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The SNP rs6508974 in *AXL* is a functional polymorphism and a promising biomarker for gefitinib treatment

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Somatic mutations in epidermal growth factor receptor (*EGFR*) found in lung adenocarcinomas are used as biomarkers for the treatment with EGFR-tyrosine kinase inhibitors, including gefitinib. The bypass tracks with amplification of AXL is one of the mechanisms underlying the resistance to gefitinib. We, therefore, carried out a candidate gene approach method to identify *AXL* polymorphisms associated with the effectiveness of gefitinib. *EGFR* mutations were first identified by mutant-enriched PCR-restriction fragment length polymorphism (RFLP), and then 2 tag single nucleotide polymorphisms (SNPs) of *AXL* were examined by PCR-RFLP in 62 Japanese patients with advanced lung adenocarcinoma and treated with gefitinib in two general hospitals in Nagasaki. Subsequently, the association of *EFGR* mutations and the *AXL* polymorphism with the effectiveness of gefitinib was examined in these patients. We next examined the effect of the *AXL* polymorphism on the expression and function of this gene. It is worthy of note that *EGFR* mutations and the *AXL* polymorphism rs6508974 independently contributed to the effectiveness of gefitinib, and the polymorphism was proved to be a possible biomarker for selecting non-responders and responders to gefitinib treatment even in the absence of *EGFR* mutations. Furthermore, this SNP increased the transcriptional activity of the *AXL* transcript variant 3, one of the three *AXL* transcript variants, which to some extent increased the epithelial-mesenchymal transition in cancer cells. Taken together, *AXL* is one of the genes that determine the effectiveness of gefitinib and a biomarker for selecting non-responders and responders among lung adenocarcinoma patients with no *EGFR* mutations, suggesting that rs6508974 in *AXL* might be a functional SNP in lung adenocarcinoma.

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Key words: AXL, functional single nucleotide polymorphism, gefitinib-responsible gene, biomarker

Introduction

Lung cancer is the leading cause of cancer-related death in Japan as well as around the world. Non-small cell lung cancers (NSCLCs) account for about 80%-90% of lung cancers, and the frequency of lung adenocarcinomas among NSCLCs has been increasing recently. Molecular targeted therapy is the mainstay of treatment for lung adenocarcinomas. In particular, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are useful for the treatment of lung adenocarcinomas with somatic mutations in the tyrosine kinase domain of *EGFR*, including 9- to 18-bp deletions in exon 19 and an L858R missense mutation in exon 21[1,2].

However, drug resistance to EGFR-TKIs is a serious problem

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for targeted therapies and reduces the quality of life of patients with lung adenocarcinomas. Resistance mechanisms include (1) a second site mutation, T790M in exon 20; (2) activation of downstream signaling; (3) bypass tracks with amplification; and (4) phenotype changes, *e.g.*, epithelial-mesenchymal transition (EMT) and small cell transformation[3-5]. Among these drug resistance mechanisms, we focused on bypass tracks with amplification of hepatic growth factor, hepatocyte growth factor receptor (MET), and AXL receptor tyrosine kinase (AXL), all of which share EGFR-downstream signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)- serine/threonine kinase (AKT)- mechanistic target of rapamycin (mTOR), janus kinase (JAK)/signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase kinase (MEK)- mitogen-activated protein kinase (ERK) pathways, thereby leading to a decrease in the inhibition of the EGFR signaling pathway by EGFR-TKIs.

AXL is a receptor tyrosine kinase and a member of the tubular aggregate myopathy (TAM) family, which includes TYRO3 protein tyrosine kinase (TYRO3) and MER tyrosine kinase proto-oncogene (MERTK)[6,7]. Its high-affinity ligand is growth arrest-specific gene-6 (GAS6)[8]. By interaction with GAS6, activated AXL undergoes homodimerization or heterodimerization with non-TAM members, transduces signals to three downstream pathways (PI3K-AKT-mTOR, JAK/STAT, and MEK-ERK), and then regulates various cellular processes, including proliferation, survival, motility, and immunologic responses[9,10]. Although AXL is expressed in normal tissues, especially in bone marrow stroma and hematopoietic stem cells[11], its overexpression in a variety of tumor cells promotes tumor invasion, progression, metastasis, resistance to anticancer therapies, leading to a poor prognosis[12-14]. In particular, EMT is involved in the resistance to anticancer agents, including EGFR-TKIs[3,5,9,10,15-17].

Three transcript variants of *AXL* have been identified: transcript variant 1, NM_021913 as the reference sequence; variant 2, NM_001699; and variant 3, NM_001278599 in GenBank database. They are splice variants from the same gene, which encodes AXL proteins with different structures. The full-length *AXL* transcript variant 1 (*AXL* v1) is composed of 20 exons and encodes 2 immunoglobulin (Ig) domains (encoded by exons 2 to 3 and 4 to 5), 2 fibronectin type III (FNIII) domains (exons 6 to 7 and 8 to 9), a transmembrane domain (exon 11), and a long kinase domain (exons 12 to 20). The *AXL* transcript variant 2 lacks exon 10 encoding the extracellular domain between the second FNIII and transmembrane domains. The *AXL* transcript variant 3 (*AXL* v3) is the shortest variant that lacks exons 1 to 4 (Ig-like domain) and has a specific first exon. The differences in the expression and function of these *AXL* transcript variants remain to be elucidated. In particular, the biological significance of the *AXL* v3 lacking the Ig-like domain, which is necessary for GAS6-ligand binding, remains uncertain.

The present study examined an association between *AXL* polymorphisms and the therapeutic effectiveness of gefitinib in Japanese patients with lung adenocarcinoma to identify a new responsible gene for gefitinib. In addition, we examined the effect of the *AXL* polymorphism responsible for the efficacy of gefitinib on the expression and function of this gene as a functional polymorphism.

Methods

Patients

In this study, 62 Japanese patients with advanced lung adenocarcinoma were enrolled and treated with gefitinib from April 2007 to September 2011 at two general hospitals in Nagasaki, Japan. The demographic and clinical characteristics of the patients are shown in Table 1.

The study protocol was approved by the Ethics Committee dealing with the Human Genome and Gene Analysis at Nagasaki University as well as another hospital. Written informed consent was obtained from all patients.

Table 1. Demographic and clinical characteristics of patients with advanced lung adenocarcinoma

Characteristics	Patients
Number of patients	62
Age, mean ± SD (years)	69.6 ± 10.7
Male/female	34/28
(%)	(54.8/45.2)

Abbreviation: SD, standard deviation

Criteria concerning the therapeutic effectiveness of gefitinib

The therapeutic responses to gefitinib were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1): complete response (CR), disappearance of all target lesions and duration of at least 4 weeks; partial response (PR), at least a 30% decrease in the sum of the diameters of the target lesions and duration of at least 4 weeks; progressive disease (PD), at least a 20% increase in the sum of the diameters of the target lesions or the appearance of 1 or more new lesions; stable disease (SD),

neither sufficient shrinkage to qualify for PR nor a sufficient increase to qualify for PD[18]. The patients with CR and PR were defined as responders and those with SD and PD were defined as non-responders.

EGFR mutation analyses

Genomic DNA was extracted from fresh frozen surgical specimens, transbronchial lung biopsy specimens, pleural fluid, and bronchoalveolar lavage fluid using a QuickGene DNA Tissue Kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol. The somatic hot-spot mutations (deletions in exon 19 and L858R mutation in exon 21) of *EGFR* (OMIM #131550) were analyzed by the mutant-enriched PCR-restriction fragment length polymorphism (RFLP) method as reported previously[19].

Selection of tag single nucleotide polymorphisms (SNPs) in AXL

AXL (OMIM #109135; encoded by *AXL*), a candidate responsible gene for gefitinib treatment, is located at 19q13.2. Based on information concerning SNPs in *AXL*, the genotyped 2 tag SNPs (rs6508974 and rs2304234; r-square of 0.092) were determined according to the same methods as reported previously[20,21]. This gene yields three transcript variants. These variants' structures and locations of the genotyped tag SNPs in *AXL* are shown in Figure 1.

SNP genotyping

Genomic DNA was extracted from the peripheral blood of each patient. Genotyping of 2 tag SNPs in *AXL*, so-called germline polymorphisms, was carried out by PCR-RFLP and PCR-direct DNA sequencing according to the protocol as reported previously[20,21]. The primers used for the amplification are shown in Table 2. PCR was performed at an annealing temperature of 55°C and in a cycle number of 35. Subsequently, the PCR products were digested with *Ban* II for rs6508974 or *Hae* III for rs2304234, and genotyping was carried out based on the band pattern of electrophoresis.

Cell culture

Human hepatocellular carcinoma (HCC) cell lines, HepG2 (JCRB1054), Huh-1 (JCRB0199), Huh-7 (JCRB0403), HLE

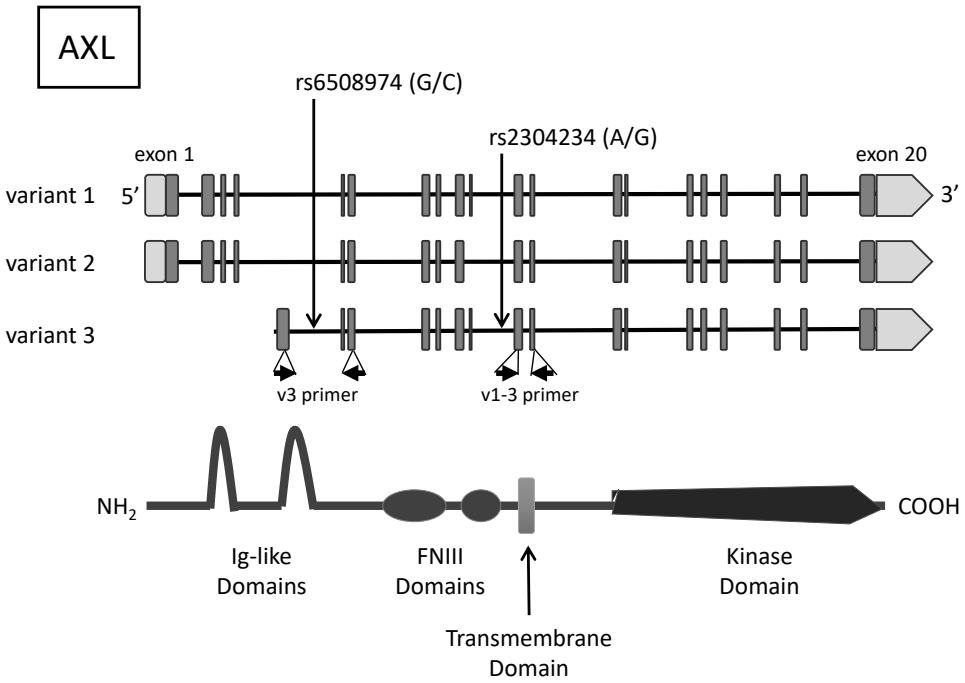

Figure 1. Location of tag SNPs and structures of *AXL* transcript variants.
The locations of the genotyped tag SNP sites (vertical arrows), primer sets for RT-PCR, genetic structure of the three *AXL* transcript variants, and functional domains of AXL are shown. In the diagram of transcript variants, boxes and lines represent exons and introns, respectively, in widths not exactly relative to the base-pair length. Boxes in dark gray show the CDS. Each exon is roughly aligned with its corresponding protein domain. Horizontal arrows in the middle represent primer sets used to detect the expression of not only the *AXL* transcript variants 1 to 3 by the v1-3 primer set, but also the *AXL* transcript variant 3 alone by the v3 primer set.

(JCRB0404), HLF (JCRB0405), and PLC/PRF/5 (JCRB0406), were purchased from JCRB Cell Bank (Osaka, Japan). These cells were cultured at 37˚C under a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium with low glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS). The human lung adenocarcinoma cell line A549 (ATCC® CCL-185) was purchased from American Type Culture Collection (Manassas, VA, USA) through Summit Pharmaceuticals International (Tokyo, Japan). Cells were cultured at 37˚C under a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution (GE Healthcare Life Sciences, Buckinghamshire, UK).

Reverse transcriptional-PCR (RT-PCR)

RNA was extracted from human HCC cells using an RNAzol® RT reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and then reverse transcribed using the PrimeScript™ RT Master Mix (Takara Bio Inc., Shiga, Japan). RNA from A549 cells was isolated using the RNAzol® RT reagent and reverse transcribed using the ThermoScript™ (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed with SYBR® Premix Ex Taq™ Tli RNaseH Plus (Takara Bio Inc.) using a LightCycler® 480 system (Roche Diagnostics, Basel, Switzerland). The primer sequences used in this study are listed in Table 2, and their locations are shown in Figure 1.

Table 2. Oligonucleotide sequences used in this study

Target		Oligonucleotide sequence (5' to 3')
PCR for polymorphisms		
rs6508974 G > C	Fw Rv	GAGGGGGGTGTCTATGTTTC ATCTGCGAAATTGTGAAGGA
rs2304234 A > G	Fw Rv	TCAGGGGGTATGAGGAACAG GCATCCATCACACACCTCAT
Quantitative PCR		
AXL transcript variants 1-3	Fw Rv	CTTGGCTCTCTTCCTTGT TCCGACGACTGTAGGACTTG
AXL transcript variant 3	Fw Rv	CTGCAGTCGCACTTACAAGAC CAGTGGGTCAGGGGGTAGAT
CDH1	Fw Rv	AAGTGCTGCAGCCAAAGACAGA AAATTGCCAGGCTCAATGACAAG
VIM	Fw Rv	GGTGGACCAGCTAACCAACGA TCAAGGTCAAGACGTGCCAGA
SNAI2	Fw Rv	AAAAGCCAAACTACAGCGAACTG AGAATCTCTGCTTGTGGTATGACA
GAPDH	Fw Rv	CATCACCATCTTCCAGGAGCG TGACCTTGCCCACAGCCTTG
Reporter plasmid construction		
v3 promoter	Fw Rv	TTTAGATCTTTGCGGTAAAGCAAGAACCT TTTAGATCTGAGGAGAGTCTCACCACGTT
rs6508974 fragment G > C	SS (G) AS (G)	CATACGTTCTCTAAGAGCCCCCACAAAAGCCTG CTAGCAGGCTTTTGTGGGGGCTCTTAGAGAACGTATGGTAC
	SS (C) AS (C)	CATACGTTCTCTAAGACCCCCCACAAAAGCCTG CTAGCAGGCTTTTGTGGGGGGTCTTAGAGAACGTATGGTAC
Expression plasmid construction		
1st PCR	Fw (v1) Fw (v3)	ATGGCGTGGCGGTGCCCCAG ATGGGCATCCAGGCGGGAGA
2nd PCR	Fw (v1) Fw (v3)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCGTGGCGGTGCCCCAGGATG GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGCATCCAGGCGGGAGAACCAG

Abbreviations: Fw, forward; Rv, reverse; SS, sense strand; AS, antisense strand; v1, the *AXL* transcript variant 1; v3, the *AXL* transcript variant 3

Plasmid DNA construction

To construct a pGL3-v3 promoter plasmid, an 894-bp *AXL* v3 promoter fragment including exon 1 of the *AXL* v3 and its upstream flanking regions was amplified from genomic DNA of HepG2 cells by PCR with a v3 promoter primer set (Table 2) using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The amplified DNA was digested with *Bgl* II and integrated into the pGL3-Basic Vector plasmid (Promega, Madison, WI, USA) at the *Bgl* II restriction site using a Rapid DNA Ligation Kit (Roche Diagnostics), as shown in Figure 2a.

In order to construct a reporter plasmid carrying the *AXL* v3 promoter and a polymorphic fragment of rs6508974, double-stranded oligonucleotides (Table 2), including this SNP, flanking sequences, and additional sticky ends of *Kpn* I or *Mlu* I, were inserted into the *Kpn* I and *Mlu* I site of the upstream region of *AXL* v3 promoter of the pGL3-v3 promoter plasmid, as shown in Figure 3a.

Expression plasmids of the *AXL* v1 or v3 were constructed via Gateway Technology™ (Thermo Fisher Scientific). In brief, the *AXL* v1 or v3 fragment was amplified from the cDNA library of A549 cells by nested-PCR using Phusion® High-Fidelity DNA Polymerase with each primer set, as described in Table 2. The obtained PCR product was inserted into a pDONR221 vector (Thermo Fisher Scientific) by the BP reaction using BP Clonase™ (Thermo Fisher Scientific). The inserted *AXL* fragment was further transferred from a pDONR221 to a pDEST26 expression vector (Thermo Fisher Scientific) by the LR reaction using LR Clonase™ (Thermo Fisher Scientific).

Reporter gene assay

For the reporter gene analyses, on the day before the transfection, 2.5×10^5 HepG2 cells were plated in each well of a 24-well plate in 500 μL of the culture medium. The constructed reporter plasmids (500 ng) were co-transfected with 20 ng of the Renilla luciferase plasmid pRL-SV40 (Promega) using 1.75 μL of the FuGENE® HD Transfection Reagent (Promega). Firefly and Renilla luciferase activities were measured 48 h after the transfection with the Dual Luciferase Assay system (Promega). The activity of the firefly luciferase was normalized to that of the Renilla luciferase.

Forced expression of AXL v1 or v3

To compare the functional activities of the *AXL* transcript variants, the expression plasmid of the *AXL* v1 or v3 was transfected into A549 cells. On the day before the transfection, 8.0×10^4 cells were plated in each well of a 24-well plate in 500 μL of culture medium. The expression plasmids (500 ng) were transfected into the cells using 1.5 μL of the Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific) with 1.0 μL of the P3000 reagent. Forty-eight hours after the transfection, cells were harvested and used for RNA extraction.

Subsequently, to examine whether each the *AXL* transcript variant promotes EMT as an oncogene, the expression of EMT-associated genes, *SNAI2* (encodes SLUG, a central regulator of EMT), *VIM* (encodes Vimentin, a marker for EMT in mesenchymal tissues), and *CDH1* (encodes E-cadherin, a marker of epithelial cells), in A549 cells transfected with the *AXL* v1 or v3 clone was determined by RT-PCR using each primer set, as described in Table 2.

Statistical analyses

Differences in the effectiveness of gefitinib between patients with and without *EGFR* mutations were evaluated by chi-square test using the Prism 5 software program (GraphPad Software Inc., San Diego, CA, USA).

To determine whether each SNP was in the Hardy-Weinberg equilibrium, chi-square test was performed based on the expectation-maximization algorithm using the SNP Alyze® ver. 7.1 standard software package (Dynacom Inc., Yokohama, Japan). The frequencies of alleles and genotypes of each SNP between responders and non-responders to gefitinib were compared by chi-squared or Fisher's exact test with odds ratio (OR) and 95% confidence interval in three different inheritance models: the minor allele, the minor allele dominant, and the minor allele recessive, without a multiple test, using the SNPAlyze® ver.7.1 standard software package.

In addition to univariate analyses, multivariate logistic regression analyses were also conducted to assess the gene-gene interaction of two genetic factors—*EGFR* mutations and *AXL* polymorphism—with the effectiveness of gefitinib using the JMP Pro 14 software program (SAS Institute Inc., Tokyo, Japan).

Finally, the data concerning the gene expression and reporter gene activity are indicated as the mean ± standard deviation. Significance was evaluated using unpaired Student's *t*-test for comparing two groups and Tukey's test for comparing three groups using the Prism 5 software program. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Association between EGFR mutations and the effectiveness of gefitinib in lung adenocarcinoma patients

The existence of somatic mutations in *EGFR* was confirmed in 40 (64.5%) out of 62 patients treated with gefitinib. Of these 40 patients, 25 had deletions in exon 19 (40.3%), and 23 had an L858R mutation in exon 21 (37.1%). Therefore, 8 patients (12.9%) had both mutations of *EGFR*. Furthermore, 23 patients (57.5%) were responders to gefitinib. Chi-square test revealed that *EGFR* mutations were positively associated with the effectiveness of gefitinib at an OR of 4.81 (P = 0.0005; data were not shown).

Association of AXL polymorphisms with the effectiveness of gefitinib in lung adenocarcinoma patients

The frequencies of alleles and genotypes of 2 tag SNPs (rs6508974 and rs2304234) in *AXL* as germline polymorphisms were identified and compared between responders (n = 29) and non-responders (n = 33) as shown in Table 3. The distributions of all SNPs in this study corresponded well to the Hardy-Weinberg equilibrium.

The frequency of a C allele of rs6508974 in *AXL* in the minor allele model was significantly lower in responders than that in non-responders (22.4% vs. 39.4%, P = 0.042, OR = 0.444; Table 3), thereby indicating a 2.3-fold resistance to gefitinib.

The frequency of a G/C or C/C genotype of rs6508974 in *AXL* in the minor allele dominant model was significantly lower in responders than that in non-responders (37.9% *vs*. 63.7%, P = 0.042, OR = 0.349; Table 3), thereby indicating a 2.9-fold resistance to gefitinib. Conversely, the possession of a G/G genotype of rs6508974 in *AXL* indicated a 2.9-fold response to gefitinib.

There were no significant differences in the frequencies of alleles or genotypes of rs2304234 in three inheritance models between responders and non-responders.

Association of the EGFR mutations plus AXL polymorphism with the effectiveness of gefitinib in lung adenocarcinoma patients

Multivariate logistic regression analyses revealed that two factors, *EGFR* mutations (somatic mutations) plus the G/G genotype of rs6508974 in *AXL* (germline polymorphism), were independently correlated with the effectiveness of gefitinib (OR = 5.193, P = 0.013 and OR = 4.665, P = 0.017, respectively), as shown in Table 4.

There were no significant differences in the frequencies of genotypes of rs6508974 in *AXL* among the *EGFR* mutation-positive patients with lung adenocarcinoma (P = 0.554; Table 5). In contrast, among the *EGFR* mutation-negative patients, the possession of the G/G genotype of rs6508974 in *AXL* was associated with responses to gefitinib by 15-fold (OR = 15.000, P = 0.023), as shown in Table 6. By contrast, the possession of the G/C or C/C genotype of rs6508974 was associated with the resistance to gefitinib by 15-fold.

Furthermore, a genetic test using rs6508974 in *AXL* as a biomarker among the *EGFR* mutation-negative patients with lung adenocarcinoma showed a sensitivity of 83.3%, a specificity of 75.0%, a positive predictive value of 55.6%, and a negative predictive value of 92.3%, as shown in Table 6.

Table 3. Association of *AXL* genetic polymorphisms with response to gefitinib in lung adenocarcinoma patients

SNP	Genotype	Number (%) of patients		Inheritance model	OR（95% CI)	P value
		Responders n = 29	Non-responders n = 33			
rs6508974	C allele*	13 (22.4)	26 (39.4)	Allele	0.444 (0.202 – 0.980)	0.042
	G/G	18 (62.1)	12 (36.4)			
	G/C	9 (31.0)	16 (48.5)	Dominant	0.349 (0.124 – 0.980)	0.042
	C/C	2 (6.9)	5 (15.2)	Recessive	0.415 (0.074 – 2.323)	0.433
rs2304234	G allele*	13 (22.4)	25 (37.9)	Allele	0.474 (0.215 – 1.047)	0.062
	A/A	17 (58.6)	12 (36.4)			
	A/G	11 (37.9)	17 (51.5)	Dominant	0.403 (0.145 – 1.124)	0.079
	G/G	1 (3.4)	4 (12.1)	Recessive	0.259 (0.027 – 2.462)	0.360

C allele* and G allele* indicate the number of minor alleles of rs6508974 and rs2304234, respectively, but not genotypes.
Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval

Table 4. Gene-gene interaction between two factors for response to gefitinib in patients with lung adenocarcinoma

Factor	Factor comparison*	
	OR (95% CI)	*P* value
EGFR mutation status	5.193 (1.409 – 19.149)	0.013
G/G genotype of rs6508974 in *AXL*	4.665 (1.323 – 16.453)	0.017

*Factors were statistically analyzed by multivariate logistic regression analysis.
Abbreviations: OR, odds ratio; CI, confidence interval

Table 5. Association of *AXL* polymorphism with response to gefitinib in the *EGFR* mutation-positive patients with lung adenocarcinoma

Genotype of rs6508974 in *AXL*	Number (%) of patients		OR（95% CI)	*P* value*
	Responders *n* = 23	Non-responders *n* = 17		
G/G	13 (56.5)	8 (47.1)	1.463 (0.415 – 5.156)	0.554
G/C or C/C	10 (43.5)	9 (52.9)		

*Genotypes were statistically analyzed by chi-square test.
Abbreviations: OR, odds ratio; CI, confidence interval

Table 6. Association of *AXL* polymorphism with response to gefitinib in the *EGFR* mutation-negative patients with lung adenocarcinoma

Genotype of rs6508974 in *AXL*	Number (%) of patients		OR（95% CI)	*P* value*
	Responders *n* = 6	Non-responders *n* = 16		
G/G	5 (83.3)	4 (25.0)	15.000 (1.324 – 170.000)	0.023
G/C or C/C	1 (16.7)	12 (75.0)		

*Genotypes were statistically analyzed by Fisher's exact test.
Abbreviations: OR, odds ratio; CI, confidence interval

Effect of the AXL v3 promoter on the expression of the gene

The *AXL* polymorphism rs6508974, which has been shown to be associated with responses to gefitinib in lung adenocarcinoma, is located within the middle part of intron 1 of the *AXL* v3 (Figure 1). We, therefore, examined the effect of the polymorphism on the expression of transcript v3. Since the *AXL* v3 (ENST00000593513.1) is expressed in normal tissues only at a marginal level according to the exon expression analysis data in GTEx (dbGaP Accession, phs000424.v8.p2; https://www.gtexportal.org/home/), we set out to determine which cancer cell lines express the *AXL* transcript variants. Although the data were not shown, HepG2, A549, and Huh-1 cells expressed a relatively higher level of the *AXL* v3. We, therefore, used HepG2 and A549 cells for the subsequent experiments.

To confirm the transcriptional activity of the *AXL* v3 promoter, a reporter gene plasmid containing the v3 promoter of a size of 894-bp was constructed (Figure 2a), which was transfected into HepG2 cells to yield a reporter cell line. The transcriptional activity of the cell line with v3 promoter was by 200 times higher than that with an empty vector ($P < 0.001$), as shown in Figure 2b. The results demonstrated that the *AXL* v3 promoter functions in some cancers.

Effect of the AXL polymorphism on the transcription of the gene

It has been reported that some functional SNPs located in the promoter regions affect the transcriptional activity of neighbor (sometimes distant) genes. Based on annotations of the non-coding variants available in the webtool HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)[22], the SNP rs6508974 associated with the effectiveness of gefitinib is located in the possible promoter region of *AXL* and thus may alter the transcription of the gene. We thus set

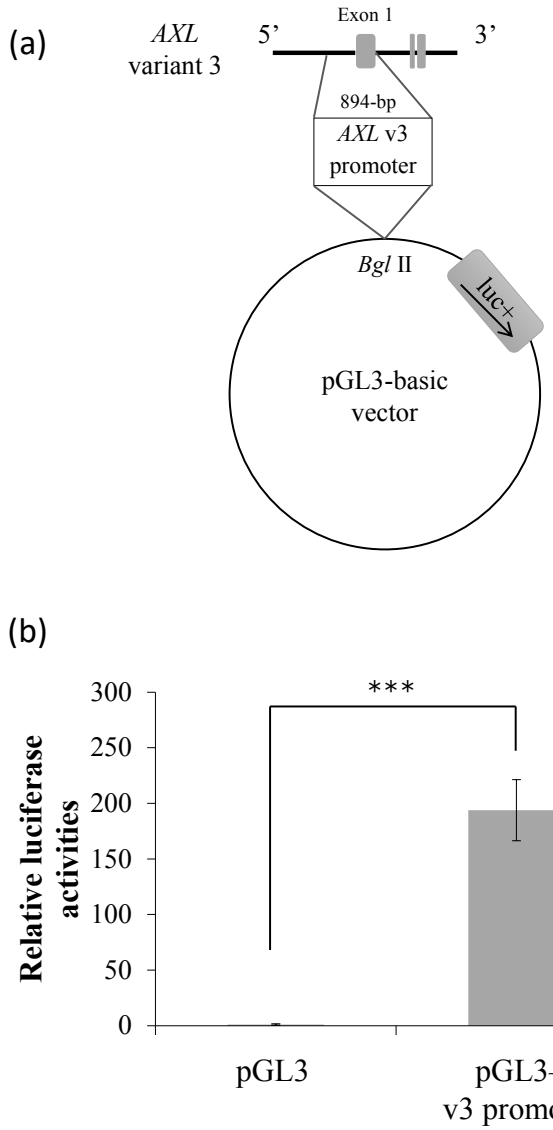

Figure 2. Transcriptional activity of the reporter gene carrying the v3 promoter in HepG2 cells.
The structure of the reporter plasmid including the first exon of the *AXL* transcript variant 3 and its upstream promoter region (894 bp) is shown (a). The results of the reporter gene assay using the pGL3-v3 promoter plasmid or pGL3-Basic empty vector plasmid in HepG2 cells are shown (b). Data presented as the mean ± standard deviation ($n = 4$). ***$P < 0.001$ by unpaired Student's *t*-test.

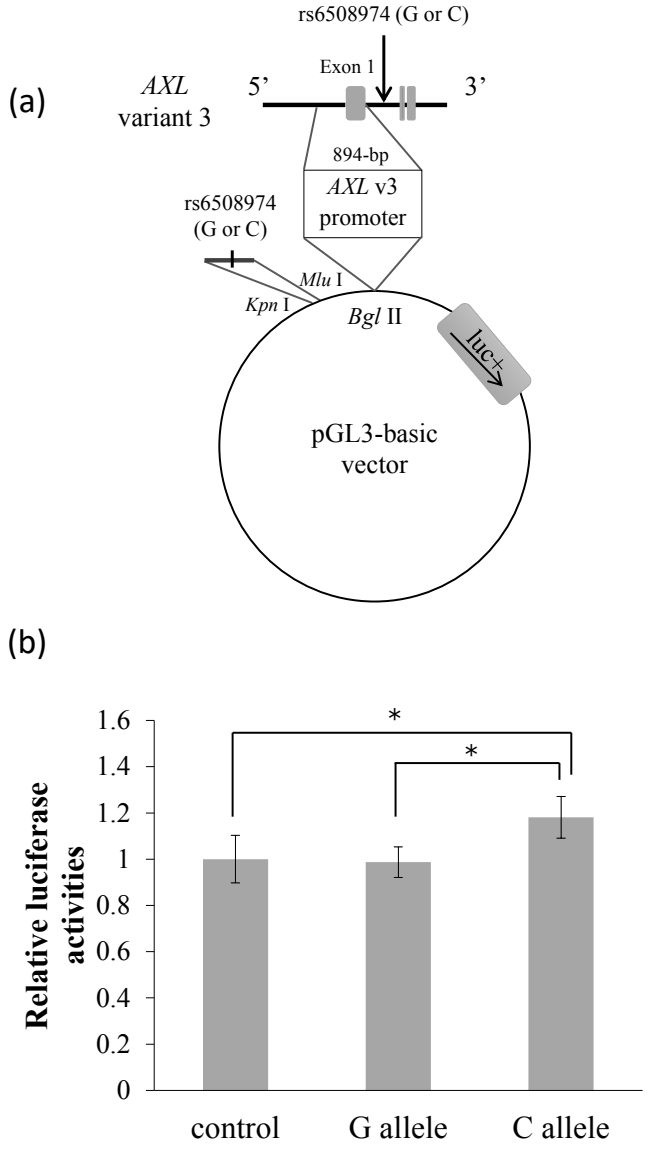

Figure 3. Transcriptional activities of the v3 promoter plasmids including each allele of rs6508974 in HepG2 cells.
Both the fragments of the v3 promoter fragment and each allele of rs6508974 were inserted into the separated sites of the reporter plasmid (a). The result of the reporter gene assay using the above plasmids including a G or C allele of rs6508974 in HepG2 cells are shown (b). Data are presented as the mean ± standard deviation ($n = 4$). *$P < 0.05$ by Tukey's test.

out to determine the transcriptional activity of the v3 promoter containing the SNP in HepG2 cells.

A short polymorphic fragment including rs6508974 was inserted into the upstream region of the pGL3-v3 promoter plasmid of the reporter gene (Figure 3a). The reporter gene assay revealed that the relative luciferase activities of the plasmid carrying the C allele of s6508974 were significantly higher than those of the G allele as well as the v3 promoter alone as a control ($P < 0.05$, respectively), as shown in Figure 3b. These results were consistent with those of the study showing that the C allele of s6508974 in *AXL* were associated with the resistance to gefitinib (Table 3).

Forced expression of the AXL v3 induced EMT

When A549 cells were transformed with the *AXL* v3 transcript, the expression of *SNAI2* was significantly increased, compared to that transfected with an empty vector ($P = 0.020$; Figure 4a).

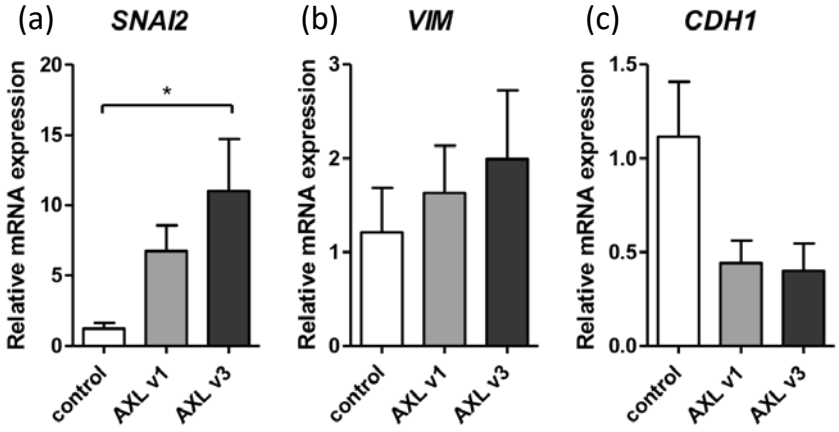

Figure 4. The expression of EMT-associated genes in A549 cells after the forced expression of each *AXL* variant.
The expression of EMT-associated genes, *SNAI2* (encodes SLUG; a), *VIM* (encodes Vimentin; b), and *CDH1* (encodes E-cadherin; c), in A549 cells transfected with the forced expression of the *AXL* transcript variant 1 or the *AXL* transcript variant 3 are shown. An empty vector (pDEST26) was used as a control. Data are presented as the mean ± standard deviation ($n = 4$). *$P < 0.05$ by Tukey's test.

The *AXL* v1 also seemed to increase the expression of *SNAI2*, but not statistically significant in multiple tests (Figure 4a). There were no statistically significant differences in the expression of *VIM* or *CDH1* in the cells that had been transfected with the *AXL* v1 or v3, when compared to those with a control plasmid (Figures 4b and 4c).

Discussion

This is the first report to show that two genetic factors—hot-spot mutations of *EGFR* (somatic mutations) and rs6508974 of *AXL* (germline polymorphism)—independently contributed to the effectiveness of gefitinib in Japanese patients with lung adenocarcinoma. In fact, the patients with not only the deletions in exon 19 and an L858R mutation in exon 21 in *EGFR*, but also the G/G genotype of rs6508974 in *AXL* showed preferable responses to gefitinib. From the pathophysiological perspective of the gefitinib-based therapy, the possession of the G/G genotype of rs6508974 in *AXL* may decrease the expression and function of *AXL* in the genetic background, thereby leading to the diminished signal transduction in the intracellular downstream pathway of AXL, as well as to a decrease in crosstalk signal transduction of the three shared pathways (PI3K-AKT-mTOR, JAK/STAT, and MEK-ERK) in the EGFR signaling pathway. Therefore, both the suppression of the EGFR signaling pathway by gefitinib therapy and the diminished signaling in the AXL pathway due to this polymorphism in the genetic background may result in preferable responses to gefitinib.

In contrast, the possession of the G/C or C/C genotype of rs6508974 in *AXL* may increase the expression and function of AXL in the genetic background, thereby leading to the acceleration of signal transduction in the AXL pathway. This accelerated AXL signaling pathway may result in an increase in crosstalk signal transduction of the three shared pathways in the EGFR signaling pathway, thereby leading to efficient signal transduction in the EGFR signaling pathway, even if the EGFR signaling pathway is inhibited by gefitinib in lung adenocarcinoma patients. As a gefitinib-resistance mechanism, elevated crosstalk signal transduction of the EGFR signaling pathway through the activation of the AXL signaling pathway due to this polymorphism in the genetic background may dominate over the suppression by gefitinib of the downstream signal transduction of the EGFR signaling pathway. Thus, patients under such conditions may eventually be non-responders to gefitinib.

Besides the pathophysiological perspective, the *AXL* polymorphism, rs6508974 might be useful as a prognostic biomarker for selecting non-responders to gefitinib. In patients with no mutations of *EGFR* and the G/C or C/C genotype of rs6508974 in *AXL*, the negative predictive value was 92.3%. Furthermore, it is intriguing that about half of patients with the G/G genotype of rs6508974 in *AXL* showed preferable responses to gefitinib, with the positive predictive value of 55.6%, even in the absence of *EGFR* mutations (Table 6). Based on the findings in this genetic test, therefore, we might be able to employ the SNP rs6508974 as a new biomarker for the gefitinib therapy in lung adenocarcinoma patients with no *EGFR* mutations. Patents with the G/C or C/C

genotype of rs6508974 in *AXL* should not be treated with gefitinib. On the contrary, patients with the G/G genotype of rs6508974 in *AXL* may benefit from the gefitinib therapy even in the absence of *EGFR* mutations, as about half of these patients showed preferable responses to gefitinib.

Finally, we emphasize that the *AXL* v3, one of the *AXL* transcript variants, might act as an oncogene in lung adenocarcinoma. Whereas this transcript lacks the Ig-like domain necessary for the GAS6-ligand binding, the ligand-independent homodimerization or heterodimerization with or without GAS6 reportedly induces the activation of AXL-dependent downstream signaling[23,24]. Furthermore, the SNP rs6508974 located in the middle part of intron 1 might be a functional SNP and an enhancer to increase the promoter activity of the *AXL* v3. In addition to the role as an enhancer, the annotation of the variant on HaploReg v4.1 revealed that rs6508974 could alter regulatory motifs of GLI family zinc finger (GLI) zinc transcription factors. The C allele of this SNP is predicted to have a stronger binding affinity to GLI family proteins than the G allele. The expression of GLI family proteins have been associated with the progression and a poor prognosis of lung cancers[25,26]. The binding of GLI proteins to this SNP site may, therefore, accelerate the expression of certain oncogenes, including the *AXL* v3. Since the forced expression of the *AXL* v3 in A549 cells upregulated the expression of *SNAI2*, an EMT-associated gene upregulated by AXL protein[27], the *AXL* v3 might partially act as an oncogene, possibly leading to the resistance to gefitinib. However, as the precise contribution of the *AXL* v3 to EMT still remains unknown, the further investigation is necessary.

Conclusions

The SNP rs6508974 associated with the effectiveness of gefitinib is likely to be a functional SNP within the enhancer of the *AXL* v3 as well as the binding site of GLI proteins. However, further investigations regarding *AXL* polymorphisms and their functions should be required for the establishment of reliable biomarkers for the gefitinib-based therapy of lung adenocarcinoma.

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Disclosure of Conflicts of Interest

There are no conflicts of interest to declare.

References

1. Pao W, Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* 23:2556-2568, 2005.
2. Sharma SV, Bell DW, Settleman J, et al. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 7:169-181, 2007.
3. Zhang Z, Lee JC, Lin L, et al. Activation of the *AXL* kinase causes resistance to *EGFR*-targeted therapy in lung cancer. Nat Genet 44:852-860, 2012.
4. Yu HA, Riely GJ, Lovly CM. Therapeutic strategies utilized in the setting of acquired resistance to *EGFR* tyrosine kinase inhibitors. *Clin Cancer Res* 20:5898-5907, 2014.
5. Huang L, Fu L. Mechanisms of resistance to *EGFR* tyrosine kinase inhibitors. *Acta Pharm Sin B* 5:390-401, 2015.
6. O'Bryan JP, Frye RA, Cogswell PC, et al. *AXL*, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Mol Cell Biol* 11:5016-5031, 1991.
7. Janssen JWG, Schulz AS, Steenvoorden ACM, et al. A novel putative tyrosine kinase receptor with oncogenic potential. *Oncogene* 6:2113-2120, 1991.
8. Varnum BC, Young C, Elliott G, et al. *AXL* receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature* 373:623-626, 1995.
9. Paccez JD, Vogelsang M, Parker MI, et al. The receptor tyrosine kinase *AXL* in cancer: biological functions and therapeutic implications. *Int J Cancer* 134:1024-1033, 2014.
10. Gay CM, Balaji K, Byers LA. Giving *AXL* the axe: targeting *AXL* in human malignancy. *Br J Cancer* 116:415-423, 2017.
11. Neubauer A, Fiebeler A, Graham DK, et al. Expression of *AXL*, a transforming receptor tyrosine kinase, in normal and malignant hematopoiesis. *Blood* 84:1931-1941, 1994.
12. Shieh Y-S, Lai C-Y, Kao Y-R, et al. Expression of *AXL* in lung adenocarcinoma and correlation with tumor progression. *Neoplasia* 7:1058-1064, 2005.
13. Hsieh M-S, Yang P-W, Wong L-F, et al. The *AXL* receptor tyrosine kinase is associated with adverse prognosis and distant metastasis in esophageal squamous cell carcinoma. *Oncotarget* 7:36956-36970, 2016.
14. Wu X, Ma W, Zhou Q, et al. *AXL*-GAS6 expression can predict for adverse prognosis in non-small cell lung cancer with brain metastases. *J Cancer Res Clin Oncol* 143:1947-1957, 2017.
15. Schoumacher M, Burbridge M. Key roles of *AXL* and MER receptor tyrosine kinases in resistance to multiple anticancer therapies. *Curr Oncol Rep* 19:19, 2017.
16. Antony J, Huang RY-J. *AXL*-driven EMT state as a targetable conduit in cancer. *Cancer Res* 77:3725-3732, 2017.
17. Tulchinsky E, Demidov O, Kriajevska M, et al. EMT: a mechanism for escape from *EGFR*-targeted therapy in lung cancer. *Biochim Biophys Acta Rev Cancer* 1871: 29-39, 2019.
18. Eisenhauer EA, Therasse P, Bogaerts L, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 45:228-247, 2009.
19. Ikeda T, Nakamura Y, Yamaguchi H, et al. Direct comparison of 3 PCR methods in detecting *EGFR* mutations in patients with advanced non-small-cell lung cancer. *Clin Lung Cancer* 13:369-374, 2012.
20. Urabe S, Isomoto H, Ishida T, et al. Genetic polymorphisms of *IL-17F* and *TRAF3IP2* could be predictive factors of the long-term effect of infliximab against Crohn's disease. *Biomed Res Int* 416838, 2015.
21. Yamashita A, Inamine T, Suzuki S, et al. Genetic variants of *SMAD2/3/4/7*

are associated with susceptibility to ulcerative colitis in a Japanese genetic background. *Immunol Lett* 207:64-72, 2019.
22. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res* 40:D930-D934. 2012.
23. Meyer AS, Miller MA, Gertler FB, et al. The receptor *AXL* diversifies *EGFR* signaling and limits the response to *EGFR*-targeted inhibitors in triple-negative breast cancer cells. *Sci Signal* 6:ra66, 2014.
24. Vouri M, Croucher DR, Kennedy SP, et al. *AXL-EGFR* receptor tyrosine kinase hetero-interaction provides *EGFR* with access to pro-invasive signalling in cancer cells. *Oncogenesis* 5:e266, 2016.
25. Ishikawa M, Sonobe M, Imamura N, et al. Expression of the *GLI* family genes is associated with tumor progression in advanced lung adenocarcinoma. *World J Surg Oncol* 12:253, 2014.
26. Cui Y, Cui C-A, Yang Z-T, et al. Gli1 expression in cancer stem-like cells predicts poor prognosis in patients with lung squamous cell carcinoma. *Exp Mol Pathol* 102:347-353, 2017.
27. Lee Y, Lee M, Kim S. Gas6 induces cancer cell migration and epithelial-mesenchymal transition through upregulation of MAPK and Slug. *Biochem Biophys Res Commun* 434:8-14, 2013.