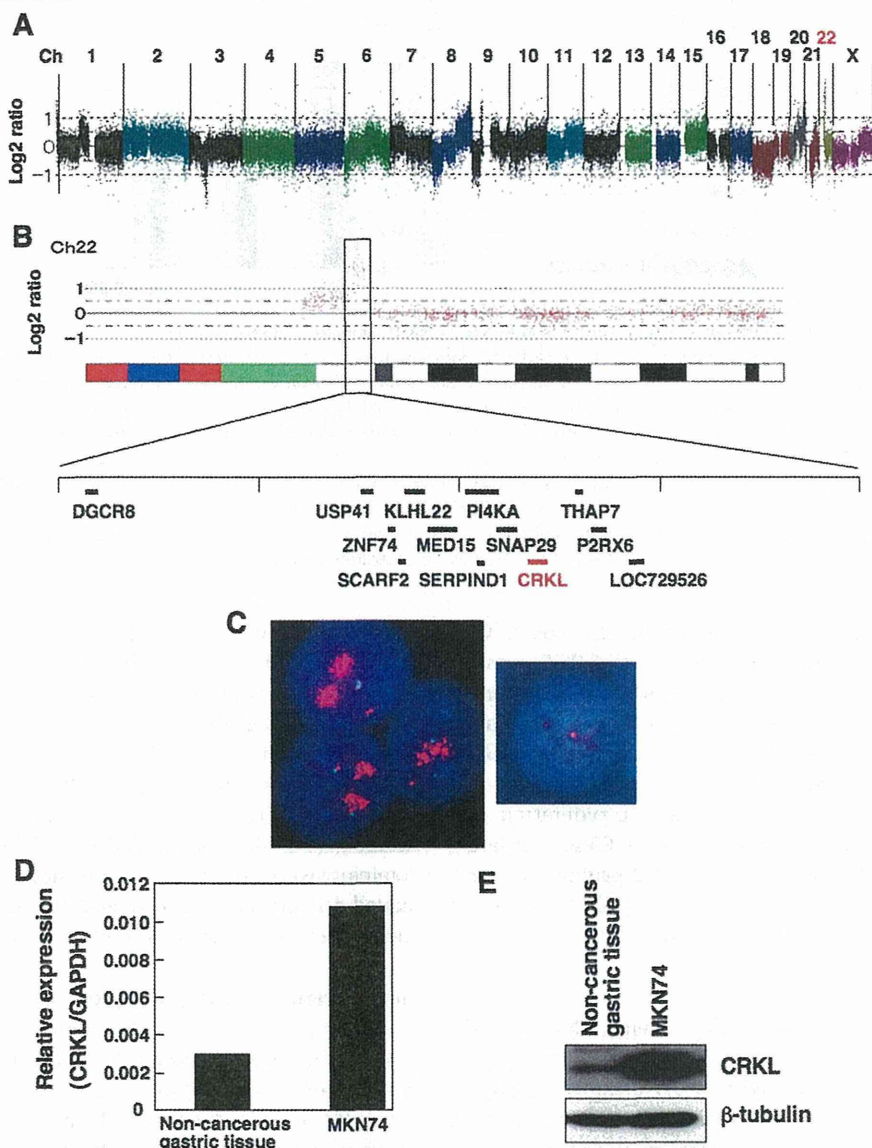

Figure 1 Identification of highly amplified chromosome regions containing the *CRKL* gene and the detection of CRKL overexpression in gastric cancer. **(A)** Genome-wide detection of copy number alterations using a high-density SNP microarray in the MKN74 gastric cancer cell line. The copy number status for the whole genome of MKN74 is shown. DNA (250 ng) was analyzed using an Affymetrix GeneChip 250 K NspI array, and the total copy numbers were determined by analyzing the microarray data using the CNAG program. The chromosome number is shown above the panel. Chromosome 22 is highlighted in red. **(B)** The copy number status of chromosome 22 of the MKN74 cells is shown. A highly amplified region of chromosome 22 is enlarged, and the genes located in this region are indicated. The *CRKL* gene is highlighted in red. **(C)** Detection of *CRKL* amplification in MKN74 cells using a FISH analysis. The left panel shows the *CRKL* signal (red) in MKN74 cells, while the right panel shows the *CRKL* (red) in non-cancerous gastric tissue cells. An extreme increase in the *CRKL* copy number was observed in the MKN74 cells, while a normal copy number (2) was seen in non-cancerous cells. Nuclei are stained with DAPI. **(D)** Detection of the increased expression of CRKL mRNA transcript in MKN74 cells using real-time QRT-PCR analysis. The amounts of CRKL transcripts normalized to the amount of GAPDH transcripts are shown in the graph. The average expression level of eight normal gastric mucosa samples was measured as a control. **(E)** Detection of the increased expression of CRKL protein in MKN74 cells using a western blot analysis. The expression of CRKL was examined using anti-CRKL monoclonal antibody (Y244; 1:500 dilution), horseradish peroxidase-coupled secondary antibody (1:5,000 dilution), and enhanced chemiluminescence detection reagents. The expression of β-tubulin protein was analyzed as an internal control.

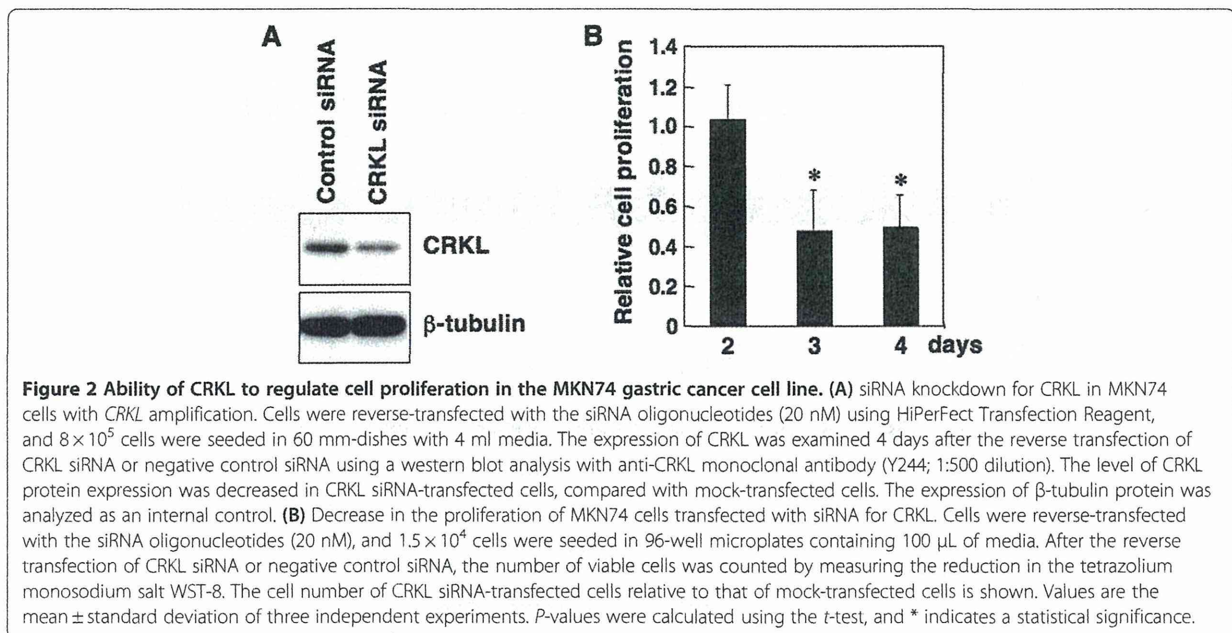

Figure 2 Ability of CRKL to regulate cell proliferation in the MKN74 gastric cancer cell line. (A) siRNA knockdown for CRKL in MKN74 cells with *CRKL* amplification. Cells were reverse-transfected with the siRNA oligonucleotides (20 nM) using HiPerFect Transfection Reagent, and 8×10^5 cells were seeded in 60 mm-dishes with 4 ml media. The expression of CRKL was examined 4 days after the reverse transfection of CRKL siRNA or negative control siRNA using a western blot analysis with anti-CRKL monoclonal antibody (Y244; 1:500 dilution). The level of CRKL protein expression was decreased in CRKL siRNA-transfected cells, compared with mock-transfected cells. The expression of β-tubulin protein was analyzed as an internal control. **(B)** Decrease in the proliferation of MKN74 cells transfected with siRNA for CRKL. Cells were reverse-transfected with the siRNA oligonucleotides (20 nM), and 1.5×10^4 cells were seeded in 96-well microplates containing 100 μL of media. After the reverse transfection of CRKL siRNA or negative control siRNA, the number of viable cells was counted by measuring the reduction in the tetrazolium monosodium salt WST-8. The cell number of CRKL siRNA-transfected cells relative to that of mock-transfected cells is shown. Values are the mean ± standard deviation of three independent experiments. *P*-values were calculated using the *t*-test, and * indicates a statistical significance.

bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO) was then added at a final concentration of 0.25 mg/mL. After incubation at 37°C for 4 h, absorbance was measured at a wavelength of 570 nm using a microplate reader. Cells grown in complete medium with DMSO alone were used as controls. The final concentration of DMSO was set to 0.2%. To assess cell proliferation in Figure 4H, the cells were cultured with CRKL targeting peptide or DMSO at 37°C for 72 h. Cell proliferation was measured by directly counting the cells using a hemocytometer, as described previously [20].

QRT-PCR

Total RNA was extracted using Isogen (Nippongene, Tokyo, Japan) or an RNeasy Plus mini kit (Qiagen, Valencia, CA) and converted to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time QRT-PCR was performed using the cDNA and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on a StepOne Real-Time PCR system (Applied Biosystems). The following PCR primers were used: 5'-CAA CCT GCC TAC AGC AGA AGA TAA-3' and 5'-CGG CAT CAT TCC CAG GAA-3' for the CRKL transcript, and 5'-GGT GGT CTC CTC TGA CTT CAA CA-3' and 5'-GTT GCT GTA GCC AAA TTC GTT GT-3' for the transcript of a housekeeping gene, *GAPDH*. The relative amounts of CRKL transcript were normalized to those of the GAPDH transcript.

Western blot analysis

Cells were lysed, and the protein concentration was quantified using a BCA protein assay kit (Pierce, Rockford, IL). The proteins were electrophoresed and transferred to a PVDF membrane (GE Healthcare Bio Science, Piscataway, NJ). After blocking with non-fat milk or Blocking One-P (Nakalai Tesque, Kyoto, Japan), the membrane was incubated with rabbit anti-CRKL monoclonal antibody (Y244; Abcam), rabbit anti-phospho CRKL polyclonal antibody (Y207; Cell Signaling, Beverly, MA), or mouse anti-β-tubulin (2-28-33, Sigma-Aldrich). The immunoreactive proteins were visualized using horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence detection reagents (GE Healthcare Bio Science) [21].

Small interfering RNA (siRNA) knockdown

A stealth siRNA duplex oligonucleotide (Invitrogen) was used for siRNA knockdown. The following CRKL sequence was used: 5'-UCG UGA AAG UCA CAA GGA UGA AUA U-3'. A low GC Duplex #2 (Invitrogen) was used as a negative control. MKN74 cells were reverse-transfected with the siRNA oligonucleotides (20 nM) using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer's instructions.

BMS354825 and AMN107 treatment

BMS354825, a dual Src/BCR-ABL kinase inhibitor, was kindly provided by Bristol-Myers Squibb (New York, NY), and AMN107, a highly selective BCR-ABL kinase inhibitor, was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) [22-25]. Stock solutions (10 mM) of BMS354825 and AMN107 were prepared in DMSO. The cells were incubated with BMS354825 or AMN107 at a final concentration of 0.01 to 1.0 μM for 72 h. The final concentration of DMSO was set to 0.1%.

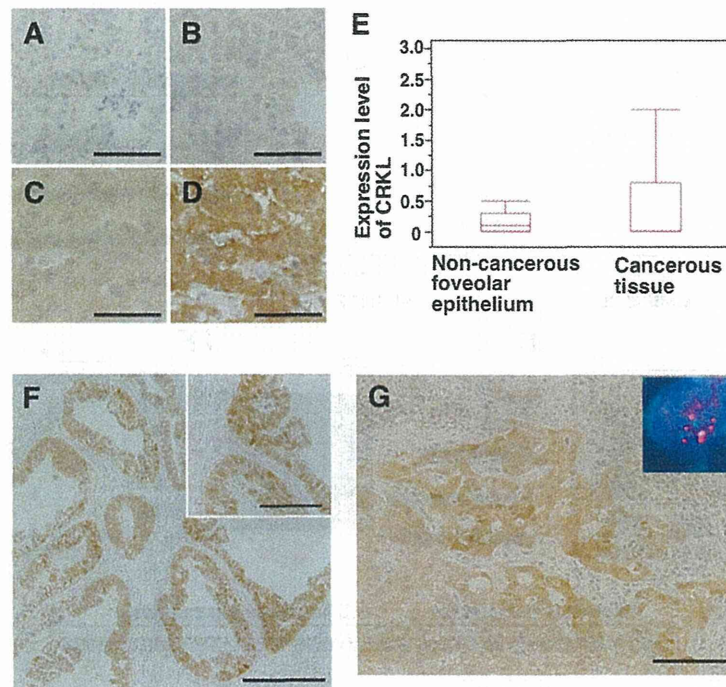

Figure 3 Immunohistochemical detection of CRKL protein in primary gastric cancer. TMA block sections were subjected to an immunohistochemical analysis using anti-CRKL monoclonal antibody (Y243; 1:100 dilution), Histofine Simple Stain Max-Po (Multi), and 3,3'-diaminobenzidine tetrahydrochloride. Intensity values of 0, 1, 2, and 3 are shown in **(A)**, **(B)**, **(C)**, and **(D)**, respectively. Bar = 50 μm. **(E)** Box-plot analysis of CRKL protein expression in gastric tissue. A statistically significant difference in the CRKL expression level was detected between non-cancerous gastric foveolar epithelium ($n = 41$) and gastric cancerous tissue ($n = 360$). **(F)** Representative result of the CRKL immunohistochemical analysis. A gastric cancer with a high CRKL expression level is shown. Bar = 500 μm. The inset is a magnified image. Bar = 50 μm. **(G)** Representative gastric cancer case showing both a high CRKL expression level and *CRKL* gene amplification. The high CRKL expression level (value = 2.6) was detected using an immunohistochemical analysis. Bar = 100 μm. The inset shows the amplification of *CRKL* (red) in the cancer cells. The *CRKL* signal (red) and the control signal for chromosome 22 (green) were detected using a FISH analysis. Nuclei are stained with DAPI.

Preparation of CRKL targeting peptide

In this study, we used the peptides, which has been reported to be disrupted complexes between BCR-ABL and CRKL depend on the SH3 domain of CRKL in CML cells [26]. Peptides used in the experiments are followed: CRKL-targeting peptide; KKW KMR RNP FWI KIQ RC – CGI RVV DNS PPP ALP PKR RRS APS PTR V, control peptide; KKW KMR RNP FWI KIQ RC – CGI RVV DNS PPG ALG PLL RRS APS PTR V. The KKW KMR RNP FWI KIQ RC was the shuttle tag sequence performing a receptor-independent cell entry. The chimeric peptide was synthesized and purified by using reverse-phase high performance liquid chromatography (HPLC) (Toray Research Center, Otsu, Japan). Peptide stocks were prepared in DMSO and stored in aliquots at -80°C.

Statistical analysis

The statistical analysis was performed using an unpaired *t*-test, chi-square test, or Dunnett's test. JMP version 7.0.1 software (SAS Institute, Cary, NC) was used for the analyses. *P* values less than 0.05 were considered statistically significant.

Results

Identification of *CRKL* amplification in gastric cancer

To search for highly amplified genes in gastric adenocarcinoma, we adopted a genome-wide high-resolution SNP microarray approach in three cell lines of differentiated gastric adenocarcinoma: MKN7, MKN28, and MKN74. Genotype calls were obtained at more than 95% of the 262,264 SNP sites on the array, meaning that the SNP microarray analysis had been performed properly. The SNP microarray data were then used to determine the chromosomal copy number using the CNAG program (Figures 1A and 1B). Five highly amplified regions with a copy number of more than 6 (9p13, 17q12-q21, 19q12, 19q13, and 22q11) were identified, as shown in Table 1. These regions contained various kinds of genes, a total of 22 genes (Table 1). Among them, we decided to focus on the *CRKL* gene at chromosome 22q11.21, the product

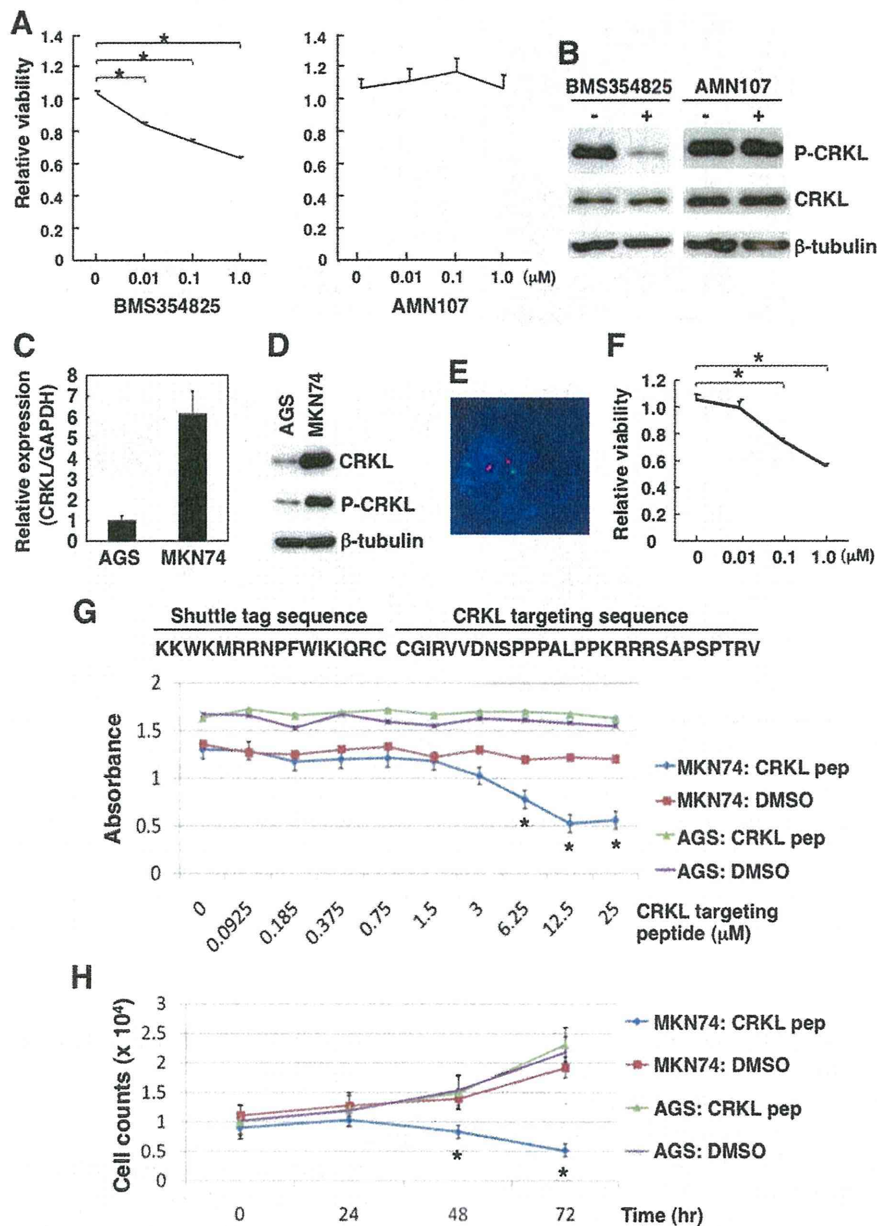

Figure 4 (See legend on next page)

of which is an SH2 and SH3 domain-containing adaptor protein that shares homology with the CRK oncoprotein, because CRKL is a known substrate of BCR-ABL kinase in Philadelphia chromosome-positive leukemia [27,28] and its role in gastric cancer has not been previously analyzed. To confirm that *CRKL* gene amplification was detectable in the MKN74 cell line, we performed a FISH analysis using a probe specific for *CRKL*. As expected, an extreme increase in the *CRKL* copy number was detected in the MKN74 cells using a FISH analysis (Figure 1C). When the level of CRKL mRNA expression was examined in MKN74 cells using a real-time QRT-PCR analysis, the level was much higher than that in non-cancerous gastric tissue (Figure 1D). Moreover, a western blot analysis showed that the level of CRKL protein expression was higher in MKN74 cells than in non-cancerous gastric tissue (Figure 1E). These results suggested that the *CRKL* gene is highly amplified and that CRKL is overexpressed in a subset of gastric cancer cell lines.

(See figure on previous page)
Figure 4 Responses of the MKN74 gastric cancer cell line with *CRKL* amplification to treatment with BMS354825 (a dual Src/BCR-ABL kinase inhibitor) and CRKL-targeting peptide. (A) Viability of MKN74 cells treated with BMS354825 but not those treated with AMN107 (a highly selective BCR-ABL kinase inhibitor) is decreased. The cells were seeded in 96-well microplates at a density of 1×10^4 per well; after 24 h, the drug (0.01–1.0 μM) or 0.1% DMSO solution was added. Viability was examined in the MKN74 cells after 72 h of treatment at the indicated concentration using WST-8 reagent. The number of viable cells after treatment with each inhibitor was normalized to the number of viable cells without treatment, and the relative viability is shown in the graph. Values are the mean ± standard error. *P* values were calculated using the Dunnett's multiple comparison test, and * indicates a statistically significant decrease. **(B)** Effective inhibition of CRKL phosphorylation in MKN74 cells treated with BMS354825. Cells were treated with each inhibitor (DMSO only or 0.01 μM of drug) for 90 min, and the expression of CRKL protein was examined using a western blot analysis with anti-phospho CRKL polyclonal antibody (Y207; 1:1,000 dilution) or anti-CRKL monoclonal antibody (Y244; 1:500 dilution). The expression of β-tubulin protein was analyzed as an internal control. **(C)** Comparison of CRKL mRNA transcripts between AGS and MKN74 cells using real-time QRT-PCR analysis. The amounts of CRKL transcripts normalized to the amount of GAPDH transcripts are shown in the graph. **(D)** Comparison of expression of CRKL protein between AGS and MKN74 cells using a western blot analysis. The expression of CRKL was examined using the primary antibodies shown in (B). The expression of β-tubulin protein was analyzed as an internal control. **(E)** Detection of *CRKL* gene copy number in AGS cells using a FISH analysis. The *CRKL* signal is red, and the control signal for chromosome 22 is green. Nuclei are stained with DAPI. **(F)** Viability of AGS cells decreased after BMS354825 treatment. Viability was examined as described in (A). Values are the mean ± standard error. *P* values were calculated using a *t*-test, and * indicates a statistically significant decrease. **(G)** MKN74 cells with *CRKL* amplification and AGS cells without *CRKL* amplification were seeded in 96-well microplates at a density of 1×10^4 per well. 24 h after seeding, cells were treated with CRKL-targeting peptide (0.0925–25 μM) or 0.2% DMSO solution at the indicated concentration. The sequence of the CRKL-targeting peptide that was used is shown above the graph. After 72 h of incubation, viability was determined using an MTT assay. The results are presented as the mean ± standard deviation of three independent experiments. *P* values were calculated using a *t*-test, and * indicates a statistically significant difference between the cells treated with CRKL-targeting peptide and those treated with DMSO. **(H)** Cell proliferation of MKN74 and AGS cells treated with CRKL-targeting peptide (6.25 μM) or DMSO as measured by counting cells using a hemocytometer. Cells (1×10^4) were seeded in 24-well plates and treated with CRKL-targeting peptide or DMSO. The cell counting was performed every 24 h for 3 days. Data are shown as the mean ± standard deviation of three independent experiments. *P* values were calculated using a *t*-test, and * indicates a statistically significant difference between the cells treated with CRKL-targeting peptide and those treated with DMSO.

Ability of CRKL to control gastric cell proliferation

To explore the functional significance of *CRKL* amplification in gastric cancer, we attempted to examine the effect of overexpressed CRKL on gastric cell proliferation. For this purpose, we prepared MKN74 cells with distinct CRKL expression levels using the siRNA knockdown of CRKL expression. CRKL-specific siRNA transfection effectively decreased the level of CRKL protein expression in MKN74 cells by approximately 70% of the levels observed in negative control siRNA-transfected cells (Figure 2A). A cell proliferation assay showed that the number of CRKL siRNA-transfected MKN74 cells was significantly lower at 3 and 4 days after transfection than the number of negative control siRNA-transfected cells (Figure 2B), meaning that CRKL has the ability to upregulate cell proliferation.

Table 1 Detection of chromosomal regions with a high copy number (more than 6) in the gastric cancer cell lines MKN7, MKN28, and MKN74 using a genome-wide SNP microarray analysis

Chromosomal regions[a]	Genes with a high copy number in the region
9p13	*PAX5*
17q12-q21	*FBXL20, MED1, PERLD1, ERBB2, IKZF3, ZPBP2*
19q12	*CCNE1*
19q13	*CD22*
22q11	*DGCR8, USP41, ZNF74, SCARF2, KLHL22, MED15, PI4KA, SERPIND1, SNAP29, CRKL, THAP7, P2RX6, LOC729526*

[a] If more than four consecutive SNP probes with a copy number of more than six were detected in either of the three cell lines, the chromosomal region was regarded as being a "highly amplified region" and was listed in this table.

Overexpression of CRKL protein in gastric cancer

Next, we investigated the expression status of CRKL protein in primary gastric cancer using an immunohistochemical analysis with anti-CRKL monoclonal antibody (Y243). CRKL was mainly observed in the cytoplasm, consistent with previous reports [29]. When we compared the level of CRKL expression between non-cancerous gastric foveolar epithelium ($n = 41$) and gastric cancer ($n = 360$), the level of CRKL expression in gastric cancer (mean ± standard deviation = 0.42 ± 0.63) was significantly higher than that in non-cancerous tissue (0.20 ± 0.26) ($P = 0.032$) (Figures 3A-3E). When an expression level of 1.00, which corresponds to a value 5-fold of the mean expression level in non-cancerous gastric foveolar epithelium, was used as a cutoff value for the expression status in gastric cancer (i.e., low expression group, 0–0.99; high expression group, 1.00–3.00), 88 (24.4%) of the 360 primary gastric cancers were included in the high expression group (Figure 3F). To examine whether CRKL overexpression is associated with *CRKL* amplification in gastric cancer, we performed a FISH analysis for the *CRKL* gene in the 360 primary gastric cancers and compared the prevalence of *CRKL* amplification between the low expression group and the high expression group. As

expected, the percentage of gastric cancer cells with *CRKL* amplification was significantly higher in the high expression group (9.1%; 8/88 cases) than in the low expression group (2.2%; 6/272 cases) ($P = 0.028$, chi-square test). This result suggests that *CRKL* amplification contributes to CRKL overexpression in primary gastric cancer. We further investigated whether the levels of CRKL expression is associated with clinicopathological features in primary gastric cancer patients, the high CRKL expression was observed significantly more often in male and differentiated-type gastric cancer (Table 2). These results suggested that CRKL protein is overexpressed partly due to *CRKL* amplification in a subset of primary gastric cancers and is associated with the gender and histopathology.

Decrease in the viability of CRKL-expressing MKN74 cells treated with BMS354825

Finally, we tested the possibility of using CRKL as a therapeutic target in MKN74 cells with *CRKL* amplification. Since Philadelphia chromosome-positive leukemia expressing BCR-ABL is responsive to BMS354825 (a dual Src/BCR-ABL kinase inhibitor) and AMN107 (a highly selective BCR-ABL kinase inhibitor) [22,24], we checked the response of MKN74 cells to both inhibitors. Cell viability was significantly decreased in BMS354825-treated (0.01–1.0 μM) MKN74 cells, compared with cells treated with the solvent only, while it was not significantly decreased in AMN107-treated cells (Figure 4A). When the status of CRKL phosphorylation was examined in the MKN74 cells using western blot analysis with an anti-phospho CRKL antibody, CRKL phosphorylation was found to be inhibited more effectively by BMS354825 than by AMN107 (Figure 4B). These results suggested that BMS354825 has the potential to suppress the viability of MKN74 cells expressing CRKL, likely via the inhibition of CRKL phosphorylation.

Table 2 Association between CRKL expression and clinicopathological factors in 360 patients with primary gastric cancer

		CRKL expression level		
Factor	Patient	Low ($n = 272$)	High ($n = 88$)	P
Age				
Year, mean ± SD[a]	62.0 ± 11.2	61.7 ± 11.7	62.9 ± 11.4	0.3936[b]
(range)	(29–86)	(29–86)	(31–85)	
Gender				
Male	255	182 (66.9%)	73 (83.0%)	0.0028[c]
Female	105	90 (33.1%)	15 (17.0%)	
Histological type				
Differentiated	172	118 (43.4%)	54 (61.4%)	0.0033[c]
Undifferentiated	188	154 (56.6%)	34 (38.6%)	
pT stage				
pT1	143	103 (37.9%)	40 (45.5%)	0.2082[c]
pT2-pT4	217	169 (62.1%)	48 (54.5%)	

[a] SD, standard deviation. [b] *t*-test. [c] Chi-square test.

To further characterize the role of CRKL in the BMS354825-induced suppression of MKN74 cell viability, we examined the effect of BMS354825 on gastric cancer cells without *CRKL* amplification. Since the AGS gastric cancer cell line had lower CRKL mRNA and CRKL protein expression levels than MKN74 cells (Figures 4C and D) and had a normal *CRKL* genomic copy number (Figure 4E), these cells were treated with BMS354825. Unexpectedly, the viability of the BMS354825-treated (0.1–1.0 μM) AGS cells decreased significantly (Figure 4F). Moreover, although the IC_{50} value (inhibitory concentration producing a 50% response) for BMS354825 was slightly higher in AGS cells than in MKN74 cells, the values were not much different between AGS and MKN74 cells (data not shown). These results suggest that BMS354825 has the potential to suppress the viability of AGS cells, likely via a CRKL-independent pathway.

Decrease in the viability/proliferation of CRKL-expressing MKN74 cells treated with a CRKL-targeting peptide

We then planned to use a more specific inhibitor of CRKL and examined the response of MKN74 and AGS cells to a CRKL-targeting peptide [26]. Cell viability decreased significantly in MKN74 cells treated with the CRKL-targeting peptide (6.25–25 μM), compared with DMSO (solvent)-treated cells, but a similar decrease was not found in AGS gastric cancer cells without *CRKL* amplification (Figure 4G). When cell proliferation was compared after treatment with 6.25 μM of the CRKL-targeting peptide, the cell proliferation was significantly suppressed in MKN74 cells treated with the peptide, compared with DMSO-treated MKN74 cells, but no inhibition of cell proliferation was seen in the AGS cells (Figure 4H). Control peptide had no effect on the gastric cancer cell proliferation. These results suggested that the CRKL-targeting peptide has the potential to suppress the viability/proliferation of gastric cells exhibiting *CRKL* amplification, but not of gastric cells that do not exhibit *CRKL* amplification.

Discussion

Through a genome-wide SNP microarray analysis performed in this study, the *CRKL* gene was identified as a highly amplified gene in gastric cancer. An increase in the copy number was confirmed in MKN74 gastric cancer cells with *CRKL* amplification using a FISH analysis, and a high CRKL expression level was also observed in these cells. The ability of CRKL to upregulate cell proliferation was shown in MKN74 cells by comparing the cell proliferation rate between CRKL siRNA-transfected cells and negative control siRNA-transfected cells. CRKL

protein was overexpressed in 24.4% of the primary gastric cancers, and its level in the gastric cancer was associated with the gender and histopathology. *CRKL* amplification was more frequently found in primary gastric cancers with high CRKL protein expression levels than in those with low CRKL expression levels. Finally, we showed that MKN74 cells with *CRKL* amplification were responsive to the kinase inhibitor BMS354825, likely via the inhibition of CRKL phosphorylation, and a CRKL-targeting peptide. Our current findings suggest that CRKL has an important role in the development of a subset of gastric cancers and has the potential to be a molecular therapy target for gastric cancer.

CRKL is an adaptor cell signaling protein that contains an SH2 domain and two tandem SH3 domains, both of which mediate protein-protein interactions [27,28,30]. CRKL is well known as a surrogate substrate of BCR-ABL kinase in chronic myeloid leukemia and acute lymphoblastic leukemia [11,27,28], and intensive studies of CRKL in Philadelphia chromosome-positive leukemia have been performed. However, only one paper by Kim *et al.* [31] has reported the CRKL status in gastric cancer. They revealed that the expression of CRKL mRNA in a cancer cell line was stimulated by proteins released by *Helicobacter pylori*, although the underlying mechanism was not resolved and the *CRKL* genomic copy number was not analyzed. Our genome-wide SNP microarray analysis successfully revealed, for the first time, that the *CRKL* gene is highly amplified in a subset of gastric cancers. We also showed that the CRKL protein can upregulate cell proliferation using the RNA-interference-mediated knockdown of CRKL in a gastric cancer cell line with *CRKL* amplification. Thus, CRKL overexpression arising from genomic amplification likely contributes to the aggressiveness of gastric cancer.

Recent progress in the development of molecular cancer therapy has revealed new molecular-targeting drugs, such as EGFR-targeting drug ZD1839 (Iressa) and HER2-targeting anti-HER2 monoclonal antibody trastuzumab (Herceptin), to be potent therapies for specific cancers [32-34]. In this study, BMS354825, a dual inhibitor for Src and BCR-ABL kinases, but not AMN107, a BCR-ABL specific inhibitor, showed an inhibitory effect on the survival of MKN74 cells with *CRKL* amplification. A decrease in CRKL phosphorylation through the inhibition of a currently unknown Src kinase seems to be one of the main mechanisms of BMS354825-mediated cytotoxicity in MKN74 cells. BMS354825 is currently being studied clinically in colorectal cancer, prostate cancer, breast cancer, lung cancer, and Philadelphia chromosome-positive leukemia [22,23,35]. Our results suggest that the CRKL protein may be a target of BMS354825-mediated therapy for a subset of gastric cancers. In our analyses, BMS354825 suppressed the viability of AGS cells without *CRKL* amplification as well as the viability of MKN74 cells with *CRKL* amplification, suggesting that a CRKL-independent pathway, which has been previously implicated [36], may also be involved in the BMS354825-mediated cytotoxicity seen in gastric cancers. We also presented the usefulness of a CRKL-targeting peptide for suppressing the proliferation of MKN74 cells with *CRKL* amplification. Our results should contribute to the establishment of CRKL-targeting therapy for a subset of gastric cancers in the future.

In the present study, a genome-wide, high-resolution SNP microarray analysis was successfully performed and five highly amplified chromosome regions containing 22 genes were identified in gastric cancers, as listed in Table 1. Although the *ERBB2* gene, a well-known oncogene that is often amplified in gastric cancer [4], was included in this list, the roles of the most of the genes in the Table have not been studied in gastric cancer. Further investigation of these roles is needed in the future.

Conclusion

We conclude that CRKL protein is overexpressed in a subset of gastric cancers and is associated with *CRKL* amplification in gastric cancer. Furthermore, we conclude that CRKL protein has the ability to regulate gastric cell proliferation and has the potential to serve as a molecular therapy target for gastric cancer.

Abbreviations

DAPI: 4',6-diamidino-2-phenylindole; DMSO: Dimethyl sulfoxide; FISH: Fluorescence *in situ* hybridization; QRT-PCR: Quantitative reverse-transcription-polymerase chain reaction; SNP: Single nucleotide polymorphism; siRNA: Small interfering RNA; TMA: Tissue microarray; SH2/SH3: Src homology 2 and 3.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We greatly appreciate the critical reading of the manuscript by Prof.Shinya Tanaka at Hokkaido University. This work was supported by grants from the Ministry of Health, Labour and Welfare (21-1), the Japan Society for the Promotion of Science (21790383, 22590356, and 22790378), the Ministry of Education, Culture, Sports, Science and Technology (20014007 and 221 S0001), and the Smoking Research Foundation.

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Authors' contributions

HN performed the experiments and wrote the paper draft. KS and SN interpreted the data and revised the paper. HT, HI, MS, KN, MG, SN,

and HY performed a part of the experiments. MM and HK provided tissue samples. SN performed a part of the experiments and was involved in the experimental design. HS conceived the research, designed the experiment, and revised the paper. All authors have read and approved the manuscript.

Received: 22 December 2011 Accepted: 16 May 2012
Published: 16 May 2012

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doi:10.1186/1479-5876-10-97
Cite this article as: Natsume *et al.*: The *CRKL* gene encoding an adaptor protein is amplified, overexpressed, and a possible therapeutic target in gastric cancer. *Journal of Translational Medicine* 2012 10:97.

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Gastric Cancer
DOI 10.1007/s10120-012-0177-y

LETTER TO THE EDITOR

How can research fields be integrated with PET imaging?

Haruhiko Sugimura

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Advances in molecular biology have reasonably driven many pathologists and many of those desiring to become pathologists to investigate the molecular characterization of human tumors. Pathologists specializing in gastric cancer are not exceptions. Microsatellite instability (MSI) in the DNA of human cancer cells has attracted the attention of the research community because MSI sometimes reflects constitutive genetic defects in mismatch repair (MMR) genes. Such information is especially important for the diagnosis of colorectal cancer in patients suspected of having hereditary nonpolyposis colorectal cancer (HNPCC) [1]. While MSI itself is characteristic of tumors, it also reflects genetic traits transmitted from one generation to the next.

MSI was previously assumed to play the same role in gastric cancer as it does in colorectal cancer: that is, many researchers expected to observe frequent MSI in familial gastric cancer cases. In most settings, however, this has not been the case. MSI in gastric cancer is known to occur in response to a deficiency in MMR genes because of promoter methylation. The methylation of *MLH1* has been identified in metaplastic gastric mucosa surrounding differentiated adenocarcinoma [2]. Epigenetic changes similar to those occurring in non-tumor gastric mucosa are now being extensively studied in light of their possible use as predictors of the recurrence of gastric cancer after endoscopic submucosal dissection [3]. Of note, the biological and clinical significance of MSI during the advanced stage of gastric cancer has been extensively studied for the past decade. MSI is a molecular marker that is involved in the pathogenesis and progression of gastric cancer either in an independent manner and/or in coordination with pivotal cancer-associated genes, such as *CDH1* and *MET* [4]. Importantly, MSI-positive tumors exhibit changes in the target genes (of the MMR genes) that control critical biological behaviors in tumors [5]. Despite these expectations regarding the use of MSI as a clinically feasible marker, neither clinicians nor pathologists have regarded MSI as a critical factor on which clinical decisions must be based. How do other members of the clinical team view the contribution of MSI? Diagnostic radiologists are usually not familiar with MSI (based on my personal experience), and imaging radiology has been one of the disciplines farthest from research fields examining genetic aberrations in tumors.

In this issue of *Gastric Cancer*, the work of an interdisciplinary team at the Konkuk University School of Medicine in Seoul, Korea, is reported (Chung et al. [6]); this team has integrated the above-mentioned research fields, combining positron emission tomography (PET) findings with MSI status for the diagnosis of gastric cancer6. Because researchers at this university have continuously reported data on MSI in colorectal and gastric cancer, their data for MSI in gastric cancer is accumulating [7]. Whether Chung et al. [6] intentionally compared PET data and MSI findings or whether their encounter with this correlation was serendipitous is not clear. The key findings of their study were that the presence of fluorodeoxyglucose (FDG) uptake on PET/computed tomography (CT) images ($P = 0.001$) and a higher maximum standardized uptake value (SUVmax) in gastric cancer was linked to the presence of MSI ($P < 0.001$). Many PET researchers have been trying to correlate PET findings with tumor phenotypes [8], but matching such findings to the molecular nature of

This editorial refers to the article doi:10.1007/s10120-012-0165-2.

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Published online: 18 July 2012