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Clinical Significance of DNA Damage Response Factors and Chromosomal Instability in Primary Lung Adenocarcinoma

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Abstract. *Aim: The purpose of this study was to investigate the biological role of DNA damage-response genes and chromosomal instability in primary lung adenocarcinoma. Materials and Methods: We investigated 60 surgically-resected lung adenocarcinomas. Levels of checkpoint kinase 2 gene (CHEK2) and breast cancer type 1 susceptibility protein gene (BRCA1) mRNA expression were evaluated by polymerase chain reaction (PCR). Epidermal growth factor receptor (EGFR) mutations (exon 19 deletion and exon 21 mutation) were detected by the PCR clamp method. Mutations in Kirsten rat sarcoma viral oncogene homolog gene (KRAS) and TP53 were examined by direct sequencing. Expression levels of p27 and p16 proteins were assessed by immunohistochemistry. Chromosomal aberrations (CA) were examined in 20 samples with single-nucleotide polymorphism–comparative genomic hybridization. Results: CHEK2 mRNA levels were significantly increased in tumor tissues compared to normal tissues (p=0.0123, paired t-test), whereas BRCA mRNA levels were not increased. TP53 mutation positivity and BRCA1 mRNA expression were positively associated with CHEK2 mRNA expression status (p=0.022 and p=0.0008). High CHEK2 mRNA expression was associated with poor recurrence-free survival (p=0.028). CHEK2 mRNA levels were higher in samples with a high CA frequency than in those with a low CA frequency (averages: 0.326 vs. 0.185; p=0.0129). Conclusion: The CHEK2 mRNA expression level was found elevated in lung adenocarcinoma and was related to a poor prognostic outcome. The CHEK2 pathway may be important for the proliferation of lung adenocarcinoma, especially in tumors with chromosomal instability.*

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Chromosomal instability (CIN) is one form of genetic variability that describes the susceptibility to developing structural and numerical alterations of chromosomes (1-3). CIN confers the capacity to develop various genetic alterations that are advantageous for cancer development and survival. Several studies have reported that CIN is a common feature of lung cancer (4, 5). Recently, it was demonstrated that activated oncogenes induce inappropriate (forced) proliferation, consequently causing DNA replication stress that induces stalling and collapse of replication forks. Although normal cells are innately equipped with the ability to mitigate this type of DNA stress through the DNA damage-response (DDR) pathway, impairment of the DDR pathway causes accumulation of DNA double-strand breaks, and these lead to CIN of the cancer cells (6, 7).

Checkpoint kinase 2 (CHEK2) is a transducer protein that is involved in the DDR pathway. Being activated by DNA damage-sensor proteins, ataxia-telangiectasia mutated (ATM) protein activates CHEK2 protein, after which CHEK2 phosphorylates effector proteins including breast cancer type 1 susceptibility protein (BRCA1), p53 and cell division cycle 25C (CDC25C) that play important roles in DNA repair, cell-cycle regulation and apoptosis (8, 9). Recently, CHEK2 was found to have a critical role in mitosis, as disruption of the CHEK2–BRCA pathway caused CIN in colon cancer cell lines (10). Despite the tumor-suppressor role of such DDR-related factors in carcinogenesis, certain studies have suggested that cancer cells may harness such DNA repair functions to contend with genetic stresses generated by unregulated proliferation and to escape apoptosis, which otherwise inhibits tumor progression (11). However, the specific behavior of such DDR-related factors in lung cancer remains to be elucidated. The purpose of this study was to investigate the biological role of DDR-related factors through its relationship with CIN in lung adenocarcinoma.

Materials and Methods

Tissue samples and patient characteristics. Primary lung adenocarcinomas and corresponding non-neoplastic lung specimens were collected from consecutive patients who underwent complete

resections (R0) for lung cancer from April 2007 to April 2010 at the Department of Surgery and Science, Kyushu University Hospital, Japan. Among them, 60 samples were available to assess DNA alterations, including the mutation status of *EGFR*, *TP53* (exons 5-9), KRAS, and the mRNA expression level of *CHEK2* and *BRCA* genes and protein expression levels of p27 and p16.

The median age of patients was 72 (range=37-85) years. The patients consisted of 26 men (43.3%) and 34 women (56.7%). Twenty-five patients (41.7%) were current or ex-smokers, while 35 (58.3%) patients were non-smokers. Tumor cell types were determined based on the WHO 2004 classification. Pathological staging was determined according to the seventh edition of the TNM classification system of the Union for International Cancer Control (UICC) (12). Patients were distributed among pathologic stages as follows: 28 IA, 13 IB, 9 IIA, 5 IIB and 5 IIIA. Tumor samples and corresponding non-neoplastic lung tissues (most distant from the tumor) were obtained immediately after resection, frozen in liquid nitrogen, and stored at −80˚C. No patient was treated with chemotherapy or radiotherapy before surgery. Following surgery, 22 (36.7%) patients received chemotherapy: 14 received oral tegafur and uracil, and eight were enrolled into a clinical trial for postoperative adjuvant chemotherapy (three received S-1 and five received cisplatin with S-1). This study was approved by the Kyushu University Institutional Review Board for Clinical Research (IRB no. 24-173).

Patient follow-up. The routine check-up procedure was described elsewhere (13). Briefly, physicians checked tumor recurrence with a physical examination, blood cell counts, serum chemistry, and serum tumor markers including carcinoembryonic antigen and cytokeratin fragment 19. Chest X-rays were performed on an outpatient basis four times a year for the first 3 years, and thereafter twice annually. Computed tomography was performed twice a year for the first 3 years, and thereafter at least annually. Brain magnetic resonance imaging, and bone scintigrams or fluorodeoxyglucose positron-emission tomography were performed annually.

DNA preparation and gene mutation analyses. Genomic DNA was extracted from the samples as follows: frozen samples were incubated in lysis buffer (0.01 mol/l Tris-HCl, pH 8.0; 0.1 mol/l EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) containing proteinase K (100 mg/ml) at 37˚C for 2 h. The samples were extracted twice in phenol then once in phenol/chloroform and once in chloroform. Following ethanol precipitation, the samples were dissolved in Tris-EDTA (0.01 mol/l Tris-HCl, pH 8.0; 0.01 mol/l EDTA, pH 8.0). *EGFR* mutation tests used the peptide nucleic acid–locked nucleic acid (PNA-LNA; Mitsubishi Chemical Medience, Tokyo, Japan) polymerase chain reaction (PCR) clamp method with formalin-fixed paraffin-embedded sections of surgical specimens (13). Mutations in TP53 (exons 5 to 9) were detected by direct sequencing PCR of all PCR products by using each forward and reverse primers and the dideoxynucleotide chain-termination method (Big-dye sequencing kit; Applied Biosystems, Foster City, CA, USA); they were sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). As previously described (14), using genomic DNA extracted from tissue samples, a 275-bp fragment containing exon 6, a 439-bp fragment containing exon 7 and a 445-bp fragment containing exons 8 and 9 of the *TP53* gene were amplified by PCR (Nippon Gene, Tokyo, Japan). Mutations in *KRAS* at codons 12 and 13 were determined by direct sequencing as previously described (15). Briefly, each region was amplified by PCR using the *c-Ki-ras*/12 primer set (Takara Bio Inc., Otsu, Japan) and purified PCR products were used as a template for cycle sequencing reactions using a BigDye terminator cycle sequencing kit as described above.

RNA extraction and quantitative RT-PCR. The procedures for RNA extraction and quantitative RT-PCR were described in our previous study (13). Briefly, total RNA was extracted from resected lung tissues and cell lines using ISOGEN (Nippon Gene) according to the manufacturer's protocol. cDNA was synthesized using a SuperScript III First-Strand Synthesis Super-Mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative PCR amplification was performed using Applied Biosystems StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA, USA). TaqMan gene expression assays (Applied Biosystems) for *CHEK2* (Hs00200485_m1) and *BRCA1* (Hs01556193_m1) were used; β-actin (Hs99999903_m1) was used as an internal control. The comparative Ct method was used to determine relative expression values. The relative mRNA expression levels of each sample were standardized to those of β-actin. Each sample was tested with triplicate measurements, and the mean value of the triplicate measurements was defined as a final value.

Immunohistochemistry. Tumor sections were assessed immunohistochemically using rabbit polyclonal anti-$p27^{KIP1}$ (#2552, 1:50; Cell Signaling Technology, Tokyo, Japan) and purified mouse anti-human $p16^{INK4}$ (551154, 1:50; BD Pharmingen, BD Biosciences, Tokyo, Japan) antibodies. Briefly, 4-μm sections were deparaffinized in xylene and dehydrated in an ethanolic series. For antigen retrieval, slides were immersed in 0.01 M sodium citrate buffer (pH 6.0) and autoclaved (121˚C for 15 min). The sections were washed and immersed in 1.5% hydrogen peroxide and absolute methanol to deactivate endogenous peroxidases. After blocking nonspecific binding of antibodies, the specimens were incubated at room temperature with primary antibodies against p16 and p27 for 60 min. Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase complex method (Histofine SAB kit; Nichirei, Tokyo, Japan), according to the manufacturer's instructions. Two investigators who were blinded to all information about the samples, including one general pathologist, evaluated the levels of expression. The expression of p16 was determined to be positive if the proportion of tumor cells exhibiting strong nuclear staining was ≥10% (16). The expression of p27 protein was evaluated according to its intensity; expression was positive if the intensity of tumor cell nuclear staining was the same as or stronger than that of infiltrating lymphocytes, and expression was negative if it was not (17).

SNP-CGH array analysis. Twenty surgically-resected specimens were genotyped by using 1,140,419 autosomal SNPs (HumanOmni1-Quad BeadChip; Illumina, Inc., Tokyo, Japan) and copy number variation was analyzed with GenomeStudio V2009.1 (Illumina, Inc.). Chromosomal aberrations (CA) were assessed using KaryoStudio v1.4. (Illumina, Inc., Tokyo, Japan) as described previously (18). The percentage defect score was the sum of the lengths of all chromosomal aberrations (including gain, loss and copy-neutral loss of heterozygosity) per sample divided by the length of the whole genome. We defined a sample with a percentage defect score ≥16% as high CA (CIN+), whereas samples with a percentage defect score <16% was defined as low CA (CIN−).

Table I. *Comparisons of mRNA expression level of DNA damage-response genes between normal lung tissue and lung adenocarcinoma.*

Factor		Normal lung (average)	Adenocarcinoma (average)	*p*-Value
Student *t*-test	n	47	60	
BRCA1 mRNA level	Average	0.1129	0.1283	0.414
	S.E.	0.0140	0.0124	
CHEK2 mRNA level	Average	0.1659	0.2223	0.0165
	S.E.	0.0173	0.0153	
Paired *t*-test	n	47		
BRCA1 mRNA level	Difference	0.0091		0.589
	S.E.	0.0168		
CHEK2 mRNA level	Difference	0.0556		0.0123
	S.E.	0.0213		

BRCA1: Breast cancer type 1 susceptibility protein gene; *CHEK2*: checkpoint kinase 2.

Statistical analyses. Qualitative variables were compared using chi-square tests and Fisher's exact test, and quantitative variables were compared using Student's *t*-tests. Survival was measured from the point of surgery. Survival curves were drawn using the Kaplan–Meier method; significant differences among subgroups were compared using the log-rank test. The Cox proportional hazard regression model was used to explore the effects of the clinicopathological variables and other factors. Factors showing prognostic significance in the univariate analyses were adopted as variables in multivariate analysis. A value of $p<0.05$ was considered statistically significant. Statistical analysis was performed using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA).

Results

Expression of CHEK2 and BRCA1 genes in lung adenocarcinomas. We first investigated the expression of *CHEK2* and *BRCA1* genes in both lung adenocarcinomas and normal lung tissues to determine if the CHEK2–BRCA1 pathway is up-regulated in lung adenocarcinoma cells (Table I). Student *t*-test demonstrated that *CHEK2* mRNA level of tumor tissues (n=60) was significantly higher than that of normal lung tissues (n=47) ($p=0.0165$). On the other hand, no significant difference was found in *BRCA* mRNA levels between the tissues ($p=0.414$). Paired *t*-tests revealed that *CHEK2* mRNA levels were significantly increased in the tumor tissues compared to the normal cells (n=47; relative expression of cancer cells and normal cells were 0.222 and 0.166, respectively, $p=0.0123$). *BRCA1* mRNA levels in the tumor were not different from those in normal cells (0.122 and 0.111, respectively, $p=0.589$).

Table II. *Clinicopathological characteristics and the status of cancer-related factors of 60 patients with lung adenocarcinoma according to expression level of checkpoint kinase 2 gene (CHEK2) mRNA*

		CHEK2		*p*-Value
Factor	Category	<0.2	≥0.2	
Age	<70	11	12	0.95
	≥70	18	19	
Gender	Male	12	14	0.77
	Female	17	17	
Smoking	No	17	18	0.97
	Yes	12	13	
T-Stage	1	16	19	0.63
	≥2	13	12	
N-Stage	0	5	5	0.91
	≥1	24	26	
Stage	I	17	24	0.12
	≥II	12	7	
EGFR mutation	–	13	14	0.89
	+	16	16	
KRAS mutation	–	26	30	0.35
	+	3	1	
TP53 mutation	–	27	21	0.022
	+	2	10	
BRCA1 mRNA	Low	21	9	0.0008
	High	8	22	
p27 protein	Low	15	12	0.31
	High	14	19	
p16 protein	Low	11	13	0.75
	High	18	18	

Genes: *EGFR*: Epidermal growth factor receptor; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *TP53*: tumor protein 53; *BRCA1*: breast cancer type 1 susceptibility protein.

Relationship between CHEK2 gene expression and clinicopathological factors and the status of cancer-related factors in patients with lung adenocarcinoma. We investigated whether *CHEK2* mRNA expression levels in the lung cancer specimens are correlated with the patients' clinicopathological factors. We divided patients according to the median *CHEK2* mRNA expression level (relative expression=0.2). There was no correlation between clinicopathological factors and *CHEK2* mRNA, as shown in

Table III. *Univariate and multivariate analyses fordisease-free survival in patients with lung adenocarcinoma.*

		Univariate analyses			Multivariate analyses		
Factor	Category	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	<70 Years	1			1		
	≥70 Years	1.80	0.73-5.05	0.21	1.42	0.42-5.09	0.57
Gender	Male	1			1		
	Female	0.57	0.24-1.35	0.20	0.71	0.28-1.97	0.55
Smoking	No	1					
	Yes	0.90	0.36-2.14	0.82			
T-Stage	1	1			1		
	≥2	3.45	1.43-9.11	0.0056	3.14	1.12-9.73	0.029
N-Stage	0	1					
	≥1	1.27	0.36-3.46	0.68			
Stage	I	1			1		
	≥II	1.94	0.79-4.65	0.14	1.39	0.52-3.72	0.50
EGFR mutation	−	1			1		
	+	0.47	0.18-1.14	0.09	0.72	0.22-2.17	0.56
KRAS mutation	−	1					
	+	1.74	0.28-6.00	0.49			
TP53 mutation	−	1					
	+	1.23	0.40-3.19	0.69			
CHEK2 mRNA	Low	1			1		
	High	2.77	1.12-7.77	0.026	3.09	1.17-9.34	0.023
BRCA1 mRNA	Low	1					
	High	0.99	0.41-2.38	0.99			
p27 protein	Low	1			1		
	High	2.09	0.86-5.59	0.10	2.02	0.73-6.20	0.18
p16 protein	Low	1					
	High	0.69	0.29-1.65	0.39			

CI: Confidence interval; HR: hazard ratio. Genes: *EGFR*: Epidermal growth factor receptor; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *TP53*: tumor protein 53; *CHEK2*: checkpoint kinase 2; *BRCA1*: breast cancer type 1 susceptibility protein.

Table II. We then examined the status of cancer-related factors in our panel of lung adenocarcinomas. We selected cell cycle-related proteins p16 and p27 since these were reported to be biologically important for lung carcinogenesis or progression (16, 17). Among the six factors we assessed, *TP53* mutation positivity and *BRCA1* mRNA expression were positively associated with *CHEK2* mRNA expression status (p=0.022 and p=0.0008) (Table II). *CHEK2* mRNA and *BRCA1* mRNA expression were significantly positively linearly correlated (ϱ=0.569, p<0.0001).

Prognostic significance of CHEK2 gene expression in lung adenocarcinoma. We assessed the relationship between *CHEK2* mRNA expression and postoperative prognosis of patients. Disease-free survival (DFS) in patients with high *CHEK2* expression was significantly poorer than that of patients with low *CHEK2* expression (5-year DFS rate: low *CHEK2*, 78.5%; high *CHEK2*, 50.4%; log-rank test: p=0.0281; Figure 1). Univariate and multivariate analyses showed that low T-stage and low *CHEK2* mRNA expression were independent prognostic factors for better DFS (Table III).

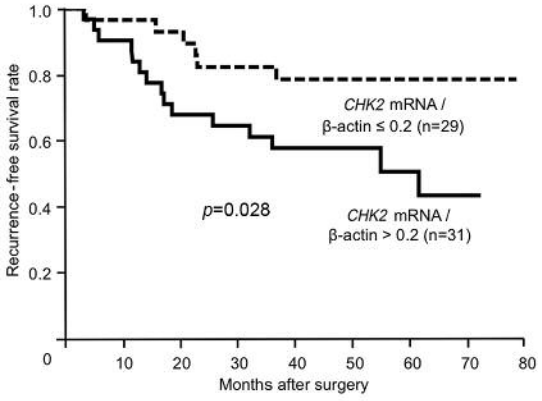

Figure 1. *Kaplan–Meier curves showing disease-free survival of patients with primary lung adenocarcinoma according to checkpoint kinase 2 gene (CHEK2) mRNA expression.*

Chromosomal aberrations and their possible relevance to lung adenocarcinoma. We performed SNP-CGH analysis of 20 lung adenocarcinoma samples. Two representative cases

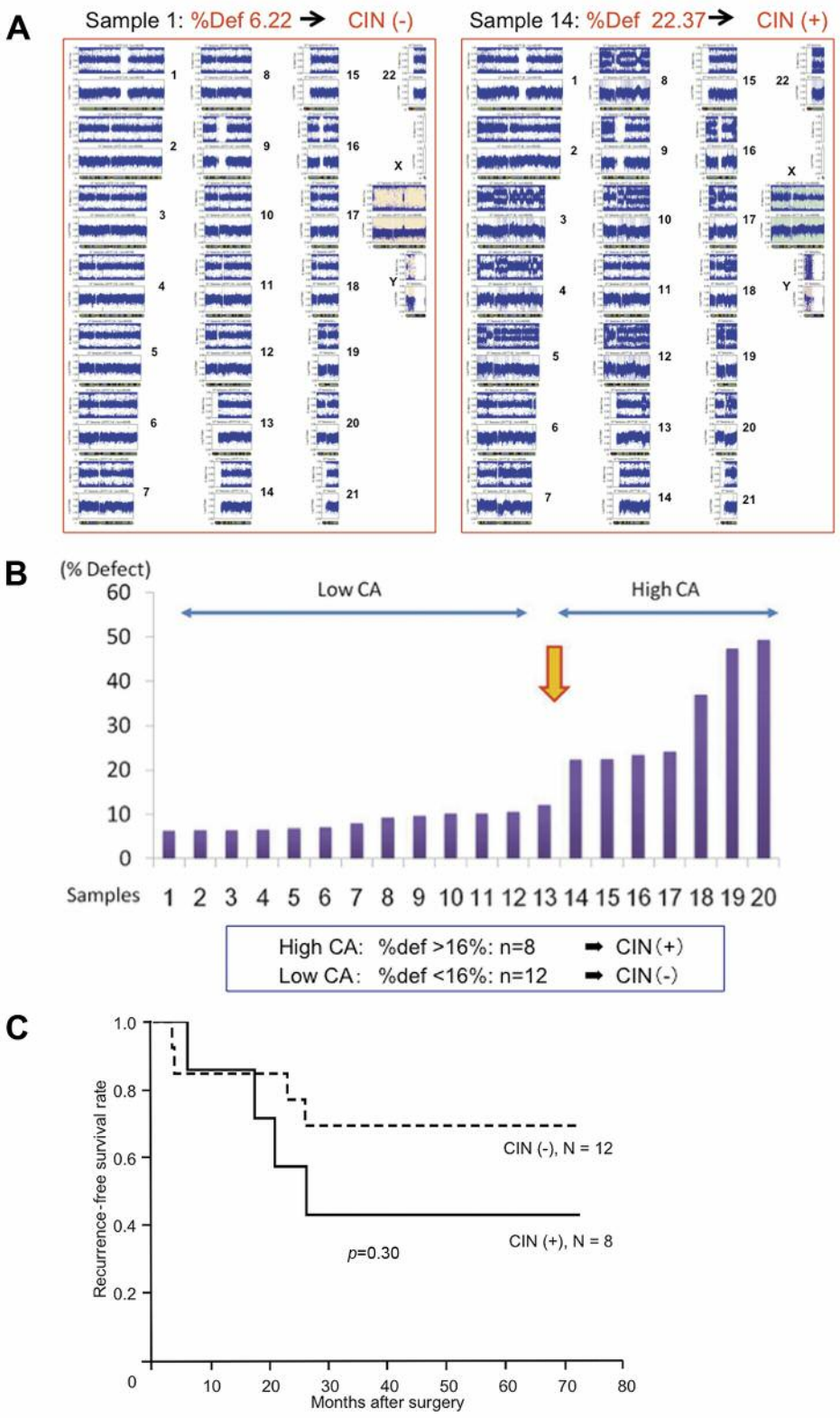

Figure 2. *Chromosomal aberrations (CA) in lung adenocarcinomas of 18 patients who underwent surgical resection. a: Two representative single-nucleotide polymorphism–comparative genomic hybridization (SNP-CGH) array analyses of lung adenocarcinoma. b: Classification of chromosomal instability (CIN) according to the percentage defect score (%Def) in SNP-CGH array analysis. c: Kaplan–Meier curves showing disease-free survival of patients with primary lung adenocarcinoma according to CIN.*

Table IV. *Relationship between chromosomal instability (CIN) status and DNA damage-response factors.*

Factor	CIN+ (average)	CIN– (average)	*p*-Value
BRCA1 mRNA level	0.142	0.115	0.463
CHEK2 mRNA level	0.326	0.185	0.0129

BRCA1: Breast cancer type 1 susceptibility protein; *CHEK2*: checkpoint kinase 2.

are shown in Figure 2a. The level of CA was assessed using the percentage defect score as described in the Material and Methods. Seven samples were determined to be high CA (CIN+), whereas 13 samples were low CA (CIN–) according to SNP-CGH (Figure 2b). Kaplan–Meier survival curves for DFS suggested that patients with CIN^+ had a trend for a poorer prognosis than those with CIN^-, although no statistical significance was demonstrated (Figure 2c). To examine the relationship between CIN status and cancer-related factors, we assessed 18 samples. Table IV shows that CIN positivity was significantly associated with high *CHEK2* mRNA level (average: 0.326 *vs.* 0.185; p=0.0129).

Discussion

Although several studies have reported an association between *CHEK2* gene expression and lung cancer, gene mutations that abrogated CHEK2 function appeared to be infrequent in primary lung cancer (19). Some studies have suggested that cancer cells utilize factors that function in the DNA repair process, allowing them to proliferate during exposure to genomic stress. In univariate and multivariate survival analyses, the present data demonstrate that high *CHEK2* gene expression was correlated with poor prognoses in patients with lung adenocarcinoma. However, there was no correlation between *CHEK2* mRNA expression and any clinicopathological characteristic of the patients. To our knowledge, this is the first study to reveal an association between *CHEK2* gene expression and cancer aggressiveness.

CHEK2 is considered to act as a transducer protein that is directly phosphorylated by ATM protein in response to DNA damage. CHEK2 activates various proteins that are involved in crucial functions that maintain the integrity of the cell, such as DNA repair, apoptosis and cell-cycle check points, both before and during mitosis (20). BRCA1 is one of the major proteins that functions with CHEK2 protein in the DDR response. Our data indicate that *BRCA1* mRNA expression was significantly correlated with *CHEK2* mRNA expression. In spite of this association, *BRCA1* expression was not related to the prognosis of the patients, thus suggesting *CHEK2* is a more sensitive factor for predicting the aggressiveness of lung adenocarcinoma than BRCA1.

Among cancer-related factors, *TP53* mutations were significantly related to *CHEK2* mRNA expression. This finding was consistent with a previous study on primary gastric cancer reported by Shigeishi *et al.* (21). They found that *CHEK2* expression was significantly elevated in tumors carrying *TP53* mutations compared to tumors with wild-type *TP53*. Since p53 generally functions downstream from CHEK2 signaling to regulate cell-Cycle arrest or apoptosis (22), our results suggest that high *CHEK2* expression in *p53* wild-type cancer might induce cell-cycle arrest or apoptosis, which are disadvantageous for tumorigenesis. Alternatively, *CHEK2* may control DNA integrity by substituting for *TP53* function.

In the SNP-CGH array analysis, we demonstrated that cancer with CIN features had higher *CHEK2* expression than cancer without CIN. Since a recent publication indicated that disruption of the DDR pathway results in CIN in cancer cells (6), the present observation seems to be in disagreement. However, on the other hand, cancer cells may depend on the function of CHEK2 for keeping their genetic instability within tolerable levels for cell proliferation. Several studies have demonstrated that cancer cells are balanced between two opposing states. That is, they are genetically flexible and can adapt to a toxic environment, yet their genetic instability can be harmful to their viability (1, 11). Considering this specific feature of cancer cells, many anticancer strategies disrupt the functions that cancer cells rely on for their proliferation, including CHK1/2 targeting (5, 23). Anti-CHEK2 treatment is also proposed in combination with radiotherapy or anticancer drugs that provoke DNA breaks (24, 25).

Our study had several limitations. Firstly, this was a retrospective study with a rather small number of patients. Secondly, several factors associated with DDR and the cell cycle were evaluated with a limited array of methodologies. Thus, the present data should be validated in other cohorts from multiple aspects. Validation of the present data will provide a useful treatment strategy that could help to prolong the survival of patients with lung cancer.

In conclusion, we showed that the *CHEK2* mRNA expression level was increased in lung adenocarcinoma and that it was related to poor prognostic outcome. The CHEK2 pathway may be important for the proliferation of lung adenocarcinoma, especially in tumors with CIN.

Conflicts of Interest

None declared.

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