.502004	RRAS2	Related RAS viral (r-ras) oncogene homologue 2	1.48	Down	3.97E-05	0.0050
212613_at	Hs.376046	BTN3A2	Butyrophilin, subfamily 3, member A2	1.73	Down	7.08E-05	0.0070
213172_at	Hs.79170	TTC9	Tetratricopeptide repeat domain 9	2.11	Up	5.37E-05	0.0057
214285_at	Hs.657242	FABP3	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	2.88	Down	8.68E-08	0.0002
218788_s_at	Hs.567571	SMYD3	SET and MYND domain containing 3	1.34	Up	1.26E-05	0.0027
219093_at	Hs.715695	PID1	Phosphotyrosine interaction domain containing 1	1.82	Down	1.56E-05	0.0029
38241_at	Hs.167741	BTN3A3	Butyrophilin, subfamily 3, member A3	1.43	Down	1.09E-04	0.0100
225307_at	Hs.422113	ZNF511	Zinc finger protein 511	1.47	Up	1.14E-05	0.0027
226438_at	Hs.46701	SNTB1	Syntrophin, beta 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	1.69	Up	7.56E-06	0.0026
228010_at	Hs.479069	PPP2R2C	Protein phosphatase 2 (formerly 2A), regulatory subunit B, gamma isoform	3.26	Up	1.26E-06	0.0011
228479_at	Hs.445588			1.48	Down	6.28E-06	0.0025
229481_at		LOC283859	Hypothetical protein LOC283859	3.16	Up	8.67E-06	0.0026
232203_at	Hs.187578			2.38	Up	1.70E-06	0.0011
232273_at	Hs.656532			1.61	Down	1.14E-05	0.0027
232320_at	Hs.665319			1.51	Up	3.64E-05	0.0048
233768_at	Hs.677217			1.65	Down	4.65E-05	0.0052
235845_at	Hs.368802	SP5	Sp5 transcription factor	2.26	Up	4.69E-05	0.0052
244174_at	Hs.301715			2.97	Up	2.49E-06	0.0011

regard to the KRAS mutational status in the present samples. Reid et al. analysed the gene expression in 38 sporadic colon cancer using an Affymetrix exon array (GSE16125). Data were downloaded from the GEO database, and were re-analysed using the GeneSpring GX software programme, versions 7.3 and 11.0 (Agilent Technologies, Santa Clara, CA).

2.6. Statistical analysis

Categorical data were statistically analysed by the χ^2 test or Fisher's exact test. Continuous data were analysed using Student's *t*-test. All differences were considered to be statistically significant for *p*-values of less than 0.05.

3. Results

3.1. KRAS mutation status in CRCs

Among 113 CRCs, KRAS mutations were present in 35 tumours and were absent in the remaining 78 tumours. The frequency of KRAS mutation was 31.0%.

3.2. Microarray data analysis

We identified 30 probes that were differentially expressed between CRCs with and without KRAS mutation (FDR, $p < 0.01$) (Table 2), and 16 expressed at higher levels and 14 were

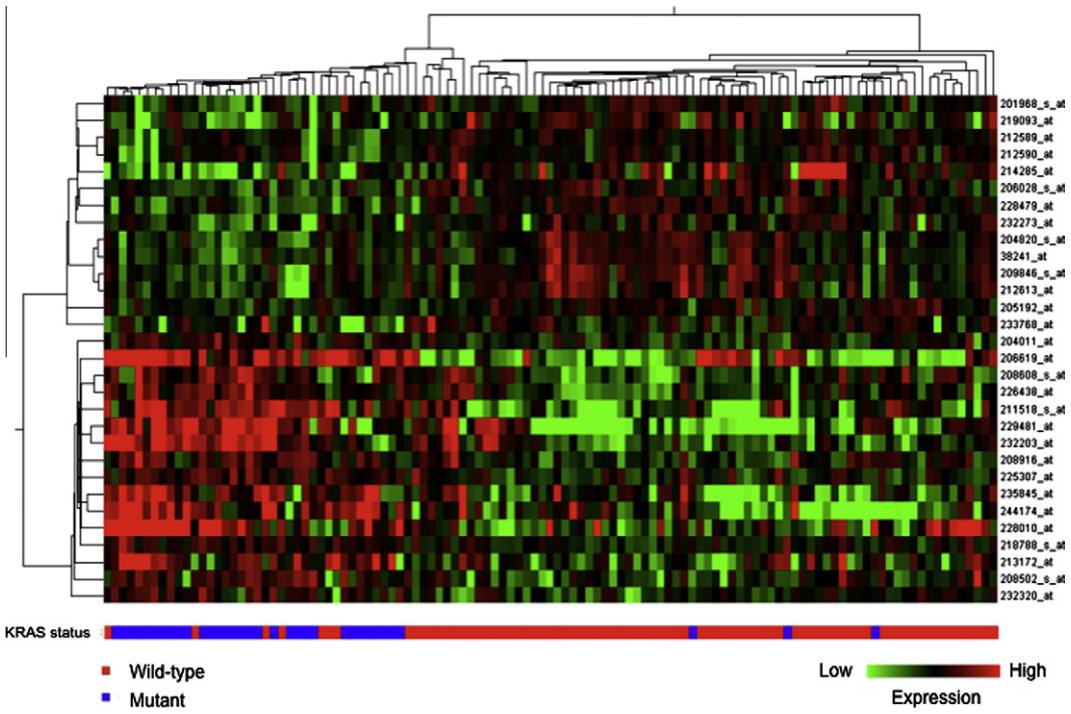

Fig. 1 – A two-way hierarchical clustering analysis was used to order samples (columns) and array targets (rows). Red indicates overexpression and green indicates an underexpression. The colorectal cancers with and without KRAS mutation were clustered into two distinct groups. At the bottom, blue indicates the colorectal cancers with KRAS mutations and red indicates those without mutations.

expressed at lower levels in CRCs with KRAS mutations. We performed a hierarchical cluster analysis (Fig. 1) and observed the clustering of CRCs with and without KRAS mutation into two distinct groups. We also generated a three-dimensional (from 30-dimensional) plot of the data (Fig. 2). PCA-based multidimensional scaling visualisation separated CRCs with and without KRAS mutation into linearly separable gene expression data spaces.

3.3. *Class prediction of KRAS status*

We carried out a supervised class prediction of all samples using the k-nearest-neighbour method and a leave-one-out cross-validation with the 30 discriminating probes. The prediction accuracy was found to be 90.3%. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the present model were 85.3% (29/34), 92.4% (73/79), 82.9% (29/35) and 93.6% (73/78), respectively.

3.4. *Comparison with GEO dataset (GSE3151 and GSE16125)*

Using the GSE3151 dataset of 348 genes as in Bild's study, we compared the levels of gene expression in cell lines and human samples according to the KRAS status (Supplementary Table S1).[14] We first examined the gene expression ratio (KRAS mutant/KRAS wild-type) of the 348 genes between Bild's cell lines and the present human samples. Among the 348 genes, 188 genes showed a concordant expression between Bild's study and the present study. Specifically, 141 genes showed higher expression and 47 genes lower expression in both KRAS mutant cell lines and human samples compared to those without KRAS mutations. However, there were no statistically significant correlations in the gene expression ratio between the cell lines and the clinical samples (Fig. 3).

We next compared the gene expression ratio (KRAS mutant/KRAS wild-type) of the present 30 discriminating genes (probes). As shown in Fig. 3, there was no statistically significant correlation in the gene expression ratio in the 30 genes between Bild's cell lines and the present human samples.

Finally, using the GSE16125 dataset of 36 patients used in Reid's analysis, we compared the gene expression ratio of the present 30 discriminating genes (probes) between the present samples and human samples in Reid's analysis.[21] Twenty-four of 30 genes (probes) were available for comparison, and a significant correlation was observed in the gene expression ratio between the present human samples and Reid's samples (Fig. 3).

4. Discussion

We identified 30 discriminating genes whose expressions differed significantly between mutant-KRAS and wild-type-KRAS CRCs. These genes were included both in the KRAS-related pathways and in other transsignalling pathways such as the Wnt-signalling pathway and the NF-kappaB-inducing signalling cascade. The present results are considered to reflect cross-talk between signalling pathways in mutant-KRAS

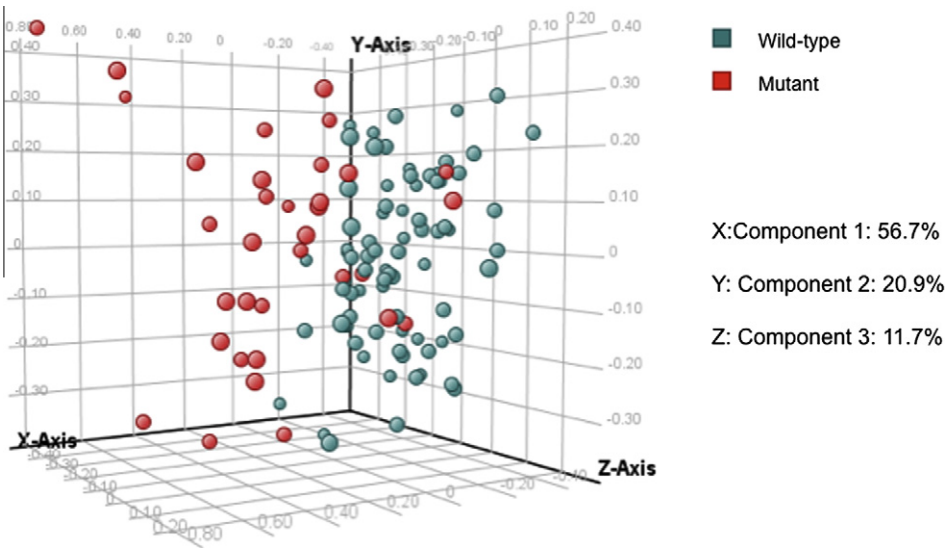

Fig. 2 – Principal component analysis. The discriminating genes were used to generate a three-dimensional (from a 30-dimensional) plot of the data. Principal component analysis (PCA)-based multidimensional scaling visualisation separated colorectal cancers with and without KRAS mutations into linearly-separable gene expression data space.

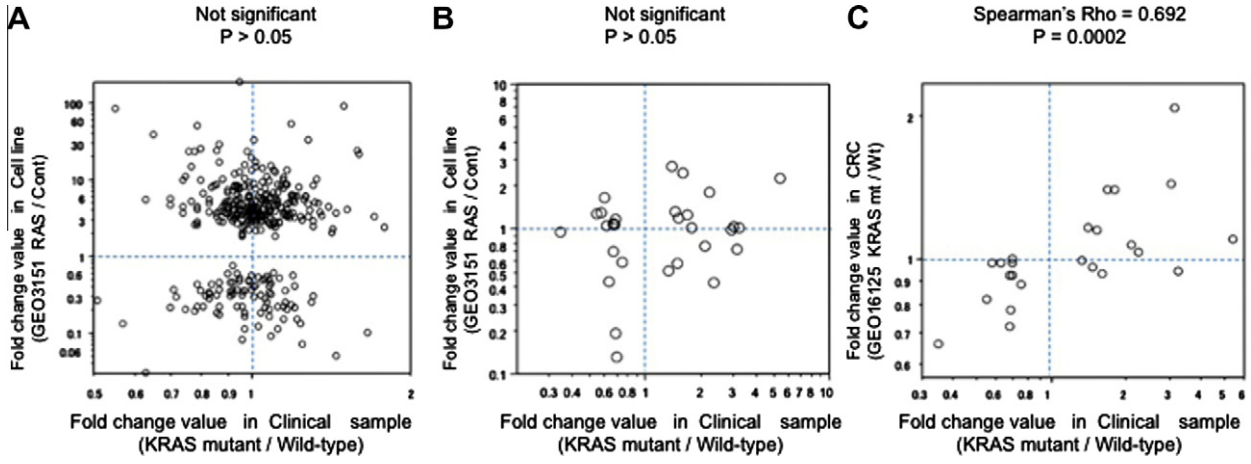

Fig. 3 – Relationship between the gene expression in the present study and GEO datasets as determined by a microarray platform. (A) Plot of gene expression levels of 348 RAS signature genes or (B) 30 RAS predictors in the present study against the GSE3151 dataset. (C) Plot of gene expression levels for 30 predictor genes in the present study against the GSE161245 dataset.

CRCs. Using 30 discriminating genes, a hierarchical analysis and a PCA analysis showed a clear separation of CRC samples with or without KRAS mutations. The CRCs were correctly classified into mutant or wild-type KRAS status with a high accuracy rate of 90.3%.

To our knowledge, there has only been one report that previously compared the levels of gene expression between human CRC tissues with and without KRAS mutations using a microarray analysis. Reid et al. examined the gene expression of 36 CRC samples by microarray according to the mutational status of KRAS.[21] However, the authors found only one gene whose expression differed significantly between CRC with and without KRAS mutation. In the present study, we identified 30 genes whose expression differed significantly between CRC with and without KRAS mutation with a 1.3-fold difference and an FDR *p*-value of less than 0.01. Reid's study examined the gene expression in 36 patients and the present study examined a larger number of patients (113 patients). Because the microarray analyses require a large number of patients to identify significant differences in gene expression, the reason for the different number of discriminating genes between Reid's study and the present study appears to be due to the difference in the number of CRC patients. However, it should be noted that the expression ratio of the present 30

discriminating genes had a significant correlation between Reid's dataset and the present results, suggesting that there is a common signature between Reid's samples and the present samples.

With regard to *in vitro* studies, few studies have examined differences in the gene expression profiles between colorectal cancer cells with and without KRAS mutations. Bild et al. identified 348 genes whose expression significantly differed depending on the KRAS mutation status by transfecting KRAS mutants into cancer cells. In the present dataset of human samples, 54% (188/348) of genes showed concordance with the results of cell lines by Bild's study. Approximately half of the genes (46%, or 160/348) exhibited discordant expression between human samples and cell lines. These results suggest that there is a clear difference in expression pattern between KRAS mutant-transfected cells and clinical CRC samples. This difference may be present because of a host response or other interactions of various signalling pathways that cannot be reconstituted in cell lines.

In the present study, 30 discriminating genes included KRAS-related genes included TC21, paired-like homeodomain 1 (PITX1) and Sprouty-2. TC21 (R-Ras2: related RAS viral (r-ras) oncogene homologue 2) is a plasma membrane-associated GTP-binding protein with GTPase activity, belongs to the Ras superfamily and is known to transform epithelial and fibroblast cell lines.[22] TC21 is upregulated in oral and oesophageal carcinomas.[23,24] In the present study, RRAS2 was downregulated in KRAS-mutant CRCs. Sprouty-2 is the Ras/MAPK antagonist and functions as a tumour suppressor in K-ras-mediated tumourigenesis.[25] Sprouty-2 plays a critical role in the regulation of oncogenic K-ras, and has been implicated in counter-regulatory mechanisms in the pathogenesis of Ras-based disease.[25] Sprouty-2 is upregulated in lung tumourigenesis and the present study showed the same tendency in colon cancer.[25] PITX1 is a tumour-suppressor gene that functions as an inhibitor of the RAS pathway. Kolfschoten et al. showed that PITX1 suppresses tumourigenicity by downregulating the RAS pathway through RASAL1 and showed a low expression of PITX1 in colon cancer cell lines containing wild-type RAS.[26] The present study exhibited similar results in human CRC tissues. CRCs expressing wild-type KRAS showed significantly lower expression levels of PITX1 than mutant KRAS.

Another important finding in the present study was that KRAS mutation also affected the gene expression of other signalling pathways, such as the Wnt-signalling pathway, NF-kappaB-inducing signalling cascade and the TGF-beta signalling pathway. DKK-4 (dickkopf homologue 4) is a Wnt-signalling modulator and upregulation of DKK-4 activates the Wnt/canonical pathway.[27] DKK-4 is upregulated in colon cancer and is associated with the acquisition of malignant properties.[27,28] Sp5 is also related to the Wnt-signalling pathway, and mediates the downstream responses to Wnt/beta-catenin signalling by directly repressing Sp1 target genes.[29] Bone morphogenetic protein-4 (BMP) is involved in the TGF-beta signalling pathway.[30] The overexpression of BMP promotes the invasion of colon cancer cells, and BMP-4 inhibits heat-induced apoptosis by modulating the MAPK pathway.[30] SET and MYND domain containing 3 (SMYD3) facilitates histone methyltransferase activity, and SMYD3 upregulation enhances the growth of tumour cells.[31,32] SMYD3 is upregulated in colorectal cancers, hepatocellular carcinomas and breast cancers.[31,32] In the present study, DKK-4, Sp5 transcription factor (SP5), BMP and SMYD3 were all upregulated in KRAS mutant CRCs and DKK-4 in particular showed the largest fold change (5.44) among 30 genes. However, mitogen-activated protein kinase kinase kinase 14 (MAP3K14) and c-mer Proto-oncogene tyrosine kinase (MerTK) were downregulated in KRAS mutant CRCs. MAP3K14, mitogen-activated protein kinase kinase, is a serine/threonine protein-kinase which plays a central role in the NF-kappaB activation pathway.[33,34] MerTK is a receptor tyrosine kinase and contributes to leukaemogenesis by the activation of Akt and ERK1/2 anti-apoptotic signals.[35] Although these discriminating genes have been reported with regard to their role in carcinogenesis, their expression has not been described in the context of KRAS status in CRCs. The present results clearly show that there is crosstalk between the KRAS-mediated RAS–RAF–MAP kinase pathway and other signalling pathways (Fig. 4).

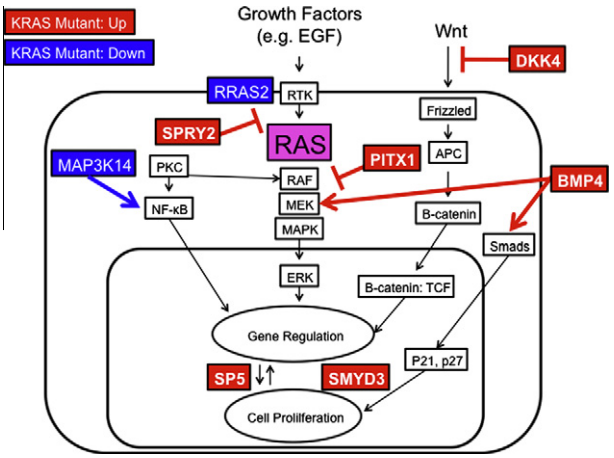

Fig. 4 – Crosstalk of the signalling pathways.

One limitation of the present study was that we did not examine the gene expression depending on the different mutational patterns of KRAS. Monticone et al. showed that within KRAS mutant cells, gene expression can differ depending on the mutational pattern.[15] Further studies may reveal subclassification of expression profiles within KRAS mutant CRCs. Another limitation was that we were unable to evaluate the actual response to various chemotherapeutic agents, such as anti-EGFR antibodies. To select appropriate chemotherapeutic agents in KRAS mutant CRC, further study will be necessary to evaluate gene expression and mutational status with regard to the response to various treatments.

In conclusion, KRAS mutant CRCs exhibited distinct gene expression signatures that were different from KRAS wild-type CRCs. Thirty differentially-expressed genes between KRAS mutant and wild-type CRCs were related to the KRAS-related pathway and other signalling pathways, including the Wnt-signalling pathway, the NF-kappaB-inducing signalling cascade and the TGF-beta signalling pathway. These results suggested that there is crosstalk between the KRAS-mediated pathway and other signalling pathways. These results should, therefore, be taken into account when establishing chemotherapeutic strategies in anti-EGFR refractory mutant KRAS CRCs.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.03.029.

REFERENCES

1. Molinari F, Martin V, Saletti P, et al. Differing deregulation of EGFR and downstream proteins in primary colorectal cancer and related metastatic sites may be clinically relevant. *Br J Cancer* 2009;**100**:1087–94.
2. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *N Engl J Med* 2008;**358**:1160–74.
3. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007;**25**:3230–7.
4. Van Cutsem E, Lang I, Dhaens G. KRAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience. *J Clin Oncol* 2008;**26**:5.
5. Amado RG, Wolf M, Peeters M, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;**26**:1626–34.
6. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;**359**:1757–65.
7. Lievre A, Bachet JB, Boige V, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008;**26**:374–9.
8. Artale S, Sartore-Bianchi A, Veronese SM, et al. Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer. *J Clin Oncol* 2008;**26**:4217–9.
9. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009;**27**:2091–6.
10. Van Cutsem E, Oliveira JESMO Guidelines Working Group. Advanced colorectal cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2009;**20**(Suppl. 4):61–3.
11. McCubrey JA, Steelman LS, Chappell WH, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 2007;**1773**:1263–84.
12. Chang F, Lee JT, Navolanic PM, et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* 2003;**17**:590–603.
13. Andreyev HJ, Norman AR, Cunningham D, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001;**85**:692–6.
14. Bild AH, Yao G, Chang JT, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;**439**:353–7.
15. Monticone M, Biollo E, Maffei M, et al. Gene expression deregulation by KRAS G12D, G12V in a BRAF V600E context. *Mol Cancer* 2008;**7**:92.
16. Li H, Cao HF, Li Y, et al. Changes in gene-expression profiles of colon carcinoma cells induced by wild type K-ras2. *World J Gastroenterol* 2007;**13**:4620–5.
17. Watanabe T, Kobunai T, Toda E, et al. Distal colorectal cancers with microsatellite instability (MSI) display distinct gene expression profiles that are different from proximal MSI cancers. *Cancer Res* 2006;**66**:9804–8.
18. Watanabe T, Komuro Y, Kiyomatsu T, et al. Prediction of sensitivity of rectal cancer cells in response to preoperative radiotherapy by DNA microarray analysis of gene-expression profiles. *Cancer Res* 2006;**66**:3370–4.
19. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 2001;**125**(1–2):279–84.
20. Cover TM, Hart PE. Nearest neighbor pattern classification. *IEEA Trans Inf Theory* 1967;**13**:21–7.
21. Reid JF, Gariboldi M, Sokolova V, et al. Integrative approach for prioritizing cancer genes in sporadic colon cancer. *Genes Chromosomes Cancer* 2009;**48**:953–62.
22. Graham SM, Oldham SM, Martin CB, et al. TC21 and Ras share indistinguishable transforming and differentiating activities. *Oncogene* 1999;**18**:2107–16.
23. Arora S, Matta A, Shukla NK, et al. Identification of differentially expressed genes in oral squamous cell carcinoma. *Mol Carcinog* 2005;**42**:97–108.
24. Sharma R, Sud N, Chattopadhyay TK, et al. TC21/R-Ras2 upregulation in esophageal tumorigenesis: potential diagnostic implications. *Oncology* 2005;**69**: 10–8.
25. Shaw AT, Meissner A, Dowdle JA, et al. Sprouty-2 regulates oncogenic K-ras in lung development and tumorigenesis. *Genes Dev* 2007;**21**:694–707.
26. Kolfschoten IG, van Leeuwen B, Berns K, et al. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell* 2005;**121**(6):849–58.
27. Matsui A, Yamaguchi T, Maekawa S, et al. DICKKOPF-4 and -2 genes are upregulated in human colorectal cancer. *Cancer Sci* 2009;**100**:1923–30.
28. Pendás-Franco N, García JM, Peña C, et al. DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1alpha, 25-dihydroxyvitamin D3. *Oncogene* 2008;**27**:4467–77.
29. Fujimura N, Vacik T, Machon O, et al. Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5. *J Biol Chem* 2007;**282**:1225–37.
30. Deng H, Makizumi R, Ravikumar TS, et al. Bone morphogenetic protein-4 is overexpressed in colonic adenocarcinomas and promotes migration and invasion of HCT116 cells. *Exp Cell Res* 2007;**313**: 1033–44.
31. Tsuge M, Hamamoto R, Silva FP, et al. A variable number of tandem repeats polymorphism in an E2F-1 binding element in the 5′ flanking region of SMYD3 is a risk factor for human cancers. *Nat Genet* 2005;**37**:1104–7.

32. Hamamoto R, Furukawa Y, Morita M, et al. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. *Nat Cell Biol* 2004;**6**:731–40.
33. Royuela M, Rodríguez-Berriguete G, Fraile B, Paniagua R. TNF-alpha/IL-1/NF-kappaB transduction pathway in human cancer prostate. *Histol Histopathol* 2008;**23**:1279–90.
34. Choudhary S, Boldogh S, Garofalo R, Jamaluddin M, Brasier AR. Respiratory syncytial virus influences NF-kappaB-dependent gene expression through a novel pathway involving MAP3K14/NIK expression and nuclear complex formation with NF-kappaB2. *J Virol* 2005;**79**:8948–59.
35. Keating AK, Salzberg DB, Sather S, et al. Lymphoblastic leukemia/lymphoma in mice overexpressing the Mer (MerTK) receptor tyrosine kinase. *Oncogene* 2006;**25**:6092–100.