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Original contribution

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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Summary New and effective treatments for advanced gastric cancer are urgently needed. Cyclins E and D1 form a complex with cyclin-dependent kinase 2, 4, or 6 to regulate 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 molecularly targeted therapies against *ERBB2* or *EGFR* when coamplified. A total of 179 formalin-fixed and paraffin-embedded gastric cancer specimens were examined for these gene amplifications by multiplex ligation-dependent probe amplification and fluorescence in situ hybridization. Amplification of at least 1 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 fluorescence in situ hybridization identified coamplification 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 coamplification of *ERBB2, EGFR*, or *KRAS* in 5 early and 3 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. Multiplex ligation-dependent probe amplification could be a useful tool for identification of patients who would benefit from such therapies.

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1. 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 (CDH) 4 or CDK6. Following accumulation of active cyclin D1/CDK4 or cyclin D1/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 involves alteration of its degradation pathway due to mutations in *hCDC4* [5,6].

Amplified genes encoding receptor tyrosine kinases (RTKs) 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 [8], have shown that the genes encoding cyclin E (*CCNE1*), cyclin D1 (*CCND1*), and CDK6 (*CDK6*) are frequently amplified in gastric cancer. Coamplification of *CCNE1* or *CCND1* with *ERBB2* reportedly reduces the antitumor 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.

2. Materials and methods

2.1. Patients and control cell lines

A total of 179 patients with gastric adenocarcinoma (84 early and 95 advanced-stage tumors) who underwent surgery in the Department of Surgery at 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 Tumors* [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 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 MKN7, A431 (both from Riken Cell Bank, Tsukuba, Japan) and SNU5 (American Type Culture Collection, Rockville, MD) cell lines were used as the positive controls for gene amplification.

2.2. Multiplex ligation-dependent probe amplification

Cancer-enriched 6-μm–thick serial sections that excluded nonneoplastic cells were selected by comparison to adjacent hematoxylin and eosin–stained sections. 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 2 to 3 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 were interpreted with GeneMapper software (Applied Biosystems). Data analysis was performed with Coffalyser MLPA-DAT version 9.4 software (MRC-Holland) to normalize peak values. Average peak values less than 0.7 were defined as "lost," between 0.7 and 1.3 as "normal," between 1.3 and 2.0 as "gain," and greater than 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 respective gene amplification by FISH.

2.3. Immunohistochemistry

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