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Gene amplification of *CCNE1*, *CCND1* and *CDK6* in gastric cancers detected by multiplex ligation-dependent probe amplification and fluorescence *in situ* hybridization

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Abstract

New and effective treatments for advanced gastric cancer are urgently needed. Cyclins E and D1 form a complex with cyclin-dependent kinase 2 (CDK2), 4 or 6, thereby regulating G1-S transition. The G1-S regulatory genes encoding cyclin E (*CCNE1*), cyclin D1 (*CCND1*) and CDK6 (*CDK6*) are frequently amplified in gastric cancer and may therefore influence molecular targeted therapies against *ERBB2* or *EGFR* when co-amplified. A total of 179 formalin-fixed and paraffin-embedded gastric cancer specimens were examined for these gene amplifications by multiplex ligation-dependent probe amplification (MLPA) and fluorescence *in situ* hybridization (FISH). Amplification of at least one G1-S regulatory gene was found in 35 tumors (*CCNE1* amplification, 15% of samples; *CCND1*, 6%; *CDK6*, 1%). In 13 of the 35 tumors, dual-color FISH identified co-amplification of the G1-S regulatory genes with *ERBB2, EGFR* and/or *KRAS* in single cancer nuclei. The observation that cells with G1-S regulatory gene amplification contained clonal subpopulations with co-amplification of *ERBB2, EGFR* or *KRAS* in five early and three advanced cancers suggests that amplification of the G1-S regulatory genes represents an early event which precedes *ERBB2, EGFR* or *KRAS* amplification. Amplified *CCNE1*, *CCND1* and *CDK6* in advanced gastric cancer may be potentially useful as direct targets for molecular therapy, or for combination therapy with ERBB2 or EGFR inhibitors. MLPA could be a useful tool for identification of patients who would benefit from such therapies.

Introduction

Gastric adenocarcinoma is the fourth most common cancer and the third leading cause of cancer-associated death worldwide [1]. In the initial stages of gastric adenocarcinoma, such as the early gastric carcinomas as defined by the Japanese Research Society for Gastric Cancer [2], the carcinoma is confined to the mucosa and the submucosa. These early tumors are usually endoscopically or surgically resectable and can be cured [3]. In contrast, a poor prognosis is associated with advanced gastric cancers that have penetrated the muscle layer [2], metastasized and/or developed an inoperable carcinoma. Thus, novel therapeutic modalities are urgently needed for the treatment of late-stage gastric carcinomas.

Under normal circumstances, growth factor signaling leads to the expression of cyclin D1 and its complexing with cyclin-dependent kinase 4 (CDK4) or CDK6. Following accumulation of active cyclin D1/CDK4 or CyclinD1/CDK6, CDK2 in combination with cyclin E then accumulates to facilitate the transition from G1 to S phase by phosphorylation of downstream targets, including the tumor suppressor RB [4]. It is generally accepted that gene amplification is the major mechanism of cyclin D1 and E overexpression. However, another possible mechanism for accumulation of cyclin E

is alterations in its degradation pathway due to mutations in *hCDC4*. [5, 6].

Amplified genes encoding receptor tyrosine kinases (RTK) such as *ERBB2*, *EGFR*, *FGFR2* and *MET* are established or potential targets of molecular therapy in advanced gastric cancers. In addition to RTK genes, recent comprehensive genomic analyses of copy number alterations using a high-resolution single nucleotide polymorphism array (SNP) [7], along with oligonucleotide array comparative genomic hybridization (aCGH) [8], have shown that the genes encoding cyclin E (*CCNE1*), cyclin D1 (*CCND1*) and CDK6 (*CDK6*) are frequently amplified in gastric cancer. Co-amplification of *CCNE1* or *CCND1* with *ERBB2* reportedly reduced the anti-tumor effects of trastuzumab, a monoclonal antibody against ERBB2, in gastric and breast cancers [9].

Multiplex ligation-dependent probe amplification (MLPA) is a new, high-resolution method for the detection of numerous copy number variations in genomic sequences in a single reaction. Using MLPA, the aims of this study were to determine the gene amplification status of *CCNE1*, *CCND1* and *CDK6* in gastric cancer specimens, and to clarify the significance of these amplifications for gastric cancer treatment.

Materials and Methods

Patients and control cell lines

A total of 179 patients with gastric adenocarcinoma (84 early- and 95 advanced-stage tumors) who underwent surgery at the Department of Surgery in Kanazawa University Hospital between 2013 and 2015 contributed tumor specimens to this study. This laboratory study was approved by the Medical Ethics Committee of Kanazawa University (Approval No. 181), and written informed consent was obtained from all patients.

Cancer staging was performed according to the TNM cancer staging system of the American Joint Committee on Cancer [10]. The World Health Organization Classification of Tumours [11] was used to determine histological classification. Serial sections cut from representative formalin-fixed and paraffin-embedded cancer specimens were used for hematoxylin and eosin (H-E) staining, MLPA, fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). When a primary tumor was positive for gene amplification of *CCNE1*, *CCND1*, *CDK6*, *ERBB2*, *EGFR* and/or *KRAS*, samples of nodal metastatic tumors, if any, were also examined for amplification

of the positive gene(s) by FISH. The cell lines MKN7, A431 (both from Riken Cell Bank, Tsukuba, Japan) and SNU5 (American Type Culture Collection, Rockville, MD) were used as the positive controls for gene amplification.

MLPA

Cancer-enriched 6 μm-thick serial section that excluded non-neoplastic cells were selected by comparison to the adjacent H-E-stained section. DNA was manually extracted from each selected section using proteinase K (Roche Diagnostics, Manheim, Germany) according to the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands).

DNA was subjected to MLPA using the SALSA MLPA probemix P458-B1 Gastric Cancer kit (MRC-Holland), which contains two to three probes for each of 16 genes including *CCNE1*, *CCND1*, *CDK6*, *KRAS*, *ERBB2*, *and EGFR*. The resulting polymerase chain reaction (PCR) products were separated on an ABI-310 capillary sequencer (Applied Biosystems, Foster City, CA) and the results interpreted with GeneMapper software (Applied Biosystems). Data analysis was performed with Coffalyser MLPA-DAT software version 9.4 (MRC-Holland) to normalize peak values.

Average peak values below 0.7 were defined as ‘lost’; between 0.7 and 1.3 as ‘normal’; between 1.3 and 2.0 as ‘gain’; and above 2.0 as ‘amplified’, as previously established.[12, 13] Both ‘amplified’ and ‘gain’ results were considered MLPA-positive and the positive tumors were further examined for the respective gene amplification by FISH.

IHC

IHC for cyclin E, cyclin D, ERBB2 and EGFR was performed on representative sections of all tumors. IHC detection of CDK6 and KRAS was also attempted but yielded unsatisfactory results. The antibodies used were a mouse monoclonal antibody against cyclin E (sc-247; Santa Cruz Biotech, Dallas, TX; working dilution, 1:200), a rabbit monoclonal antibody against cyclin D1 (SP4; Nichirei, Tokyo, Japan; prediluted), a polyclonal antibody against the internal domain of human ERBB2 (Nichirei; working dilution, 1:400), and mouse monoclonal antibodies against the external domain of the human EGFR (Novocastra Lab, Newcastle, UK; working dilution, 1:20). Antibody binding was visualized using the LSAB^{TM} system (Dako, Glostrup, Denmark).

For evaluation of cyclin E and D1 staining, only nuclear immunostaining in each tumor section was scored using a four-tier system: 0, no staining; 1+, staining with an

intensity and frequency similar to the pattern occasionally observed in the neck zone of normal gastric mucosa; 2+, intermediate staining intensity; 3+, intense staining in more than 80% of tumor cells. Immunostaining of ERBB2 and EGFR was scored using a four-tier system (0, 1+, 2+, 3+) according to the criteria recommended by Dako for the HercepTest™, except that the number of positive cells was not considered. Staining was evaluated by two observers (TO and AO) and discordance was resolved by discussion. Tumors with 2+ and 3+ staining were considered positive, and were further examined for amplification of the corresponding gene(s) by FISH.

FISH

The FISH probes used in this study were acquired from BACPAC Resources (Oakland, CA) and are summarized in Table 1. The probes were labeled with SpectrumOrange™ or SpectrumGreen™ using a nick translation kit (Abbott Laboratories, Abbott Park, IL). For quantification of gene amplification, a pericentromeric probe (Abbott) specific to each gene's chromosome, or a BAC probe specific to sequences near the particular gene, was co-hybridized to standardize the chromosome number. Tumors demonstrating co-amplification of multiple genes were further examined by simultaneous hybridization using two probes to the genes that were labeled with different fluorescent

markers, in order to assess the co-existence of the amplified genes in single nuclei or single amplicons.

Removal of protein from the tissue sections, denaturation, hybridization and post-hybridization washing were performed as described previously [13]. The tissue sections were counterstained with DAPI II (Abbott) and examined using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter set (Abbott) for DAPI II, SpectrumOrange^{TM} and SpectrumGreen^{TM}. Scoring and evaluation of FISH slides was performed manually by counting the target gene signals and control signals in 20 tumor cell nuclei per sample. Gene amplification was determined according to the American Society of Clinical Oncology/College of American Pathologists criteria for *ERBB2* amplification except that the number of positive cells was not considered in this study [14]. Low-level amplification was defined as two or more additional copies of genes compared to control signals. In cases of apparent intratumoral heterogeneity in gene amplification, the fractions (%) of tumor cells positive for gene amplification were scored and categorized into broad tiers of <10%, 10-30%, 30-50%, 50-70% and >70%.

Direct sequencing

Mutations in exons 8 and 12 of *hCDC4*, which represent the locations of most reported *hCDC4* mutations in gastric carcinomas [15] were analyzed by direct sequencing in tumors with overexpression but not amplification of *CCNE1*. In tumors with *KRAS* amplification, codons 12, 13 and 61 were also analyzed, as point mutations have been reported only in these locations. The PCR conditions for amplification of exon-specific regions from tumor genomic DNA and for mutation identification are detailed in the supplemental data.

Statistical analysis

The association between gene amplification and early or advanced cancer was analyzed using Fisher's exact probability test.

Results

MLPA analysis

All 179 gastric adenocarcinoma tissue samples and all three cell lines were successfully analyzed by MLPA for the expression of 16 genes. The status of each gene in each of the 179 tumors was categorized as 'amplified', 'gain' or 'normal' based on its mean

MLPA peak value. There were no instances of 'lost' genes. Subsequent FISH analyses confirmed gene amplification in 45 of the 49 (92%) tumors that were classified as 'amplified', and 48 of the 148 (32%) tumors that were classified as 'gain', for any of these 13 genes. However, gene amplification of *FGFR1*, *GATA4* and *GATA6* was not observed in any tumor. The number of tumors categorized as 'amplified' or 'gain' for specific genes by MLPA analysis and FISH-proven amplification is summarized in Table 1.

IHC analysis

Analysis of the expression of cyclin E and cyclin D1 using IHC identified positive immunostaining for these proteins in 58 and 59 of the 179 tumors, respectively. IHC analysis of ERBB2 and EGFR expression was also performed. By combining the results of IHC and subsequent FISH analysis, gene amplification of *CCNE1*, *CCND1*, *ERBB2* and *EGFR* was observed among tumors with 'normal' MLPA values, as shown in Table 1. A total of 20 tumors had positive immunostaining for cyclin E but were negative for amplification of its gene *CCNE1*. Although *hCDC* was successfully sequenced in all of these tumors, no instance of *hCDC* mutations was found.

FISH analysis

The same 16 genes that were analyzed by MLPA were also examined with FISH. In FISH images, most of the samples with highly amplified genes displayed either tightly clustered or numerous scattered signals, suggesting amplicons in homogeneously staining regions (HSRs) or double minute chromosomes (DMs), respectively [16]. Cancer nuclei with co-localization of an amplified gene and the amplified centromeric lesion on which the gene is located, which results in a yellow fusion signal [17], were rarely observed. FISH analysis of the cell lines used as positive controls for gene amplification of *CCNE1*, *CDK6* and *CCND1* indicated HSR-type amplification of *CCNE1* and *CDK6* in MKN7 and SNU5 cells, respectively, and low-level amplification of *CCND1* in A431 cells, as shown in Suppl. Fig. 1.

FISH analysis indicated that *CCNE1* was amplified in 12 of the 84 (14%) early gastric cancer tumors and in 14 of the 95 (15%) advanced tumors, and was the most frequently amplified gene among the 16 genes examined. Amplification of *CCND1* and *CDK6* was found in ten and two tumors, respectively. Table 2 shows the clinicopathological characteristics of the 35 tumors with gene amplification of *CCNE1*, *CCND1* and/or *CDK6*. No significant differences between early and advanced cancers were observed in the amplification frequencies of *CCNE1*, *CCND1* or *CDK6* (p=0.87).

FISH was further used to determine co-amplification of genes within the tumors. Co-amplification of *CCNE1* and *CCND1* was found in three early gastric cancers (Cases 1, 12 and 15) (Table 2); *CCNE1*-amplified cancer cells and *CCND1*-amplified cells were present in different areas of these cancers. However, no cancer cell had simultaneous amplification of these two genes, as shown in Fig. 1. Analysis of 19 advanced gastric cancer tumors indicated that *CCNE1*, *CCND1* or *CDK6* was amplified in each tumor and that, in most instances, the majority of cells demonstrated the gene amplification.

Co-amplification of *CCNE1*, *CCND1* and/or *CDK6* with *ERBB2*, *EGFR* and/or *KRAS* in various combinations was found in 18 out of 35 tumors, as shown in Table 2. More precise examination by dual-color FISH revealed that these co-amplifications occurred in single cancer nuclei in five early gastric cancers (Cases 3, 5-7 and 14) and in eight advanced gastric cancers (Cases 18, 23, 24, 26-28, 34 and 35). Case 5 was a non-invasive mucosal adenocarcinoma in which almost all cancer cells had amplification of *CCND1* and three separate areas of the tumor also had co-amplification of *ERBB2*, *EGFR* and *KRAS*, as shown in Fig. 2. The other two mucosal cancers, which were predominantly composed of *CCNE1*-amplified cells (Case 3) or *CDK6*-amplified cells (Case 6), demonstrated co-amplified *EGFR* in only a fraction of the tumors. Two

submucosal invasive cancers that consisted exclusively of *CCNE1*-amplified cells (Case 7) or *CCND1*-amplified cells (Case 14) had a clonal subpopulation of *ERBB2*-amplified and *EGFR*-amplified cells, respectively.

In the remaining five early gastric cancer tumors (Cases 2, 10, 13, 15 and 16), gene amplification of the cyclins and RTKs occurred in different cells; however, in three tumors (Cases 2, 10 and 13) the cells with amplified cyclins and those with amplified RTKs were located adjacent to each other. Co-amplification of one of the G1-S regulatory genes and *ERBB2*, *EGFR* and/or *KRAS* was found in eight advanced gastric cancer tumors; however, due to the large size of the tumors, the precise distribution of the amplified cells could only be determined in three tumors (Cases 18, 28 and 34). Case 28 consisted predominantly of *CCNE1*-amplified cells but had a small area with co-amplified *EGFR*. In Case 18, cancer cells that had co-amplified *ERBB2* were found to occupy small areas of the tumor, which was mostly composed of *CCND1*-amplified cells. Case 34 predominantly composed of *CCND1*-amplified cells showed two small subclonal populations of co-amplified *CCND1* and *ERBB2*, and *CCND1* and *EGFR* in the vicinity (Fig. 3A&B)

Although Case 23 had a *KRAS*-amplified clonal subpopulation that was distinct from the majority *CCNE1*-amplified population, Case 27 demonstrated co-amplification

of *KRAS* and *CCNE1* in single nuclei. Direct sequencing did not detect any point mutations in *KRAS* codons 12, 13 or 61 in either of these two tumors, the tumor of Case 5, or an early gastric cancer tumor. Dual-color FISH analysis of the co-amplified genes showed that the fluorescent signals corresponding to the amplified genes were separated, thus suggesting that they were located on different amplicons. In Case 35, however, *CDK6* signals and *ERBB2* signals always overlapped, which suggested that these two genes were located in the same amplicons in most cancer cells (Fig. 3C).

Among the 19 cases of advanced gastric cancer with gene amplification of the G1-S regulatory genes in the primary tumor, 15 cases had lymph node metastasis. FISH analysis showed that the gene amplification pattern was consistent between the primary and nodal tumors in 14 of these cases (Table 2)

Discussion

In the present study, gene amplification of *CCNE1* was the most frequent amplification found among the 16 genes examined in tumor samples from patients with gastric cancers, with *CCNE1* amplification confirmed by FISH analysis in 26 out of 179 (15%)

tumor samples. Amplification of *CCND1* and *CDK6* was found in ten (6%) and two (1%) gastric cancer tumor samples, respectively.

We did not observe co-amplification of the G1-S regulatory genes *CCNE1*, *CCND1* and *CDK6* in single tumor cells. However, three early gastric cancer tumors contained *CCNE1*-amplified cells located in the vicinity of *CCND1*-amplified cells. The lack of observed co-amplification in single cells is likely due to only one perturbation in early G1 control being sufficient for gastric carcinogenesis. [18]. KRAS is a downstream mediator of RTK signaling and is activated by point mutations in many cancers. However, these activating point mutations are strikingly rare in gastric cancers compared to other gastrointestinal cancers.[7] Our results show that gene amplification of *KRAS* occurred with greater frequency than expected in gastric cancers, and was mutually exclusive with RTK gene amplification.

In the present study, more than half of the tumors with amplification of at least one G1-S regulatory gene had co-amplification of *ERBB2, EGFR* and/or *KRAS* (10/16 early and 8/19 advanced cancers). This co-amplification occurred in single nuclei in five of the early cancers and in all eight of the advanced cancers. Co-amplification of these genes in various frequencies has been reported in studies of gastric cancers using SNP, DNA microarray or FISH analyses [19-21]. However, our use of dual-color FISH

enabled us to clearly demonstrate for the first time that these co-amplifications can occur in single cancer nuclei.

It remains to be clarified why these co-amplified genes, which were originally located on different chromosomes or on different arms of the same chromosome (e.g., *CDK6* and *EGFR*), are preferentially co-amplified. We observed in five early gastric cancers (including three intramucosal cancers) and at least three advanced cancers that cells with amplification of *CCNE1*, *CCND1* or *CDK6* contained clonal subpopulations of concomitantly amplified *ERBB2*, *EGFR* or *KRAS*. This combination of cancer staging, histological typing and gene amplification patterns suggests that genetic instability initiates the amplification of G1-S regulatory genes during the preclinical stage of non-invasive and/or mucosal cancers. The resulting deregulation of the cell cycle may then lead to further genomic instability, driving *ERBB2* or *EGFR* gene amplification in these tumors. Subsequent selection then produces separated, heterogeneous clonal subpopulations, as shown in Fig. 4. Unlike RTK genes, the G1-S regulatory genes are likely necessary for later tumor progression. Therefore, these genes are maintained in large populations within most advanced cancers and metastatic foci. This observation supports possible use of the cyclins and CDK6 as targets of molecular therapy. However, cyclins are generally regarded as difficult to target directly with

therapies, as they lack intrinsic enzymatic activity and are intracellular. Thus, their functionality may most readily be targeted via their partner kinases, i.e., CDK2 for cyclin E [22] and CDK4 or CDK6 for cyclin D1 [21]. Currently, there are an estimated ten small-molecule pan-CDK inhibitors or highly selective inhibitors in clinical trials for various cancer types [23].

Information regarding the co-amplification of *CCNE1* and *CCND1* may be further useful for current molecular targeted therapies. One study of patients with *ERBB2*-positive gastric cancer found that individuals with concomitant amplification of *CCNE1* demonstrated a significantly shorter progression-free survival than those without concomitant *CCNE1* amplification [20]. *CCND1* amplification has been linked to resistance to the EGFR inhibitor gefitinib in experimental models of head and neck cancer [24] [25].-

In molecular targeting therapy, it is critical to establish a feasible screening method in order to identify eligible patients. MLPA is, compared to SNP, aCGH and next-generation sequencing techniques, a relatively cheap, and easy–to-perform method that allows simultaneous detection of multiple gene copy number aberrations in small amount of fragments DNA derived from formalin-fixed material [26]. It is, however, based on PCR and averages many different cells, thus theoretically validation by

morphological method, such as FISH is mandatory. The present study not only showed MLPA can be used as an efficient screening method for searching tumors with most gene amplification but also in several genes the confirmatory FISH may be unnecessary by setting approximate threshholds.

In conclusion, the common and relatively homogeneous amplification of *CCNE1*, *CCND1*, and *CDK6* in primary and metastatic nodes of advanced gastric cancer may offer direct targets for molecular therapy. Co-amplification of these genes with *ERBB2* or *EGFR* may also render them targets for combination therapy with ERBB2 or EGFR inhibitors. MLPA is a useful tool to identify patients who would benefit from such therapies.

References

Legends

Table 1.

Results of the MLPA and FISH analyses of tumor samples from 179 gastric cancer

patients. Values in parentheses are the numbers of tumors with gene amplification verified by FISH.

Table 2.

Characteristics of gastric cancer tumors with amplification of *CCNE1*, *CCND1* and/or *CDK6*. ‘Cases 1-16 are early gastric cancers; Cases 17-35, advanced gastric cancers.

Abbreviations: co-amp, co-amplification of a gene and the centromeric lesion in HSR; DCS, combination chemotherapy with docetaxel, cisplatin and S-1; DM, double-minute chromosome-type amplification; DOC, docetaxel; HSR, homogeneously staining region-type amplification; low amp, low-level amplification; mixed, mixed carcinoma; MP, muscularis propria; NE, neuroendocrine carcinoma; por, poorly cohesive adenocarcinoma; SE, serosa exposed; sig, signet-ring cell carcinoma; SM, submucosa; SS, subserosa; tub, tubular adenocarcinoma.

Fig. 1.

A representative intramucosal gastric cancer tumor sample with co-amplification of *CCNE1* and *CCND1* (Case 1). (A) Hematoxylin and eosin stain. The areas indicated as (I) and (II) show overexpression and amplification of cyclins E and D1, respectively.

The region enclosed by the black rectangle labelled (B) corresponds to the fields in panels (B) and (D); the black rectangle labelled (C) corresponds to panels (C) and (E). (B and C) Immunohistochemistry of cyclin E (B) and cyclin D1 (C). (D and E) Dual-color FISH analysis: orange fluorescence, *CCNE1*; green fluorescence, *CCND1*.

Fig. 2.

Early gastric cancer tumor with amplification of *CCND1* (Case 5). This intramucosal cancer with homogenously amplified *CCND1* contained small, separated areas with co-amplification of *ERBB2*, *KRAS* or *EGFR*. (A) Hematoxylin and eosin stain. (B, C and E) Immunohistochemistry showed homogeneous staining of cyclin D1 (B) and indicated that the small, separated areas were positive for ERBB2 (C) and EGFR (E). (D, F, & G) The region enclosed by the black rectangle labelled D in panel (C) corresponds to panel (D), and in panel (E) the rectangles labelled F and G correspond to panels (F) and (G), respectively. FISH analysis showed that ERBB2- and EGFR-overexpressing cells had amplification of the corresponding gene (D and G, respectively) and focal *KRAS*-amplification (F). In (D), orange fluorescence corresponded to *ERBB2;* green fluorescence, *CCND1*; (F) orange fluorescence, *KRAS*; green fluorescence, *CCND1*. (G) orange fluorescence, *EGFR;* green fluorescence,

centromere 7.

Fig. 3.

Advanced gastric cancer tumors with amplified *CCND1* (Case 34, panels A and B) or *CDK6* (Case 35, C). (A, B) Case 34 had an area of *CCND1* amplification where amplification of *ERBB2* and *EGFR* was observed in nearby cells, but their amplification was mutually exclusive. Adjacent sections were alternatively stained for ERBB2 (A) and EGFR (B) using immunohistochemistry. Dual-color FISH showed co-amplification of *CCND1* (orange signals) and *ERBB2* (green signals) (A, inset) and co-amplification of *CCND1* (orange signals) and *EGFR* (green signals) (B, inset). (C) Dual-color FISH analysis of Case 35 demonstrated overlap (yellow) of amplified HSR-like signals for *CDK6* (orange) and *ERBB2* (green).

Fig. 4.

A potential mechanism for the gene co-amplification observed in gastric cancer. This model speculates that gene amplification of *CCNE1*, *CCND1* and *CDK6* occurs in early gastric carcinogenesis. Such amplification induces genomic instability, which drives *ERBB2*, *EGFR* or *KRAS* gene amplification. The 35 tumors with *CCNE1*, *CCND1* and

CDK6 are sorted according to this pathway. The numbers in normal font and the numbers in bold font correspond to the case numbers of the early gastric cancers and the advanced gastric cancers, respectively. Circles: amplified *CCNE1*, *CCND1* or *CDK6*; rectangles: amplified *ERBB2*, *EGFR* or *KRAS*.

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Table 1 Results of the MLPA and FISH analyses of 179 gastric cancers

Name of genes	Chromosomal locus of gene	FISH probe	No. of amplified ' tumors	No. of gain ' tumors	No. of 'normal' tumors with IHC-positive	Frequency of tumors with gene amplification
CCNE1	19p12	RP11-345J21	16 (16)	11 (7)	31(3)	15% (26)
CCND1	11q13.3	RP11-775J10	5 (5)	7 (3)	47 (2)	6% (10)
CDK6	7q21.2	RP11-316P4	2 (2)	8 (0)		1% (2)
ERBB2	17q12	RP11-62N23	6 (6)	10 (7)	4 (4)	9%(17)
EGFR	7p11.2	RP11-339F13	2 (1)	9 (3)	10 (9)	7% (13)
MET	7q31.2	RP11-75I20	1 (1)	9 (4)		3% (5)
FGFR2	10q26.13	RP11-62L18	1(1)	4 (2)		2% (3)
KRAS	12p12.1	RP11-1119I8	2 (2)	11 (2)		2% (4)
MYC	8q24.21	RP11-440N18	8 (6)	20 (10)		9% (16)
PTP4A3	8q24.3	RP11-240D7	1 (1)	15 (8)		5% (9)
PIK3CA	3q26.32	RP11-1115H8	0	6 (1)		0.5% (1)
KLF5	13q22.1	RP11-179I20	2 (2)	16 (0)		1% (2)
TOP2A	17q21.2	RP11-48O10	2 (2)	6 (1)		2% (3)
FGFR1	8p11.23	RP11-148D21	0	4 (0)		0
GATA4	8p23.1	RP11-235I4	1 (0)	5 (0)		0
GATA6	18q11.2	RP11-18K7	1 (0)	7 (0)		0

Values in parentheses are the numbers of tumors with gene amplification validated by FISH.

Table 2 Gastric cancers with gene amplification of *CCNE1*, *CCND1*, and/or *CDK6*

Case No.	Macroscopic classification[c]	Histological classification[c]	Depth of invasion[d]	Anatomic stage /prognostic groups[d]	Neoadjuvant therapy	*CCNE1*	*CCND1*	*CDK6*	*ERBB2*	*EGFR*	*KRAS*	Amplified genes in metastatic nodes
1	0-IIc	tub	non	0		HSR (10-30) [g]	HSR (10-30) [f]					-
2[b]	0-IIb+IIc	tub	non	0		DM (50-70) [f]	DM (10-30) [f]		DM (<10) [g]			-
3[a]	0-IIb	tub	non	0		HSR, (50-70) [f]				HSR (10-30) [f]		-
4	0-IIc	tub	non	0		HSR (>70) [e]						-
5[a]	0-IIa	tub	non	0			HSR (>70) [e]		HSR (10-30) [f]	DM (<10) [g]	DM (<10) [g]	-
6[a]	0-IIc	tub	non	0				DM (>70) [e]		DM (30-50) [e]		-
7[a]	0-IIc	tub	SM	IA		HSR (>70) [e]			HSR (10-30) [f]			-
8	0-IIa+IIc	tub	SM	IA		HSR & low Amp (>70) [e]						-
9	0-IIc	tub	SM	IA		HSR (>70) [e]						-
10[b]	0-I+IIa	tub	SM	IA		HSR, (10-30) [f]				DM&HSR, (<10) [f]		-
11	0-IIb+IIc	tub	SM	IA		HSR (>70) [e]						-
12	0-IIa+IIc	tub	SM	IA		HSR (>70) [e]						-
13[b]	0-IIc+IIb	tub	SM	IA			co-amp & Low amp (50-70) [e]			co-amp (<10) [g]		-
14[a]	0-IIc	tub	SM	IA			HSR (>70) [e]			HSR (<10) [g]		-
15	0-III	tub	SM	IB		Low amp (<10) [g]	Low amp (10<) [g]			co-amp (10-30) [f]		-
16	0-IIc	tub	SM	IB		Low amp (50-70) [f]			co-amp (10-30) [f]			-
17	2	tub	MP	IB		HSR (>70) [e]						-
18[a]	3	tub	MP	IB			HSR (>70) [f]		HSR (10-30) [f]			-
19	2	tub	MP	IIA			HSR (>70) [e]					*CCND1*
20	1	tub	SS	IIA		Low amp (10-30) [f]						-
21	3	por	SS	IIA		Low amp (30-50) [f]						-
22	2	mixed	SS	IIIA		HSR (>70) [e]						*CCNE1*
23[a]	3	mixed	SS	IIIB	S-1	Low amp (50-70) [f]			HSR (10-30) [f]		HSR (30-50) [e]	*KRAS*
24[a]	2	tub	SS	IIIB		HSR (>70) [e]			HSR & Low amp (>70) [e]			*CCNE1, ERBB2,*
25	3	mixed	SS	IIIB	DCS	HSR (>70) [e]						*CCNE1*
26[a]	2	tub	SS	IIIB			HSR (>70) [e]		HSR (<10) [g]			*CCND1*
27[a]	3	sig	SE	IIIB		Low amp>HSR, (>70) [e]					HSR (50-70) [e]	*CCNE1, KRAS*
28[a]	3	tub	SE	IIIB		HSR (>70) [e]				HSR, (<10) g		*CCNE1*
29	3	por	SE	IIIB		HSR (>70) [e]						*CCNE1*
30	3	tub	SE	IIIC	DCS	Low amp (50-70) [g]						*CCNE1*
31	4	mixed	SE	IV		HSR (>70) [e]						Failed
32	2	NE	SE	IV	DCS	HSR (>70) [e]						*CCNE1*
33	5	mixed	SE	IV		co-amp, (>70) [f]						*CCNE 1*
34[a]	3	mixed	SE	IV	DCS		HSR (30-50) [g]		DM (<10) g	HSR (<10) g		*CCND1, EGFR*
35[a]	4	tub	SE	IV				HSR (>70) [e]	HSR (>70) e			*CDK6, ERBB2*

a: Co-amplification of at least of one of CCNE1, CCND1, or CDK6 with at least one of ERBB2, EGFR, or KRAS in single cancer nuclei.

b: The co-amplification of the cyclins and RTKs occurred in different cells, however, in they were located adjacent to each other.

c: Accoding to The World Health Organization Classification of Tumors[17]

d: According to the TNM cancer staging system of the American Joint Committee of Cancer.[16]

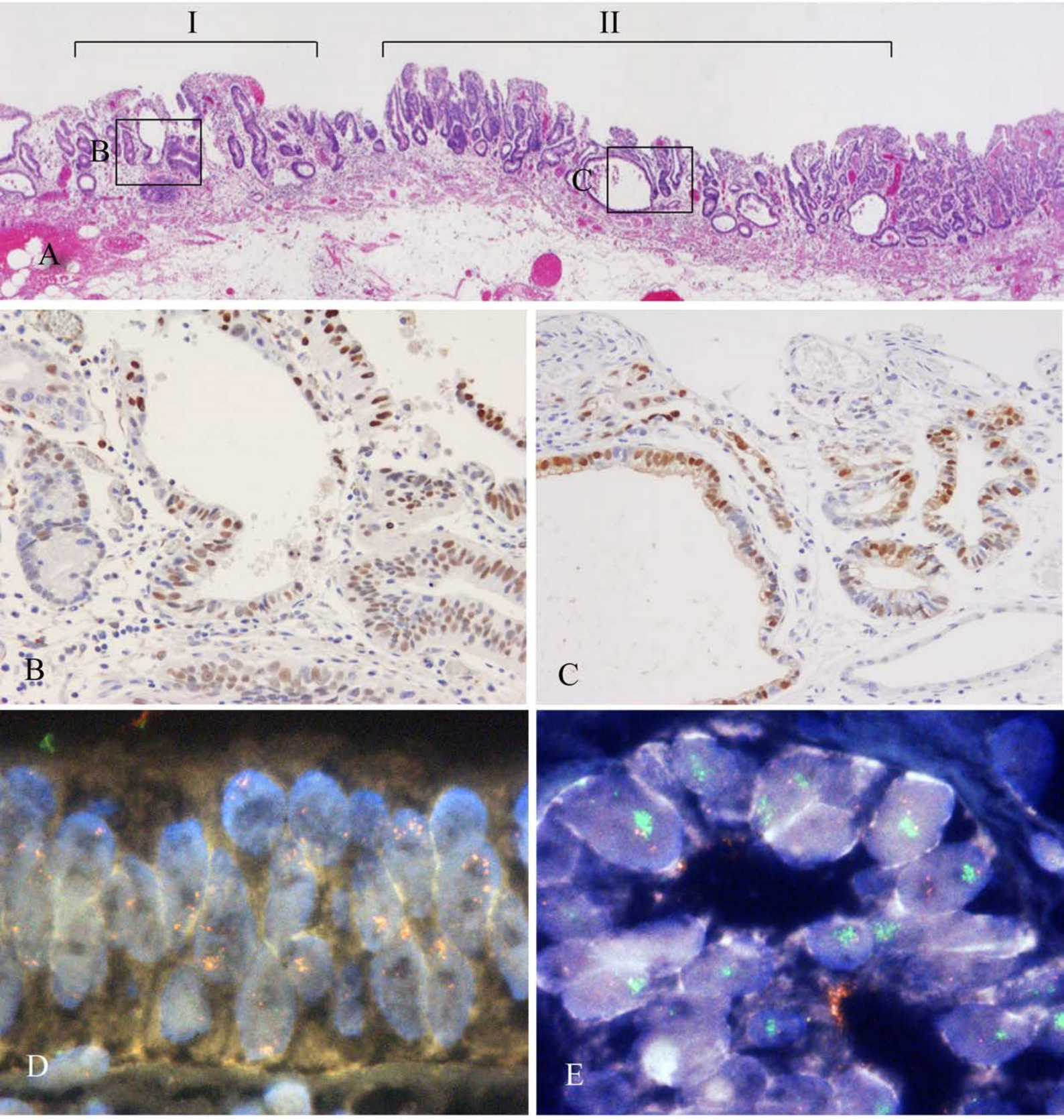
e: 'amplified' by MLPA.

f: 'gained' by MLPA.

g: 'normal' by MLPA.

Cases 1-16, early gastric cancers; cases 17-35, advanced gastric cancers.

Abbreviations: tub, tubular adenocarcinoma; mixed, mixed carcinoma; sig, signet-ring cell carcinoma; por, poorly cohesive adenocarcinoma; NE, neuroendocrine carcinoma; SM, submucosa; MP, muscularis propria; SS, subserosa; SE, serosa exposed; DCS, combination chemotherapy with docetaxel, cisplatin and S-1; DOC, docetaxel; HSR, homogeneously staining region type amplification; DM, double minute chromosome type amplification; co-amp, co-amplification of a gene and the centromeric lesion in HSR; Low amp, low-level amplification.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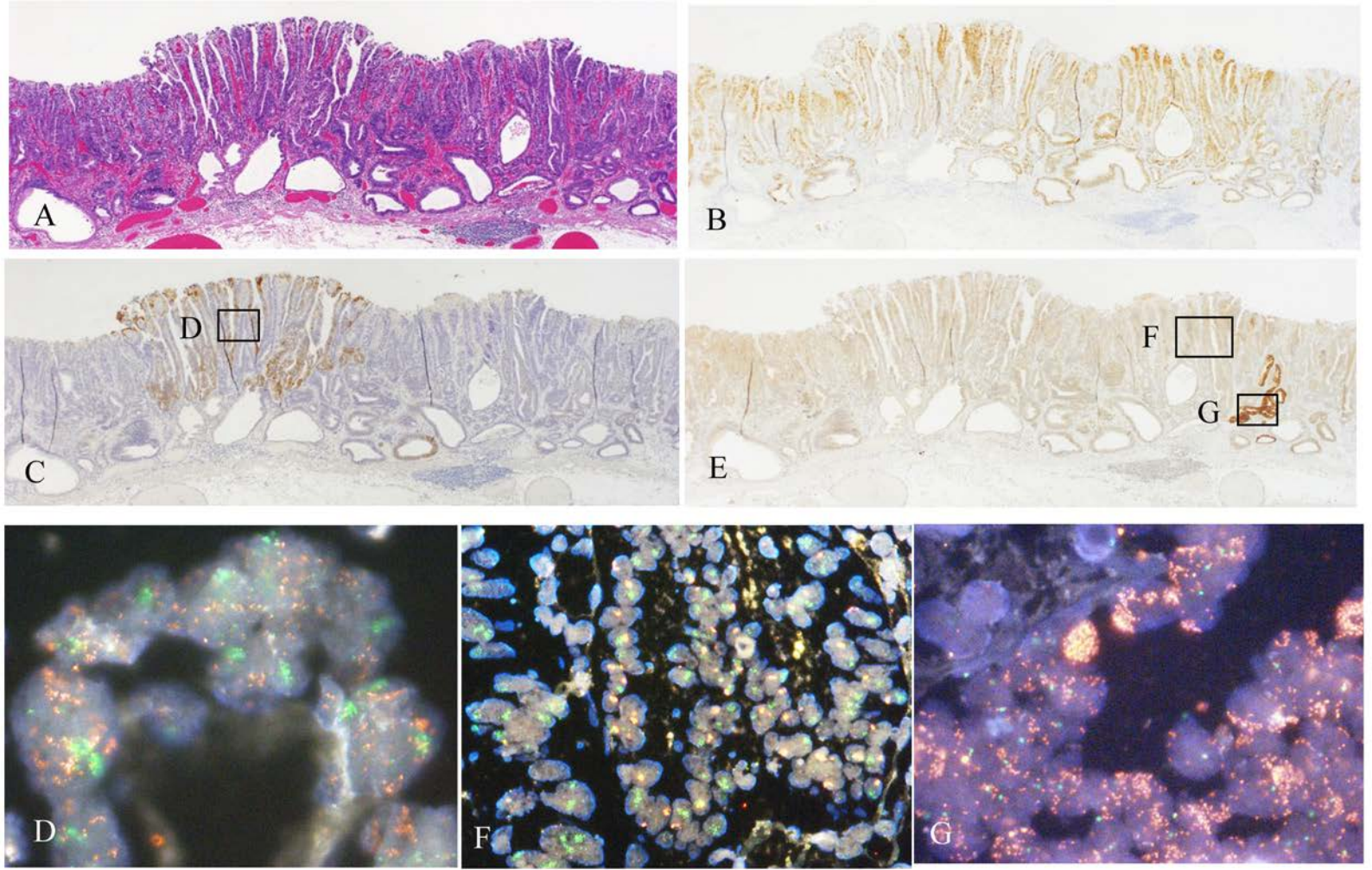
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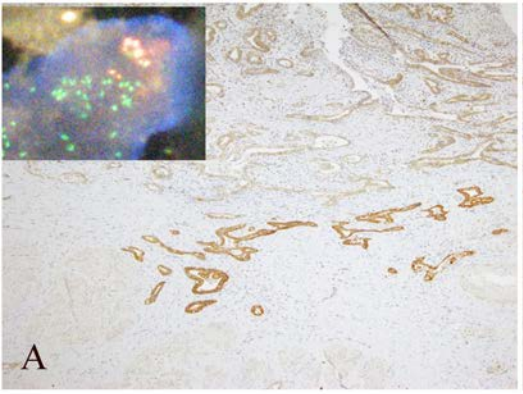
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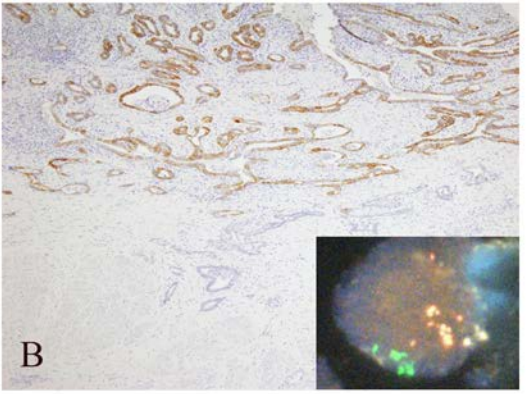
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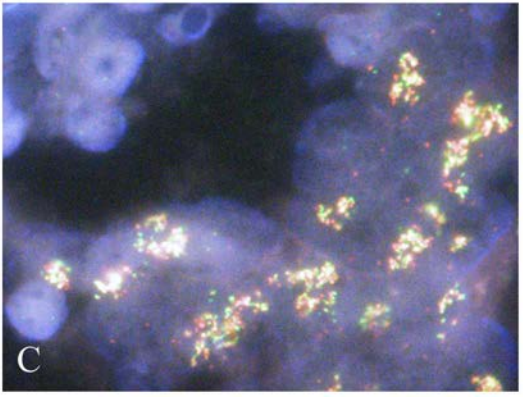
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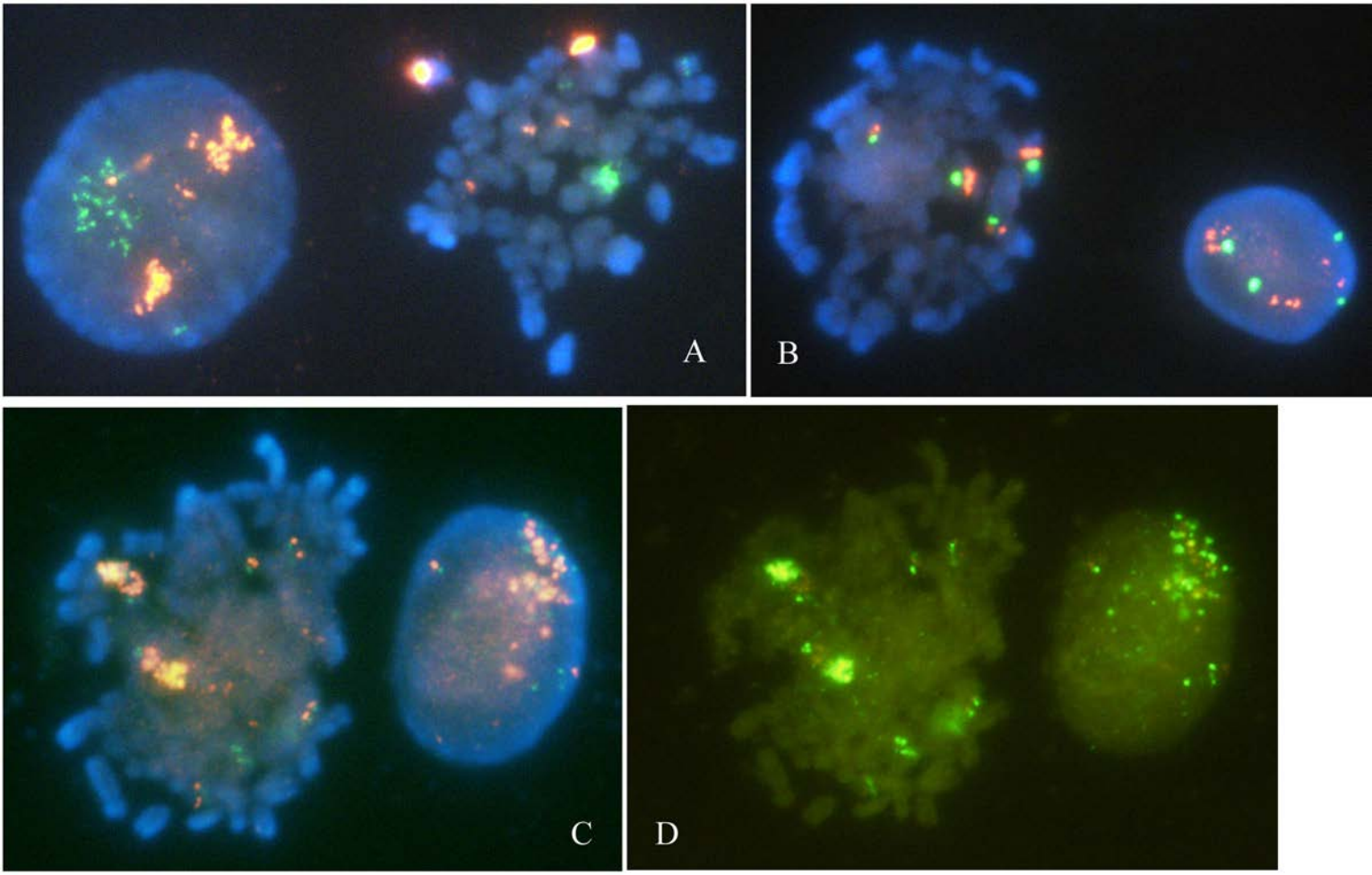
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