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Original Article

Chromogenic *in situ* hybridization (CISH) to detect *HER2* gene amplification in breast and gastric cancer: Comparison with immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH)

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The chromogenic *in situ* hybridization (CISH) assay, designed to detect the amplification of the *HER2* gene in formalin-fixed, paraffin-embedded (FFPE) breast cancer (BC) and gastric cancer (GC) tissue specimens, was evaluated in 125 FFPE BC cases and 198 FFPE GC cases for which the HER2 status had been predetermined using immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). In the 125 BC cases and the 198 gastric cases, we found a very good concordance (98.4% and 99.0%, respectively) between CISH and FISH. In particular, we evaluated the polysomy cases, as these cases often have ambiguous treatment options in clinical practice. The polysomy of chromosome 17 was defined as the presence of three or more CEP17 signals in at least 10% of the tumor cells. In the 50 BC cases and 54 GC cases displaying chromosome 17 polysomy, the concordance between FISH and CISH was 98.0% and 98.1%, respectively. These results indicate that CISH could provide an accurate and practical alternative to FISH for the clinical diagnosis of *HER2* gene amplification in FFPE BC and FFPE GC samples.

Key words: breast cancer, chromogenic *in situ* hybridization (CISH), fluorescence *in situ* hybridization (FISH), gastric cancer, HER2, immunohistochemistry (IHC)

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The human *HER2* oncogene is located on chromosome 17 and encodes a 185-kDa membrane receptor-like protein with tyrosine kinase activity. The *HER2* gene is a member of the human epidermal growth factor receptor gene family.[1,2] Amplification of the *HER2* gene and overexpression of its protein have been demonstrated in 15–25% of breast cancers. This up-regulation is associated with a poor prognosis.[3–5] Trastuzumab (Herceptin) is a monoclonal antibody specific for the HER2 protein. Trastuzumab has been shown to be an effective therapy only in patients whose tumors show *HER2* gene amplification and/or HER2 protein overexpression. Two types of assays can be used for HER2 evaluation: immunohistochemistry (IHC), which detects protein overexpression, and fluorescence *in situ* hybridization (FISH), which assesses gene amplification. The most frequently used method to ascertain the HER2 status in breast cancer specimens is IHC. However, discrepant IHC results are sometimes obtained using formalin-fixed, paraffin-embedded (FFPE) tissues because technical factors, fixation, and the subjective scoring system can affect the quality of the results. Although FISH is a very accurate and sensitive assay for detecting *HER2* amplification, the evaluation of FISH results requires an expensive fluorescence microscope with high-magnification oil immersion objective lenses (x60-100) as well as a digital camera to record the results. FISH can be performed as a dual-color hybridization allowing the simultaneous enumeration of *HER2* and chromosome 17, which supposedly makes it easier to distinguish true *HER2* amplification from an increase in *HER2* copies arising from chromosomal aneusomy. Recently, chromogenic *in situ* hybridization (CISH) has been introduced by several groups

as an alternative to FISH, and assessments of these commercialized products have been reported in previous studies.[6–9] CISH is similar to FISH, except that the *HER2* gene copies are detected using a permanent peroxidase reaction instead of a fluorescent dye. The hybridization results can be viewed using an ordinary transmitted light microscope. Here, we report our experience with CISH using the HISTRA HER2 CISH kit (JOKOH, Tokyo, Japan) for the determination of *HER2* gene amplification in FFPE breast cancer (BC), and gastric cancer (GC) tissue specimens.

MATERIALS AND METHODS

Tumors and tissue microarray

In total, 125 BC and 198 GC were collected from Hamamatsu University Hospital, Hamamatsu Medical Center, and Toyohashi Municipal Hospital. The CISH, FISH, and IHC evaluations were performed in a blinded fashion with the observer unaware of the results of the other assays.

The tissue microarrays were constructed using 3 mm tissue cores according to a previously reported method.[10–12] One core of tumor from the middle portion of a single tissue block was sampled.

IHC

The IHC analyses were performed according to the manufacturer's protocol for the HercepTest™ (DAKO, Glostrup, Denmark). HER2 overexpression was determined as defined in the HercepTest™ kit guide: a score of 0 or 1+ was considered negative, a score of 2+ was considered weakly positive, and a score of 3+ was considered strongly positive.

FISH

The FISH analysis was performed using the PathVysion™ HER-2 DNA probe kit (Abbott Laboratories, Abbott Park, Des Plaines, IL, USA), which includes two directly labeled DNA probes: a locus-specific probe for the *HER2* gene labeled with SpectrumOrange, and an alpha satellite probe that targets the centromere region of chromosome 17 labeled with SpectrumGreen (CEP17). The mean *HER2*/CEP17 ratio was counted in at least 20 tumor cells for each case.

CISH

The CISH analyses were performed according to the protocol provided with the composition reagent in the HISTRA HER2 CISH kit (JOKOH). After deparaffinization, the sections were incubated in the pretreatment solution at 94°C for 20 min, followed by washing with distilled water and digestion with protease solution at 37°C for 5 min. The slides were then washed with distilled water, and ready-to-use digoxigenin-labeled HER2 DNA probe (probe size, 450 kb) was applied to the slides, which were covered with 22 × 22-mm coverslips (10 μL of probe mixture per slide). The sections were denatured on a thermal plate at 94°C for 5 min, and hybridization was performed overnight at 37°C. After hybridization, the slides were washed with SSC at 75°C for 5 min. The endogenous peroxidase activity and unspecific staining were blocked by applying 3% H_2O_2 and blocking reagent, respectively. A mouse anti-digoxigenin antibody was added to the slides hybridized with the HER2 DNA probe for 30 min at room temperature followed by incubation with antimouse-peroxidase polymer for 30 min at room temperature. A 3,3′-diaminobenzidine (DAB) chromogen substrate was used for chromogenic visualization for 10 min at room temperature. The antibody reaction and the peroxidase reaction were performed using automatic dyeing equipment (Autostainer Universal Staining System™, DAKO). The tissue sections were lightly counterstained with hematoxylin and were embedded. The CISH sections were evaluated using a x40 dry objective lens. The non-amplified gene copy number was defined as 1–5 dots per nucleus. Amplification was defined as more than five dots per nucleus or large or small clusters or mixtures of multiple dots and clusters of *HER2* gene per nucleus in >50% of the cancer cells in the tissue area selected for enumeration (we evaluated at least 30 tumor cells for each case).

RESULTS

Performance of CISH

CISH was performed for 125 BC cases and 198 GC cases. Gene copies visualized using CISH were clearly distinguishable using a x40 objective lens in tissue sections counterstained with hematoxylin. Tumors without *HER2* amplification typically showed 1–2 dots per nucleus (when diploid) (Fig. 1a,b) or 3–5 dots in cases of chromosomal aneuploidy. Amplified gene copies typically presented as large intranuclear gene copy clusters (Fig. 1c,d) or as a mixture of multiple dots and clusters (Fig. 1e,f) or as small gene copy clusters. In GC, heterogeneity of the *HER2* gene copy number was observed. Multiple dots (clusters) and a smaller number of dots per nucleus were observed in the same tissue section (Fig. 2).

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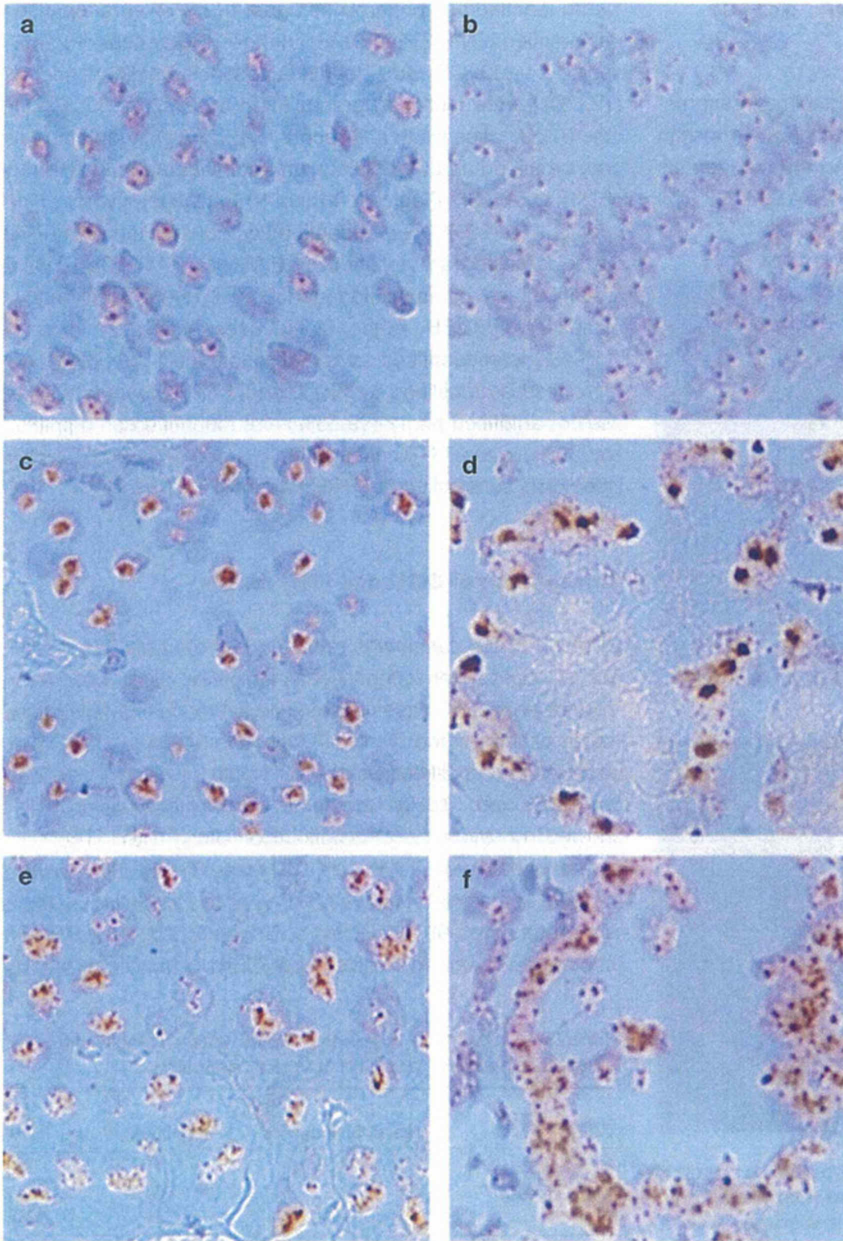

Figure 1 *HER2* chromogenic *in situ* hybridization (CISH) in breast cancers (BC) and gastric cancers (GC). Tumors with no amplification of *HER2* typically showed 1–2 dots per nucleus (BC, **a**; GC, **b**). Amplified gene copies presented typically as large intranuclear gene copy clusters (BC, **c**; GC, **d**) or as a mixture of multiple dots and clusters (BC, **e**; GC, **f**).

Table 1 Correlation between HER2 overexpression as detected using IHC and *HER2* gene amplification as detected using FISH and CISH in breast cancers

	FISH		CISH		
IHC score	No amplification (%)	Amplification (%)	No amplification (%)	Amplification (%)	Total
0	55 (100)	0 (0)	55 (100)	0 (0)	55
1 +	14 (87.5)	2 (12.5)	14 (87.5)	2 (12.5)	16
2 +	4 (23.5)	13 (76.5)	4 (23.5)	13 (76.5)	17
3 +	1 (2.7)	36 (97.3)	1 (2.7)	36 (97.3)	37
Total	74	51	74	51	125

CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

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IHC analysis, CISH, and FISH results for histological specimens

As summarized in Tables 1 and 2, HER2 protein overexpression detected using the HercepTest was correlated with *HER2* gene amplification as detected using the two genetic assays. In the 71 BC cases with a 0/1+ score, 69 (97.2%) were identified as not amplified and 2 (2.8%) were identified as amplified by FISH and CISH. In the 17 BC cases with a 2+ score, 4 (23.5%) were identified as not amplified and 13 (76.5%) were identified as amplified by FISH and CISH. In the 37 BC cases with a 3+ score, 1 (2.7%) was identified as not amplified and 36 (97.3%) were identified as amplified by FISH and CISH (Table 1). In the 148 GC cases with a 0/1+ score, 146 (98.6%) were identified as not amplified, 2 (1.4%) were identified as amplified by FISH, and 145 (98%) were identified as not amplified and 3 (2%) were identified as amplified by CISH. In the 13 GC cases with a 2+ score, 2 (15.4%) were identified as not amplified and 11 (84.6%) were identified as amplified by FISH, and 1 (7.7%) was identified as not amplified and 12 (92.3%) were identified as amplified by CISH. In the 37 GC cases with a 3+ score, 37 (100%) were identified as amplified by FISH and CISH (Table 2).

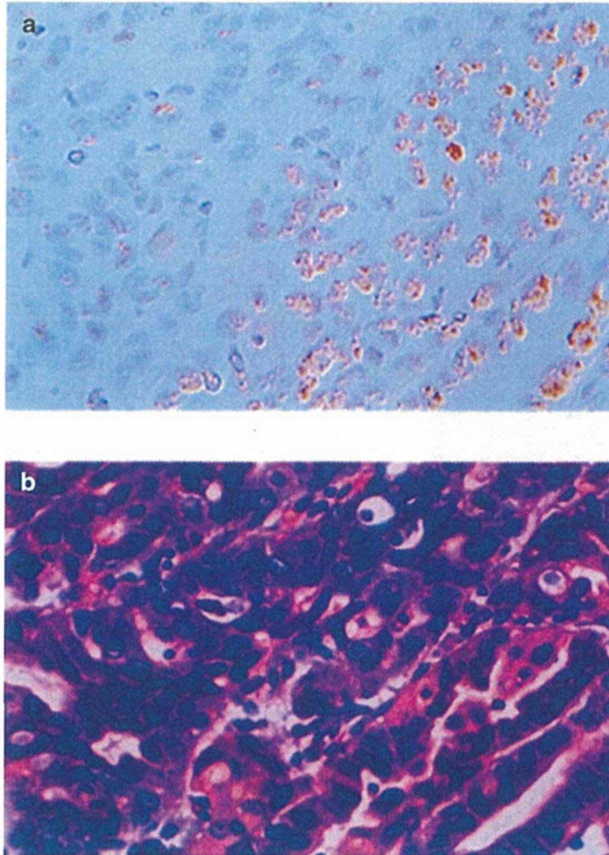

Figure 2 *HER2* chromogenic *in situ* hybridization (CISH) in gastric cancer. (**a**) Heterogeneity of the *HER2* gene copy number: multiple dots (clusters) are visible in the right half of this image, while a smaller number of dots per nucleus is visible in the left part of the tissue section. (**b**) Hematoxylin and eosin (H&E) staining of the same tissue section. The histological heterogeneity is barely detectable with the H&E staining.

Comparison of CISH and FISH results

Of the 125 BC cases with results from both CISH and FISH, 50 BC cases were identified as amplified using both techniques and 73 BC cases showed no evidence of amplification using either method (Table 3). Only one BC case (0.8%) was identified as amplified using CISH but not using FISH, and one BC case (0.8%) identified as amplified using FISH showed no evidence of amplification using CISH. The concordance between FISH and CISH was 98.4%. The positive percent agreement was 98.0%, and the negative percent agreement was 98.6% (Table 3). A detailed characterization of those BC cases with discordant CISH versus FISH results

Table 3 Comparison between *HER2* gene amplification as detected using FISH and CISH in breast cancers

	FISH		
CISH	No amplification	Amplification	Total
No amplification	73	1	74
Amplification	1	50	51
Total	74	51	125

Concordance, 98.4%; Positive percent agreement, 98.0%; Negative percent agreement, 98.6%.

CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization.

Table 2 Correlation between HER2 overexpression as detected using IHC and *HER2* gene amplification as detected using FISH and CISH in gastric cancers

	FISH		CISH		
IHC score	No amplification (%)	Amplification (%)	No amplification (%)	Amplification (%)	Total
0	139 (99.3)	1 (0.7)	139 (99.3)	1 (0.7)	140
1 +	7 (87.5)	1 (12.5)	6 (75)	2 (25)	8
2 +	2 (15.4)	11 (84.6)	1 (7.7)	12 (92.3)	13
3 +	0 (0)	37 (100)	0 (0)	37 (100)	37
Total	148	50	146	52	198

CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

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Table 4 Characterization of the two breast cancers with discrepant results for the *HER2* oncogene status between FISH and CISH analyses

Tumor	HER2 IHC score	Mean *HER2* copy number by FISH	*HER2* : CEP17 ratio by FISH	CISH (ampl status; mean copy number)	Possible explanation for discrepancy
1	2+	6.42	1.12	Amplified; mean 8.4	chr17-related discrepancy; chr17 = 5.75 copies
2	2+	5.45	2.02	No ampl; mean 4.5	*HER2* equivocal-related discrepancy; chr17 = 2.7 copies

ampl, amplification; chr, chromosome; CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry. Amplification if ratio ⩾2.0 in FISH. Amplification if mean copy number >5.0 in CISH.

Table 5 Comparison between *HER2* gene amplification as detected using FISH and CISH in gastric cancers

	FISH		
CISH	No amplification	Amplification	Total
No amplification	146	0	146
Amplification	2	50	52
Total	148	50	198

Concordance, 99.0%; Positive percent agreement, 100%; Negative percent agreement, 98.6%.

CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization.

is shown in Table 4. The differences between the two methods were related to the lack of chromosome 17 information in this CISH kit and an equivocal value in FISH and CISH. Actually, one of these cases (case 1) that had elevated copy numbers of *HER2* according to both methods was interpreted as not amplified because a FISH analysis of this case showed a high centromere count. The discrepancy in case 2 was caused by an equivocal signal value. Of the 198 GC cases with results from both CISH and FISH, 50 GC cases were identified as amplified using both techniques and 146 GC cases showed no evidence of amplification using either method (Table 5). Only two GC cases (1.0%) were identified as amplified using CISH but not using FISH. The concordance between FISH and CISH was 99.0%. The positive percent agreement was 100%, and the negative percent agreement was 98.6% (Table 5). A detailed characterization of the GC cases with discordant CISH versus FISH results is shown in Table 6.

In summary, the differences between the two methods were caused by the lack of information regarding chromosome 17 in the CISH method and equivocal values obtained using the FISH and CISH methods. Our experience indicated that such discrepancies occurred in around 1% of the cases.

Comparison of CISH and FISH results in the polysomy cases

We evaluated the polysomy cases based on our institutional clinical practices. Polysomy of chromosome 17 was defined as the presence of three or more CEP17 signals. In the 50 BC cases displaying polysomy of chromosome 17, the concordance between the FISH and CISH results for the *HER2* signals was 98.0%. The positive percent agreement was 100%, and the negative percent agreement was 96.0% (Table 7). In the 54 GC cases displaying polysomy of chromosome 17, the concordance between the FISH and CISH results for the *HER2* signals was 98.1%. The positive percent agreement was 100%, and the negative percent agreement was 95.5% (Table 8). In the 50 BC cases and the 54 GC cases displaying polysomy of chromosome 17, the IHC 0/1+ score was 22 (44%) and 21 (38.9%), the IHC 2+ score was 8 (16%) and 9 (16.7%), and the IHC 3+ score was 20 (40%) and 24 (44.4%).

DISCUSSION

FISH is used as a standard method for measuring *HER2* gene amplification. However, a fluorescence microscope and a powerful camera are required for FISH procedure. In addition to this economical burden, there are other problems; for example, FISH slides are not fit for long-term preservation, and the image obtained through a fluorescence microscope does not provide an overview of the tumor tissues such as the contours of the tumor cells and tumor heterogeneity. In contrast CISH can be performed using a light microscope and allows the amplification signals to be correlated with the morphological features. In addition, the staining results can be easily photographed and the results can be saved for long periods. In GC, since heterogeneity in the HER2 protein expression level or *HER2* gene amplification is often observed, it is important to be able to observe the whole tumor at a glance and to save and keep the slides for long periods. Therefore, the practical need for the use of CISH for the detection of *HER2* gene amplification may be greater for GC than for other tumors, such as BC.[13–15]

Here, CISH evaluated in the present research was optimized for FFPE tissue specimens of BC and GC. The main sample pretreatments consisted of heat and enzymatic

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Table 6 Characterization of the two gastric cancers with discrepant results for the *HER2* oncogene status between FISH and CISH analyses

Tumor	HER2 IHC score	Mean *HER2* copy number by FISH	*HER2* : CEP17 ratio by FISH	CISH (ampl status; mean copy number)	possible explanation for discrepancy
1	2+	5.65	1.55	Amplified; mean 5.2	chr17-related discrepancy; chr17 = 3.65 copies
2	1+	3.75	1.97	No ampl; mean 5.1	*HER2* equivocal-related discrepancy; chr17 = 1.9 copies

CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.
ampl, amplification; chr, chromosome; Amplification if ratio ≥2.0 in FISH. Amplification if mean copy number >5.0 in CISH.

Table 7 Comparison between *HER2* gene amplification as detected using FISH and CISH in breast cancers with polysomy

	FISH		
CISH	No amplification	Amplification	Total
No amplification	24	0	24
Amplification	1	25	26
Total	25	25	50

Concordance, 98.0%; Positive percent agreement, 100%; Negative percent agreement,96.0%.
CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization.

Table 8 Comparison between *HER2* gene amplification as detected using FISH and CISH in gastric cancers with polysomy

	FISH		
CISH	No amplification	Amplification	Total
No amplification	21	0	21
Amplification	1	32	33
Total	22	32	54

Concordance, 98.1%; Positive percent agreement, 100%; Negative percent agreement,95.5%.
CISH, chromogenic *in situ* hybridization; FISH, fluorescence *in situ* hybridization.

treatment, and since the antibody reaction and the peroxidase reaction were performed using automatic dyeing equipment, the analysis work was completed in a short period of time. Moreover, since the size of the HER2 DNA probe was relatively large (450 kb), the sensitivity and reproducibility were thought to be satisfactory.

A disagreement between *HER2* gene amplification and the protein expression level was seen in some of the BC and GC samples in the present study. A relation between *HER2* gene amplification and the protein expression level has been reported in BC and GC.[16–18] Such disagreement is known to occur for BC, and the state of the sample and technical problems are considered to be causes. There were more examples of IHC 2+ with *HER2* gene amplification than in previous reports. One of the reasons would be that IHC positivity became weaker in the cases which had longer fixation time. Therefore, it is thought that some examples judged to be IHC 2+ and IHC 1+ may be actually IHC 3+ and IHC 2+. The HER2 status in GC has been investigated in a phase III randomized study (ToGA trial). In the ToGA trial, the modified HER2-scoring system showed a concordance of 87.5% between the IHC and FISH results. In our BC samples, most of the IHC 0/1+ samples were FISH negative. In contrast, in ToGA trial, the frequency of IHC 0/1+ samples that tested FISH positive was almost as high as the IHC 2+/FISH-positive samples (23% vs. 26%).[19] Heterogeneity in the *HER2* gene amplification was observed 3–5% in GC cases. Because of the small size of tissue core in tissue microarrays, there is a possibility of having overlooked the part of heterogeneity.

We checked the high concordance between the FISH and CISH results in both BC and GC. The judgment regarding *HER2* gene amplification was performed using FISH based on the ratio of *HER2* gene signals to the CEP17 signals in the centromere domain of chromosome 17. Since CISH evaluated in the present research judges *HER2* gene amplification based on a single color for only the *HER2* gene signals, information regarding the polysomy of chromosome 17 cannot be acquired using CEP17 signals. However, a high concordance between FISH using dual colors and CISH using a single color has been reported in previous reports.[7,9,20] As a cause of disagreement, cases where the FISH and CISH values were near the cutoff value may have generated false-positive or false-negative interpretation. Disagreement for the same reasons as that reported in these previous reports was also observed in the present research.

In conclusion, even though CISH uses a single color, the practical feasibility did not suffer greatly because a strong correlation between the FISH and CISH results was observed. We confirmed that CISH enables results to be observed using only an ordinary light microscope, and CISH was successfully used to evaluate *HER2* gene amplification in BC and GC, similar to FISH. Considering that a wide variety of kinase genes and others are amplified in human GC and that these amplifications are detectable using FISH in FFPE,[21–23] CISH kits targeting these other genes are likely to become available soon. Here, we did not compare a single color CISH kit with the recently released dual color HER2 kit;[24] however, in practical settings, a single color CISH kit may be a feasible choice for pathologists, considering the easiness of the procedures. At any rate, CISH is likely to become a major modality that can be easily used in community hospitals and reference laboratories in the near future.

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DISCLOSURE OF CONFLICT OF INTERESTS

S.K., K.K., H.H., and N.H. are employees of JOKOH Co. Ltd. This fact does not alter the authors' adherence to all the *Pathology International* policies on sharing data and materials. This paper does not recommend any particular brand as a CISH assay.

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Original Article

Detection of kinase amplifications in gastric cancer archives using fluorescence *in situ* hybridization

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To test the feasibility of using bacterial artificial chromosomes (BAC) containing kinases for pathological diagnosis using fluorescence *in situ* hybridization (FISH), 10 BAC probes containing a gene amplified in 5% or more of a pilot cohort were selected from a previous survey using arbitrarily selected BAC clones harboring 100 kinases. In this report, we describe the prevalence and association with the clinico-pathological profile of these selected 10 BAC probes in 365 gastric cancer tissues. FISH analyses using these 10 BAC probes containing loci encoding EGFR, ERBB2(HER2), EPHB3, PIK3CA, MET, PTK7, ACK1, STK15, SRC, and HCK showed detectable amplifications in paraffin-embedded tissue in 2.83% to 13.6% of the gastric cancer tissues. Considerable numbers of the cases showed the co-amplification of two or more of the probes that were tested. BAC probes located within a genome neighborhood, such as PIK3CA, EPHB3, and ACK1 at 3q26-29 or HCK, SRC, and STK15 at 20q11-13.1, were often co-amplified in the same cases, but non-random co-amplifications of genes at distant genomic loci were also observed. These findings provide basic information regarding the creation of a strategy for personalizing gastric cancer therapy, especially when using multiple kinase inhibitors.

Key words: amplification, companion diagnosis, fluorescence *in situ* hybridization, kinase, kinase inhibitor

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Kinases are popular targets for pharmacological intervention in human cancers, and particular receptor kinases such as ERBB2(HER2), epidermal growth factor receptor (EGFR) (HER1), and vascular endothelial growth factor receptor (VEGFR) have been practical targets of currently used drugs. Mutations, overexpression, and copy number alterations in human tumors are important indicators of sensitivity to specific kinase inhibitors or therapeutic antibodies and can act as outcome modifiers when specific therapy is performed. There are 518 kinases in the genome,[1,2] and a considerable number of them have genetic changes, such as mutations and amplifications, as revealed by a recent kinome study.[3] The aim of this study was to test the practical feasibility of bacterial artificial chromosome (BAC) probes harboring kinases to detect amplifications of kinases, which may lead to the development of targeted therapy for amplified kinases. BAC probes representing 100 kinase genes were arbitrarily selected, and fluorescence *in situ* hybridization (FISH) using these BAC probes (usually four probes) was performed for 20 cases of lung, colorectal, and gastric cancers during an initial screening.[4] The 10 loci were revealed to be amplified in 5% or more of the first screening cases, and we subsequently used these 10 BAC probes to analyze 365 gastric cancer cases and assessed the clinico-pathological significance of alterations at these loci.

MATERIALS AND METHODS

Tissue microarray and FISH

One hundred BACs harboring the loci of kinases (Table 1) were labeled and hybridized to a tissue microarray consisting of 60 cancers (20 colorectal, 20 lung, and 20 gastric). We arbitrarily selected 100 kinases for which reports of

Table 1 List of the BAC clones tested in the discovery block

Gene	Group†	Chr.	Position	Tested BAC DNA	Gene	Group†	Chr.	Position	Tested BAC DNA
CDC42BPA	AGC	1	p36.2	RP1-224A6	MET	TK	7	q31	RP11-95I20
FRAP1	Atypical	1	p36.2	RP4-647M16	BRAF	TKL	7	q34	RP5-839B19
EPHB2	TK	1	p36-35	RP11-69E9	EPHB6	TK	7	q34-35	RP11-114L10
EPHA2	TK	1	p36	RP11-276H7	EPHA1	TK	7	q35	RP11-811J9
LCK	TK	1	p35.1	RP4-675E8	CDK5	CMGC	7	q36	RP11-148K1
EPHA10	TK	1	p34.3	RP4-783C10	BLK(GATA4)	TK	8	p23	RP11-235I5
ROR1	TK	1	p32	RP4-597J3	FGFR1	TK	8	p11.2–12	RP11-100B16
TRIM33	Atypical	1	p13.1	RP4-591B8	LYN	TK	8	q12	RP11-446E9
TRK(NTRK1)	TK	1	q21-22	RP11-107D16	STK3	TK	8	q22	RP11-125O21
INSRR	TK	1	q21-23	RP11-180B22	PTK2(FAK)	TK	8	q24.3	RP11-642A1
DDR2	TK	1	q22	RP11-572K18	TAF1L	Atypical	9	p13-20	RP11-205M20
RNASEL	Other	1	q25	RP11-20H6	NTRK2	TK	9	q21-22	RP11-301F14
ARG(ABL2)	TK	1	q25-31	RP11-177A2	ABL	TK	9	q34.1	RP11-83J21
NEK7	Other	1	q31.3	RP11-135A15	RET	TK	10	q11.2	RP11-351D16
RPS6KC1	AGC	1	q32-41	RP11-161F11	MAPK8	CMGC	10	q11.2	RP11-541M12
MLK4(KIAA1804)	TKL	1	q42-43	RP5-862P8	BMPR1A	TKL	10	q23.2	RP11-9M11
ALK	TK	2	p23	RP11-328L16	CHUK	Other	10	q24	RP11-316M21
EIF2AK3	Other	2	p12	RP11-450E9	FGFR2	TK	10	q26	RP11-62L18
FLJ23074(YSK4)	STE	2	q21-22	RP11-341H1	ATM	Atypical	11	q22-23	RP11-241D13
TTN	CAMK	2	q31	RP11-88L24	ANKK1	TKL	11	q23.1	RP11-79I17
ERBB4	TK	2	q33-34	RP11-84F8	CDK2	CMGC	12	q13	RP11-973D8
RAF1	TKL	3	p25	RP11-275J11	CDK4	CMGC	12	q14	RP11-571M6
DCAMKL3	CAMK	3	p22.3	RP11-640L9	IRAK3	TKL	12	q14.3	RP11-335I12
SNRK	CAMK	3	p21-22	RP11-188P20	DYRK2	CMGC	12	q15	RP11-335O4
MGC8407(CAMKV)	CAMK	3	p21	RP11-78O10	CDK8	CMGC	13	q12	RP11-88J11
EPHA6	TK	3	q11.2	RP11-79K12	FLT1	TK	13	q12	RP11-502P18
EPHB1	TK	3	q21	RP11-452H12	FLT3	TK	13	q12	RP11-9D14
PIK3CA	Atypical	3	q26.3	RP11-245C23 + RP11-355N16	DCAMKL1	CAMK	13	q13	RP11-157B21
EPHB3	TK	3	q27-28	RP11-328G15	AKT1	AGC	14	q32	RP11-982M15
ACK1(TNK2)	TK	3	q29	RP11-436M6	NTRK3	TK	15	q25	RP11-62D2
FGFR3	TK	4	p16.3	RP11-572O17	FES	TK	15	q26.2	RP11-405A15
STK32B	AGC	4	p16.2	RP11-326O23	ERBB2	TK	17	q11	RP11-94L15 + RP11-62N23
KDR	TK	4	q11-12	RP11-463H12	TEX14	Other	17	q22-23	RP11-142B17
KIT	TK	4	q11-12	RP11-586A2	ERN1	Other	17	q24	RP11-89H15
PDGFRA	TK	4	q11-12	RP11-231C18	AATK	TK	17	q25	RP11-149I9
EPHA5	TK	4	q13	RP11-641A24	YES1	TK	18	p11	RP11-769O8
PLK4	AGC	4	q27-q28	RP11-398H1	DAPK3	CAMK	19	p13.3	RP11-109H2
TRIO	CAMK	5	p15.3	RP11-81P9	MATK	TK	19	p13.3	RP11-383B15
MGC42105	CAMK	5	p12	RP11-447H19	STK11	CAMK	19	p13.3	RP11-75H6
FGFR4	TK	5	q35	RP11-627M5	AXL	TK	19	q13.1	RP11-551E10
BRD2	Atypical	6	p21.2–21.3	RP11-79J17	PAK7	STE	20	p12	RP5-873P14
MAPK14	CMGC	6	p21	RP1-179N16	HCK	TK	20	q11-q12	RP5-836N17
STK19	Atypical	6	p21	RP1-34F7	SRC	TK	20	q12-q13	RP5-1141E15
PTK7(CCK4)	TK	6	p12-21	RP11-387M24	PTK6,SRMS	TK	20	q13	RP4-697K14
EPHA7	TK	6	q15-16	RP11-346N8	STK15(STK6)	AGC	20	q13.2	RP11-65K20
ROS1	TK	6	q22	RP1-179P9	BCR	Atypical	22	q11.23	RP11-164N13
RPS6KA2	AGC	6	q26-27	RP3-427A4	BMX	TK	x	p22.2	RP11-478H11
EGFR	TK	7	p12	RP11-339F13 + RP5-1091E12	BTK	TK	x	q21-23	RP1-164F3
LMTK2	TK	7	q21.3	RP11-445E22	GUCY2F	RGC	x	q23	RP4-596C15
EPHB4	TK	7	q22	RP11-126L15	IRAK1	TKL	x	q28	RP11-54I20

†Group of kinases: AGC, cyclic-nucleotide-dependent family (PKA and PKG) and the protein kinase C family; TK, tyrosine kinase family; TKL, tyrosine kinase like; RGC, receptor guanlylate cyclases; STE, containing the homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases; CMGC, containing CDK, MAPK, GSK3 and CLK kinases; CK1, containing the casein kinase 1 group; CAMK, containing the calcium/calmodulin-dependent protein kinases.

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amplification or mutation in at least some tumor types were available. Ten BAC probes that detected amplification in 5% or more of 60 pilot cases were selected; the symbol genes for these BACs were PIK3CA, EPHB3, ACK1, PTK7, EGFR, MET, ERBB2 (HER2), HCK, SRC, and STK15. The FISH analysis procedures have been described previously.[4–7] Briefly, for each FISH analysis, two BAC probes, two kinase probes or one kinase probe and the corresponding centromere probe, were labeled with different colors. Tissue microarrays were constructed using KIN-1 (Azumaya, Tokyo, Japan) using 365 gastric cancer cases.[8] The 3 mm cores of the tumor portion in the middle of the tumor area (neither in the surface nor in the invasive front) were obtained and used in the tissue microarray. The amplification of each probe was evaluated according to the University of Colorado Cancer Center[9] stratification system. Briefly, signals in 30 to 50 representative tumor cell nuclei without nuclear truncation were counted and the ratios of kinase signal to the corresponding centromere signal in each tumor cells were recorded. The ratio of 3 or greater in a considerable amount of the tumors was defined as kinase amplification, and 6 or more kinase signals was defined as extensive polysomy independent of the kinase to centromere ratio. Large and bright kinase signals of 15 or more copies were referred to as homogeneous staining region like staining (HSR type).[10] The exact standard picture is shown in figure 2 in the study by Varella-Garcia *et al.* in 2006.[9]

Table 2 Clinicopathological profiles of the subjects

	Male (260)	Female (105)	Total (365)
Ages			
Mean (standard deviation)	62.50 (10.48)	61.02 (12.76)	62.07 (11.19)
Median	65	61	64
Range	29–84	27–86	27–86
Histological subtype†			
pap	3	1	4
tub1	37	7	40
tub2	103	23	126
por1	18	4	22
por2	50	32	82
sig	36	36	72
muc	9	2	11
others	4	0	4
Differentiated type	143	31	174
Undifferentiated type	117	74	191
Depth†			
m	30	19	49
sm1	16	3	19
sm2	58	20	78
mp	39	21	60
ss	61	13	74
se	55	28	83
si	1	1	2
Early stage	104	42	146
Advanced stage	156	63	219
Lymph node involvement			
N0 (negative)	139	65	204
N1	51	13	64
N2	41	17	58
N3a	24	7	31
N3b	5	2	7
Node positive	121	39	160
NX	0	1	1

†Abbreviations are according to the Japanese Classification System, 1995.

Clinicopathological profiles

The clinicopathological profiles are summarized in Table 2.

A total of 365 cases of resected gastric cancer (260 men, 105 women; age range, 29–84 years for men and 27–86 years for women) were examined. Forty percent of the cases were early-stage diseases in both sexes. Histological typing was performed according to the Japanese Classification System.[11] Differentiated-type cancers were seen in 55% of the men and 30% of the women; 46.5% of the men and 38% of the women had lymph node metastasis at the time of operation.

Statistical analysis

The FISH results were categorized into two groups: normal and near-normal numbers of loci tested vs. extensive polysomy, amplification and HSR-type amplification.[9]

The clinicopathological subgroups were also dichotomized: advanced vs. early stage (T factor), lymph node positive vs. negative (N factor), and differentiated vs. undifferentiated histological type. The Fisher exact test was used to analyze the results of 2 × 2 tables (Table 3).

The Fisher independency test was performed for two pairs of amplifications. Forty-five combinations were calculated, and the *P*-values are shown in Table 4.

All the calculations were performed using the statistics package software JMP7 (SAS Institute, Tokyo, Japan).

RESULTS

Amplification status and clinicopathological characteristics of gastric cancer

The results are shown in Table 3. Overall, the success rates for the FISH analyses were 333/365 (91.2%) to 356/365 (97.5%), depending on the probes and the tissue conditions. The amplification rate of these probes in gastric cancer ranged from 2.83% (PIK3CA) to 13.6% (HER2).

Amplification and extensive polysomy of the BAC probes that were tested, except for MET, were more frequent among differentiated-type gastric cancer than among undifferentiated-type cancer. Amplification and extensive polysomy of the BAC probes containing EGFR and SRC had a statistically significant higher prevalence in advanced cancer than in early cancer. The other eight BAC probes showed no difference in prevalence according to the T factor

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Table 3 Prevalence of polysomy and amplification in association with clinicopathological factors

	PIK3CA	EPHB3	ACK1	PTK7	EGFR	MET	HER2	HCK	SRC	STK15
Chromosome locus	3q26.3	3q27-28	3q29	6p12-21	7p12	7q31	17q11	20q11-12	20q12-13	20q13.1
Amplification (total)	10/353	22/354	21/355	21/352	17/347	10/333	46/338	25/348	28/356	27/351
	2.83%	6.21%	5.92%	5.97%	4.90%	3.00%	13.61%	7.18%	7.87%	7.69%
Histological subtype										
Differentiated	9/193	16/190	16/192	18/187	15/192	7/182	40/185	19/186	20/190	23/189
Undifferentiated	1/160	6/164	5/163	3/165	2/155	3/151	6/153	6/162	8/166	4/162
P-value*	0.0208**	0.0497**	0.0286**	0.0014**	0.0037**	0.2553	<0.0001**	0.0147**	0.0346**	0.0004**
Early vs advanced										
Early	2/142	10/138	6/141	7/137	2/135	1/129	19/132	9/136	5/141	7/137
Advanced	8/211	12/216	15/214	14/215	15/212	9/204	27/206	16/212	23/215	20/214
P-value*	0.9572	0.3343	0.9066	0.7777	0.0136**	0.0519	0.6933	0.4597	0.0098**	0.1044
Lymphnode metastasis										
N0	5/193	8/194	6/196	10/194	6/191	3/180	25/184	12/189	7/196	11/192
Node positive	5/160	14/160	15/159	11/158	11/156	7/153	21/154	13/159	21/160	16/159
P-value*	0.5044	0.0580	0.0104**	0.3122	0.0768	0.1098	0.5566	0.3256	0.0008**	0.0944

**P*-value by Fisher's exact test. **At the figures means statistically significant.

Table 4 Independency test of 10 choromosome loci on amplification status

Choromosome locus		PI3KCA	EPHB3	ACK1	PTK7	EGFR	MET	ERBB2(HER2)	HCK	SRC	STK15
Chr 3q26 –29	PI3KCA		**0.0001**	**0.0001**	0.4699	0.4074	0.2479	**0.0336**	**0.0300**	0.1588	**0.0047**
	EPHB3			**<0.0001**	0.1402	0.3024	1.0000	0.0939	**0.0022**	**0.016**	**0.0041**
	ACK1				0.3682	0.0793	1.0000	0.1631	0.1727	0.6604	0.0713
Chr 6p12-21	PTK7					0.0799	**0.0017**	**0.0006**	**0.0131**	0.1963	0.0615
Chr 7p12	EGFR						0.4018	0.4822	0.3719	**0.037**	**0.0333**
Chr 7q31	MET							0.3187	0.5491	0.5817	**0.0381**
Chr 17q11	HER2								**0.0022**	0.0752	**0.0011**
Chr 20q11-13.1	HCK									**<0.0001**	**<0.0001**
	SRC										**<0.0001**
	STK15										

The bold means amplification status of the two loci is not independent.
The numbers in the table is *P*-values of Fisher's exact test (two-sided)

(depth) of the gastric cancer. Alterations at the MET locus tended to be associated with the further progression of the T factors ($P = 0.0519$).

Amplification and extensive polysomy of ACK1 and SRC were associated with lymph node involvement. The other eight probes did not exhibit any statistically significant associations.

Representative pictures of amplification or extensive polysomy are shown in Figure 1.

Co-amplification of kinases in gastric cancer

Among the 365 cases, 246 cases (67.3%) had no amplification at any of the 10 loci. Sixty-six cases (18.1%), 26 cases (7.1%), 13 cases (3.6%), eight cases (2.2%), four cases (1.1%), and two cases (0.6%) had one, two, three, four, five and six loci amplified, respectively. An independency test disclosed that loci in the same neighborhood had been amplified in the same individual at a greater than expected frequency, as shown in Table 4. Three probes on chromosome 3q26-29 (PIK3CA, EPHB3, and ACK1) were often co-amplified, while HCK, SRC, and STK15 on chromosome 20q11-13.1 were also co-amplified. However, the co-amplifications of probes on different loci were also observed. Representative pictures of co-amplification are shown in Figure 2.

DISCUSSION

The recent successful introduction of the Hercep test (DAKO, Carpinteria, CA, USA) to gastric cancer therapy[12] has encouraged pathologists to pursue this type of 'companion diagnostics' in various clinical settings. Kinases are general drug targets, and the genes at the loci examined in the present study were mostly well-known target-candidates or already-in-use targets.

In contrast to the enthusiasm toward the application of kinase gene alterations for targeted therapy, almost no information or theories about how these changes in amplification or mutations occur exists, with the exception of a few reports.[13] Our observations of the numbers of amplified loci (Fig. 3) obeyed an exponential decrease according to the numbers of

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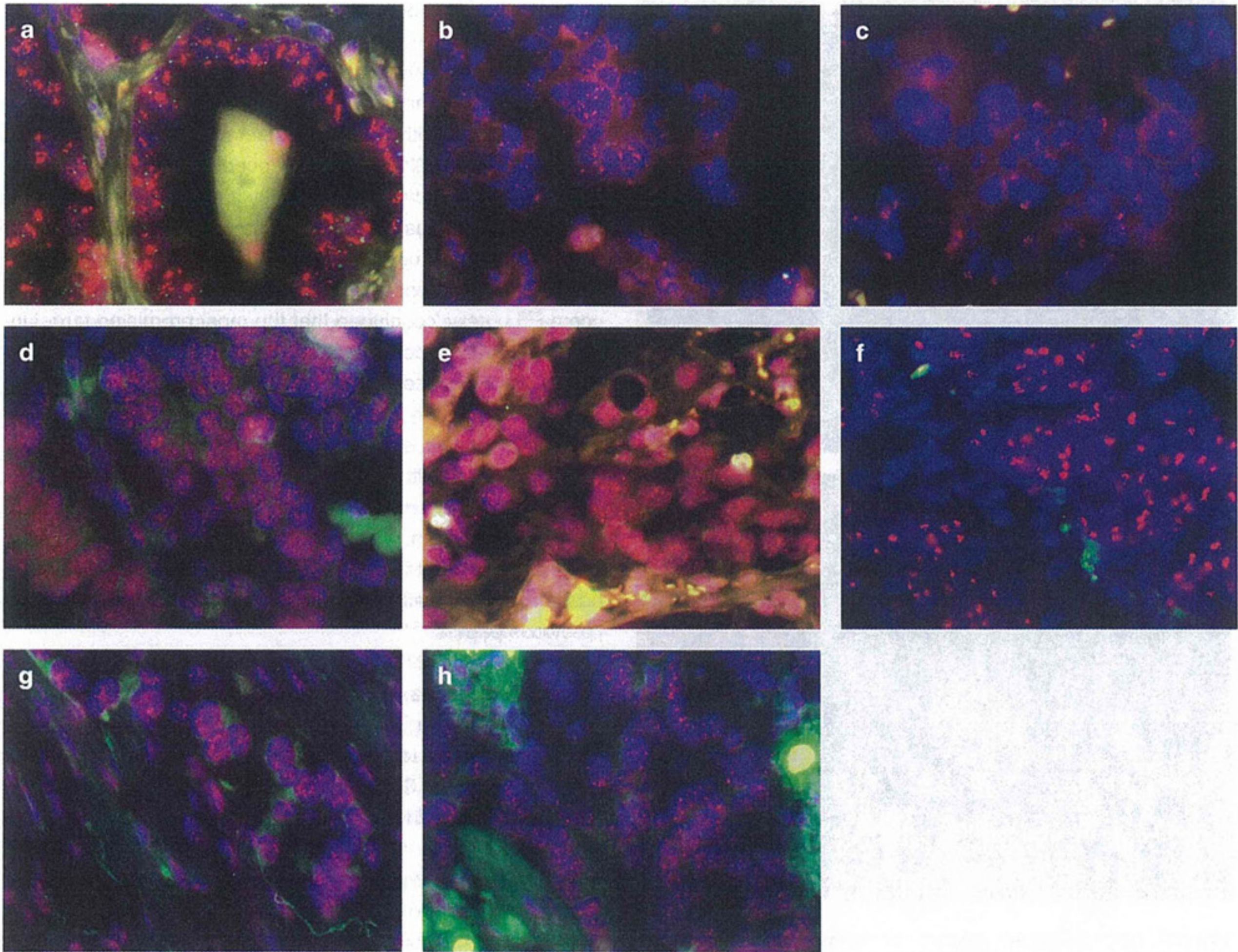

Figure 1 Representative pictures of amplification. All the probes are labeled with SpectrumOrange-dUTP (**a**). HER2 amplification in the cancerous gland in case of differentiated type carcinoma. (**b**). EGFR. (**c**). MET amplification. (**d**). STK15 amplification. (**e**). HCK amplification. (**f**). PTK7 amplification grade 6. (**g**). SRC amplification. (**h**). EPHB3 amplification.

amplification. This situation probably reflects the amplification of particular loci as a stochastic phenomenon. As far as we could determine, no 'amplification phenotype' analogous to a mutator phenotype exists.[14] Not mutually exclusively, however, these multiple amplifications may sometimes cause tumor progression (lymph node metastases) and/or may be accompanied by the various progressive features of clinical tumor. Furthermore, these multiple amplifications of different loci probably reflect drastic structural changes of the genome in cancer cells, chromothripsis.[15–18]

The difference of the prevalence of kinase gene amplification in different histological type was of notice and preference for intestinal type (differentiated type) in HER2 expression is consistent with the previous paper.[19] The prevalence of EGFR overexpression seems to be the same in two subtypes (40 intestinal vs. 47 diffuse cases) in one report.[20] Though kinase and gastric cancer is a seasoned topic,[21] the answer to the question on possible histopathological difference in prevalence of kinase amplification warrants further exploration.

PIK3CA amplification has been examined in malignant lymphoma,[22] lung cancer,[23,24] and colorectal cancer,[25] and inhibitors of the phosphoinositol-3-kinase (PI3K)-mTOR pathway have been extensively investigated.[26]

EPHB3 is sparsely mentioned as a therapeutic target, but EPH family kinase receptors are associated with gastrointestinal tract cancers.[27] Overexpression in lung cancer has been reported, and a correlation with metastasis has been suggested.[28] In our data, though statistically not significant, EPHB3 amplification was more frequent in node-positive cases. On the other hand, EPHB3 has been reported as a possible target together with the other genes, MASP1 and SST, at 3q26.2-q29.[29]

The regulation and inhibition of ACK1 have been investigated in terms of cross-talk with other kinases, but

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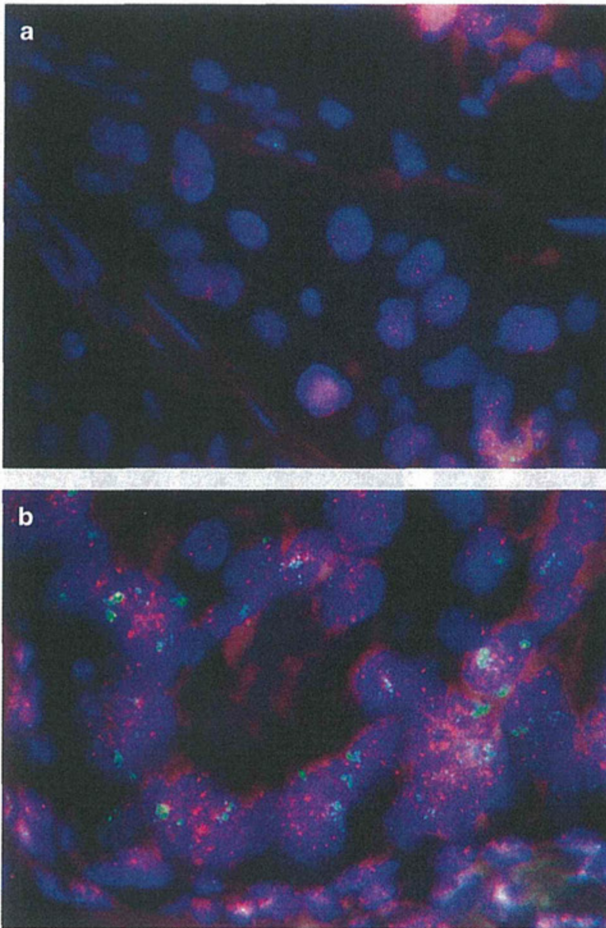

Figure 2 Representative pictures of co-amplifications. (**a**). A case showing co-amplification of *HCK* (SpectrumOrange-dUTP) and *PTK7* (SpectrumGreen-dUTP). (**b**). Another case showing co-amplification of *HCK* and *SRC*.

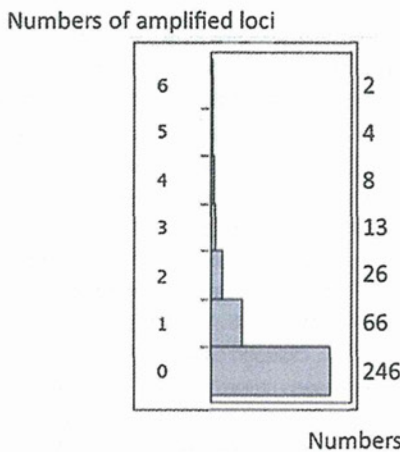

Figure 3 Numbers of cases having no, 1, 2, 3, 4, 5, and 6 amplifications out of 10 kinases. The frequency of amplification decreases exponentially.

amplification has been studied only in one previous report.[30] Our data strongly support the association between ACK1 amplification and lymph node metastasis in gastric cancer based on a FISH analysis of primary cancer tissues, a clinically feasible method. Further investigation of the clinical significance of ACK1 in gastric cancer would be interesting.

PTK7 is located within a recently identified genetic area of amplification in gastric cancer that was discovered using array comparative genomic hybridization.[31] The amplification was related to poor prognosis in patients with osteosarcoma.[32] Lu *et al.* concluded that the most promising target in the 6p12-p21 region was CDC5L, not PTK7. PTK7 amplification is associated with the amplification of MET, HER2, and HCK (Table 4); thus, PTK7 amplification may reflect the extensive genetic derangement of gastric cancer cells.

Epidermal growth factor receptor (EGFR) amplification has been well documented in some human cancers, including lung and stomach.[33–36] In our dataset, EGFR amplification was more frequent in advanced gastric cancer than in early gastric cancer, which is compatible with the results of many previous papers.[33–36]

The frequency of MET amplification detected using FISH in gastric cancer was low in this paper, unlike the classical observations using Southern blotting,[37] though the MET axis is expected to be a target of chemotherapy in various cancers and sarcoma.[38–40] Okamoto *et al.*[41] proposed that MET amplification is responsible for c-Src inhibition in gastric cancer cell lines. Interestingly, unlike the other kinases that were tested, no difference in the prevalence of MET amplification was seen according to the histological type of gastric cancer, which is compatible with the results of a previously mentioned report.[37]

HER2 is the most recently introduced histological diagnostic test for gastric cancer. The FISH data and accompanying immunohistochemical data (data not shown) obtained in our paper were mostly compatible with the currently available information.[42,43] The prevalence was somewhat lower than that in other reports,[44] but we do not think that this difference is significant considering that the materials had been handled in a rural community hospital in an ordinary clinical setting and that our samples included a considerable number of cases with undifferentiated-type cancer. Co-amplification with three of nine other kinases was noted. This may partially explain the resistance to Herceptin.

The amplification and mutation of HCK in gastric cancer has been reported in one previous report.[3] HCK has been investigated in the context of treatment-resistance in leukemia,[45] but the characterization and significance of HCK alterations in human gastrointestinal cancer have not been fully investigated.

SRC is a prototype of a non-receptor kinase, and extensive biological investigations have been performed over the past few decades. Dasatinib is an SRC inhibitor and is used as a

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second-line kinase inhibitor against chronic myelogenous leukemia. Preclinical and clinical studies have been conducted for lung cancer,[46] urothelial cancer,[47] and ovarian cancer.[48] A few researchers have considered SRC as a target for gastric cancer therapy.[41] As our data showed, SRC amplification is more prevalent in node-positive and more advanced cases; thus, SRC amplification is likely to be an important clinical determinant of the outcomes of gastric cancer patients.

STK15 is known as Aurora kinase A and has also been considered as a target of many cancers including the brain,[49] esophagus,[50] larynx,[51] and colon,[52] but the use of STK15 as a therapeutic target of gastric cancer has not been reported. Several targeting drugs against Aurora A kinase are presently available including VX-680;[53,54] thus, some patients with STK15 amplification may benefit from these drugs.

Since we adopted a tissue microarray (TMA) approach, the results here cannot escape the problems of sampling biases, especially when heterogeneous genetic change is considered.[55] Our core sizes taken are larger than ordinary ones (0.6, 1.0 and 2.0 mm) that the most popular tissue microarrayer generates, but we were aware that we missed some genetic information in heterogeneous cancer tissue. We are currently duplicating these samples, that is two and more cores are taken from the same block, and the trend is basically same (data not shown).

In conclusion, our study suggests the following: (i) a FISH analysis of 10 kinases is feasible as a routine diagnostic measure; (ii) the co-amplification status of kinases may further help to modify therapeutic strategies, especially when dual inhibitors or pan-inhibitors of kinases are included in the treatment regimen; (iii) the amplifications of kinases occur in a stochastic manner, that is, there is no 'amplification phenotype' analogous to a 'mutator phenotype'; and (iv) some kinase amplifications start at an early disease stage, while others are correlated with metastasis and progression.

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The *CRKL* gene encoding an adaptor protein is amplified, overexpressed, and a possible therapeutic target in gastric cancer

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Abstract

Background: Genomic DNA amplification is a genetic factor involved in cancer, and some oncogenes, such as *ERBB2*, are highly amplified in gastric cancer. We searched for the possible amplification of other genes in gastric cancer.

Methods and Results: A genome-wide single nucleotide polymorphism microarray analysis was performed using three cell lines of differentiated gastric cancers, and 22 genes (including *ERBB2*) in five highly amplified chromosome regions (with a copy number of more than 6) were identified. Particular attention was paid to the *CRKL* gene, the product of which is an adaptor protein containing Src homology 2 and 3 (SH2/SH3) domains. An extremely high *CRKL* copy number was confirmed in the MKN74 gastric cancer cell line using fluorescence *in situ* hybridization (FISH), and a high level of CRKL expression was also observed in the cells. The RNA-interference-mediated knockdown of CRKL in MKN74 disclosed the ability of CRKL to upregulate gastric cell proliferation. An immunohistochemical analysis revealed that CRKL protein was overexpressed in 24.4% (88/360) of the primary gastric cancers that were analyzed. The *CRKL* copy number was also examined in 360 primary gastric cancers using a FISH analysis, and *CRKL* amplification was found to be associated with CRKL overexpression. Finally, we showed that MKN74 cells with *CRKL* amplification were responsive to the dual Src/BCR-ABL kinase inhibitor BMS354825, likely via the inhibition of CRKL phosphorylation, and that the proliferation of MKN74 cells was suppressed by treatment with a CRKL-targeting peptide.

Conclusion: These results suggested that CRKL protein is overexpressed in a subset of gastric cancers and is associated with *CRKL* amplification in gastric cancer. Furthermore, our results suggested 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.

Keywords: CRKL, Gastric cancer, Cell proliferation, Overexpression, Copy number amplification

Background

Although the overall incidence of gastric cancer is decreasing in many countries, the high incidence of gastric cancer remains a serious health problem, and gastric cancer continues to be the second-leading cause of cancer-related death worldwide [1,2]. Gastric carcinogenesis is a multi-step process in which environmental and genetic factors interact [1–8]. Among the genetic changes observed in cancerous cells, genomic DNA amplification is a well-known alteration that is involved in gastric cancer [4,5,7]. Amplification is often associated with increased expression levels of the genes contained in the amplified loci [5]. Oncogenes in gastric cancer, such as *MYC* (mapped to chromosome 8q24), *KRAS* (12p12), and *ERBB2* (17q12), are located in such amplified regions [4,5,7,9]. We considered the possibility that there exist genes whose amplification in gastric cancer has not been revealed to date. To uncover such novel gene alterations, we searched for highly amplified genes in

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gastric cancer using a genome-wide single nucleotide polymorphism (SNP) microarray analysis and found that the *CRKL* [*v-crk sarcoma virus CT10 oncogene homolog (avian)-like*] gene (22q11) is highly amplified in gastric cancer. The CRKL, a member of the CRK family of adapter proteins, consists of an NH2-terminal Src homology 2 (SH2) domain followed by two SH3 domains: SH3n and SH3c [10], and participates in signal transduction in response to growth factors, cytokines, and the oncogenic BCR-ABL fusion protein, resulting in cell proliferation, survival, adhesion, and migration [10,11]. We hypothesized that CRKL might play an important role in gastric carcinogenesis and investigated whether CRKL expression and the function of CRKL protein affect the regulation of cell proliferation in gastric cancer. We also investigated responsiveness of a gastric cancer cell line containing *CRKL* amplification to a kinase inhibitor, BMS354825, and a CRKL-targeting peptide.

Materials and Methods

Cell lines and surgical specimens

The gastric adenocarcinoma cell lines MKN7, MKN28, MKN74, and AGS were purchased from the Human Science Research Resource Bank (Osaka, Japan) or from American Type Culture Collection (Manassas, VA). Cells were cultured and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) under a 5% CO_2 atmosphere at 37°C. Paraffin-embedded gastric tissues obtained from gastric cancer patients who underwent surgery at Toyohashi Municipal Hospital (Japan) were used for the immunohistochemical analysis. Gastric tissue samples obtained from gastric cancer patients who underwent surgery at Hamamatsu University Hospital (Japan) were used for the quantitative reverse-transcription (QRT)-polymerase chain reaction (PCR) analysis. The study design was approved by the Institutional Review Boards (IRBs).

Genome-wide SNP microarray

DNA (250 ng) was digested with *Nsp*I restriction enzyme (New England Biolabs, Hertfordshire, UK) and ligated to a universal adaptor sequence. The ligated DNA was PCR-amplified using primers complementary to the universal adaptors, and the PCR products were purified, quantified, and normalized. The products were then fragmented, end-labeled using terminal deoxynucleotidyl transferase, and hybridized to the Affymetrix GeneChip human mapping 250 K NspI arrays (Affymetrix Japan, Tokyo, Japan). After hybridization, the arrays were washed, stained using Affymetrix fluidics station 450, and scanned with a GeneChip Scanner 3000 7 G. Raw SNP call data were extracted using Affymetrix GeneChip Genotyping Analysis software (GTYPE) 4.1. The SNP microarray data were analyzed to determine the total copy number using the CNAG program, as previously described [12,13] (Figure 1).

WST-8 assay

Cell proliferation and viability were quantified using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions [14]. The assay was based on the extracellular reduction of the tetrazolium salt WST-8 by NADH produced in the mitochondria of living cells. The cells were incubated with the WST-8 reagent for 1 hr at 37°C, and the absorbance was measured at 450 nm using an EL340I microplate reader (BIO-TEK Instruments, Winooski, VT) (Figure 2).

Immunohistochemistry

Tissue microarray (TMA) blocks were prepared as previously described [14-16]. TMA block sections were deparaffinized, rehydrated, and boiled in Tris-EDTA buffer (pH 9.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation in a hydrogen peroxide solution. Next, the sections were incubated with a rabbit anti-CRKL monoclonal antibody (Y243; Abcam, Cambridge, UK). The antigen-antibody complex was visualized using Histofine Simple Stain Max-Po (Multi) (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine tetrahydrochloride. Counterstaining was performed using hematoxylin. The intensity values of the cells were determined using a 4-point scale according to the color of the cell cytoplasm after CRKL immunostaining as follows: 0, blue; 1, blue-brown; 2, light brown; and 3, brown. The percentage of cells with each intensity value was then multiplied by the intensity value, as described previously [14]. The scores obtained for CRKL immunostaining were classified as either a low expression level (0–0.99) or a high expression level (1.00–3.00) (Figure 3).

DNA fluorescence *in situ* hybridization (FISH)

FISH was performed as previously described [16-19]. Tissue slides were hybridized with a Spectrum Orange-labeled BAC clone (RP11-801O20 and RP11-1058B20) for the *CRKL* locus (Advanced GenoTechs Co., Tsukuba, Japan) and a Spectrum Green-labeled control probe for the near centromere locus on chromosome 22 (BAC clone: RP11-232E17). 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used for nuclear staining (Figure 3).

MTT assay and direct cell counting

In the experiment involving treatment with the CRKL-targeting peptide, an MTT assay was performed to assess cell viability in Figure 4G. The cells were cultured with the indicated concentration of CRKL-targeting peptide or dimethyl sulfoxide (DMSO) at 37°C for 72 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

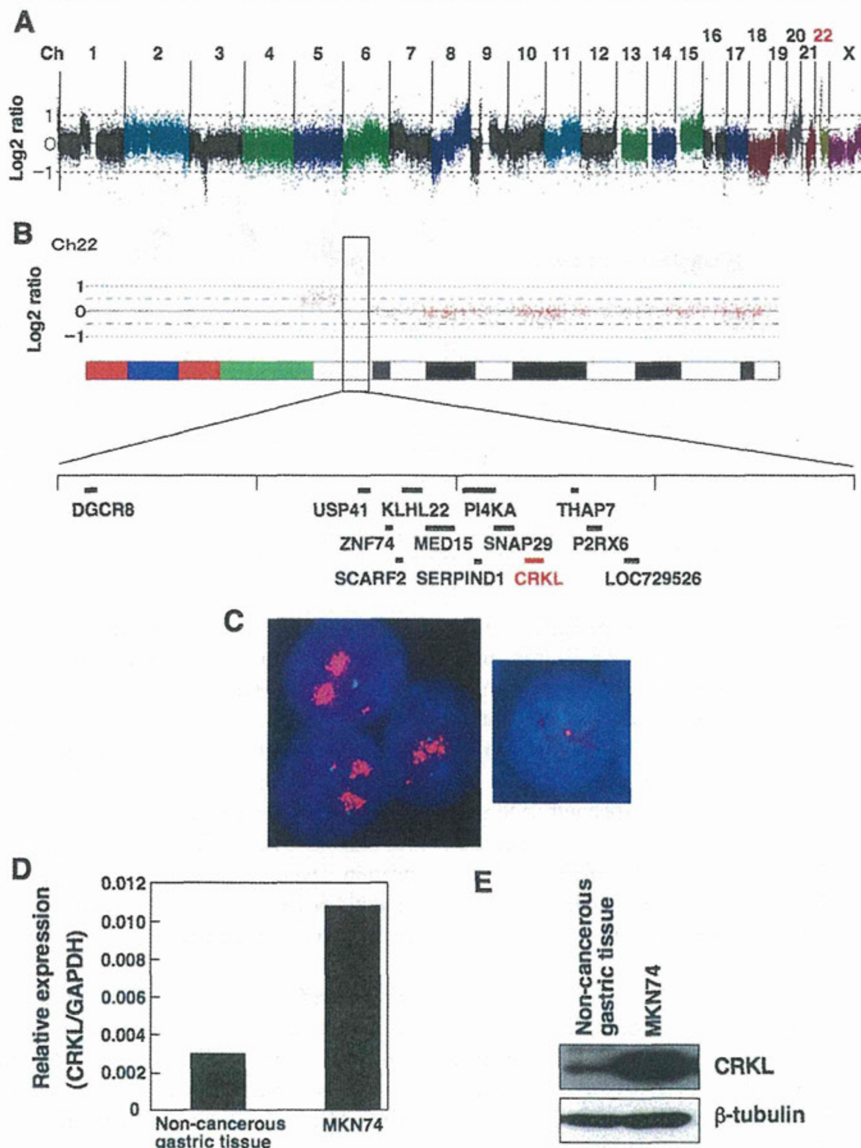

Figure 1 Identification of highly amplified chromosome regions containing the *CRKL* gene and the detection of CRKL overexpression in gastric cancer. (A) Genome-wide detection of copy number alterations using a high-density SNP microarray in the MKN74 gastric cancer cell line. The copy number status for the whole genome of MKN74 is shown. DNA (250 ng) was analyzed using an Affymetrix GeneChip 250 K Nspl 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.

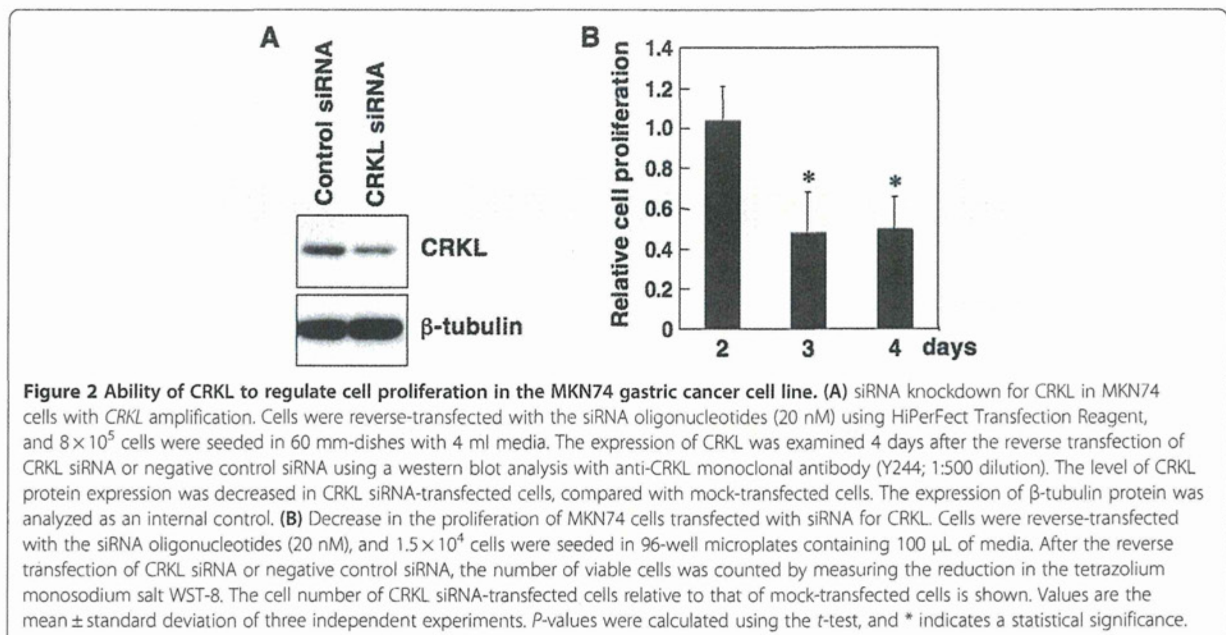

Figure 2 Ability of CRKL to regulate cell proliferation in the MKN74 gastric cancer cell line. (A) siRNA knockdown for CRKL in MKN74 cells with *CRKL* amplification. Cells were reverse-transfected with the siRNA oligonucleotides (20 nM) using HiPerFect Transfection Reagent, and 8×10^5 cells were seeded 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%.