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Lack of Association between the *BIM* Deletion Polymorphism and the Risk of Lung Cancer with and without *EGFR* Mutations

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Introduction: The *BIM* deletion polymorphism in intron 2 was found in a significant percent of the Asian population. Patients with epidermal growth factor receptor (*EGFR*) mutant lung cancers harboring this *BIM* polymorphism have shorter progression free survival and overall response rates to EGFR tyrosine kinase inhibitors. However, the association between the *BIM* deletion polymorphism and lung cancer risk is unknown.

Methods: The *BIM* deletion polymorphism was screened by polymerase chain reaction in 765 lung cancer cases and 942 healthy individuals.

Results: Carriers possessing one allele of the *BIM* polymorphism were observed in 13.0% of control cases and 12.8% of lung cancer cases, similar to incidence rates reported earlier in healthy individuals. Homozygote for the *BIM* polymorphism was observed in four of 942 healthy controls and three of 765 lung cancer cases. The frequency of the *BIM* deletion polymorphism in lung cancer patients was not related to age, sex, smoking history, or family history of lung cancer. The *BIM* deletion polymorphism was found in 30 of 212 patients with *EGFR* wild type lung cancers and 16 of 120 patients with *EGFR* mutant lung cancers. The frequency of the *BIM* polymorphism is similar between cancers with wild type *EGFR* and mutated *EGFR* ($p = 0.78$).

Conclusion: The *BIM* deletion polymorphism was not associated with lung cancer susceptibility. Furthermore, the *BIM* polymorphism is not associated with *EGFR* mutant lung cancer.

Key Words: BIM polymorphism, Lung cancer, Susceptibility, *EGFR* mutation.

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Lung cancer is a leading cause of cancer death in developed countries. Loss of apoptosis is critical for both tumorigenesis and resistance to drug therapies. The BCL-2 family member proteins play important roles in regulating apoptosis in response to a wide variety of cellular signals, including DNA damage and growth factor withdrawal.[1,2] The BCL-2 family consists of three subfamilies[1,2]: pro-survival members (e.g., BCL-2 and MCL1), pro-apoptotic members (i.e., BCL-2 homology domain 3 [BH3]-only proteins including BIM and PUMA, and the pro-apoptotic BAX and BCL-2 antagonist/killer [BAK]). BIM is a member of the BH3-only proteins that binds and neutralizes the anti-apoptotic BCL2 family members, as well as directly activating BAX and BAK to induce apoptosis. In a number of different cancer types, both in vitro and in vivo studies have evidenced that BIM is essential for apoptosis following targeted therapy administration.[2–10]

Activating mutations in the epidermal growth factor receptor (*EGFR*) renders EGFR the primary driver oncogene in lung cancer. *EGFR* tyrosine kinase inhibitors (EGFR-TKIs) have provided significant survival benefit in patients harboring *EGFR* mutations. However, these studies have indicated that 20 to 40% of patients are primarily resistant to EGFR-TKIs.[11–13] In oncogene addicted cancers like *EGFR* mutant lung cancers, survival signals derived from the oncogene regulate the expression and the interaction of BCL-2 family members. In particular, BIM is a key mediator of apoptosis in response to EGFR-TKIs.[3–5,10,14–16] EGFR-TKIs downregulate MAPK signaling that leads to upregulation of BIM expression in these cancers. Importantly, several studies have shown that low levels of pretreatment, functional BIM in tumor cell lines and patients' tumors is related to a mitigated apoptotic

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response and lack of efficacy following EGFR-TKI treatment.[4,5,10,15–21] Although degradation of BIM is mainly regulated by MAPK signaling, a number of other mechanisms, such as alternative splicing, transcriptional and posttranscriptional regulation, posttranslational modification, and epigenetic silencing also effects BIM expression.[18] Recently, paired-end DNA sequencing identified a deletion polymorphism in *BIM*.[14] This polymorphism is located in intron 2 of the *BIM* gene that results in the expression of BIM isoforms lacking BH3 domain. This polymorphism is commonly found in the East Asian population yet absent in the Caucasian population. Intriguingly, lung cancer patients harboring this *BIM* germ line polymorphism have shorter progression free survival (PFS) to EGFR-TKIs[14,19–22] thus contributing substantially to primary resistance. Tests to identify the *BIM* polymorphism in the clinic are being developed. Given the clinical significance of the *BIM* polymorphism, we sought to investigate the relationship between this *BIM* polymorphism and the risk to develop lung cancer in general and lung cancer specifically with *EGFR* activating mutations.

PATIENTS AND METHODS

Subjects

All subjects were first-visit outpatients at the Aichi Cancer Center Hospital (ACCH) aged 18 to 79 who gave written informed consent for enrollment in the Hospital-based Epidemiological Research Program at Aichi Cancer Center (HERPACC) during 2001–2005. Information on lifestyle factors was collected using a self-administered questionnaire, checked by a trained interviewer. The outpatients were also asked to provide blood samples. Approximately 95% of eligible subjects completed the questionnaire and 60% provided blood samples. Details of this program have been described elsewhere.[23,24] The lung cancer cases consisted of 765 patients who were newly and histologically diagnosed as having lung cancer. Controls (n = 942) were randomly selected from outpatients who completed the questionnaire, provided blood samples, and were confirmed cancer free.[25] The study protocol was approved by the ethics committee of Aichi Cancer Center and complied with the declaration of Helsinki.

Genotyping of the BIM Deletion Polymorphism and Other Polymorphisms

DNA of each subject was extracted from the buffy coat fraction using a DNA Blood mini kit (Qiagen, Tokyo, Japan) for the use of genotyping. Primers detecting wild type *BIM* and *BIM* deletion polymorphism were developed previously.[26] The primer sequences are F: 5′-CCACCAATGGAAAAGGTTCA-3′, R: 5′-CTGTCATTT CTCCCCACCAC-3′ for detecting wild-type *BIM* and F: 5′-CTGTCATTTCTCCCCACCAC-3′, R: 5′- GGCACAGCC TCTATGGAGAA-3′ for identifying the *BIM* deletion polymorphism. The primer pairs yield a 362 bp and 284 bp of PCR products, respectively. Screening was performed by primer sets identifying the *BIM* deletion polymorphism. DNA from PC-3 cells, known to harbor the *BIM* deletion polymorphism, was used as a positive control. Positive samples were then determined to be homozygote or heterozygote by performing PCR with both primer sets. In addition to the *BIM* deletion polymorphism, genotyping data on 14 other polymorphisms (rs2289321, rs1439287, rs2015454, rs1837369, rs17041869, rs13396983, rs1877330, rs724710, rs3789068, rs17041887, rs616130, rs13405741, rs726430, rs9308742) that locate ±30,000-bp to the *BIM* polymorphism was adopted from previously genotyped data by an Illumina Human 610-Quad BeadChip (Illumina, San Diego, CA). Briefly, 576,736 SNP markers were examined at the Center for Genomic Medicine of Kyoto University Graduate School of Medicine. After removing SNPs that failed the quality control criteria (Hardy–Weinberg equilibrium $p < 1 \times 10^{-6}$ [excluded SNPs: n = 277]; SNP call rate > 0.95 [n = 2921]; and minor allele frequency [MAF] < 0.01 [n = 82,414]), 491,738 markers were selected as a source for this analysis (some SNPs were excluded based on two or more criteria).

Assessment of Smoking and Fruits and Green–Yellow Vegetable Intake

All exposures were assessed from the self-administered questionnaire, as completed at the first visit to ACCH before the diagnostic procedure was conducted. Subjects were questioned specifically about their lifestyle before the onset of the symptoms that prompted their visit to ACCH. Smoking status was divided into three categories: never, former, and current. Former smokers were defined as those who quit smoking at least 1 year before the time of the survey. The intake of fruits and green–yellow vegetables was determined using a food frequency questionnaire (FFQ), described in detail elsewhere.[25] Briefly, FFQ enables estimating quantity of intake by the information of frequency of the intake in eight categories: never or seldom, 1 to 3 times/month, 1 to 2 times/week, 3 to 4 times/week, 5 to 6 times/week, once/day, twice/day, and three or more times/day. The intake was adjusted for total energy intake, and was classified into tertiles.

Clinicopathological Information

Clinicopathological information was obtained by linking clinical cohort data[27] with HERPACC database. Pathological staging was based on UICC version 7. Mutation status of *EGFR* (exon 18 to 21) and *KRAS* (exon 1 and 2) were examined by sequencing of PCR products as previously described.[28] EML4-ALK fusion was screened with RT-PCR and immunohistochemistry as described elsewhere.[27]

Statistical Analysis

Differences in categorized demographic variables between cases and controls were tested by a chi-squared test or Fisher's exact test as appropriate. To verify that the allele distribution for each SNP was in the Hardy–Weinberg equilibrium (HWE), we used a chi-squared test with one degree of freedom.

We applied odds ratios as measures of association and they and their 95% confidence intervals were estimated using unconditional logistic regression models adjusted for potential confounders. Potential confounders considered in this analysis were age, sex, smoking evaluated as pack-years (PY), and the energy-adjusted intake of fruit and green–yellow vegetables.

We evaluated the association of the *BIM* deletion and selected polymorphisms within the major histological subtypes of lung cancer (adenocarcinoma, squamous-cell carcinoma, and small-cell carcinoma) and *EGFR* mutation status for those with information available. Survival probabilities were estimated by the Kaplan–Meier product limit method and comparisons between groups were tested by the log-rank test.

We used STATA version 13 (STATA Corporation, College Station, TX) for all analyses and adopted p value of less than 0.05 as statistically significant.

RESULTS

Patients Characteristics

Table 1 shows the difference in characteristics among cases and controls. Older subjects, males and heavier smokers made up a significantly higher number of the cases. Lower intake of fruit and vegetable trended higher in lung cancer cases but did not reach statistical significance. There is no difference between family history between cases and controls.

TABLE 1. Characteristics of Subjects

	Case (n = 765) (%)	Controls (n = 942) (%)	p
Age			
<40	21 (2.7)	339 (36.0)	
40–49	60 (7.8)	155 (16.5)	
50–59	210 (27.5)	179 (19.0)	
60–69	295 (38.6)	176 (18.7)	
70-	179 (23.4)	93 (9.9)	<0.001
Sex			
Male	564 (73.7)	492 (52.2)	
Female	201 (26.3)	450 (47.8)	<0.001
Smoking			
Never	197 (25.8)	551 (58.5)	
Low	56 (7.3)	159 (16.9)	
Moderate	145 (19)	113 (12.0)	
Heavy	362 (47.3)	111 (11.8)	
Unknown	5 (0.7)	8 (0.8)	<0.001
Fruit/Vegetable consumption			
Tertile 1	278 (36.3)	306 (32.5)	
Tertile 2	226 (29.5)	306 (32.5)	
Tertile 3	246 (32.2)	305 (32.4)	
Unknown	15 (2.0)	25 (2.7)	0.28
Family history of lung cancer			
No	731 (95.6)	896 (95.1)	
Yes	34 (4.4)	46 (4.9)	0.67
Histology			
Adenocarcinoma	450		
SCC	132		
SCLC	69		
Large	49		
Other/unknown	65		

SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

Association between BIM Deletion Polymorphism and Neighboring SNPs

The association between the *BIM* deletion polymorphism and neighboring SNPs and lung cancer risk are shown in Table 2. There is no violation of HWE among controls except rs13405741. As shown in Figure 1, there is a strong linkage disequilibrium in this region. The *BIM* deletion polymorphism as well as neighboring SNPs was shown to be a lack of statistically significant association with lung cancer risk (Table 2). These results suggest that a lung cancer susceptibility locus is less likely to be included in this region.

No Difference of Frequency of the BIM Deletion Polymorphism between Controls and Lung Cancer Patients

We screened for the *BIM* deletion polymorphism in 765 lung cancer cases and 942 healthy individuals. Carrier possessing one allele of the *BIM* polymorphism was observed in 13.0% of control and 12.8% of lung cancer cases. Homozygosity for the *BIM* polymorphism was observed in four of 942 controls and three of 765 lung cancer cases. The frequency of *BIM* polymorphism in lung cancer patients was not related to age, sex, smoking history or family history of lung cancer. Furthermore, these characteristics were not different between control and lung cancer cases (Table 3).

Lack of Association between the BIM Polymorphism and Histology and EGFR Mutation Status of Lung Cancer

To determine the association between lung cancer subtype and the *BIM* polymorphism, we examined the *BIM* polymorphism with histological lung cancer subtype (Table 4). Although the frequency of the *BIM* polymorphism was slightly lower in the small cell lung cancer subtype, no significant association of the *BIM* polymorphism and histological type was observed. Importantly, the *BIM* polymorpism was not associated with the risk of any histological subtype in lung cancer cases (Table 5). These results suggest a lack of association between lung cancer susceptibility and this *BIM* polymorphism. Furthermore, frequency of the *BIM* polymorphism was comparable among *EGFR* wild-type and *EGFR* mutant lung cancer patients, suggesting lack of association between the *BIM* polymorphism and *EGFR* mutations in lung cancer (Table 4).

Impact of the BIM Polymorphism on the Survival of Early Stage Lung Cancer

To determine the natural history of lung cancers harboring the *BIM* polymorphism, we analyzed 139 stage I lung cancer cases who received complete surgical resection. The *BIM* polymorphism was identified in 15 patients, all of which are heterozygote. Clinical characteristics are shown in Table 6. Survival of these stage I lung cancer patients was similar regardless of *BIM* polymorphism status (Fig. 2).

DISCUSSION

In this case-control study, we have shown that the frequency of the *BIM* deletion polymorphism is approximately

TABLE 2. Association between SNPs around *BIM* Deletion Polymorphism and Lung Cancer Risk

Rs#	Location	Gene	Miscellaneous		MAF in Cases	MAF in Controls	*p* Values for HWE Test in Controls	*p*[a]
rs2289321	111870220	*FIJ44006*	In gene	Intron 1	0.1538	0.1576	0.0094	0.543
rs1439287	111871897	FIJ44006	In gene	5′flk	0.3979	0.4091	0.4568	0.801
rs2015454	111872148	FIJ44006	In gene	5′flk	0.4483	0.4294	0.9264	0.324
rs1837369	111874276	LOC642268	Not in gene	nearest 5′	0.398	0.4119	0.4343	0.984
BIM deletion		BCL2L11	In gene		0.068	0.069	0.8056	0.812
rs17041869	111896243	BCL2L11	In gene	Intron 1	0.2346	0.2471	0.192	0.726
rs13396983	111900598	BCL2L11	In gene	Intron 1	0.4516	0.4315	0.9559	0.338
rs1877330	111906762	BCL2L11	In gene	Intron 1	0.2349	0.2442	0.1059	0.903
rs724710	111907691	BCL2L11	In gene	Exon 2	0.0903	0.0961	0.1657	0.29
rs3789068	111909247	BCL2L11	In gene	Intron 2	0.3986	0.4117	0.651	0.899
rs17041887	111910459	BCL2L11	In gene	Intron 2	0	0	—	NE[b]
rs616130	111912681	BCL2L11	In gene	Intron 3	0.4541	0.4384	0.6983	0.238
rs13405741	111913056	BCL2L11	In gene	Intron 3	0.0007	0.0048	0.8829	0.486
rs726430	111931421	BCL2L11	Not in gene	—	0.2314	0.2463	0.2103	0.162
rs9308742	111943621	BCL2L11	Not in gene	—	0.3889	0.4071	0.774	0.641

[a]*p* values for loci in logistic regression models including age, sex, smoking, fruit/vegetable consumption in tertile, and family history of lung cancers covariates with multiple imputations.
[b]NE indicates not estimated because of lack of subjects.
MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium.

13% in Japanese population, comparable with the occurrence rate in the Chinese population.[14,19,20] This *BIM* polymorphism was not associated with lung cancer susceptibility. Furthermore, the *BIM* polymorphism is not enriched in *EGFR* mutant lung cancers, nor does it appear to increase the risk of death of patients with stage I resected lung cancer.

Despite the lack of association between this *BIM* polymorphism and the acquisition of lung cancer, several studies have shown that SNPs in the apoptotic machinery are related to the risk of lung cancer. Multi-cohort genome wide association studies have identified genetic variants mapped to chromosomal regions 15q25 [nicotinic acetylcholine receptor (nAChR) subunits: CHRNA3, CHRNA5], 5p15

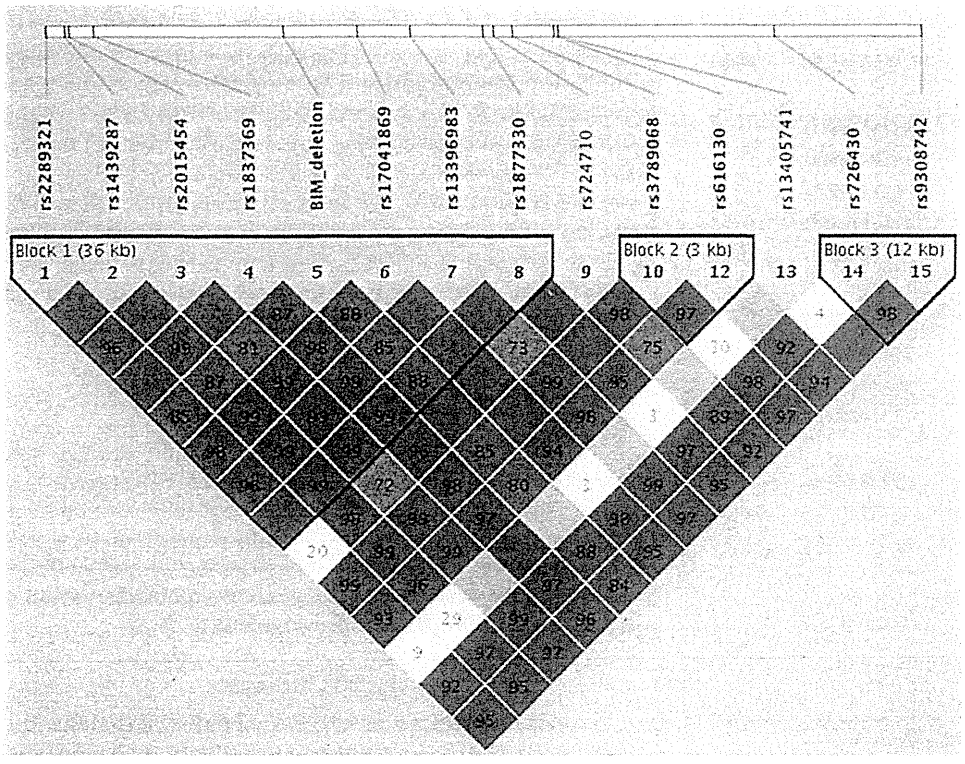

FIGURE 1. Linkage disequilibrium plot of polymorphisms around *BIM* deletion polymorphism. LD (D′) plot of SNPs in *BIM* and adjacent regions. The color scheme is based on D′ and logarithm of the odds of linkage (LOD) score values: white, D′ < 1 and LOD < 2; blue, D′ = 1 and LOD < 2; shades of pink/red, D′ < 1 and LOD ≥ 2; and bright red, D′ = 1 and LOD ≥ 2. The numbers in squares are D′ values (values of 1.0 are not shown). The map was drawn using Haploview. Haplotype blocks were identified by the software.

TABLE 3. Distribution of *BIM* Deletion Polymorphism Genotype According to Characteristics

	Case (n = 765)				Controls (n = 942)				
	Wild-Type	Heterozygote	Homozygote		Wild-Type	Heterozygote	Homozygote		
No. Cases (%)	664 (86.8)	98 (12.8)	3 (0.4)		816 (86.6)	122 (13.0)	4 (0.42)		
Characteristics				p^a				p^a	Case-Control p Values
Age									
<40	19	1	1		301	37	1		0.095
40–49	52	8	0		135	20	0		1
50–59	178	32	0		154	25	0		0.78
60–69	258	36	1		145	29	2		0.19
70-	157	21	1	0.34	81	11	1	0.39	1
Sex									
Male	487	75	2		433	59	0		0.46
Female	177	23	1	0.65	383	63	4	0.058	0.61
Smoking									
Never	171	25	1		475	73	3		0.96
Low	44	12	0		142	16	1		0.055
Moderate	128	17	0		99	14	0		1
Heavy	318	42	2		94	17	0		0.52
Unknown	3	2	0	0.25	6	2	0	0.73	1
Fruit/vegetable consumption									
Tertile 1	243	34	1		270	35	1		0.90
Tertile 2	190	34	2		267	38	1		0.47
Tertile 3	217	29	0		254	49	2		0.15
Unknown	14	1	0	0.63	25	0	0	0.15	0.38
Family history of lung cancer									
No	632	96	3		780	112	4		0.92
Yes	32	2	0	0.39	36	10	0	0.67	0.062

[a]Fisher's exact test.

(TERT-CLPTM1L locus) and 6p21 (BAT3-MSH5) were associated with lung cancer risk,[29,30] which was confirmed in the Japanese population as well.[31] Some of these genes such as *CLPTM1L* and *BAT3* may be involved in apoptosis.[32] In addition, associations between SNPs in BCL2 family member proteins and lung cancer risk have also been suggested.[32] However, loss of proapoptotic BCL2 family members itself does not appear sufficient to transform cells. Moreover, the level of BIM expression in *EGFR* mutant lung cancer did not affect the magnitude of apoptosis induction by DNA damaging agents such as cisplatin,[6] nor does it affect the PFS to chemotherapy.[16,33] Understanding the precise role of apoptotic proteins in lung carcinogenesis might help to provide a strategy for potential lung cancer therapeutics and chemoprevention. Although the *BIM* polymorphism was not associated with lung cancer risk in this study, it does not exclude the possibility that the *BIM* polymorphism increases the risk of other cancers, especially hematological malignancies. The *BIM* polymorphism was originally found in chronic myeloid leukemia (CML) cells and associated with clinical resistance to BCR-ABL inhibitors in patients with BCR-ABL positive CML.[14] Furthermore, *BIM* knockout mice showed accumulation of lymphoid and myeloid cells, and resistance to apoptotic stimuli in lymphocytes.[34]

In this study, the incidence of *BIM* polymorphism was not related to *EGFR* mutation status in 332 patients. While there has been strong evidence from mouse experiments that BIM mitigates oncogene-induced tumors such as MYC[35] and cyclin D1,[36] other oncogenes directly downregulate BIM, like BCR-ABL, through the MEK/ERK pathway. Similarly, EGFR downregulates BIM directly through the MEK/ERK pathway, particularly the BIMEL isoform, therefore offering a different way to downregulate functional BIM that may phenocopy the *BIM* polymorphism. Furthermore, numerous reports have highlighted differential ways cancers downregulate BIM at the RNA level, including through overexpression of microRNAs, genetic deletion, and epigenetic silencing. In *EGFR* mutant lung cancer cell lines, genetic LOH and micro-RNA-mediated downregulation was shown to lead to low BIM expression.[6,37] Additionally, other *BIM* polymorphisms may contribute to reduced BIM levels and efficacy of TKIs.[38] Therefore, functional BIM is downregulated via different mechanisms in *EGFR* mutant lung cancers, which would be overlooked by sole evaluation of the *BIM* deletion polymorphism.

TABLE 4. Prevalence of *BIM* Polymorphism Based on Histology and *EGFR* Mutation Status among Lung Cancer Cases

	Number of Subjects	Wild-Type	Heterozygote	Homozygote	*p*
Histology					
Adenocarcinoma	450	380	69	1	
SCC	132	119	13	0	
SCLC	69	62	6	1	
Large	49	43	6	0	
Other/unknown	65	60	4	1	0.17
EGFR mutation					
Wild-type	212	182	30	0	
Mutant	120	104	16	0	
Unchecked	433	378	52	3	0.78

SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma; Large, large cell carcinoma.

TABLE 5. Impact of *BIM* Polymorphism on the Risk of Lung Cancer According to Histologic Subtype

	Wild-Type	Heterozygote	Homozygote	Hetero or Homo
Controls (*n*)	816	122	4	126
Case overall (*n*)	664	98	3	101
Adjusted OR[a]	Reference	0.97	0.78	0.96
95% CI	—	0.69–1.36	0.13–4.58	0.69–1.34
p	—	0.86	0.79	0.83
Adenocarcinoma				
Number of case	380	69	1	70
Adjusted OR[a]	Reference	1.15	0.55	1.13
95% CI	—	0.81–1.64	0.06–5.45	0.80–1.60
p	—	0.44	0.61	0.49
SCC				
Number of case	119	13	0	13
Adjusted OR[a]	Reference	0.69	NE[b]	0.69
95% CI	—	0.33–1.43	—	0.33–1.42
p	—	0.32	—	0.31
SCLC				
Number of case	62	6	1	7
Adjusted OR[a]	Reference	0.56	5.73	0.65
95% CI	—	0.22–1.45	0.28–116.8	0.26–1.59
p	—	0.23	0.26	0.35

[a]Adjusted for age, sex, smoking, fruit/vegetable consumption in tertile, and family history of lung cancer with multiple imputation.
[b]NE indicates not estimated because of lack of subjects.
OR, odds ratio; CI, confidence interval, SCC, squamous cell carcinoma; SCLC, small cell carcinoma.

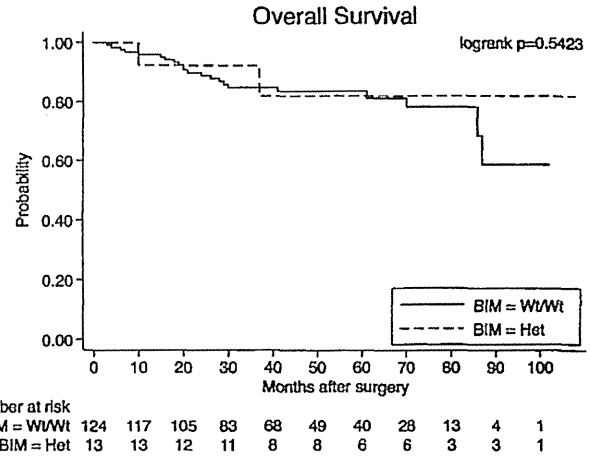

FIGURE 2. Overall survival according to *BIM* genotype. Overall survival for *BIM* deletion wt/wt and heterozygotes are drawn. No significant difference was shown by logrank test.

TABLE 6. Characteristics of Stage IA/IB Patients According to *BIM* Deletion Genotypes

	Wild-Type	Heterozygote
Number of subjects	124	15
Median age	62	57
(min, max)	(26, 78)	(47, 77)
Sex		
Male	66	8
Female	58	7
pStage		
IA	41	4
IB	83	11
EGFR mutation		
Wild-type	56	7
Mutant	57	7
Unknown	11	1
KRAS mutation		
Wild-type	68	9
Mutant	6	2
Unknown	50	4
ALK		
Wild-type	120	15
Mutant	4	0

This study has several strengths and limitations. A notable strength is that this study was conducted in a single region in central Japan within the framework of the HERPACC study, with a substantial number of subjects and a high response rate to the completion of questionnaires and provision of blood samples. One limitation of the study is the problem of multiple testing although none of test for the association between *BIM* deletion polymorphism and susceptibility as well as survival showed statistical significance. The second limitation is the selection of controls: hospital-based outpatients who did not have a diagnosis of cancer. Nevertheless, both cases and controls were selected from the same framework, and most were residents of the same area (Aichi and its adjacent prefectures), warranting the internal validity of this study.

Lastly, we did not find an association with survival of patients with Stage I lung cancer and the *BIM* polymorphism.

Low BIM expression does affect the survival time for patients with *EGFR* mutant advanced lung cancer, where surgical resection is not possible.[16] Thus, the *BIM* polymorphism may similarly influence survival in advanced lung cancers.

Our study provides evidence that lung cancer risk and *BIM* polymorphisms are not significantly linked, indicating that genetic test of *BIM* deletion polymorphism is not necessary for the screening of lung cancer among healthy individuals in the Japanese population. However, this *BIM* deletion polymorphism is a negative predictive factor of response to EGFR-TKI therapy.[14,19,20] We have recently reported histone deacetylase inhibitor could restore functional BIM expression and circumvent EGFR-TKI resistance in *EGFR* mutant PC-3 and HCC2279 cells with the *BIM* polymorphism.[26] This combination is going to be assessed in a clinical trial (NCT02151721). Therefore, while this *BIM* polymorphism does not appear to be associated with a higher risk to develop lung cancer, its clinical utility to determine best treatment options appears quite significant.

In conclusion, in a large Japanese population, we report that the *BIM* polymorphism does not appear to increase the risk of *EGFR* mutant or *EGFR* wild type lung cancer, nor does it negatively impact the survival of stage I lung cancer patients.

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Review

Clinical significance of epidermal growth factor receptor tyrosine kinase inhibitors: Sensitivity and resistance

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ABSTRACT

Gefitinib and erlotinib, which are epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs), are highly effective against lung tumors with EGFR activating mutations. However, in 20–30% of cases, there is intrinsic resistance, and even if the treatment is effective, resistance is acquired in one to several years. Possible mechanisms of acquired resistance to EGFR-TKI, thus far, include a gatekeeper mutation of EGFR, activation of an alternate pathway, activation of EGFR downstream signals, transformation to small cell lung cancer, and epithelial–mesenchymal transition (EMT). Recently, BIM (BCL2L11), which is a BH3-only proapoptotic member of the Bcl-2 protein family, was shown to play a central role in inducing apoptosis in response to EGFR-TKI treatment in EGFR mutant lung cancer cells. Moreover, when the expression of active BIM protein was low, there was resistance to apoptosis induction by EGFR-TKI treatment and early disease progression.

A polymorphism of the BIM gene unique to East Asian people has been detected and is now attracting attention as a factor causing resistance to EGFR-TKI due to decreased BIM activity.

Abbreviations: EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; MST, median survival time; HGF, hepatocyte growth factor; FISH, fluorescence *in situ* hybridization; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; EMT, epithelial-to-mesenchymal transition; SRC, sarcoma viral oncogene homolog; APAF-1, apoptotic peptidase activating factor 1; PKC-ε, protein kinase Cε; ABC transporter, adenosine triphosphate-binding cassette transporter; BCRP, breast cancer resistance protein; NFκβ, nuclear factor kappa B; TGF-β, transforming growth factor-β; IL-6, interleukin-6; BCL2L11, Bcl-2-like protein 11; PFS, progression free survival; mRNA, messenger RNA; Hsp90, heat shock protein 90; HDAC, histone deacetylace; BH3, Bcl-2 homology domain 3; PBMC, peripheral blood mononuclear cell; OS, overall survival; NA, not applicable; ORR, overall response rate; NS, not significant

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Contents

1. Introduction

EGFR tyrosine kinase inhibitors (EGFR-TKIs) are dramatically effective in lung cancer with epidermal growth factor receptor (EGFR) activating mutations. However, some cases are inherently resistant, and even in cases where there is high effectiveness, tolerance is acquired within several months to several years, leading to recurrence. Recently, many studies have been performed to examine TKI-resistance, and many clinical treatments are being developed to overcome it. In this paper, we summarize the latest knowledge of the molecular mechanisms of resistance to gefitinib and erlotinib, which are EGFR-TKIs, in EGFR mutant lung cancer, and strategies to overcome this resistance.

2. EGFR-TKI efficacy in patients with EGFR mutant lung cancer

EGFR is overexpressed in many solid cancers. In lung cancer with EGFR activating mutations, EGFR-TKIs like gefitinib and erlotinib show dramatic efficacy. EGFR activating mutations include deletion of exon 19 and L858R point mutation in exon 21, and these account for 90% or more of EGFR mutations [1]. In lung cancer with EGFR activating mutations, gefitinib and erlotinib show a marked response, with a response rate of 70–80% [2].

When EGFR-TKI treatment is utilized for treating lung cancer with EGFR activating mutations, the median survival time (MST) of patients is approximately 30 months, and considering that the MST for platinum-based chemotherapy is around 12 months, this is clearly a breakthrough. However, even if there is a complete response, the cancer will recur in several years due to acquired resistance, almost without exception. Moreover, in 20–30% of cases with an EGFR mutation, EGFR-TKI has no effect, known as intrinsic resistance. To better understand and use EGFR-TKI therapy, these two types of resistance need to be resolved.

3. Major mechanisms of resistance to EGFR-TKIs

3.1. *EGFR T790M gatekeeper mutation*

T790M was first reported to be an acquired mutation that leads to TKI-resistance and is known as the gatekeeper mutation in EGFR. Threonine, which is the 790th amino acid located in exon 20 of the EGFR, undergoes mutation to methionine, and T790M is detected in about 50% of tumors with acquired resistance [1,3,4].

If this T790M genetic mutation occurs in addition to the deletion of exon 19 or the L858R mutation in exon 21, the affinity of EGFR for ATP increases and affinity for EGFR-TKIs decreases, and resistance develops [5]. A few cancer cells that have the T790M mutation and EGFR activating mutations are already present before EGFR-TKI treatment, and they are thought to gradually become predominant during EGFR-TKI treatment. Due to the T790M mutation, the kinase activity of EGFR and tumor-forming ability of cancer cells have been

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reported to increase, but according to the latest report, the growth rate of cancer cells with the T790M mutation is slower, and it is possible that this slows tumor progression [6,7].

3.2. *Activation of bypass signaling*

3.2.1. *Met amplification*

Due to genetic amplification, Met proteins undergo autophosphorylation, and due to association with ErbB3, the PI3K/Akt pathway is activated downstream and induces resistance [8]. Although it was initially reported that this could be detected in 20–25% of tumors with acquired resistance, the cutoff value for genetic amplification has not been determined. According to the latest report, which declare 5 copies or more as positive, amplification can be detected in about 4–10% of cases [9]. A few cancer cells which have Met amplification and EGFR activating mutations are already present before EGFR-TKI treatment, and they are thought to gradually become predominant during EGFR-TKI treatment [10].

3.2.2. *High-level expression of HGF*

Hepatocyte growth factor (HGF), a Met ligand, activates the Met/PI3K/Akt pathway and induces resistance [11]. Unlike Met amplification, resistance mediated by HGF is transmitted downstream *via* Gab1, a Met adapter protein. There are two methods of increased HGF expression, including autocrine production by cancer cells and paracrine production from interstitial fibroblasts. In a Japanese cohort of lung cancer with EGFR-TKI acquired resistance, HGF is highly expressed in 61% of tumor tissues from patients who acquired resistance [12], and this resistance mechanism is thought to occur with high frequency clinically (Fig. 2B). Moreover, although there may be an EGFR mutation, in a study of intrinsically resistant cases where EGFR-TKIs did not show a marked response, HGF was highly expressed in 29% of cases, suggesting that it is an intrinsic resistance factor (Fig. 2A). The clinical application of HGF quantification or cutoff values as biomarkers is debated. However, previous studies have suggested that the sensitivity to EGFR-TKIs can be predicted by measuring HGF levels in peripheral blood [13–14], and more future promising studies are underway.

3.2.3. *HER2 amplification*

The results of the FISH test indicated that HER2 genetic amplification occurred in 12% of cases (3 of 26 samples) in which resistance to gefitinib or erlotinib was acquired [15]. Since HER2 genetic amplification occurred in 199 (1%) lung adenocarcinoma samples prior to treatment, it was detected at high frequency in resistant tumors and suggested as a clinically important resistance factor. Interestingly, this was mutually exclusive to the EGFR T790M mutation.

3.2.4. *Activation of AXL kinase*

Preclinically, AXL has been shown to be overexpressed and activated by Gas6 (its ligand) to induce resistance to EGFR-TKIs in EGFR mutant lung cancer [16]. Further, in studies of clinical samples before and after acquisition of EGFR-TKI resistance, AXL was highly expressed in tumors after acquisition of resistance. In resistance to EGFR-TKI due to AXL, epithelial-to-mesenchymal transition (EMT) is also suggested to be involved.

3.2.5. *Integrin β1 overexpression*

Integrins are major mediators of cellular adhesion to extracellular matrix proteins. Integrins also play important roles in cell–cell adhesion. In addition to cellular adhesion, integrins facilitate transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways [17]. Recently, erlotinib-resistant sub-clones of EGFR mutant lung cancer cells were reported to express elevated levels of β1 and α2/α5 integrins as well as Src, resulting in Akt activation. Integrin β1 or Src knockdown in erlotinib-resistant clones markedly suppresses Akt activation and restores erlotinib sensitivity to the cells. Moreover, in four clinical samples assessed before and after acquisition of EGFR-TKI resistance, integrin β1 expression was particularly increased in the EGFR-TKI-resistant tumor samples from patients with EGFR mutant lung cancer [18].

4. Activation of downstream signaling

4.1. *PI3K/AKT signaling*

PTEN is an enzyme that catalyzes the dephosphorylation reaction of PI3K. Phosphorylation of PI3K increases when PTEN is deleted, and induces EGFR-TKI resistance by activating the PI3K/Akt pathway.

Moreover, PTEN expression reportedly decreases because of the decrease in intranuclear translocation of the transcription factor EGR1, which controls the expression of PTEN; this causes EGFR-TKI resistance [19].

4.2. *MAPK signaling*

Although PI3K/AKT signaling was reported to be important for proliferation and EGFR-TKI resistance of EGFR mutant lung cancer cells, the involvement of MAPK signaling in EGFR-TKI resistance induction was unclear. However, *in vitro* studies on the resistance mechanism of WZ4002, a mutant-selective EGFR-TKI, showed amplification of the MAPK1 gene, which encodes ERK2 [20]. In a study of clinical samples, MAPK1 amplification was detected in tumor tissues of EGFR mutant lung cancer resistant to erlotinib. Further, in an analysis of 200 cases of EGFR-TKI acquired resistance, BRAF mutations (V600E and G469A) were observed in 2 of 195 cases. In cases where BRAF G469A was detected, this mutation was not detected in samples before EGFR-TKI administration, suggesting that this was a secondary mutation [21].

5. Others

5.1. *Epithelial-to-mesenchymal transition (EMT)*

There is a change of morphology from epithelial cells to mesenchymal cells during EGFR-TKI resistance, and an epithelial-to-mesenchymal transition (EMT) with decreased

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expression of epithelial markers or increased expression of mesenchymal markers occurs [22]. This is not a single mechanism, and thus far, AXL activation [16], decreased expression of MED12 [23], and activation of the TGF-β/IL-6 axis [24] have been reported. No strategy has yet been established to overcome resistance due to EMT, but this may become possible in the future when the molecular mechanism of EMT induction is elucidated.

5.1.1. *Transformation to small cell lung cancer*

Cases with EGFR activating mutations have been reported wherein there was a transformation to small cell lung cancer and acquisition of resistance [9]. However, the frequency with which this occurs varies depending on the report, and the molecular mechanism whereby resistance is acquired is not understood. Moreover, it is not clear if a few small cell lung cancer cells were originally present and proliferated into larger numbers, or if lung cancer cells with the EGFR mutation themselves underwent a morphological transformation to small cell lung cancer. Clinically, a therapeutic effect can be obtained using ordinary chemotherapy for small cell lung cancer.

5.1.2. *microRNAs*

Reportedly, microRNAs mediate EGFR-TKI resistance [25]. Whereas expression levels of miR-30b, miR-30c, miR-221, and miR-222 are controlled by both EGFR and Met, miR-103 and miR-203 expression levels are exclusively controlled by Met. These microRNAs suppress genetic expression of BIM, apoptotic peptidase activating factor 1 (APAF-1), protein kinase C-ε (PKC-ε), and sarcoma viral oncogene homolog (SRC), which are all important in cancer cell apoptosis in response to gefitinib and EMT. Although these results interestingly suggest the possibility that sensitivity of lung cancer to EGFR-TKI can be improved by controlling microRNAs, the drugs used for analysis were at a concentration far exceeding the clinical level, and therefore, further studies are required to examine clinical significance.

5.1.3. *Chromatin modification*

In cases which showed a marked response to gefitinib but subsequently acquired resistance, if gefitinib was withdrawn (drug holiday) and another treatment was administered for a time period prior to gefitinib re-challenge, a curative effect was again obtained [26]. Therefore, resistance to gefitinib in this case was reversible. We surmise that HGF is a factor that induces reversible resistance, and a reversible tolerance mechanism due to chromatin modification has also been proposed [27]. In this mechanism of reversible resistance, activation of IGF-1R signaling occurs due to chromatin modification, expression of RBP2/KDM5A/Jarid1A which has histone demethylating activity increases, and methylation of the target, H3K4, decreases.

5.1.4. *ABC transporters*

Adenosine triphosphate (ATP)-binding cassette (ABC) transporter proteins, such as the ABCB1/P-glycoprotein (P-gp) and ABCG2/breast cancer resistance protein (BCRP) cause multidrug resistance in tumors; this is mainly because they transport various compounds out of the cell [28]. One of the key multidrug transporters, ABCG2/BRCP, interacts with many recently developed molecularly targeted drugs such as gefitinib and imatinib. Elkind et al. [29] reported that the expression of ABCG2, but not that of its nonfunctional mutant, protects EGFR signaling-dependent cancer cells from death when exposed to gefitinib. This protection is reversed by treatment with an ABCG2-specific inhibitor, suggesting that ABCG2 may cause resistance through active efflux of gefitinib in cancer cells.

6. Heterogeneity of resistance mechanisms

Although there are many reports in which T790M and Met genetic amplification occurred in a mutually exclusive manner, one report describes both mutations detected in the same tumor. We performed a study of a Japanese cohort of lung cancer patients with EGFR mutant lung cancer who acquired resistance to EGFR-TKIs, and found that 14 of the 23 tumors (61%), which were obtained from those patients who acquired resistance, showed high expression levels of HGF. There was no tumor that expressed both T790M and Met amplification simultaneously. However, of the 12 tumors that expressed T790M, 6 tumors highly expressed HGF, and of the 2 tumors that had Met amplification, one also highly expressed HGF [12]. Therefore, it was clear that high HGF expression often coexists with other resistance factors, such as T790M and Met amplification (Fig. 2B). Recently, it has become generally accepted that several resistance factors are present together in one individual or one tumor, which supports our report, and this is an important consideration in overcoming EGFR-TKI resistance.

7. Resistance to apoptosis

Recently, resistance to apoptosis has attracted attention as a factor that leads to EGFR-TKI resistance in EGFR mutant lung cancer cells. Decrease in BIM activity and activation of Fas and NFκβ signaling have been reported as causative factors, and for BIM, there have also been studies using clinical samples. At present, it has not yet been determined what mechanism is responsible for the proliferation of tumors that are resistant to apoptosis (Fig. 1). BIM (BCL2L11) is a BH3-only proapoptotic member of the Bcl-2 protein family, and gene products with BH3 domains are required to induce apoptosis. Mainly BIM_{EL}, but also BIM_L and BIM_S, block apoptosis suppression factors such as Bcl-2, Bcl-xL, and Mcl-1, and activate BAX and BAK, which are apoptosis promotion factors. In EGFR mutant lung cancer, BIM plays a central role in the induction of apoptosis in response to EGFR-TKIs, and low BIM expression in a tumor was reported to induce resistance to apoptosis in response to EGFR-TKIs and lead to shorter progression-free survival (PFS) in EGFR-TKI treatment [30,31].

Further, in a recent analysis using samples from the EURTAC study, it was reported that in cases where BIM expression in EGFR mutant lung cancer tumors was low, PFS of patients treated with erlotinib was shorter, and overall survival (OS) was also significantly shorter, making it clear

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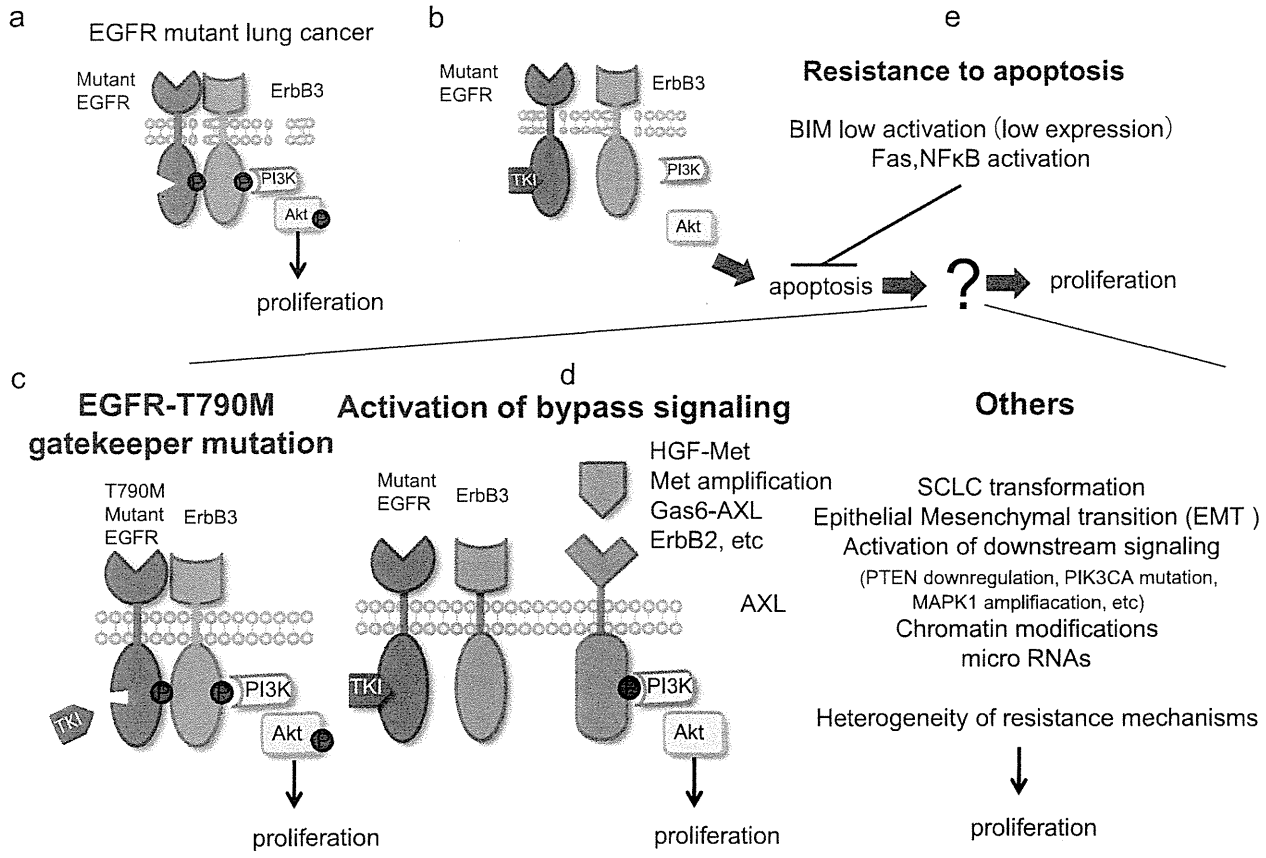

Fig. 1 – Mechanisms of resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in EGFR mutant lung cancer cells. (a) Mutant EGFR associates with ErbB3 and transduces a survival signal through the PI3K/Akt pathway. "P" indicates phosphorylation. (b) EGFR-TKIs such as gefitinib and erlotinib bind to the tyrosine kinase domain of mutant EGFR, shut off signaling, and induce apoptosis. (c) The EGFR-T790M gatekeeper mutation prevents EGFR-TKIs from binding to EGFR and thereby induces resistance. (d) Amplified Met associates with ErbB3, transactivates the downstream PI3K-Akt signaling pathway, and thereby induces resistance. Hepatocyte growth factor (HGF) phosphorylates Met and activates the PI3K-Akt pathway independently of EGFR or ErbB3 and thereby induces resistance. (e) Apoptosis resistance has recently been reported as a factor that mediates acquired resistance to EGFR-TKIs at early stages. Mechanisms that have been reported include decreased BIM activity and activation of Fas and NFκβ signaling. At present, the mechanism responsible for proliferation of tumors resistant to apoptosis is still not understood.

that this is an important factor in resistance [32]. However, these reports utilized BIM mRNA expression in the tumor. To apply these results clinically, a reference level must be set. Additionally, the quantification of mRNA is influenced by the quality of samples, and therefore, a biomarker that can be evaluated more objectively is desired.

In 2012, the BIM gene was reported to have a specific polymorphism that decreased BIM activity [33]. Wild-type BIM is mostly active, having the BH3 domain, but the polymorphism leads to expression of the BIM protein BIMγ in which 2903 bases are deleted in intron 2 of the BIM gene leading to loss of the BH3 domain, which cannot induce apoptosis. This causes resistance to apoptosis in response to EGFR-TKIs. This genetic polymorphism is not seen in Caucasians and Africans (German: 0/595 persons, African: 0/60 persons), but it is specifically detected in East Asians. In many cases, the polymorphism is heterozygous, but in rare cases, the deletion polymorphism was homozygous (0.5%). In a study of 141 EGFR mutant lung cancers, the 115 cases expressing wild-type BIM had a median PFS with EGFR-TKI of 11.9 months, but in the 26 cases that were positive for BIM genetic polymorphism, PFS was significantly shorter at 6.6 months, suggesting that this BIM genetic polymorphism can serve as a biomarker of EGFR-TKI resistance [33]. In 4 of 5 reports correlating BIM genetic polymorphism and EGFR-TKI therapeutic effects [33–37], BIM genetic polymorphism led to significantly shorter PFS (Table 1), which suggests that it is important as a biomarker of EGFR-TKI resistance. Moreover, since BIM genetic polymorphism can be measured accurately and simply using peripheral blood mononuclear cells (PBMCs), it is a very promising biomarker. On the other hand, Lee et al. [34] reported that BIM genetic polymorphism was not a predictive biomarker of EGFR-TKI resistance. Including this study, 4 reports [33–36] were retrospective studies with a limited population. Prospective studies with a larger number of cases will be necessary in the future.

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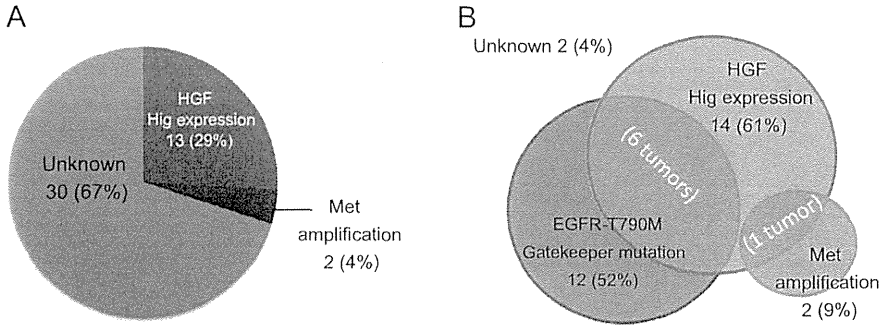

Fig. 2 – Incidence of resistance factors in EGFR mutant lung cancer resistant to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs). Presented are the results of a joint study of Japanese patients with EGFR-mutant lung cancer conducted at 12 facilities to determine the clinical significance of resistance triggered by hepatocyte growth factor (HGF). (A) Of 23 tumors with acquired resistance, 14 had high levels of HGF expression (61%), 12 had T790M mutations (52%), and 2 had Met amplification (9%). High levels of HGF expression were detected most often. T790M mutation and HGF were often both present in tumors that acquired resistance to gefitinib and erlotinib. (B) Of 45 tumors that did not respond to EGFR-TKIs despite having EGFR mutations, 13 had high levels of HGF expression (29%), 0 had T790M mutation (0%), and 2 had Met amplification (4%). High levels of HGF expression were again detected most often. These results suggest that HGF induces acquired and intrinsic resistance to EGFR-TKIs, and it is the most prevalent resistance mechanism.

Table 1 – Summary of reports correlating the BIM polymorphism and EGFR-TKI therapeutic effects in EGFR mutant lung cancer.

References	No. of patients (treatment of EGFR-TKI)	BIM polymorphism	No. of patients	PFS (months)	ORR (%)	OS (months)
Ng et al. [33]	141 (136: treated with gefitinib) (5: treated with erlotinib)	–	115	11.9	NA	NA
		+	26	6.6 (p=0.0027)	NA	NA
Lee et al. [34]	197 (179: treated with gefitinib) (18: treated with erlotinib) (4: unknown)	–	172	11.3	NA	NA
		+	21	11.9 (p=0.791)	NA	NA
Lee et al. [36]	153 (135: treated with gefitinib) (12: treated with erlotinib) (6: treated with afatinib)	–	126	8.6	57 (n=118) (8 patients: NA)	24.8
		+	27	4.6 (p=0.004)	38 (n=26) (1 patient: NA) (p=0.09: NS)	16.8 (p=0.005)
Isobe et al. [35]	70 (65: treated with gefitinib) (5: treated with erlotinib)	–	57	17.7	64.9	45.5
		+	13	7.5 (p<0.001)	61.5 (NS)	39.2 (p=0.27)
Zhao et al. [37]	166 (26: treated with gefitinib) (140: treated with erlotinib)	–	150	11	66	NA
		+	16	4.7 (p<0.001)	25 (p=0.001)	NA

In 4 of 5 reports, progression free survival (PFS) in patients treated with EGFR-TKI was significantly shorter when the BIM polymorphism was present, validating its significance as a biomarker of EGFR-TKI resistance in EGFR mutant lung cancer. On the other hand, regarding its effect on overall survival (OS), it remains necessary to study a larger number of cases in the future. "ORR" indicates the overall response rate. "p" indicates p-values. "NA" indicates not applicable. "NS" indicates not significant.

8. Treatments for overcoming resistance

For T790M, there are many promising treatments. Mutant-selective EGFR-TKIs have a low affinity for wild type EGFR and a high affinity for mutant EGFR (exon 19 deletion, exon 21 L858R, and exon 20 T790M) [38]. Combination therapy with irreversible EGFR-TKIs can be combined with EGFR T790M and anti-EGFR antibodies [39]. Heat shock protein 90 (Hsp90) inhibitors block Hsp90, which participates in stabilizing mutated EGFR protein [40].

For HER2 gene amplification, afatinib, which blocks both EGFR and HER2, is effective [15].

For treatment of resistance due to ligand stimulation by HGF, anti-HGF antibody, anti-Met antibody, and Met-TKI, in combination with EGFR-TKI, are expected to be effective. In cases in which transformation to small cell lung cancer has occurred, remission has been obtained by performing chemotherapy effective for small cell lung cancer [9]. For apoptosis resistance resulting from the BIM gene polymorphism, we showed that vorinostat, a histone deacetylace (HDAC) inhibitor, increases the expression of active BIM

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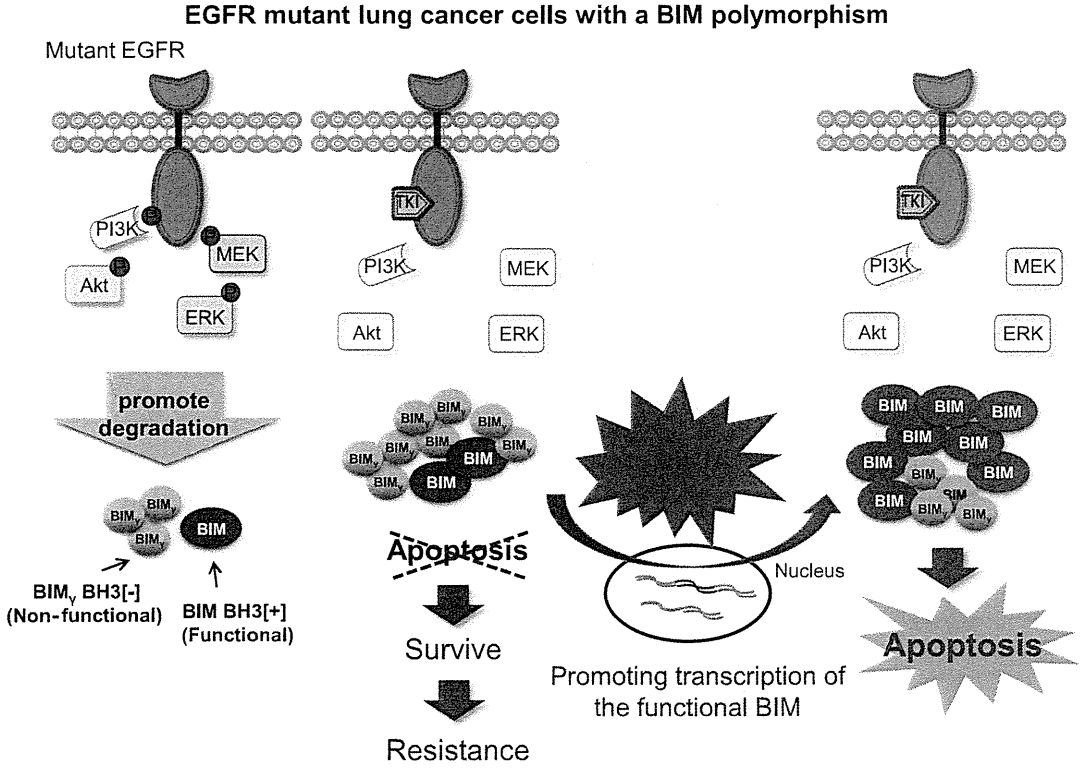

Fig. 3 – Using HDAC inhibition to overcome resistance due to BIM polymorphism. In EGFR mutated lung cancer cells that have BIM polymorphism, since BIM$_\gamma$, which cannot induce apoptosis, is predominantly expressed, there was resistance to apoptosis even if EGFR signaling is inhibited by EGFR-TKIs. When vorinostat, an HDAC inhibitor, was used in combination, apoptosis was induced, and resistance was overcome due to expression of the active BIM.

protein in EGFR mutant lung cancer cells with BIM genetic polymorphism. Apoptosis induction was also clearly shown using *in vitro* and *in vivo* studies to be promoted when vorinostat is used together with an EGFR-TKI (Fig. 3) [41]. Currently, in a multi-institutional study of EGFR mutant lung cancer patients who have a BIM genetic polymorphism, an investigator-initiated Phase I trial using vorinostat and gefitinib together is under way (ClinicalTrials.gov Identifier: NCT02151721), and a therapy is being developed to overcome resistance using BIM genetic polymorphism as a biomarker. Various factors are involved in decreased expression and decreased activity of BIM, but in addition to selective splicing due to genetic polymorphism, although we have no data, the efficacy of HDAC inhibition is apparently due to the degree of deacetylation. For decreased BIM activity due to other factors, clinical development of BH3 mimetic drugs is desired, but they are still in the early stages of clinical trials.

9. Conclusions

Regarding EGFR-TKI resistance, many resistance mechanisms involving secondary mutations of EGFR and proliferation signaling such as bypass signaling via HGF-MET have been reported thus far. Specific inhibitors for these various resistance mechanisms are now being developed. When resistance does occur, it is now increasingly important to perform an analysis of the tumor cells. On the other hand, apoptosis resistance has recently attracted attention as a factor resulting in resistance to therapy. It is now clear that in EGFR mutant lung cancer cells, BIM plays a central role in the induction of apoptosis by EGFR-TKIs, and when BIM activity declines, resistance to apoptosis in response to EGFR-TKIs will occur. Since BIM genetic polymorphism, which is considered to cause declining activity, can be measured using PBMCs, this is a very promising biomarker of BIM activity decline. Since this genetic polymorphism is specific to East Asians, it is hoped that more clinical research will be done, and studies and treatments to overcome resistance will be developed in Japan and the rest of East Asia.

Conflict of interest

Seiji Yano received honoraria and research funding from AstraZeneca and Chugai Pharmaceutical Co., Ltd. Shinji Takeuchi has no conflict of interest.

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Review Article

Not just gRASping at flaws: Finding vulnerabilities to develop novel therapies for treating *KRAS* mutant cancers

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Key words

Apoptosis, Kirsten rat-sarcoma, MEK, phosphatidylinositol 3-kinase, synthetic lethality

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Mutations in Kirsten rat-sarcoma (KRAS) are well appreciated to be major drivers of human cancers through dysregulation of multiple growth and survival pathways. Similar to many other non-kinase oncogenes and tumor suppressors, efforts to directly target KRAS pharmaceutically have not yet materialized. As a result, there is broad interest in an alternative approach to develop therapies that induce synthetic lethality in cancers with mutant KRAS, therefore exposing the particular vulnerabilities of these cancers. Fueling these efforts is our increased understanding into the biology driving *KRAS* mutant cancers, in particular the important pathways that mutant KRAS governs to promote survival. In this mini-review, we summarize the latest approaches to treat *KRAS* mutant cancers and the rationale behind them.

Oncogenic mutations in Kirsten rat-sarcoma (*KRAS*) occur in up to 25% of human cancers, positioning them as the most common gain-of-function mutations in human cancer.[1–3] Despite the development of small-molecule inhibitors that interfere with the localization of KRAS or inhibit the activity of mutant KRAS,[4,5] oncogenic KRAS remains a largely elusive target of drug development. Thus, blocking mutant KRAS may require a strategy more akin to one designed to counter the loss of a tumor suppressor – via targeting of vital downstream effector pathways. Along these lines, a number of studies in *KRAS* mutant cancers have led to strategies to target these pathways. Below, we will discuss the main effector pathways of KRAS and current approaches to develop combination therapies targeting these KRAS-effector pathways. Also, other approaches targeting KRAS, including synthetic lethal screening, will be summarized.

Downstream Effectors of KRAS

Kirsten rat-sarcoma protein cycles between an inactive GDP-bound state and an active GTP-bound state. A number of stimuli, including ligands that activate growth factor receptors and G-protein coupled receptors on the cell membrane, lead to the activation of RAS guanine exchange factors (GEFs).[6] This, in turn, results in the formation of active GTP-bound KRAS. In wild-type KRAS cells, KRAS is subsequently inactivated by Ras-GTPase activating proteins (RasGAPs). However, oncogenic *KRAS* mutations, which occur most frequently at amino acids 12, 13, and 61, render KRAS proteins resistant to RasGAP-mediated GTP-hydrolysis. This leads to constitutive activation of KRAS protein. Mutant KRAS activates multiple downstream effector pathways, resulting in the uncontrolled growth, proliferation, and survival of cancer cells (Fig. 1). Amongst these, three major effector pathways have emerged as being critical to mutant *KRAS*-mediated transformation and will be discussed in greater detail: the RAF-MEK-ERK pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the Ral-NF-kB pathway.

RAF-MEK-ERK pathway. The RAF serine/threonine kinases bind KRAS via their RAS Binding Domain (RBD). RAF activation in turn activates the serine/threonine kinases MEK1 and MEK2, which in turn activate ERK. The requirement for the RAF-MEK-ERK (MAPK) pathway in *KRAS*-mediated transformation and tumorigenesis has been well established.[7] However, inhibition of the MAPK pathway alone is not sufficient to eradicate *KRAS* mutant tumors. MEK inhibitors exhibit cytostatic rather than cytotoxic activity, inhibiting proliferation but not inducing significant apoptosis.[8,9] In accordance with these preclinical studies, the MEK inhibitor selumetinib (Astra-