Original Article

Cyclin-dependent kinase 4 upregulation mediates acquired resistance of dabrafenib plus trametinib in *BRAF* V600E-mutated lung cancer

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Background: Combination therapy with the B-Raf inhibitor, dabrafenib, and the MEK inhibitor, trametinib (DT) is commonly used to treat patients with B-Raf proto-oncogene, serine/threonine kinase V600E (*BRAF* V600E)-mutated non-small cell lung cancer (NSCLC). However, the mechanisms through which cancer develops DT resistance are unclear. Here, we investigated new mechanisms underlying acquired DT-resistant NSCLC with the *BRAF* V600E mutation.

Methods: We compared genomic signatures before and after DT treatment in patients with NSCLC.

Results: Two of four patients treated with DT developed carcinomatous pleuritis within 3 months. Target DNA sequencing and quantitative polymerase chain reaction (PCR) analyses revealed the increased expression level of cyclin-dependent kinase 4 (*CDK4*). We also found prominent protein expression of *CDK4* after DT treatment. Induction of *CDK4* expression in a cell line derived from a patient with the *BRAF* V600E mutation resulted in partial resistance to dabrafenib.

Conclusions: Our findings suggest a possible relationship between *CDK4* upregulation and acquired resistance to DT therapy.

Keywords: B-Raf proto-oncogene, serine/threonine kinase V600E (*BRAF* V600E); cell cycle; cyclin-dependent kinase 4 (*CDK4*); dabrafenib; trametinib

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Introduction

Since the discovery of the oncogenic mutation in the B-Raf proto-oncogene, serine/threonine kinase V600E (*BRAF* V600E), combination therapy with the B-Raf inhibitor, dabrafenib, and the MEK inhibitor, trametinib (DT) has been used for treating *BRAF* V600E-mutated non-small cell lung cancer (NSCLC) (1). Although mutations in the mitogen-activated protein kinase (MAPK) or phosphatidylinositol

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3-kinase (PI3K)/AKT pathways have been reported, the mechanisms of resistance to DT therapy are unclear (2-4).

Cyclin-dependent kinase 4 (*CDK4*) is involved in regulating cell proliferation during the G1 phase. *CDK4* forms a complex with cyclin D, which is regulated by MAPK activity, thereby mediating retinoblastoma (Rb) phosphorylation and resulting in cell cycle progression (5). *CDK4/6* inhibitors have been used as anticancer agents in hormone receptor-positive breast cancer and *KRAS*-mutated NSCLC (6). Moreover, combined inhibition of *RAF* and *CDK4/6* may have synergistic effects on tumors with *RAS* or *RAF* mutations (7). Additionally, CD4/6 inhibitors can overcome resistance to *BRAF* inhibitors caused by cyclin D1 upregulation in *BRAF* V600E-mutated melanoma (8). Thus, targeting CDK may overcome resistance to *RAS*- and *RAF*-mutant cancers. *CDK4* may be implicated in the development of resistance in *BRAF* V600E-mutant NSCLC; however, the involvement of *CDK4* in acquired resistance is unclear.

Here, we established clinically resistant cell lines derived from pleural effusion samples of patients with NSCLC. We report two cases of clinically DT-resistant *BRAF* V600E-mutated NSCLC and compared the expression levels of cell cycle-related genes, including *CDK4*, in DT-naïve and DT-resistant cases. We present the following article in accordance with the STROBE reporting checklist (available at https://dx.doi.org/10.21037/tlcr-21-415).

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All study participants provided informed consent and this study was approved by the Institutional Review Board of Asahikawa Medical University (No.: 18185/19081). Written consent was obtained from all patients whose samples were analyzed, including pleural effusions.

Patients and treatments

Four patients were treated with DT, two of whom developed carcinomatous pleuritis after treatment. Pleural fluid from these two patients (LC-1, LC-6) was used for post-DT samples; lung tissue from corresponding patients collected before DT treatment was used for DT-naïve samples. The *BRAF* V600E mutant (IG: pleural effusion; ON: transtracheal lymph node biopsy) and the *BRAF* K601E mutant (MY: pleural effusion) were also subjected to the Oncomine Dx target test (Thermo Fisher Scientific, Waltham, MA, USA), and cancer cells derived from DT-naïve patients harboring these genetic mutations were used as a reference.

Establishment of cell line and expression constructs

Lymph node biopsies of patients with the *BRAF*_V600E mutation (ON) were performed, and the excess specimens were used. The isolated lung cancer cells were cultured in RPMI 1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin and 1 mmol/L sodium pyruvate as the primary culture medium. After confirming that the tumor cells were proliferating, single cells were cultured in 96-well plates, and only the proliferating lung cancer cells were passaged as cell lines.

The *CDK4* cDNA vector was a gift from Sander van den Heuvel (Addgene, plasmid #1876, Watertown, MA, USA). The *CDK4* cDNA vector or empty vector was transfected by electrophoresis (Neon system, Thermo Fisher Scientific, Waltham, MA, USA). Dissociated ON cells were resuspended in 8 μL of Neon Resuspension Buffer R for every one million cells. For each electroporation, cells and 2 μL of vector were aliquoted into a sterile microcentrifuge tube. A Neon Tip was inserted into the Neon Pipette and the cell-DNA mixture was aspirated into the tip avoiding air bubbles. The Neon Pipette was then inserted into the Neon Tube containing 3 mL of Neon Electrolytic Buffer E in the Neon Pipette Station. Cells were pulsed twice with a voltage of 1,400 and a width of 20. After the pulse, cells were quickly transferred into a RPMI1640 medium without antibiotics.

Cell proliferation and growth assays

Dabrafenib and erlotinib were purchased from Selleckchem (Boston, MA, USA) and inhibition of growth was assessed by MTS assay according to previously established methods (9). All experimental points were set up in 6 to 12 wells and all experiments were repeated 2 times.

Genetic analysis

BRAF mutations were identified by the droplet digital polymerase chain reaction (PCR) or OncomineDx Target Test (Thermo Fisher Scientific, Waltham, MA, USA) in 12 of 233 patients with NSCLC over a 5-year period (2015–2020) in Asahikawa Medical University Hospital. DNA was extracted from bronchial lavage fluid (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany). Digital

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PCR was performed using a *BRAF* V600E-specific probe (Taqman cfDNA assay *BRAF* V600E, A44177 Thermo Fisher Scientific, Waltham, MA, USA) according to the QuantStudio3D (Thermo Fisher Scientific, Waltham, MA, USA) protocol. Genomic DNA mutations and copy number variations (CNVs) were assessed by a comprehensive cancer panel (160 genes, GeneRead DNAseq Targeted HC panel v2; QIAGEN, Hilden, Germany) on an Illumina MiSeq platform (Appendix 1).

mRNA expression analysis

mRNA was extracted from formalin-fixed paraffin-embedded samples of LC-1 and LC-6 samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany). mRNA expression was assessed by the real-time PCR using a TaqMan Array human anticancer drug panel (RAPRJ2Y, Thermo Fisher Scientific, Waltham, MA, USA).

Antibodies and Western blotting

Cell lysis, Western blotting and immunohistochemistry (IHC) was done as previously described (9,10). The following antibodies were used for immunostaining and Western blotting: anti-CDK4 (D9G3E), anti-cyclin D1 (92G2) and anti-β-actin (13E5) (Cell Signaling Technology, Danvers, MA, USA). The receptor tyrosine kinase (RTK) array (Proteome Profiler Human Phospho-RTK Array Kit, R&D Systems, Minneapolis, MN, USA) was purchased and used according to the manufacturer's recommended conditions as previously described (9). List of 49 RTKs measuring relative phosphorylation status on antibody arrays were shown in Appendix 2.

Statistical analysis

In all cell growth assays, the mean and standard deviation were calculated from 6 wells of sample and 12 wells of control. The statistical analysis was carried out with two-sample unpaired Student *t*-test performed using R (version 3.4.1; The R Foundation, Vienna, Austria) and 95% CI of the mean difference was plotted.

Results

Case LC-1

A 61-year-old man was diagnosed with lung adenocarcinoma and underwent surgical resection of the right middle lobe (pathological stage 1A). Four years later, the adenocarcinoma recurred in the right upper lobe and metastasized to multiple lymph nodes, bone and the adrenal glands. The patient received four cycles of cisplatin/pemetrexed/bevacizumab, followed by maintenance therapy with pemetrexed/bevacizumab. After five cycles of maintenance therapy, new metastases were found in the cervical lymph nodes. Following a cycle of pembrolizumab, the patient developed fever and hypoxemia owing to drug-induced pneumonia. The drug was discontinued and the patient was treated with oral steroids for 3 months. The *BRAF* V600E mutation was detected in the surgical specimen (LC-1 pre); thus, DT was administered as a third-line therapy. DT caused a temporary reduction in tumor size; however, the disease progressed to brain metastasis, meningitis and pleural effusion (LC-1 post) within 3 months. Whole-brain irradiation was performed, but the patient died 1 month after discontinuing DT.

Case LC-6

A 62-year-old man was diagnosed with lung adenocarcinoma by transbronchial lymph node biopsy with bronchoscopy. Because the patient had clinical stage 3B disease, four cycles of cisplatin/pemetrexed/bevacizumab were administered, followed by maintenance therapy with pemetrexed/bevacizumab. After seven cycles of maintenance therapy, the patient was switched to nivolumab as second-line therapy owing to right lung progression. After 19 cycles of nivolumab, the patient was switched to docetaxel/ramucirumab owing to right lung progression and lymph node metastasis. After 10 cycles of docetaxel/ramucirumab, the disease progressed with carcinomatous lymphangitis and bone metastasis. *BRAF* V600E was detected in a re-biopsy specimen of the mediastinal lymph nodes (LC-6 pre); thus, DT was started as a fourth-line therapy. After 11 months of DT, the disease progressed with pleural effusion (LC-6 post). The patient died 4 months after discontinuing DT.

Genomic analysis and protein expression

Next-generation sequencing was performed for LC-1 and LC-6 pre/post to evaluate acquired DT resistance (*Figure 1*, *Table 1*, Table S1). In LC-1, *BRAF* V600E was identified before and after DT treatment. The copy number variation (CNV) analysis showed that *CDK4* on chromosome (chr) 12 was amplified after DT treatment. In LC-6, *BRAF* V600E was confirmed by SNV analysis before and after treatment,

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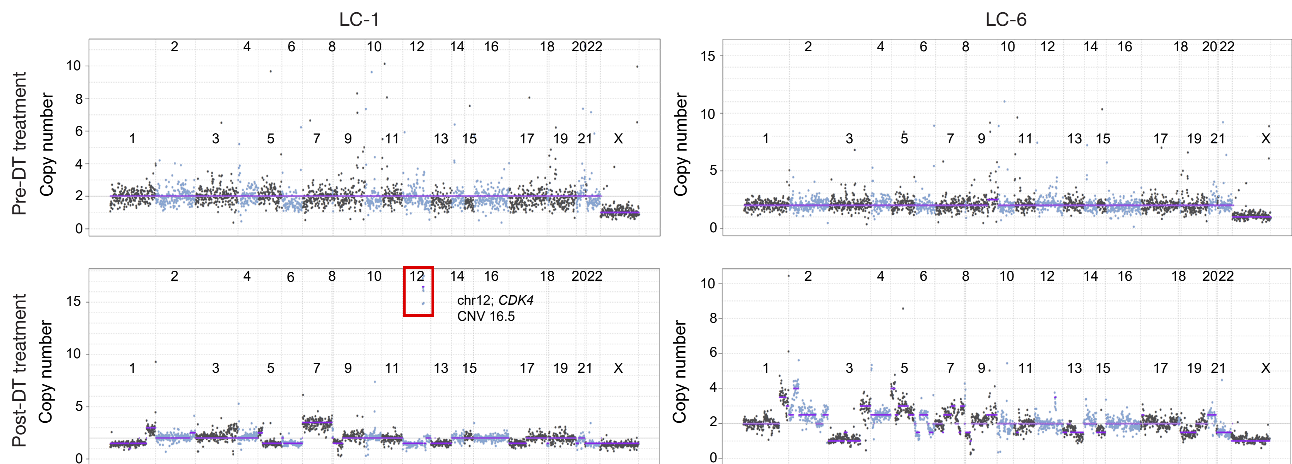

Figure 1 Changes in DNA copy number before and after treatment with DT in patients with *BRAF* V600E-mutated lung adenocarcinoma. Left panel: focal amplification (CNV =16.5) of the gene locus encoding *CDK4* at chr12 in specimens collected after the acquisition of resistance to DT in patient LC-1. Right panel: four copies of the gene on chrs 2 and 5, and copy number loss on chr 3 were observed in specimens collected after the acquisition of resistance in patient LC-6. DT, dabrafenib and trametinib; chr, chromosome; *CDK4*, cyclin-dependent kinase 4; *BRAF* V600E, B-Raf proto-oncogene, serine/threonine kinase V600E.

Table 1 CNV in patients LC-1 and LC-6

Cases	Chr	Gene name	Variant type	CNV change	Pre-DT treatment (copy number)	Post-DT treatment (copy number)
LC-1	Chr12	*CDK4*	CNV	Gain	2	16.5
	Chr7	*CARD11* *PMS2* *RAC1* *IKZF1* *EGFR* *MET* *SMO* *BRAF* *EZH2*	CNV	Gain	2	3.5
	Chr1	*MUTYH*	CNV	Loss	2	1.0
LC-6	Chr2	*ALK*	CNV	Gain	2	4.0
	Chr5	*TERT* *IL7R*	CNV	Gain	2	4.0
	Chr1	*DDR2* *CDC73*	CNV	Gain	2	3.5
	Chr3	*FANCD2* *VHL* *XPC* *MLH1* *SETD2* *BAP1* *PBRM1*	CNV	Loss	2	1

CNV, copy number variation; chr, chromosome; DT, dabrafenib and trametinib.

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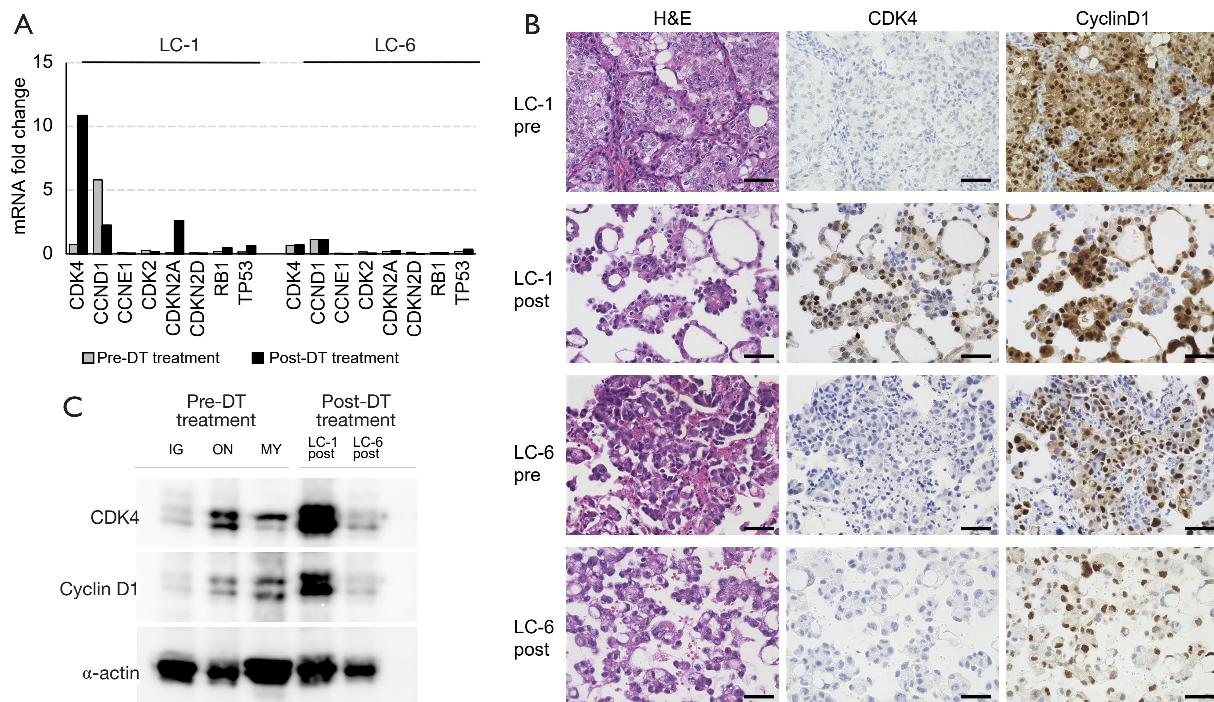

Figure 2 Investigation of mRNA expression and protein expression (using IHC and Western blotting) in samples from patients LC-1 and LC-6 before and after DT treatment. (A) Transcript levels of cell cycle-related genes determined using quantitative real-time PCR. (B) Microscopy-based images of H&E, anti-CDK4 and anti-cyclin D1 staining before and after treatment with DT in samples from patients LC-1 and LC-6 (scale bar: 50 µm). (C) Protein expression in pleural effusion-derived cells from *BRAF* mutation-positive patients before and after DT treatment (IG: pleural effusion; ON: transtracheal lymph node biopsy; MY: pleural effusion). *CDK4*, cyclin-dependent kinase 4; H&E, hematoxylin and eosin; DT, dabrafenib and trametinib; IHC, immunohistochemistry; PCR, polymerase chain reaction; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase.

and missense S2264L and nonsense S2269* mutations in the *AT-rich interaction domain 1A* (*ARID1A*) were detected on chr 1 in the post-DT sample. In both LC-1 and LC-6, other copy number aberrations were detected; however, there was no focal amplification directly associated with resistance.

Next, we performed quantitative PCR and IHC analyses on LC-1 and LC-6 pre/post samples (*Figure 2A,2B*). In the LC-1 post sample, *CDK4* mRNA was upregulated, as were the tumor suppressors *CDKN2A* (encoding p16), *TP53*, and *Rb1*. The *CDK4* protein was upregulated after DT therapy in LC-1, whereas *CCND1* mRNA (encoding cyclin D1) was downregulated. However, cyclin D1 expression was observed before and after treatment, and cell cycle signatures were not markedly altered in LC-6 (*Figure 2A,2B*). Western blotting results (*Figure 2C*) showed that *CDK4* was expressed abundantly in LC-1, but only marginally in the other samples. Finally, we tested *in vitro* experiments to determine whether cells in the *BRAF* V600E cell line expressing the *CDK4* protein are resistant to *BRAF* inhibitors. ON cells were transfected with a vector encoding *CDK4* and treated with dabrafenib; at a concentration of 100 nM dabrafenib, ON cells transfected with the *CDK4* gene were more resistant than controls (*Figure 3A*). On the other hand, cells treated with erlotinib, an *EGFR* inhibitor, did not show any suppression of proliferation. The expression of *CDK4* protein was increased in ON cells transfected with a vector encoding *CDK4* (*Figure 3B*). We further examined whether overexpression of *CDK4* causes a bypass pathway for *BRAF* inhibitor resistance by increasing the phosphorylation of RTKs. The results showed that the phosphorylation of RTKs was not altered with or without forced expression of *CDK4* protein (*Figure 3C*).

Discussion

Increased *CDK4* DNA copy numbers followed by increased

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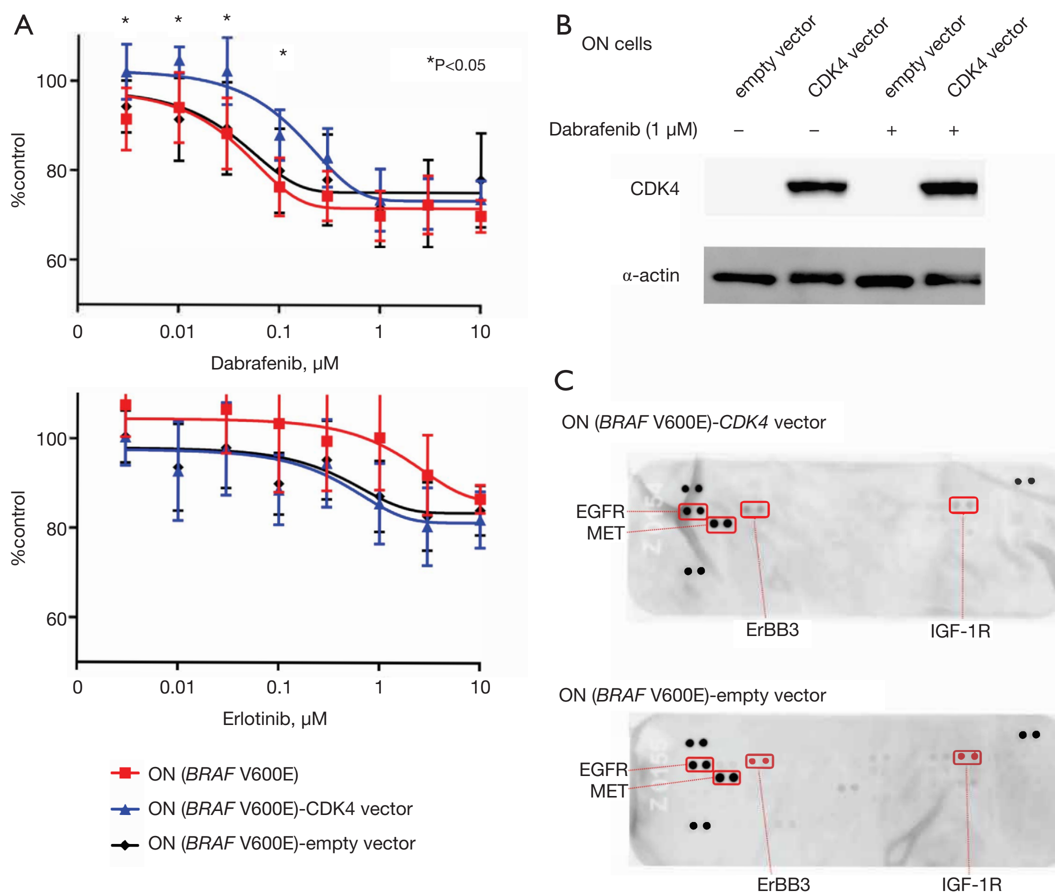

Figure 3 ON cell growth assays and RTK phosphorylation. (A) ON cells were treated with dabrafenib or erlotinib and cell proliferation was assessed by MTS assay (ON: transtracheal lymph node biopsy). *CDK4* protein expressing cells were statistically resistance to dabrafenib at the concentration of 3–100 nM compared to control cells. (B) Protein expression of *CDK4* was confirmed by Western blotting. (C) Changes in RTK phosphorylation status in ON cells expressing *CDK4*. Phosphorylation of *EGFR*, *MET*, *ErBB3* and *IGF-1R* was also observed in control and *CDK4* expressing cells. We did not observe any RTKs altered by *CDK4* expression. *, P<0.05. *CDK4*, cyclin-dependent kinase 4; *BRAF* V600E, B-Raf proto-oncogene, serine/threonine kinase V600E; *EGFR*, epidermal growth factor receptor; *MET*, MET proto-oncogene, receptor tyrosine kinase; *ErBB3*, erb-b2 receptor tyrosine kinase 3; *IGF-1R*, insulin like growth factor 1 receptor; RTK, receptor tyrosine kinase.

mRNA and protein levels may be associated with the early acquisition of DT resistance in *BRAF* V600E-mutated lung adenocarcinoma. Data from clinical trials show that the median duration of response to the DT combination DT is 9.8 months in previously treated patients with *BRAF* V600E-mutated NSCLC, and the clinical course of LC-6 was similar. In contrast, LC-1 showed a short response period of about 3 months and rapidly worsened, exhibiting carcinomatous pleurisy, brain metastasis, and meningitis at relapse. Thus, there may be an association with an unknown resistance mechanism involving *CDK4*.

Rudin *et al.* first reported resistance to dabrafenib in clinical specimens in *BRAF* V600E-mutated adenocarcinoma in 2013 (4), *KRAS* G12D mutations and *CDKN2A* nonsense mutations were the main causes of resistance. Previous case studies reporting acquired resistance to DT therapy in *BRAF* V600E-mutated adenocarcinoma have detected *KRAS* G12V and *NRAS* Q61K mutations. Facchinetti *et al.* (3) performed a genetic analysis of eight *BRAF* V600E-mutated lung cancers resistant to trametinib or dabrafenib and found mutations in *MEK1*, *NRAS*, *KRAS* and *PTEN* in four cases. Additionally, comparative genomic hybridization arrays were performed in the four cases in which no novel genetic mutations were identified by targeted next-generation

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sequencing, and deletions of *CDKN2A/B* were found in two of these cases. Thus, the mechanisms of resistance to *BRAF* or *BRAF*/MEK inhibition include reactivation of the RAS/MAPK pathway, supplementing signaling through other RAF family members, and activation of the PI3K/AKT pathway by *PTEN* deficiency. *CDKN2* deletion or nonsense mutations are common in many cancers, although the significance of these mutations in the acquisition of DT resistance is unclear. Reactivation of the MAPK pathway and suppression of p16ink4 lead to positive regulation of the cell cycle; however, abnormalities in *RAS* and *CDKN2* were not detected in our resistant cases.

Cyclin D-dependent kinases 4 and 6 are core proteins in the cell cycle machinery. In response to mitogenic signals, the cyclin D *CDK4*/*CDK6*/Cip/Kip complex is formed, resulting in the isolation of the Cip/Kip proteins from cyclin E-CDK2. Cyclin D- and E-dependent kinases phosphorylate Rb protein, releasing E2F, which is required for the transcription of genes necessary for S phase progression, including cyclin E itself, forming a positive feedback loop at the G1-S boundary. In malignant cells, alterations in the expression of CDK and its regulators, such as cyclin overexpression, regulate CDK activity and selectively promote proliferation (5). *CDK4/6* inhibitors inhibit cell cycle progression and shows anti-tumor effects (6). Smalley *et al.* reported *CDK4* mutations or *CCND1* amplification in *BRAF* V600E-mutated melanomas and evaluated their roles in resistance to *BRAF* inhibition (11). They found that *CCND1* amplification contributes to resistance to *BRAF* inhibition, particularly in the presence of *CDK4* mutations or overexpression. However, because LC-1 harbored only *CDK4* amplification, it is unclear whether the same resistance mechanism occurs in lung cancer.

The present *in vitro* study suggests that induction of *CDK4* protein in *BRAF* V600E mutant cells results in dabrafenib resistance. The mechanism of resistance in lung cancer includes protein mutation due to the acquisition of gene mutation of therapeutic target and expression of bypass pathway mainly by RTKs. In these cells, the absence of secondary resistance mutations in *BRAF* has been confirmed by the results of comprehensive genetic analysis. Therefore, we examined the phosphorylation of RTKs with an antibody array and did not find any specific changes. A weakness of this *in vitro* study is that ON cells are partially resistant to *BRAF* inhibitors; the inhibition of cell proliferation by dabrafenib above 100 nM was about 70%, suggesting that this is a naturally acquired function during cell line establishment.

Taken together, these findings in LC-1 suggested that tumor cells originally harboring *BRAF* V600E mutations, and *de novo* or early acquired *CDK4* amplification, selectively survived under simultaneous *BRAF*/MEK inhibition. We are currently investigating how *CDK4* contributes to the mechanism of resistance. Unfortunately, it was not possible to establish cell lines with pre-DT-treated samples. In IHC, cell blocks were used for post-DT samples because tissue biopsy was clinically difficult in these cases. Immunostaining of cell blocks may overestimate the intensity of staining. However, changes in *CDK4* staining of LC-1 were evident.

In summary, *CDK4* may be involved in the early acquisition of resistance and a new target for overcoming such resistance.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are

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appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All study participants provided informed consent and this study was approved by the Institutional Review Board of Asahikawa Medical University (No: 18185/19081). Written consent was obtained from all patients whose samples were analyzed, including pleural effusions.

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Supplementary

Appendix 1—method

List of 160 genes involved in cancer to be analyzed by GeneRead DNAseq Targeted HC panel v2

ABL1	AKT1	AKT2	ALK	AMER1	APC	AR	ARID1A	ARID2	ASXL1
ATM	ATRX	BAP1	BCL6	BCOR	BRAF	BRCA1	BRCA2	BRIP1	BTK
BUB1B	CARD11	CBL	CBLB	CD79A	CD79B	CDC73	CDH1	CDK12	CDK4
CDKN2A	CHEK2	CIC	CREBBP	CRLF2	CSF1R	CTNNB1	CYLD	DAXX	DDB2
DDR2	DICER1	DNMT3A	ECT2L	EGFR	EP300	EPCAM	ERBB2	ERBB3	ERBB4
ERCC5	ESR1	EZH2	FAM46C	FANCA	FANCD2	FANCE	FAS	FBXO11	FBXW7
FGFR2	FGFR3	FH	FLCN	FLT3	FUBP1	GATA1	GATA2	GATA3	GNA11
GNAQ	GNAS	GPC3	GRIN2A	H3F3A	HIST1H3B	HNF1A	HRAS	HSPH1	IDH1
IDH2	IKZF1	IL6ST	IL7R	JAK1	JAK2	JAK3	KDM6A	KDR	KIT
KLF6	KMT2D	KRAS	MAP2K1	MAP2K2	MAP2K4	MAP3K1	MAP4K3	MDM2	MED12
MEN1	MET	MLH1	MSH2	MSH6	MTOR	MUTYH	MYC	MYD88	NF1
NF2	NFE2L2	NFKBIA	NOTCH1	NOTCH2	NPM1	NRAS	PALB2	PAX5	PBRM1
PDGFRA	PHF6	PIK3CA	PIK3R1	PMS2	PPP2R1A	PRDM1	PRKAR1A	PTCH1	PTEN
PTPN11	RAC1	RB1	RET	ROS1	SDHB	SETD2	SF3B1	SLC7A8	SMAD4
SMARCA4	SMARCB1	SMO	SPOP	SRC	STK11	SUFU	TERT	TNFAIP3	TNFRSF14
TP53	TSC1	TSC2	TSHR	U2AF1	VHL	WT1	XPC	ZNF2	ZRSR2

Appendix 2—method

List of 49 RTKs measuring relative phosphorylation status on antibody arrays

ALK/CD246	Axl	DDR1	DDR2	Dtk	EGF R	EphA1	EphA2	EphA3	EphA4
EphA5	EphA6	EphA7	EphA10	EphB1	EphB2	EphB3	EphB4	EphB6	ErbB2
ErbB3	ErbB4	FGF R1	FGF R2 α	FGF R3	FGF R4	Flt-3/Flk-2	HGF R/c-MET	IGF-I R	Insulin R/CD220
M-CSF R	Mer	MSP R/Ron	MuSK	PDGF R α	PDGF R β	c-Ret	ROR1	ROR2	Ryk
SCF R/c-kit	Tie-1	Tie-2	TrkA	TrkB	TrkC	VEGF R1/Flt-1	VEGF R2/KDR	VEGF R3/Flt-4	

RTK, receptor tyrosine kinase.

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Table S1 Mutations and variants in patients LC-1 and LC-6

Cases	chr	Gene name	Variant type	Amino acid change	Pre-DT treatment (mutant/wild-type allele fraction)	Post-DT treatment (mutant/wild-type allele fraction)
LC-1	chr7	*BRAF*	SNP	p.V600E	0.177	0.570
	chr17	*TP53*	SNP	p.V272M	0.221	0.866
	chr1	*H3F3A*	SNP	p.R43W	0.172	0.191
	chr16	*GRIN2A*	SNP	p.N1085Y	0.174	0.590
	chr12	*HNF1A*	SNP	p.S487N	0.341	–
LC-6	chr7	*BRAF*	SNP	p.V600E	0.060	0.776
	chr1	*ARID1A*	SNP	p.S2264L	–	0.462
	chr1	*ARID1A*	SNP	p.S2269*	–	0.391

chr, chromosome; DT, dabrafenib and trametinib; SNP, single nucleotide polymorphism.

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