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

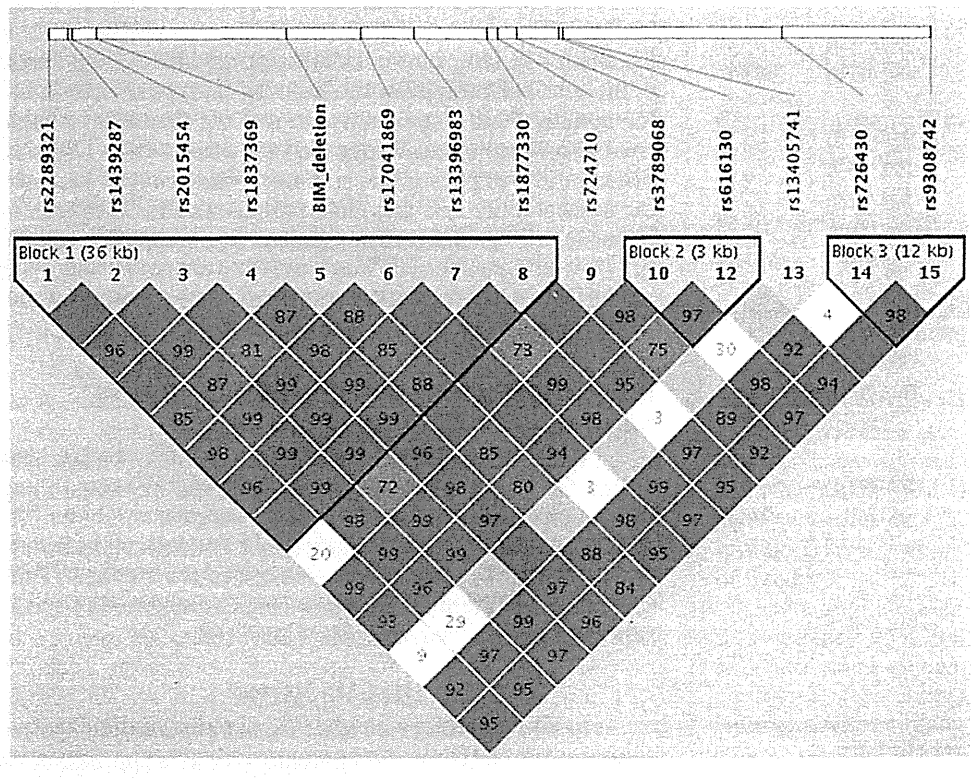

FIGURE 1. Linkage disequilibrium plot of polymorphisms around *BIM* deletion polymorphism. LD (D′) plot of SNPs in *BIM* and adjacent regions. The color scheme is based on D′ and logarithm of the odds of linkage (LOD) score values: white, D′ < 1 and LOD < 2; blue, D′ = 1 and LOD < 2; shades of pink/red, 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.

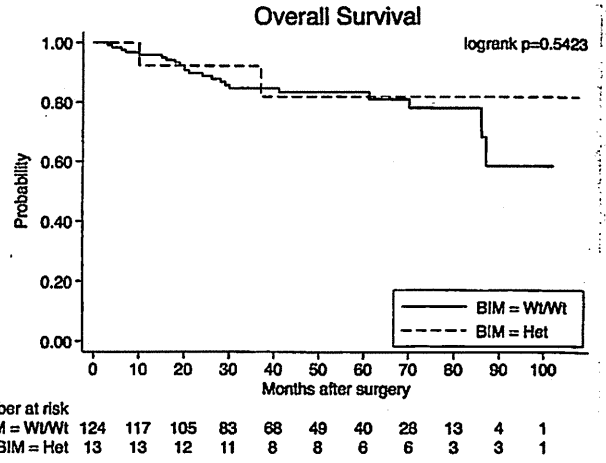

FIGURE 2. Overall survival according to *BIM* genotype. Overall survival for *BIM* deletion wt/wt and heterozygotes are drawn. No significant difference was shown by logrank test.

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.

ACKNOWLEDGMENTS

This work was supported by Ministry of Education, Science, Sports, Culture, and Technology of Japan (Grants-in-Aid for Scientific Research on Priority and Innovative Areas); Ministry of Health, Labor, and Welfare of Japan (Third-Term Comprehensive 10-Year Strategy for Cancer Control; National Cancer Center Research and Development Fund [23-A-4]; Health and Labour Sciences Research Grants for Research on Applying Health Technology; and Research on Development of New Drugs, Health and Labour Sciences Research Grants).

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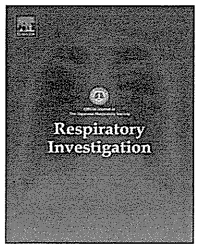

Review

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

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ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form
16 September 2014
Accepted 14 October 2014

Keywords:
EGFR mutation
EGFR-TKI
Acquired resistance
Apoptosis
BIM

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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http://dx.doi.org/10.1016/j.resinv.2014.10.002
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Please cite this article as: Takeuchi S, Yano S. Clinical significance of epidermal growth factor receptor tyrosine kinase inhibitors: Sensitivity and resistance. Respiratory Investigation (2014), http://dx.doi.org/10.1016/j.resinv.2014.10.002

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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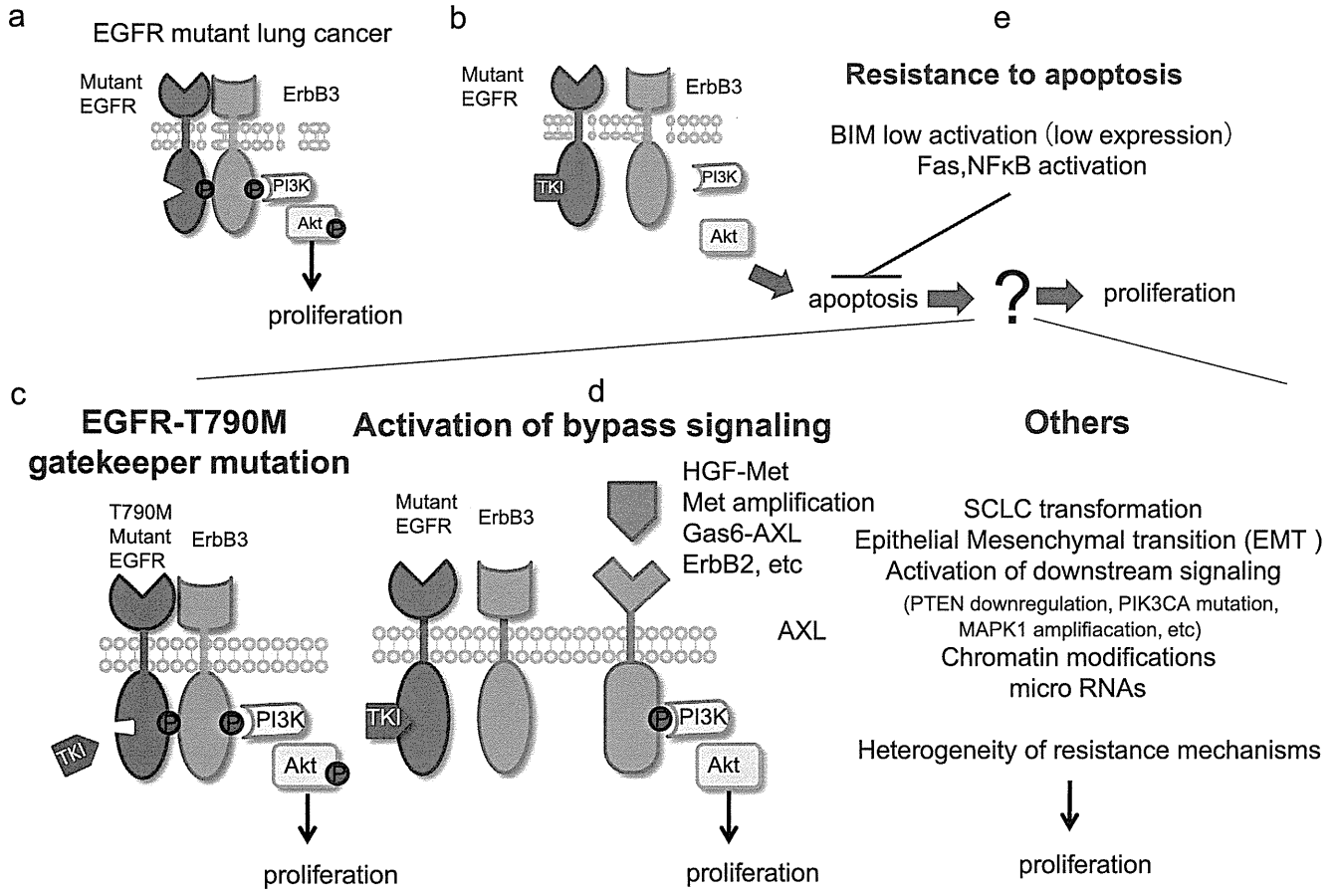

Fig. 1 – Mechanisms of resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in EGFR mutant lung cancer cells. (a) Mutant EGFR associates with ErbB3 and transduces a survival signal through the PI3K/Akt pathway. "P" indicates phosphorylation. (b) EGFR-TKIs such as gefitinib 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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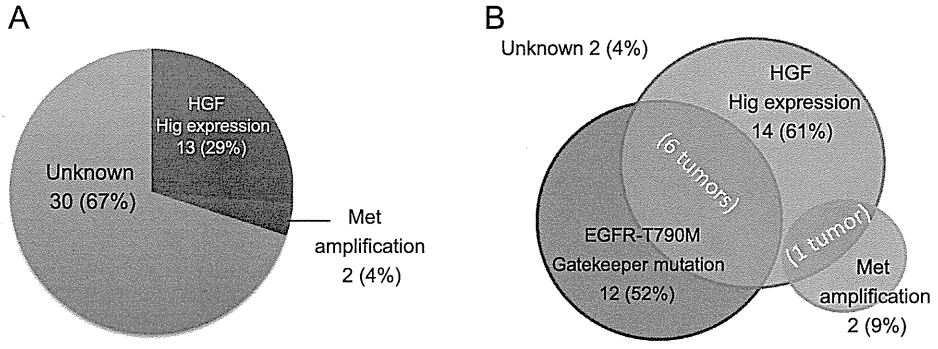

Fig. 2 – Incidence of resistance factors in EGFR mutant lung cancer resistant to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs). Presented are the results of a joint study of Japanese patients with EGFR-mutant lung cancer conducted at 12 facilities to determine the clinical significance 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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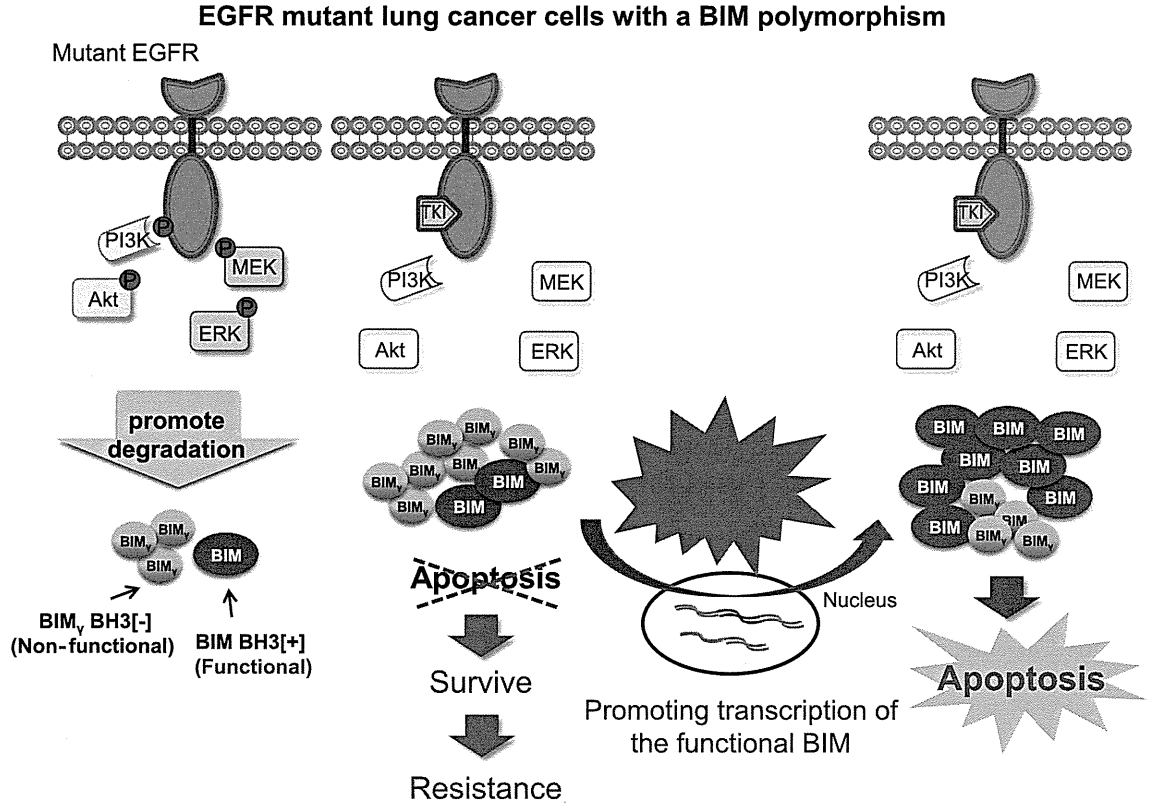

Fig. 3 – Using HDAC inhibition to overcome resistance due to BIM polymorphism. In EGFR mutated lung cancer cells that have BIM polymorphism, since BIMγ, which cannot induce apoptosis, is predominantly expressed, there was resistance to apoptosis even if EGFR signaling is inhibited by EGFR-TKIs. When 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.

Acknowledgments

This work was supported by Grants-in-Aid for Cancer Research (13274949 to S. Takeuchi and 21390256 to S. Yano), Scientific Research on Innovative Areas "Integrative Research on Cancer

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Microenvironment Network" (S. Yano, 22112010A01), and P-DIRECT from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by Grant-in-Aid from the Ministry of Health, Labor and Welfare (S. Yano).

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Please cite this article as: Takeuchi S, Yano S. Clinical significance of epidermal growth factor receptor tyrosine kinase inhibitors: Sensitivity and resistance. Respiratory Investigation (2014), http://dx.doi.org/10.1016/j.resinv.2014.10.002

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Please cite this article as: Takeuchi S, Yano S. Clinical significance of epidermal growth factor receptor tyrosine kinase inhibitors: Sensitivity and resistance. Respiratory Investigation (2014), http://dx.doi.org/10.1016/j.resinv.2014.10.002

Cancer Science
Japanese Cancer Association JCA
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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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Funding information

None declared.

Received January 15, 2014; Revised February 14, 2014; Accepted February 17, 2014

Cancer Sci **105** (2014) 499–505

doi: 10.1111/cas.12383

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-

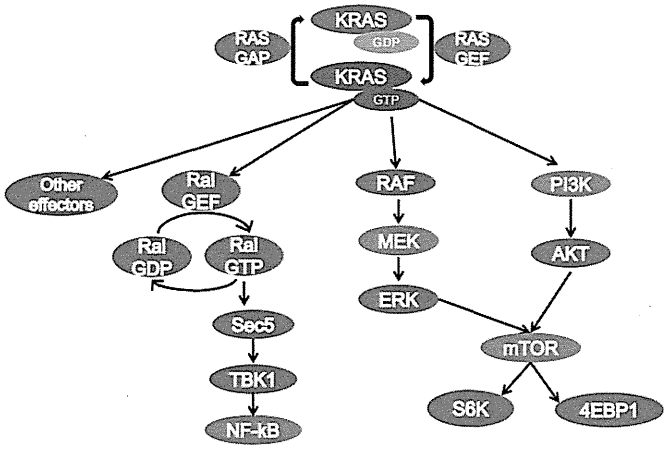

Fig. 1. Effector pathways of Kirsten rat-sarcoma (KRAS). Proteins highlighted green are pharmacologically targetable.

Zeneca, Macclesfield, UK) failed to show clinical activity in an unselected pretreated patient population with a high-rate of *KRAS* mutations.[10–12]

PI3K pathway. The precise role of KRAS in regulating PI3K has been difficult to elucidate because PI3K can be activated by multiple upstream signals, not all of which integrate KRAS to promote downstream signaling. Several lines of evidence suggest PI3K associates with, and is activated by KRAS, thus serving as a principal mechanism of PI3K regulation. The binding of KRAS to p110α induces a conformational change in p110α, which opens and orients the active site of KRAS toward its substrate. Although RBD mutants of p110α fail to bind KRAS, they still maintain enzymatic activity. Interestingly, mice engineered to express RBD-mutant p110α cannot develop mutant *Kras*-driven lung tumors.[13] Furthermore, by using an inducible mouse model of mutant *Kras*-driven lung cancer, Downward and colleagues showed that loss of Kras-p110α binding leads to long-term tumor stasis and partial regression.[14] These elegant studies showed that the interaction between mutant KRAS and p110α is not only required for tumorigenesis but also for tumor maintenance.

In addition to direct activation by KRAS, PI3K can also be activated by receptor tyrosine kinases (RTKs) in *KRAS* mutant cancers. We have reported in colorectal cancers that insulin-like growth factor 1 receptor (IGF-IR) exerts dominant control over PI3K signaling through binding to insulin receptor substrate (IRS) adaptor proteins even in the presence of mutant *KRAS*.[15] PI3K activity is also dependent on basal IGF-IR activity in *KRAS* mutant lung cancer, although in this context mutant KRAS is still thought to be involved in PI3K activation. It has been shown that IGF-IR activation causes IRS-1:p85 complex formation, which in turn relieves an inhibitory effect of p85 on PI3K signaling.[16] Additionally, a recent study showed the *KRAS* mutant NCI-H358 non-small cell lung cancer (NSCLC) cell line still remains dependent on ERBB3 for PI3K signaling.[17] Altogether, these studies suggest numerous contributors, including mutant KRAS and RTKs, activate PI3K signaling in *KRAS* mutant cancers. Another confounding issue is that the role of mutant KRAS may further differ depending on other mutations that may be more or less prevalent among the different tissue types of origin. For example, oncogenic mutations in *KRAS* and *PIK3CA* often coexist in colorectal cancer but less often in pancreatic cancer.[18] The coexistence of *KRAS* and *PIK3CA* mutations in colorectal cancers suggests that mutant KRAS is not sufficient for robust PI3K activity. Similar to MEK inhibitors, single agent PI3K inhibitors are also ineffective for treatment of *KRAS* mutant cancers; murine lung cancers driven by oncogenic *Kras* do not respond to the PI3K/mammalian target of rapamycin (mTOR) inhibitor, NVP-BEZ235.[19] Furthermore, *KRAS* mutations predict resistance to PI3K inhibitors in cell culture experiments.[20,21]

Ral-NF-κB pathway. While the RAF-MEK-ERK and PI3K pathways have been established as key KRAS-effector pathways, KRAS has a number of additional effectors. Among them, the guanine exchange factors of the Ras-like (Ral) GTPases (RalGEFs) have emerged as important effectors of KRAS. Ras-like GTPases directly interact with RAS, and subsequently activates Ral small GTPases.[22,23] Two Ral small GTPases, RalA and RalB, appear to have distinct biological roles in *KRAS* mutant cancers. For instance, inhibition of RalA alone is enough to inhibit tumor initiation, while RalB is vital for tumor invasion and metastasis.[24–26] Similar to KRAS, activated Ral-GTP interacts with multiple downstream effector proteins including RalBP1, which promotes membrane ruffling and filopodia formation through Rac1 and CDC42, as well as receptor trafficking via endocytic regulation.[27] Additional effectors of Ral are the octometric exocyst subunits Sec5 and Exo84, important for secretory vesicle delivery to different membrane compartments.[28,29] Lastly, active RalB signaling causes the association of Sec5 complex with the atypical IkB-related protein kinase TBK1 to promote cell survival through activation of the oncogenic transcription factor NF-κB.[30]

Targeting PI3K-AKT and MEK-ERK Signaling by Combinatorial Approaches

The lack of efficacy seen following suppression of single effector pathway (e.g. use of MEK inhibitors or PI3K inhibitors) in *KRAS* mutant cancers suggests that a combinatorial approach targeting multiple effector pathways is needed. When cancer cells exhibit dependency on a single oncogene ("oncogene addiction"), inhibition of the oncogene leads to downregulation of both PI3K/AKT and MEK/ERK signaling in most instances. Importantly, combination of both a PI3K inhibitor and a MEK inhibitor is sufficient to recapitulate much of the apoptosis and suppression of tumor growth induced by EGFR inhibitors in *EGFR* mutant NSCLC.[31] Moreover, *HER2* amplified and/or *PIK3CA* mutant breast cancers are particularly sensitive to single agent PI3K inhibitors, which surprisingly downregulate both PI3K and MEK/ERK signaling in these cancers, resulting in apoptosis.[32] These results suggest that concomitant disruption of PI3K/AKT and MEK/ERK signaling may underlie much of the antitumor effects observed with targeted therapies in oncogene-addicted models. Consistent with this concept, pharmaceutical inhibition of both the MEK and PI3K pathways has shown durable responses in *KRAS* mutant cancers *in vivo*.[8,19]

Currently, a large number of clinical trials to assess the combination of PI3K inhibitors and MEK inhibitors are ongoing (Table 1). A recent dose-escalation trial tested the combination of the dual PI3K/mTOR inhibitor SAR245409 (Sanofi, Paris, France) with the MEK1/2 inhibitor pimasertib (Merck KGAA, Darmstadt, Germany) in 46 cancer patients. Among the patients, two partial responses were observed: one in a patient with *KRAS* mutant colorectal cancer whose tumor exhibited neuroendocrine features, and a low-grade ovarian cancer patient with simultaneous *KRAS* and *PI3KCA* muta-

Table 1. Currently ongoing trials combining phosphatidylinositol 3-kinase (PI3K) inhibitor and MEK inhibitor

NCT no.	Phase	Company	PI3K inhibitor	MEK inhibitor	Patient selection
01347866	I	Pfizer (New York, NY, USA)	PF-05212384 (PI3K/mTOR inhibitor)	PD-0325901	At the MTD dose, further assessment of these combinations will be done in patients with KRAS mutated colorectal cancer
01363232	Ib	Novartis	BKM120 (pan PI3K inhibitor)	MEK162	At the MTD dose, this combination is explored in patients with EGFR mutant NSCLC, whom have progressed on EGFR inhibitors and triple negative breast cancer, as well as other advanced solid tumors with KRAS, NRAS, and/or BRAF mutations
01390818	I	EMD Serono (Rockland, MA, USA)	SAR245409 (PI3K/mTOR inhibitor)	Pimasertib	Locally advanced or metastatic solid tumors
01155453	Ib	Novartis	BKM120 (pan PI3K inhibitor)	Trametinib	At the MTD dose, further assessment will be done in patients with KRAS or BRAF mutated NSCLC, ovarian, and pancreatic cancer
01859351	I	Wilex (München, Germany)	WX-037 (pan PI3K inhibitor)	WX-554	Solid tumor
01337765	Ib	Novartis	BEZ235 (PI3K/mTOR inhibitor)	MEK162	At the MTD dose, this combination was assessed in patients with EGFR mutant NSCLC, whom have progressed on EGFR inhibitors and triple negative breast cancer, as well as other advanced solid tumors with KRAS, NRAS, and/or BRAF mutations
01392521	Ib	Bayer (Leverkusen, Germany)	BAY80-6946 (pan class I PI3K inhibitor)	BAY86-9766	Advanced cancer
00996892	Ib	Genentech (San Francisco, CA, USA)	GDC-0941 (Pan PI3K inhibitor)	GDC-0973	Locally advanced or metastatic solid tumors
01449058	Ib	Novartis	BYL719 (PI3K alpha-specific inhibitor)	MEK162	Advanced solid tumors or AML or high risk and very high risk MDS, with documented RAS or BRAF mutations
01248858	I	GlaxoSmithKline	GSK2126458 (pan PI3K/mTOR inhibitor)	Trametinib	Advanced solid tumors

AML, acute myeloid leukemia; EGFR, epidermal growth factor receptor; MDS, myelodysplastic syndromes; MEK, mitogen-activated protein kinase kinase; MTD, Maximum Tolerated Dose; mTOR, mammalian target of rapamycin; NCT, national clinical trial that is given to each registered clinical trial; NSCLC, non–small-cell lung cancer; PI3K, phosphatidylinositol 3-kinase.

tions. Grade 3 and 4 toxicities were infrequent, with the most common grade 3 event being skin rash in 14% of patients.[33] In a separate trial combining the PI3K inhibitor BKM120 (Novartis, Basel, Switzerland) and the MEK inhibitor trametinib (GlaxoSmithKline, Brentford, UK), three patients with *KRAS* mutant ovarian cancer achieved partial responses among 66 patients in an unselected population.[34] Based on these three responses, this trial is expanding cohorts to specifically include patients with *KRAS* or *BRAF* mutant tumors. These results suggest that the combination of PI3K and MEK inhibitors has activity, but the activity appears relatively limited. This lack of robust activity seems to be attributed to the difficulty of sufficiently suppressing both pathways without toxicities in a given patient. For example, a trial combining MK-2206 (Merck), an AKT inhibitor, and selumetinib, four of eight patients demonstrated biologically significant inhibition in one marker; however, at the maximum tolerated dose no patient had ≥70% inhibition of both targets.[35]

Alternative therapeutic strategies targeting RTKs that indirectly suppress the PI3K pathway in combination with MEK inhibition may be more tolerable, and as a consequence more effective. As mentioned, the IGF-IR is largely responsible for PI3K activation in *KRAS* mutant colorectal and lung cancer cell lines, and the combination of IGF-IR and MEK inhibitors results in tumor regressions in these xenografts.[15,16] This approach is currently being evaluated in a phase I/II trial of IGF-IR antibody ganitumab (Amgen, Thousand Oaks, CA, USA) combined with the MEK inhibitor MEK162 (Novartis) in *KRAS* mutant colorectal and pancreatic cancer and *BRAF* mutant melanoma (ClinicalTrilas.gov registry number, NCT01562899).

Targeting the Apoptotic Machinery

As mentioned above, in cancers addicted to a single oncogene, effective target inhibition generally results in apoptosis. This process involves the downstream BCL-2 family of proteins, which act as guardians of mitochondria-mediated apoptosis. For example, in *EGFR* mutant NSCLCs, treatment with an EGFR inhibitor shifts the balance of pro- and anti-apoptotic BCL-2 family members, reducing the expression of anti-apoptotic MCL-1 as a result of PI3K/mTORC1 inhibition,[31] and increasing the expression of pro-apoptotic BIM as a result of MEK/ERK suppression, leading to apoptosis.[31,36] In addition,

a recent study using engineered mice deficient for the pro-apoptotic BCL-2 family members BIM or PUMA provided evidence that BIM and PUMA are both key apoptotic effectors of tyrosine kinase inhibitors in *EGFR* mutant NSCLC and *HER2* amplified breast cancer.[37]

The TBK1/BCL-XL pathway. In addition to the PI3K and MEK/ERK pathway, mutant KRAS maintains proliferation and evades apoptosis through other pathways. For instance, shRNA screening using *KRAS* mutant cancer cell lines identified TBK1 as a synthetic lethal partner of oncogenic KRAS. Interestingly, BCL-XL, a known NF-κB target, was identified as a TBK1-regulated gene. Overexpression of BCL-XL rescued apoptosis induced by KRAS or TBK1 knockdown in the NCI-H23 KRAS mutant cell line.[38]

Combination of MEK inhibitor with BCL-XL inhibitor. Pharmacological inhibition of the MEK/ERK pathway is relatively more achievable compared with the PI3K pathway.[39,40] Therefore, MEK inhibitor therapy could be a backbone for combinatorial approaches for *KRAS* mutant cancers. To this point, shRNA screening was performed to identify genes that, when inhibited, cooperate with MEK inhibitors to reduce cell survival in *KRAS* mutant cell lines.[41] BCL-XL emerged as a top hit through this approach. That is, BIM induction following MEK inhibition is not enough to cause apoptosis, but BCL-XL knockdown disrupts an inhibitory complex between BIM and BCL-XL, leading to apoptosis in the presence of MEK inhibitor. Induction of apoptosis is recapitulated by combining the BCL-2/BCL-XL inhibitor navitoclax (ABT-263) with a MEK inhibitor. Two additional studies have also shown the efficacy of this combination.[42,43]

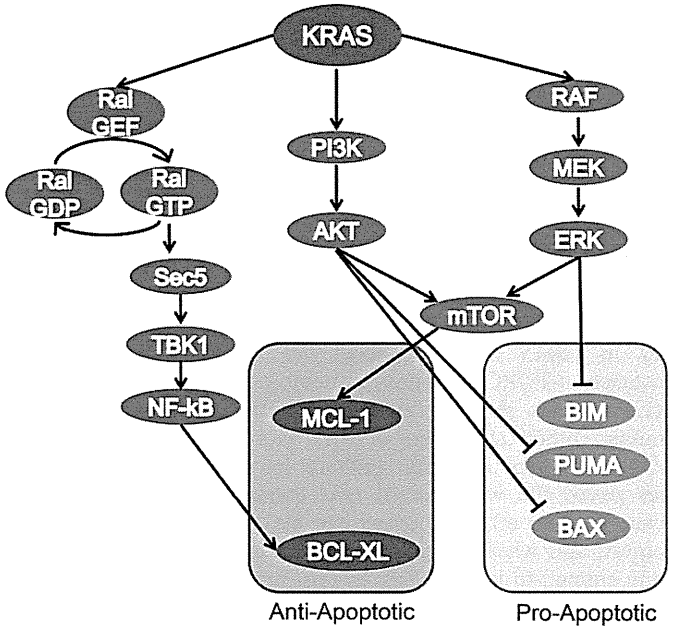

Fig. 2. Effector proteins of Kirsten rat-sarcoma (KRAS) and apoptosis. The BCL-2 family of proteins regulates mitochondrial-driven apoptosis in KRAS mutant cancers. The BCL-2 family consists of three subfamilies: the pro-survival members such as BCL-2 or MCL1, the pro-apoptotic BCL-2 homology domain 3 (BH3)-only proteins such as BIM and PUMA, and the pro-apoptotic BAX and BCL-2 antagonist/killer (BAK; not shown in this figure). The anti-apoptotic function of oncogenic KRAS is mediated by several effector pathways that converge on the BCL-2 family of proteins. The PI3K effector pathway suppresses pro-apoptotic protein PUMA and BAX, the RAS–RAF pathway downregulates the pro-apoptotic protein BIM, and the mTORC1 pathway regulates MCL-1. In addition, the Ral-NF-κB pathway has been implicated in the regulation of BCL-XL. Thus, KRAS suppresses cell death responses through regulation of both pro-apoptotic and anti-apoptotic BCL-2 family proteins.

Combination of mTORC1/2 inhibitor and BCL-2/BCL-XL inhibitor. We have recently showed *KRAS* mutant colorectal cancers are particularly vulnerable to simultaneous inhibition of the BCL-2 anti-apoptotic proteins BCL-2, BCL-XL and MCL-1.[44] Pure mTORC catalytic site inhibitors downregulated MCL-1 in *KRAS* mutant colorectal cancers, and targeting *KRAS* with shRNA similarly reduced mTORC1 signaling and MCL-1 levels, suggesting MCL-1 to be a vital KRAS-effector molecule in these cancers. When combined with the BCL-2/BCL-XL inhibitor navitoclax, the mTORC1/2 inhibitor AZD8055 induced tumor regressions in *KRAS* mutant human colorectal cancer xenografts and *Kras* mutant genetically engineered mouse models of colorectal cancers. In all, this study provides the rationale to use mTORC inhibitors in combination with BCL-2/BCL-XL inhibitors in *KRAS* mutant colorectal cancers. Altogether, these data mark the apoptotic machinery as an attractive target to treat *KRAS* mutant cancers (Fig. 2).

Combination of MEK inhibitor and docetaxel. Several studies have demonstrated that cytotoxic agents, including microtubule stabilizing drugs, stimulate MAPK signaling upon administration. Combining inhibitors of MAPK signaling with one such drug, docetaxel, results in an enhanced anti-tumorigenic phenotype.[45] One of the key mechanisms of this synergy is induction of pro-apoptotic proteins by inhibiting MAPK signaling, which reduces the threshold for apoptosis induction by cytotoxic agents. In fact, prolonged exposure to the MEK inhibitor selumetinib induced BIM expression in the *KRAS* mutant HCT-116 xenograft model. A prospective randomized phase II study assessing the impact of adding selumetinib to docetaxel in previously treated patients with advanced *KRAS* mutant NSCLC was conducted based on these pre-clinical results. Despite no differences in median overall survival, there was significant improvements in both progression-free survival and objective response rate in patients administered selumetinib.[46]

Concurrently with the clinical trials in human subjects, a *Kras* mutant transgenic mouse model was used to optimize treatment modalities, a so-called "co-clinical" trial.[47] This mouse study revealed that adding selumetinib was beneficial for mice with *Kras* or *Kras / p53* mutant lung cancer, but not with *Kras* and *Lkb1* mutations. Interestingly, *Kras/Lkb1* tumors show substantially less phosphorylation of ERK, suggesting that the ERK pathway is less active in these cancers. Furthermore, integrated genomic and proteomic profiles revealed SRC is activated in *Kras/Lkb1* tumors,[48] suggesting that *Kras/Lkb1* mutant tumors are a distinct subset of *KRAS* mutant cancers that may be less dependent on ERK signaling and more dependent on other pathways. Intriguingly, another recent report suggests that NSCLCs harboring mutations both in *KRAS* and *LKB1* are addicted to coatomer complex I (COPI)-dependent lysosome acidification, which participates in retrograde transport, is required for endosome maturation and is a CDC42 effector required for CDC42 transformation.[49]

Identifying Synthetic Lethal Interaction with KRAS

Recent high-throughput screening has provided an expanded list of targets for *KRAS* mutant tumors (Table 2). For example, siRNA screening in *KRAS* mutant NSCLC cell lines identified the transcription factor GATA2 as necessary for the survival

Table 2. Candidate genes showing synthetic lethal interaction with Kirsten rat-sarcoma (KRAS)

Synthetic lethal genes or pathways	Methodology	Pharmacological inhibition	References
TBK1	shRNA screening	Not assessed	38
Coatomer complex I (COPI)	Parallel screening of chemical and genetic perturbations	Saliphenylhalamide A	49
GATA2	siRNA screening	Bortezomib with Fasudil	50
CDC6	siRNA screening	Bortezomib and topotecan	51
STK33	shRNA screening	Specific inhibitor was subsequently developed, but failed to suppress growth of cells	52, 57
TAK1	Expression data based bioinfomatic analysis	5Z-7-oxozeaenol	53
Polo-like kinase (PLK) 1 and 2	shRNA screening and outlier kinase analysis	BI-2536	54, 58
CDK4	Mouse genetic studies	PD0332991	55
Reactive oxygen species	Chemical screening	Lanperisone	56

Fasudil is a Rho signaling inhibitor, approved for the treatment of cerebrovascular spasm in Japan.

of these cancers.[50] GATA2 maintains cell survival via the proteasome machinery, the IL-1/NF-κB signaling pathway, and the Rho-signaling cascade. Combined inhibition of the proteasome and Rho signaling recapitulates the effect of GATA2 loss on *KRAS*-driven tumorigenesis. CDC6, a critical regulator of DNA replication, has also been identified as a synthetic lethal protein with mutant *KRAS*.[51] Bioinformatic analysis suggests proteasome components functionally interact with CDC6, and knockdown of CDC6 showed additional synthetic lethal effects with proteasome inhibitor treatment. Other targets identified by synthetic lethal approaches include, as discussed above, TBK1,[38] as well as COPI,[49] STK33,[52] TAK1,[53] APC/C,[54] CDK4,[55] Polo-like kinase (PLK) 1,[54] and reactive oxygen species (ROS).[56] It should be cautioned that a major caveat associated with RNAi screening is potential off-target effects and the potential disconnect between reduction of total expression and inhibition of kinase function. For example, while STK33 knockdown was synthetic lethal for *KRAS* mutant cancers, inhibition of STK33 kinase activity does not appear to be effective therapy for *KRAS* mutant cancers.[57]

Other Means to Target KRAS

"Outlier kinase" approach. Using an innovative approach of identifying "outlier kinase" expression through analysis of transcriptome sequencing data from a large number of cancers, polo-like kinases (PLKs) were noted to be overexpressed in a subset of *KRAS* mutant pancreatic cancers, and these cancers had specific sensitivity to the PLK-pan inhibitor, BI-6727.[58]

HSP90 inhibitor combinations. Pharmaceutically targeting HSP90 has attracted significant interest. HSP90 inhibitors target HSP90 client proteins resulting in their rapid degradation. Although KRAS is not a client protein of HSP90, *KRAS* mutant NSCLCs are exquisitely sensitive to HSP90 inhibition,[59] most likely through the HSP90-inhibitor-mediated degradation of downstream signaling proteins such as C-RAF[60] as well as the production of ROS.[61] Interestingly, HSP90 inhibitors may have particular activity in combination with the mTOR inhibitor rapamycin in *KRAS/p53* mutant NSCLCs through rapamycin-mediated suppression of glutathione in the presence of HSP90-inhibitor induced ROS.[61]

Targeting posttranslational modification of KRAS. Lastly, targeting mutant *KRAS* by interfering with important KRAS post-translational modifications has recently been explored. The phosphorylation of KRAS on Serine 181, which is mediated by PKC,[62] is indispensable for full KRAS oncogenic activity.[63,64] As such, treatment of *KRAS* mutant cancers with PKC inhibitors has anti-proliferative and pro-apoptotic activity,[63,64] marking PKC as an intriguing therapeutic target.

Conclusion

Targeted therapies that directly disrupt oncogene function have changed the way cancers are treated. While one of the most obvious targets is oncogenic *KRAS*, mutated in roughly one-fourth of all cancers, direct targeting of KRAS has remained largely elusive. Instead, co-targeting pathways downstream of mutant KRAS has emerged in pre-clinical studies as a promising therapeutic strategy. However, validation of these pre-clinical studies has been hindered by unanticipated challenges, such as dose-limiting toxicity of combinatorial inhibition of PI3K and MEK/ERK signaling. Alternatively, blocking upstream activators of PI3K, such as IGF-IR, in combination with MEK inhibition, may be a less toxic and thus more successful strategy. More recently, targeting the apoptotic machinery in *KRAS* mutant cancers has garnered attention. For instance, mTORC inhibitors in combination with BCL-2/BCL-XL inhibitors showed dramatic pre-clinical efficacy in *KRAS* mutant colorectal cancers *in vivo*. Moreover, the identification of novel targets that offer synthetic lethality with mutant *KRAS* has paved the way toward new therapeutic strategies. However, whether effective drugs can be designed to disrupt these targets, and whether these drugs can be administered at doses high enough to inhibit their targets, remains to be seen. Lastly, the identification of already clinically available drugs that show efficacy in subsets of *KRAS* mutant cancers, such as the combination of docetaxel and selumetinib in *KRAS* mutant NSCLC with wild type *LKB1*, may speed up the implementation of much needed novel therapies.

Acknowledgments

We thank Drs Matt Niederst and Erin Coffee for critical reading of the manuscript.

Disclosure Statement

The authors have no conflict of interest.