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CCNE1 amplification is associated with liver metastasis in gastric carcinoma

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ABSTRACT

With targeted therapies becoming the new standard of care in oncology, next generation sequencing (NGS) is emerging as a valuable method for analyzing the molecular underpinnings of individual tumors. Cyclin E1, encoded by CCNE1 causes activation of E2F mediated transcription and drives cells from G1 into S phase with cyclin-dependent kinase 2 (CDK2). CCNE1 amplification has been found in 11–12% of gastric cancers, but the clinical significance of this amplification remains controversial, and its association with liver metastasis has not been studied.

This study included 226 patients diagnosed with advanced gastric adenocarcinoma. We performed multi-gene panel tests containing 143 genes using DNA and RNA obtained from primary (n = 197; 120 endoscopic biopsies and 77 resections) or metastatic cancer tissues (n = 29; 26 biopsies, 2 excisions, and 1 fin. needle aspiration). Among the 226 cases, 28 cases (12.4%) had CCNE1 amplification, almost half of which (n = 13, 46.4%) showed liver metastasis. In patients with CCNE1 amplification (n = 28), TP53 mutations (n = 23, 82.1%) and ERBB2 amplification (n = 8, 28.6%) were the most frequent concurrent genetic alterations. In contrast, 42 (21.2%) of 198 patients without CCNE1 amplification showed liver metastasis. CCNE1 amplification was significantly associated with liver metastasis (p = 0.004; odds ratio, 3.219).

Our results show that CCNE1 amplification is significantly associated with liver metastasis in a TP53-mutated gastric cancer subtype. Given the frequent association of CCNE1 amplification with liver metastasis, close follow up for liver metastasis and further clinical trials targeting CDK2 inhibitors are warranted.

1. Introduction

Next generation sequencing (NGS), a method of simultaneous sequencing of fragmented DNAs, is a fast and efficient technique to analyze large amounts of genetic information at once. As a result of this breakthrough technology, precision medicine has become a reality. Precision medicine in oncology integrates clinical, histological, and molecular information to obtain the genetic backgrounds and predict the biologic behavior of tumors to identify appropriate treatments for each patient. Therefore, analysis and application of NGS data are important for management of cancers.

Cyclin E1, encoded by CCNE1, with cyclin-dependent kinase 2 (CDK2) phosphorylates and inactivates Rb, which binds to E2F, resulting in activation of E2F-mediated transcription and development of cells from G1 into S phase to initiate DNA synthesis [1]. CCNE1 amplification has been observed in breast, ovary, and endometrial cancers and is related to poor prognosis [2–4]. According to TCGA data and previous studies, CCNE1 amplification is found in 11–12% of gastric cancers and is more frequent in microsatellite stable (MSS) tumors compared to microsatellite instability-high (MSI-H) cancers [5,6]. In gastric and breast cancers, co-amplification of CCNE1 and ERBB2 has been suggested to be associated with trastuzumab resistance [7–9]. However, the clinical significance of CCNE1 amplification remains controversial, and its association with liver metastasis has not been reported [10].

For treatment of cancers with CCNE1 amplification, a combination of CDK inhibitor and AKT inhibitor was suggested as a therapy in high-grade serous ovarian carcinoma [2]. In CCNE1-amplified and PIK3CA-

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mutated uterine serous carcinomas, blocking both CCNE1 and PI3K pathways showed the efficacy [11]. In HER2-positive and cyclin E-overexpressed breast cancers with trastuzumab resistance, an in vivo xenograft study showed that CDK2 inhibition significantly reduced tumor growth, suggesting that cyclin E overexpression causes increased CDK2 activity, and that the treatment with CDK2 inhibitors may be a valid strategy in these patients [12]. Although no clinical trials have examined patients with CCNE1-amplified gastric cancer, CDK2 inhibitor could be a new treatment option.

In the present study, we performed NGS cancer panel tests in gastric cancer patients receiving palliative care to clarify the clinical significance of CCNE1 amplification and the associated genetic alterations in this subset.

2. Materials and methods

2.1. Patient samples

A total of 238 patients diagnosed as advanced gastric carcinoma in palliative setting was requested for NGS cancer panel test from September 2017 to August 2018. In 12 cases (5.0%), NGS test was not successful due to low tumor volume (n = 6) and no remaining tissue (n = 6). Finally, 226 cases were analyzed consisting 197 primary tumor (120 endoscopic biopsies and 77 resections) and 29 metastatic cancer tissues (26 biopsies, 2 excisions, and 1 fin. needle aspiration). We performed multi-gene panel tests containing 143 genes using DNA and RNA obtained from these specimens. Liver metastasis was considered to be present when a suspicious lesion in liver was confirmed by biopsy or appeared newly on abdominal computed tomography. In one case, the NGS test was performed in both primary and hepatic metastatic tumors during follow-up.

In CCNE1-amplified gastric adenocarcinoma with liver metastasis, the male to female ratio was 12:1, and the median patient age was 65 years. Eight cases showed hepatic metastasis in the first visit to our hospital. The time to liver metastasis after clinical manifestation of gastric cancer ranged from 0 to 24 months, with an average of 4 months (Table 1).

2.2. NGS analysis

We performed NGS in all cases to detect copy number alterations (CNAs), single nucleotide variants (SNVs), small insertions and deletions (indels), and gene fusions using the Oncomine comprehensive cancer panel v1 (Thermo Fisher Scientific, Waltham, MA), which examines 143 oncogenes and tumor suppressor genes. Our Oncomine comprehensive cancer panel v1 showed sensitivity of 99% for SNVs and 93% for indels at 10% allele frequency on the validation of analytical procedures using Acrometrix® Oncology Hotspot Control (Thermo Fisher Scientific. MA, USA) and 5-Fusion Multiplex FFPE RNA Reference Standard from Horizon Discovery (Waterbeach, UK). It also showed 100% sensitivity when the sample was diluted to 23% (CNAs) and 10% (fusions). We used formalin-fixed paraffin-embedded (FFPE) tissue samples and the minimum tumor volume was 20%. After deparaffinization and nucleic acid extraction, targeted DNA and RNA amplification of the tumor was performed using the Ion AmpliSeq Library kit 2.0 (Thermo Fisher Scientific). Partially digested primer sequences, ligation adapters to amplicons, and purification and quantitation of the libraries were performed using the Ion Xpress Barcode Adapter 1–96 kit (Thermo Fisher Scientific), Ion AmpliSeq Library Kit, and the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). We loaded 8 constructed libraries to an Ion 540 chip, and sequencing was performed on the Ion S5XL system. We used Ion Torrent software (Ion ReporterTM 5.2) and Oncomine Knowledgebase for automated data analysis. We set the following criteria and evaluated the sequencing quality accordingly: mapped reads > 5,000,000, on-target rate > 90%, mean depth > 1,200, and uniformity > 90%.

Table 1
Clinicopathologic characteristics of *CCNE1*-amplified gastric adenocarcinomas.

	With liver metastasis (n = 13)	Without liver metastasis (n = 15)
Age (median, range)	65 (49–81)*	55 (39-82)*
Sex (M:F)	12:1	11:4
Liver metastasis at presentation	8	
Time to liver metastasis	4 (0–24) months	
Other metastasis		
Abdominal lymph node	6	9
Peritoneum	4	8
Supraclavicular lymph node	2	3
Lung	0	2
Bone	0	2
Spleen	1	0
Others**	0	1
Tumor samples		
Stomach, biopsy	5	9
Liver, biopsy	3	0
Lymph node, biopsy	0	1
Stomach, resection	5	5
Type		
Papillary	1	1
Tubular	12	14
Differentiation		
Good	1	0
Moderate	6	4
Poor	5	10

*p = 0.036 by Student's *t*-test; **Others include pleura, bone marrow, breast, pancreas, ureter, ovary, brain, leptomeninges, axillary lymph node, and hilar lymph node.

A sequencing coverage of 250X, variant coverage of 25X and variant allelic frequency of 5% were set as cutoffs to avoid any false positive and false negative results. An average copy number ≥ 4 was interpreted as a gain (amplification) and < 1 as a loss (deletion). For translocations, read counts ≥ 20 and total valid mapped reads ≥ 50,000 were interpreted as positive results. We excluded germline mutation based on COSMIC and ExAC datas. All CCNE1 amplification results were confirmed by Integrative Genomics Viewer (IGV) (Fig. 1).

2.3. Immunohistochemistry for CCNE1

To prove activation of CCNE1 by amplifications [8], which reported high correlation of CCNE1 amplification and IHC, we performed immunohistochemistry with anti−CCNE1 primary antibody (clone HE12; Thermo Fisher Scientific) in 20 CCNE1-amplified gastric cancer where tissue was available. The intensity (0 to 3+ scale) and percentage of the stained tumor cells were measured and the results with 3+ in > 75% of tumor cell nuclei were considered positive.

2.4. Statistical analysis

The χ^2 test or Fisher exact test was used to compare categorical variables and the association of CCNE1 amplification with liver metastasis. Student's *t*-test was used to compare continuous variables after a normality test. The p value was two-sided, and $P < 0.05$ was considered significant using SPSS software version 25.

3. Results

Of the 226 gastric adenocarcinoma cases included in this study, 28 cases (12.4%) had CCNE1 amplification, and almost half of these cases (n = 13, 46.4%) showed liver metastasis. Among the patients with CCNE1 amplification (n = 28), TP53 mutations (n = 23, 82.1%) and ERBB2 amplification (n = 8, 28.6%) were the most frequent concurrent genetic alterations. In contrast, 42 (21.2%) of 198 patients without CCNE1 amplification showed liver metastasis. Statistical analysis

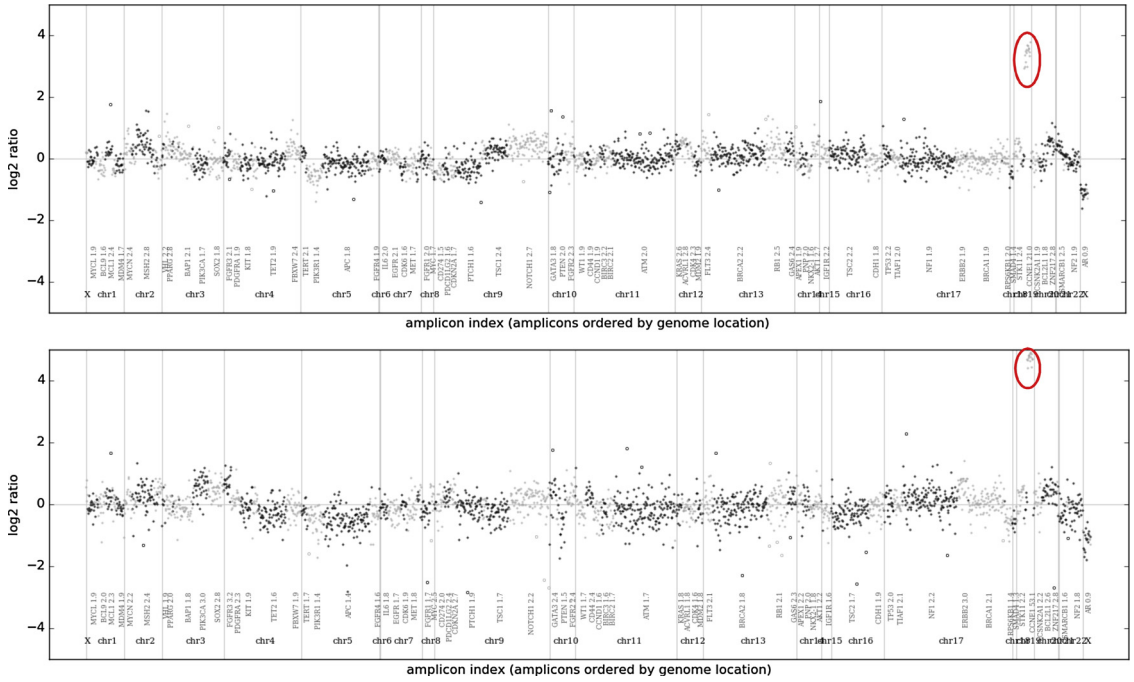

Fig. 1. The amplification of *CCNE1* by Integrative Genomics Viewer (IGV).

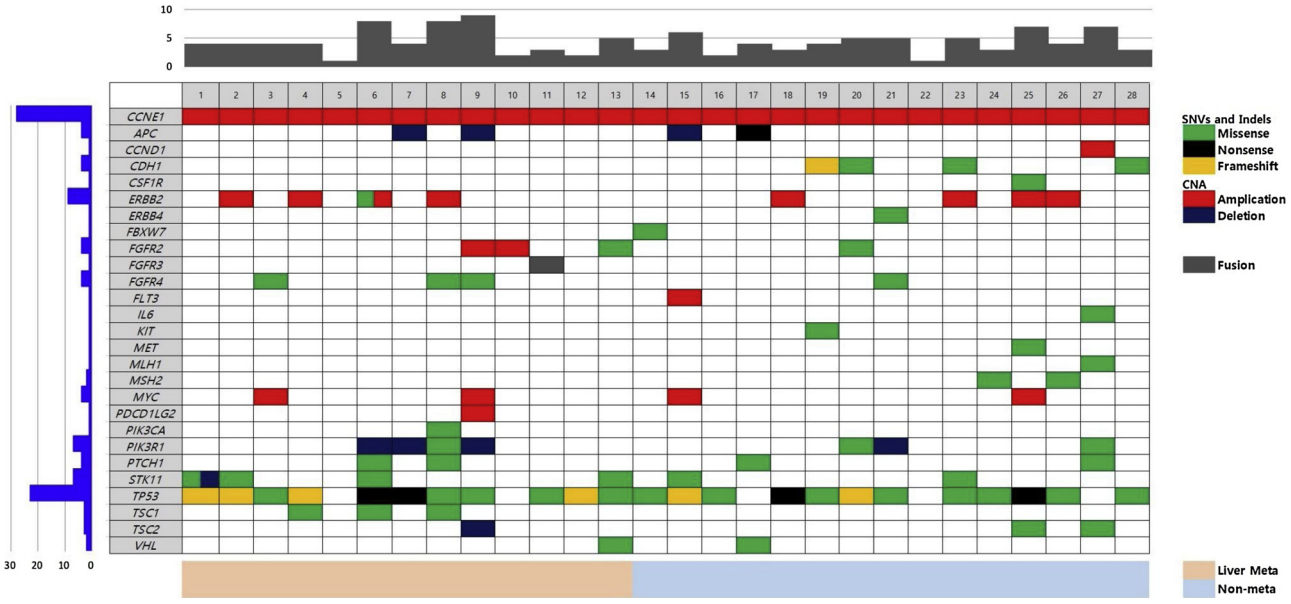

Fig. 2. Oncoplots of genetic alteration found in *CCNE1*-amplified gastric adenocarcinomas.

showed that CCNE1 amplification was significantly associated with liver metastasis (p = 0.004; odds ratio, 3.219).

In the 13 gastric cancer cases with CCNE1 amplification and liver metastasis, 9 experienced distant metastases to a location other than the liver, comprising abdominal distant lymph node (n = 6), peritoneum (n = 4), supraclavicular lymph node (n = 2), and spleen (n = 1). Three cases showed liver metastasis only, and one case recurred at the remnant gastric stump after the surgery. In these 13 patients, the tissues used for NGS analysis were obtained by endoscopic biopsy (n = 5), liver biopsy (n = 3), or gastrectomy (n = 5). The most common histologic type of gastric cancer with liver metastasis was tubular adenocarcinoma with moderate (n = 5), poor (n = 5), and well differentiation (n = 1), and one case was papillary adenocarcinoma.

In 15 CCNE1-amplified gastric cancers without liver metastases, 14 showed distant metastases to a location other than the liver: abdominal distant lymph node (n = 9), peritoneum (n = 8), supraclavicular lymph node (n = 3), lung (n = 2), bone (n = 2), and other organs. In these 15 patients, the tissues used for NGS analysis were obtained by endoscopic biopsy (n = 9), lymph node biopsy (n = 1), or gastrectomy (n = 5). The most common histologic type of gastric cancer without liver metastasis was tubular adenocarcinoma with moderate (n = 4) and poor differentiation (n = 10), and one case was papillary adenocarcinoma. The clinical and pathologic characteristics of patients with CCNE1-amplified gastric adenocarcinomas are summarized in Table 1.

The overall genetic alterations associated with CCNE1 amplifications are depicted in Fig. 2. The genetic alterations in gastric adenocarcinomas with CCNE1 amplifications and liver metastasis are listed in Table 2. The concomitant SNVs/indels was detected in TP53 (11/13,

Table 2
Detected mutations in *CCNE1*-amplified gastric adenocarcinomas with liver metastasis.

Case	MR	TR	MD	UF	TV	SNV1	AA	TM	TD	VAF	SNV2	AA	TM	TD	VAF	CNA1	CN	CNA2	CN	CNA3	CN	CNA4	CN
1	9,541,282	97.91	3,876	95.27	50	TP53	N263fs	Del	1992	58.4						CCNE1	13	STK11	0.1				
2	8,632,973	98.59	3,471	85.82	50	TP53	R333fs	Del	1990	21.3						CCNE1	7.4	HER2	12.4				
3	8,143,424	98.83	3,346	94.26	80	TP53	Y234N	Mis	1995	81.6						CCNE1	8.04	MYC	6.46				
4	7,490,110	98.03	3,096	97.16	90	TP53	R333fs	Del	1984	41.3						CCNE1	8.32	HER2	15.52				
5	10,924,383	97.55	4,518	98.22	70											CCNE1	14.26						
6	9,375,308	87.11	2,935	35.6	80	TP53	R213	Non	1903	61.3						CCNE1	98.9	HER2	83.69	PIK3R1	0.85		
7	14,963,026	98.24	6,238	95.93	60	TP53	E204	Non	1999	53.3						CCNE1	45.47	PIK3R1	0.78				
8	9,900,971	98.27	4,162	93.57	80	TP53	M237I	Mis	2000	43.8	PIK3CA	E542K	Mis	1981	10.7	CCNE1	34.2	HER2	45			TSC2	0.25
9	13,553,175	98.45	5,641	96.04	20	TP53	R337L	Mis	1989	47.1						CCNE1	149.45	MYC	6.8	PIK3R1	0		
10	12,065,904	98.38	5,101	95.94	30											CCNE1	16.8						
11*	11,976,689	98.36	4,820	97.17	30	TP53	R175G	Mis	1995	8.4						CCNE1	7.17						
12	10,528,803	98.41	4,502	96.07	40	TP53	G112fs	Del	1971	19.1						CCNE1	5.5						
13	4,909,000	98.00	2,057	93.22	30	TP53	S215I	Mis	1991	31.59	STK11	P281 L	Mis	1993	45.96	CCNE1	33.5						

Abbreviations: MRmapped reads; TRtarget rate; MDmean depth; UFuniformity; TVtumor volume; AAamino acid; TMtype of mutation; Deldeletion; Mismissense; Nonnonsense; TDtotal depth; VAFvariant allele frequency; CNcopy number; *with GFR3-TACC3 fusion (264,608 reads).

84.6%), PIK3CA (1/13, 7.7%) and STK11 (1/13, 7.7%). All SNVs was searched on COSMIC and has been confirmed somatic mutation. The most frequent CNA associated with CCNE1 amplification was ERBB2 amplification (n = 4), which was found in 30.8% of cases. MYC amplification (2/13, 15.4%) and PIK3R1 deletion (3/13, 23.1%) were observed. STK11 deletion and TSC2 deletion was found in one case each (7.7%), and FGFR3-TACC3 fusion was detected in one case. The genetic alterations in CCNE1-amplified gastric adenocarcinomas without liver metastasis are listed in Table 3. The concomitant SNVs/indels were detected in TP53 (12/15, 80.0%) and FBXW7 (1/15, 6.7%). There were two mutations of TP53 (p.L206fs, p.A138 G) which was not searched on COMIC. The most frequent CNA associated with CCNE1 amplification was ERBB2 amplification (n = 4), which was found in 26.7% of cases. MYC amplification was observed in 2 cases (13.3%). FLT3 amplification, CCND1 amplification, and PIK3R1 deletion were found in one case each (6.7%).

In immunohistochemistry for anti − CCNE1 in 20 available CCNE1-amplified cases, we could obtain valuable results in 14 samples and 6 cases were unavailable due to lack of tumor cells. Eleven of 14 cases showed strong (3 +) and diffuse (> 75%) positivity in tumor cell nuclei. One biopsy sample with CCNE1 copy number of 16.8 by NGS cancer panel test showed moderate staining (2 +) of CCNE1 in 60% of tumor cells. In two biopsy samples with CCNE1 copy numbers with 8.04 and 10.45, respectively, CCNE1 was weakly stained (1 +) in about 5% of tumor cells. Overall, 78.6% of cases with CCNE1 amplification showed strong and diffuse CCNE1 overexpression.

4. Discussion

We performed multi-gene panel tests of 143 genes in 226 patients diagnosed at advanced disease stage and receiving palliative care for gastric adenocarcinoma. We found CCNE1 amplification in 28 cases (12.4%), and CCNE1 amplification was significantly associated with liver metastasis (p = 0.004; odds ratio, 3.219). The most frequent concurrent genetic alterations were found in TP53 and ERBB2.

A previous study reported that the frequency of CCNE1 amplification in gastric cancer was 11%, and most of these patients showed AJCC stage II or III [5]. Furthermore, focal amplification of CCNE1 in gastric cancer was significantly associated with mRNA expression [6]. In this study, the prevalence of CCNE1 amplification in gastric cancer cases in a palliative clinical setting was 12.4%, which is similar to the previous result [5]. Another report using mRNA expression arrays and protein-protein interaction network analyses showed that CCNE1 was upregulated in primary gastric carcinomas but was not significantly different between early versus advanced stage or primary versus metastatic liver [13]. One case in our study tested using NGS in both primary and hepatic metastatic tumors showed concurrent CCNE1 amplification. These observations support CCNE1 amplification as an early event during progression of gastric carcinoma.

To explore the genetic alterations associated with liver metastasis in gastric carcinoma, we examined other genetic alterations associated with CCNE1 amplifications. Out of 28 gastric carcinomas with CCNE1 amplification, 23 (82.1%) cases showed mutations of TP53, suggesting chromosomal instability subtype and these results are consistent with previous study [5].

Among the CCNE1-amplified cases in our study, 8 cases (28.6%) showed co-amplification of ERBB2. Significant co-amplification between ERBB2 and CCNE1 had been previously reported and suggested to reduce the effects of trastuzumab in breast and gastric cancers [7,12]. A previous study showed that amplifications of cell cycle-related genes including CCNE1 coexist in approximately 40% of ERBB2-amplified gastric and esophageal adenocarcinomas [8]. Like CCNE1, ERBB2 amplifications are frequent in the chromosomal instability subtype and MSS/TP53- subtype of gastric cancer [5,6]. In our recent study, we found that CCNE1 amplifications were more frequent in HER2-overexpressing gastric cancers with intratumoral heterogeneity

Table 3
Detected mutations in *CCNE1*-amplified gastric adenocarcinomas without liver metastasis.

Case	MR	TR	MD	UF	TV	SNV1	AA	TM	TD	VAF	SNV2	AA	TM	TD	VAF	CNA1	CN	CNA2	CN	CNA3	CN
1	6,395,020	98.28	2,526	95.01	80	TP53	C242Y	Mis	1495	35.7	FBXW7	R465H	Mis	2000	20.7	CCNE1	18.55				
2	9,167,168	98.37	3,703	92.63	30	TP53	N131fs	Del	2000	20						CCNE1	13.67	MYC	9.27	FLT3	10.4
3	8,477,205	98.05	3,491	96.37	40	TP53	R273H	Mis	1998	8.2						CCNE1	15.7				
4	8,427,295	98.12	3,496	94.64	70											CCNE1	5.43				
5	11,220,543	98.45	4,614	96.59	30	TP53	E258	Non	1816	19.8						CCNE1	23.33	HER2	77.27		
6	8,117,467	97.65	3,289	97.79	70	TP53	H193R	Mis	1292	14.8						CCNE1	6.28				
7	13,142,196	98.11	5,434	96.94	90	TP53	L206fs	Ins	1995	31.6						CCNE1	10.01				
8	11,530,445	97.83	4,697	97.88	30	TP53	G262V	Mis	1996	48.9						CCNE1	65.23	PIK3R1	0		
9	9,233,126	98.07	3,854	97.74	50											CCNE1	16.1				
10	13,570,753	98.36	5,662	97.04	80	TP53	V173M	Mis	1996	30.7						CCNE1	10.45	HER2	37.84		
11	10,978,851	98.11	4,558	97.84	60	TP53	G266R	Mis	1998	18.9						CCNE1	6.48				
12	12,396,083	98.86	4,994	90.72	70	TP53	W53	Non	2000	18.3						CCNE1	14.11	HER2	19.46	MYC	17.43
13	9,925,640	98.55	4,159	93.71	70	TP53	P278S	Mis	2000	59.5	TP53	A138G	Mis	1930	7.9	CCNE1	74.94	HER2	3.49		
14	9,637,383	97.98	4,044	95.8	70											CCNE1	18.17	CCND1	7.01		
15	10,261,187	97.9	4,292	97.01	30	TP53	R273C	Mis	2000	10						CCNE1	12.73				

Abbreviations: MR, mapped reads; TR, target rate; MD, mean depth; UF, uniformity; TV, tumor volume; AA, amino acid; TM, type of mutation; Mis, missense; Del, deletion; Non, nonsense; Ins, insertion; TD, total depth; VAF, variant allele frequency; CN, copy number.

compared to cancers with homogeneous HER2 overexpression [14]. These observations indicate that the presence of CCNE1 amplification should be examined in gastric cancer patients with ERBB2 amplification to predict response and select an effective treatment option.

Among CCNE1-amplified gastric cancers, we observed PIK3CA mutation in one MSS, EBV-negative, TP53 mutated and HER2 amplified case (3.6%), and this case was considered as a chromosomal instability subtype [5]. Previous in vitro studies suggested PIK3CA mutation as a predictive marker of everolimus (mTOR inhibitor) sensitivity, and one gastric carcinoma with PIK3CA mutation actually showed a good response to everolimus [15]. Several previous studies have reported poor response to trastuzumab in PIK3CA mutated and ERBB2-posivie breast cancer, however, the response to tratuzumab in PIK3CA mutated and ERBB2-positive gastric cancer is still under study [16]. We also detected MYC amplification in 4 cases (14.3%) and this result is consistent with the previous study reporting a significant co-amplification and interaction between CCNE1 and MYC [7]. MYC is involved in a wide range of biological functions such as cell proliferation, survival, and differentiation by regulating the transcription of at least 15% of the entire genome [17]. Although there are many targets of MYC inhibition [18], BET inhibitor is suggested as a target therapy of MYC altered cancers [19] and GSK525762, a specific BET inhibitor, is in an early-phase clinical trial for relapsed or refractory hematologic malignancies (NCT01943851).

Cyclin E1, encoded by CCNE1, complexes with CDK2 to play a critical role in regulation of the G1-S phase transition. Many researchers have attempted to inhibit the activity of Cyclin E1/CDK2 using CDK inhibitors [20]. A combination of CDK inhibitor and AKT inhibitor was suggested as a target therapy in CCNE1-amplified, high-grade serous ovarian carcinoma [2]. Moreover, simultaneously blocking CCNE1 and PI3K pathways showed the efficacy in CCNE1-amplified/PIK3CA-mutated uterine serous carcinoma [11]. In HER2- and cyclin E-overexpressed breast cancers, CDK2 inhibitors were effective in a xenograft study and suggested as a valid treatment strategy [12]. Although no clinical trials have examined CDK inhibitors in gastric cancer with aberrant CCNE1 expression, CDK2 inhibitor may be considered as a treatment option in gastric cancer with CCNE1 amplification.

Our NGS results showed that nearly half of the CCNE1-amplified gastric adenocarcinoma (46.4%) showed liver metastasis. Although the precise contributions of CCNE1 to liver metastasis are unknown, we speculate that high proliferation in the background of chromosomal instability plays a major role. However, the similar prevalence of CCNE1 amplification in our cohort compared with previous studies suggests that CCNE1 amplification occurs early in the progression of gastric carcinomas. In cases of gastric carcinoma with liver metastasis, it is important to determine CCNE1 amplification for consideration of target therapy such as CDK2 inhibitor in addition to trastuzumab in patients with ERBB2 co-amplified gastric carcinomas.

In conclusion, CCNE1 amplification is significantly associated with liver metastasis in TP53-mutated gastric cancer.

Disclosure of potential conflicts of interest

None.

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