Determining EGFR-TKI sensitivity of G719X and other uncommon EGFR mutations in non-small cell lung cancer: Perplexity and solution (Review)

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Abstract. Mutations in epidermal growth factor receptor (EGFR) play critical roles in the pathogenesis of non-small cell lung cancer (NSCLC), and they are highly associated with sensitivity to tyrosine kinase inhibitors (TKIs). While the pathogenic and pharmacological characteristics of common mutations in EGFR have been thoroughly investigated, those of uncommon mutations remain to be elucidated. Traditional approaches to study common mutations by randomized controlled trials are not feasible for uncommon mutations owing to their rarity. Therefore, by systematically reviewing laboratory and clinical studies of the G719X mutation, one of the uncommon mutations, we concluded that the G719X mutation was intermediately sensitive to TKIs, with an average response rate of 35.1% (47/134). Moreover, accordingly, we proposed a comprehensive model to investigate uncommon mutations in EGFR. The model involves both basic and clinical components, composed of structural analyses, functional alterations, cell viabilities and animal models with various types of clinical studies. In this review, we systematically reviewed studies of the G719X mutation and put forward a research model that could be generalized to explore uncommon mutations in diseases associated with gene mutations.

Contents

1. Introduction

Lung cancer is the leading cause of death among all cancer deaths (1). It has the highest morbidity and mortality among all malignancies worldwide (1,2). NSCLC accounts for 70-85% of all lung cancers, and most cases are of advanced stage or metastatic condition when diagnosed (3,4). As a targeted therapy, EGFR tyrosine kinase inhibitors (EGFR-TKIs) have been approved by the FDA for the treatment of advanced NSCLC since 2003 (5). EGFR-TKIs have produced encouraging results by postponing tumor progression and prolonging the progression-free survival (PFS) of advanced NSCLC patients for approximately 5 months compared to platinum-based doublet chemotherapy (6,7).

However, only 15% of NSCLC patients responded to TKI (8), and clinical trials on gefitinib or erlotinib failed in unselected patient populations, as they were not able to significantly prolong patient overall survival (OS) compared to traditional chemotherapy (9-11). Based on known EGFR mutations, researchers eventually discovered the association between TKI sensitivity and EGFR mutations (12). Moreover, they also observed considerable ethnic differences in the frequencies of EGFR mutations in NSCLC patients. EGFR mutations were detected in approximately 50% of Asian patients with NSCLC, but only in 10% of patients in the

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Abbreviations: Del19, in-frame deletions in exon 19 of EGFR; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; G719X, point mutations that result in substitutions of the glycine at position 719 to other residues; L858R, point mutations that result in substitutions of the leucine at position 858 to arginine; NSCLC, non-small cell lung cancer; RCT, randomized controlled trial; RR, response rate; TKI, tyrosine kinase inhibitor; wt, wild-type

Key words: epidermal growth factor receptor, non-small cell lung cancer, uncommon mutations, G719X mutation, tyrosine kinase inhibitor, tyrosine kinase inhibitor sensitivity, targeted therapy, methodology

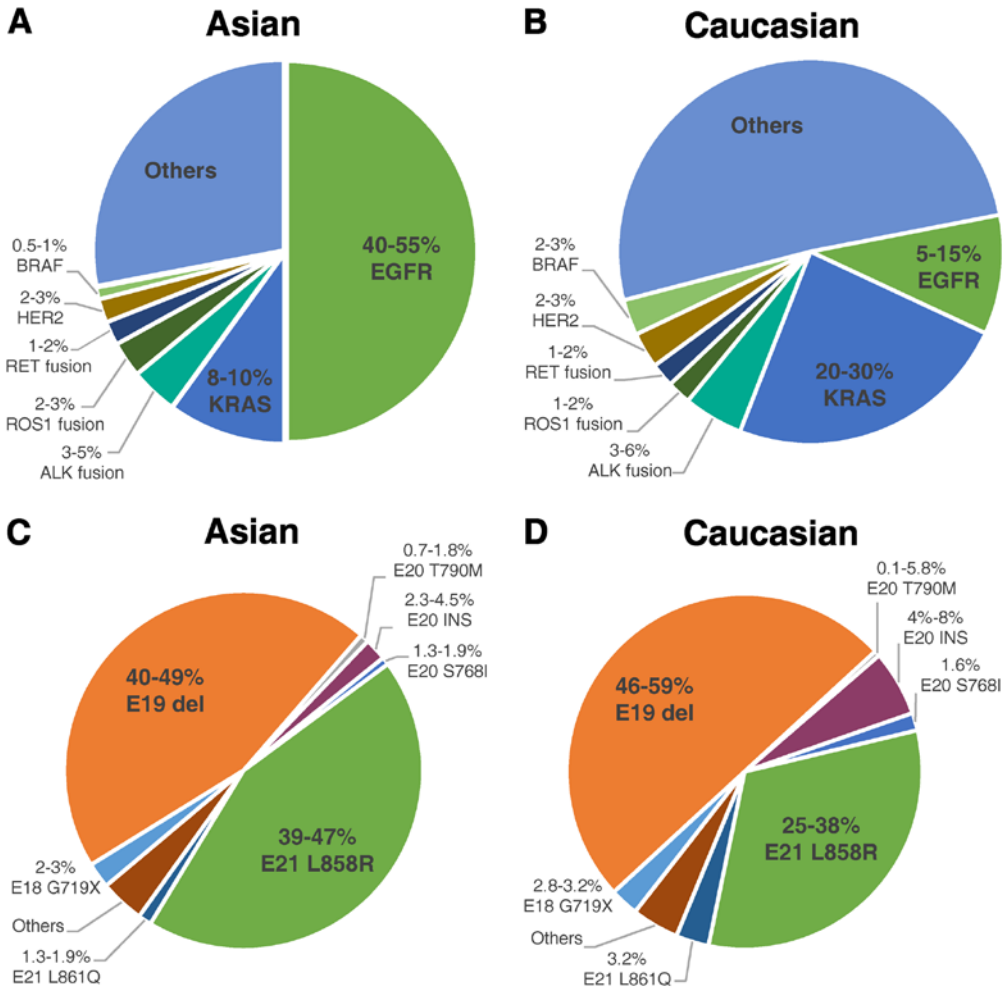

Figure 1. Mutation frequency and distribution in Asian and Caucasian populations. (A and B) Frequencies of various driver mutations in NSCLC patients of Asian and Caucasian populations; the data are referred from Kohno 2015 (14). (C and D) Distribution of different mutations among EGFR mutations in NSCLC patients of Asian and Caucasian populations; the data were generated by summarizing the results from previous studies (14-22).

western world (13-15) (Fig. 1). Two types of EGFR mutations, in-frame deletions in exon 19 (Del19) and point mutations in exon 21 causing a leucine-to-arginine substitution at codon 858 (L858R), which are established to be definitely sensitive to TKIs, comprise approximately 90% of all EGFR mutations (15-18). The remaining 10% of EGFR mutations are defined as uncommon mutations (Fig. 1). Therefore, for NSCLC patients with uncommon EGFR mutations of unknown clinical significance, it is dubious whether they can benefit from TKI targeted therapy.

To answer this important question, there is an urgent need to determine the clinical significance of uncommon mutations in EGFR, particularly their sensitivity to TKIs. Although they only account for a small proportion of patients with EGFR mutations, they are still a large population due to the high incidence of NSCLC.

It seems apparent that we could try to pattern the methodologies of the Del19 and L858R mutations, which have been proven to be sensitive, mostly by means of clinical randomized controlled trials (RCTs) with large sample sizes. However, this is not a practical approach for uncommon mutations, as cases of uncommon mutations are too rare to conduct RCTs. Therefore, we have to take advantage of basic studies or other types of clinical studies. In other words, the ultimate problem is how we determine the sensitivity of uncommon EGFR mutations. Then, we can make clinical decisions regarding whether to apply TKI targeted therapy to NSCLC patients with certain uncommon mutations.

The G719X mutation in EGFR refers to point mutations that result in substitutions of the glycine at position 719 to other residues, primarily alanine (G719A), cysteine (G719C) and serine (G719S). The G719X mutation accounts for approximately 3% among all EGFR mutations in both Asian and Caucasian populations (14-22) (Fig. 1). It is the most commonly seen and most thoroughly studied EGFR uncommon mutation, and it is considered a sensitive mutation.

In this study, by reviewing studies of the G719X mutation, we propose a comprehensive research model to explore the laboratory and clinical characteristics of uncommon mutations (Fig. 3). We also systematically summarized the studies of the G719X mutation and discovered the missing components to form a complete research system. The conclusions regarding the G719X mutation would be much more convincing if the evidence was complete. More importantly, the research model can be generalized to direct researchers to explore other uncommon mutations in patients with diseases associated with

Table I. Summary of studies of G719X responses to 1G-TKIs.[a]

Study	Mutation	TKI	Total	Response	RR/%	Sensitivity	Ref.
Lynch 2004	G719C	G	1	1	100	Sensitive	23
Han 2005	G719A	G	2	1	50	Intermediate	24
Takano 2005	G719X	G	2	1	50	Intermediate	25
Eberhard 2005	G719A	E	1	0	0	Resistant	26
Janne 2006	G719C	G	1	1	100	Sensitive	27
Ichihara 2007	G719X	G	1	0	0	Resistant	28
Pallis 2007	G719D	G	1	0	0	Resistant	29
Sequist 2008	G719A	G	1	0	0	Resistant	30
Wu 2008	G719A	E/G	2	1	50	Intermediate	31
Wu 2011	G719X	E/G	8	4	50	Intermediate	32
De Pas 2011	G719S	E	1	1	100	Sensitive	33
Takahashi 2012	G719A	G	1	0	0	Resistant	34
Lee 2013	G719A	E	1	0	0	Resistant	35
Umekawa 2013	G719A	E	1	0	0	Resistant	36
Locatelli-Sanchez 2013	G719A	E/G	1	1	0	Resistant	37
Keam 2014	G719A	G	1	0	0	Resistant	38
Beau-Faller 2014	G719X	E/G	10	1	10	Resistant	18
Guan 2014	G719A	E	1	0	0	Resistant	39
Watanabe 2014	G719X	G	3	0	0	Resistant	40
Fukihara 2014	G719A	E/G	4	1	25	Intermediate	41
Chiu 2015	G719X	E/G	76	28	36.8	Intermediate	42
Xu 2016	G719X	E/G/I	14	6	42.9	Intermediate	43
Total			134	47	35.1	Intermediate	

[a]1G TKIs, 1st generation EGFR-TKIs mainly refers to gefitinib, erlotinib and icotinib. Data were extracted from corresponding studies. G, gefitinib; E, erlotinib; I, icotinib; RR, response rate. Sensitivity cut-off values: ≥0 RR<25%, resistant; ≥25% RR <75%, intermediately sensitive; ≥75% RR ≤100%, sensitive.

gene mutations and to ascertain their pharmacologic properties efficiently.

2. Current studies of the G719X mutation in EGFR in NSCLC

The first observation of the G719X mutation in EGFR in NSCLC patients was reported by Lynch *et al* in 2004 (23). The patient harbored a G719C mutation and presented with partial response to gefitinib, with an OS of 17.9 months. Based on studies conducted over the following two years, the NCCN guidelines for NSCLC (version 2.2011) described the G719X mutation in EGFR as associated with response to TKIs. This conclusion was supported by subsequent investigations in general. Herein, the studies of the G719X mutation are reviewed comprehensively from both clinical and laboratory perspectives. The history of studies of the G719X mutation in EGFR is presented in Fig. 2.

Clinical studies of the G719X mutation in NSCLC

Case reports and retrospective studies. Since Lynch reported the first case (23), more and more cases have been reported either in the form of case reports or retrospective studies. However, most of them involved no more than ten patients. Only one retrospective study by Chiu *et al* (42) in 2015 enrolled a relatively large sample size of 76 patients with the G719X mutation, of which 28 responded to TKIs, indicating a response rate (RR) of 36.8%. To overcome the limitation of sample size, we summarized all of these studies and combined the results to obtain an average RR. We enrolled 22 relative studies from 2004 to 2016 and excluded all reviews to avoid possible data overlap (18,23-43). Then, we had a total of 134 G719X patients, of which 47 patients responded to 1st generation EGFR-TKIs (Table I). The average RR is 35.1% (47/134), indicating that G719X is a mutation of intermediate sensitivity, which is in accordance with previous reviews (16,44-46) (Table II).

Reviews and meta-analyses. Four reviews concerned responses of G719X to TKIs, with response rates of 50-66.7% (16,44-46) (Table II). Still, in these reviews, the numbers of cases were too small to be convincing. No meta-analyses were found.

Prospective studies. Due to the rarity of uncommon mutations, we could not enroll enough patients to conduct a prospective randomized controlled trial. Observational studies are probably a feasible way to investigate the sensitivity of uncommon mutations in a prospective manner. Arrieta *et al* analyzed 188 NSCLC patients in their cohorts and found 11 patients with the G719X mutation who received TKIs, including a single

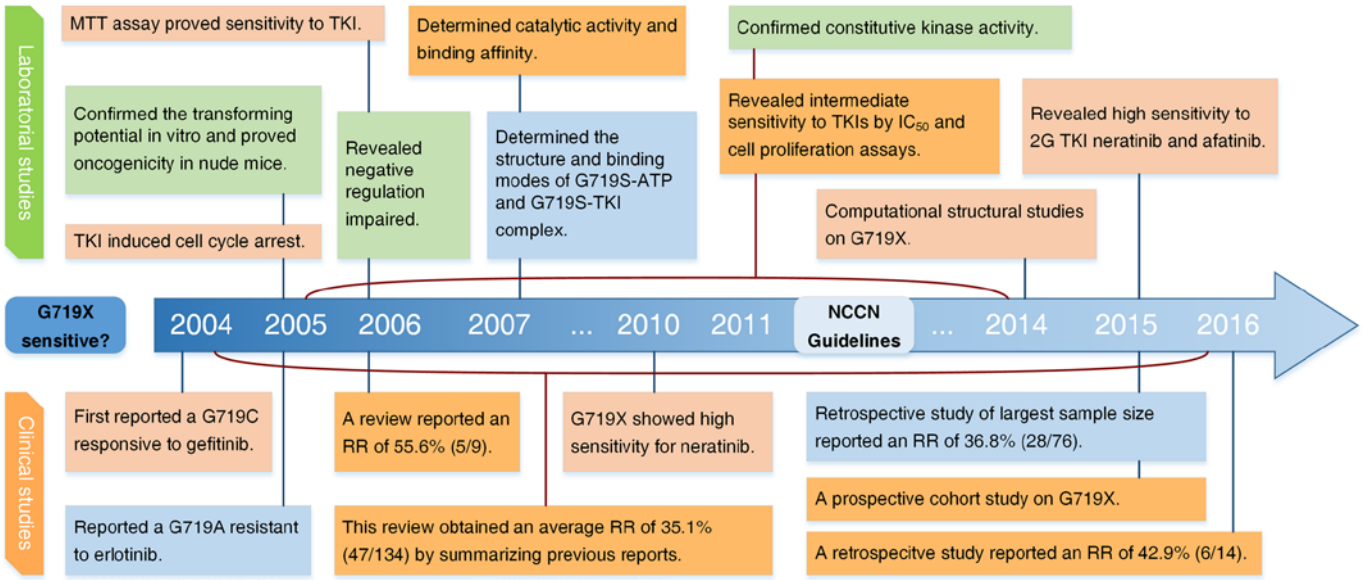

Figure 2. The history of studies of G719X mutation in EGFR. 2G TKI, second generation of tyrosine kinase inhibitor; RR, response rate; wt, wild-type EGFR. Green, oncogenicity; red, TKI sensitive; orange, TKI intermediately sensitive; blue, TKI resistant.

Table II. Summary of clinical studies of the G719X mutation in EGFR.

Research type	Article	Mutation	TKI	Total cases	Response	RR/%	Sensitivity	Ref.
Retrospective studies								
Summary of cases	This article	G719X	E/G	134	47	35.1%	Intermediate	Table I
Reviews	Mistudomi 2006 and 2007	G719X	E/G	9	5	55.6	Intermediate	44,45
	Kobayashi 2015	G719X	G	3	2	66.7	Intermediate	16
	Klughammer 2016	G719X	E	2	1	50	Intermediate	46
Meta-analysis	Not found							
Prospective studies								
Observational	Arrieta 2015	G719X	E/G/A	11	NA[a]	NA	Not known	47
Clinical trials	Not found							

[a]The response rate of the G719X mutation were not discussed separately. G, gefitinib; E, erlotinib; A, Afatinib; RR, response rate; NA, not accessible. Sensitivity cut-off values: ≥0 RR <25%, resistant; ≥25% RR <75%, intermediately sensitive; ≥75% RR ≤100%, sensitive.

G719X mutation and complex mutations. Although G719X was not discussed separately, they found the rare mutation group to be intermediately sensitive with an RR of 32.4% (47).

Summary of clinical studies. All clinical studies enrolled are summarized in Table II. As stated above, because of limitations in sample size, it is not adequately convincing to determine the sensitivity of the G719X mutation based only on clinical studies. Given the circumstances, it is necessary to seek supporting evidence from laboratory studies. With both clinical and basic studies to form a complete evidence system and logic network, we could have sufficient cause to consider G719X a sensitive mutation.

Laboratory studies of the G719X mutation in EGFR in NSCLC. In general, the laboratory studies mainly focused on alterations caused by the G719X mutation, regarding the protein structure, protein function, cell viability and animal experiments. Thus, the laboratory studies were reviewed in these four perspectives.

Functional alterations. The activation of EGFR is initiated after binding to its ligand, epidermal growth factor (EGF) or transforming growth factor-α (TGF-α). The receptor changed its conformation and then dimerized with another ligand-bound EGFR or other ErbB family members to form homodimers or heterodimers, respectively. The dimer harbored kinase activity and would phosphorylate itself at specific sites (48,49), which could act as catalytic sites to activate downstream signaling pathways, such as MAPK or PI3K/Akt, by phosphorylation of the corresponding molecules. Afterwards, the activated EGFRs were internalized into the cell plasma by endocytosis,

and then they were either recycled onto the cell membrane or degraded by fusion with lysosomes (50). This is one way of negative regulation in EGFR signaling pathway. A series of studies revealed the influences of the G719X mutation and TKI treatment on all of the functional processes.

Ligand binding and dimerization. Choi *et al* explored how the G719S mutation affected ligand binding using a ^{125}I-labelled EGF binding assay. Moreover, they also used antibodies against the EGFR extracellular region to label EGFR, and they observed dimerization of receptors with immunofluorescence microscopy. No differences were discovered in ligand binding or dimerization between G719S mutants and wild-type (wt), while they indeed observed EGF-independent dimerization of EGFRs in Del19 and L858R mutants (51).

Kinase activity. Greulich *et al* systematically investigated the kinase activity of G719S mutants (52). Using immunoblotting, they discovered the ligand-independent constitutive phosphorylation activity on both the receptor itself and on downstream signal molecules, such as Shc, STAT3 and Akt (52). Chen and Choi further compared the auto-phosphorylation levels of G719S with that of Del19 and L858R. They found that the auto-phosphorylation level of G719S was lower, indicating that the oncogenicity of G719S was weaker than that of the other two common mutants (51,53). Subsequent studies further confirmed the conclusions using western blotting and immunofluorescence staining (51,53-57).

As the transforming potential of G719X was determined, the question arose as to what extent EGFR-TKI can inhibit the uncontrolled kinase activity of G719X mutant EGFR. Jiang *et al* investigated gefitinib in their kinase assay of G719X and found that gefitinib was able to inhibit the auto-phosphorylation of G719S in a dose-dependent manner. However, compared with L858R, G719S required a higher concentration of gefitinib (54). Their conclusions were further validated by subsequent studies using other techniques (53,57-59). In general, based on the studies mentioned above, G719X was found to have moderate oncogenicity and intermediate sensitivity to TKIs regarding kinase function.

Kinetics and binding affinity. Why would the G719X mutation cause weaker oncogenicity and lower sensitivity to TKIs than L858R? Is it because the mutation affected the catalytic properties of the kinase and the interactions between EGFR and TKIs? As TKIs are competitive inhibitors of ATP, studies comparing the binding affinities of TKI-EGFR complexes with ATP-EGFR complexes may answer these questions.

Yun *et al* investigated the kinetics and affinities of G719X mutants using a continuous colorimetric assay and a fluorescence-quenching assay. The mutation in the tyrosine kinase domain was found to dramatically elevate catalytic activity by approximately 50-fold in L858R and 10-fold in G719S compared to wild-type (60). In terms of affinities, they determined the dissociation constants (Kd) of EGFR-gefitinib complexes and EGFR-AMPPNP (an analogue of ATP) complexes. Although the G719S mutation decreased the affinity to gefitinib, it lowered the affinity to ATP much more, indicating that the inhibiting potential (Kd_{TKI}/Km_{ATP}) of gefitinib was 5-fold stronger than wt, while L858R was approximately 100 times stronger (60). This might explain why G719S mutants were less sensitive to gefitinib than L858R mutants.

Negative regulation. There are mainly two ways to negatively regulate the activated wild-type EGFR after the activated receptor has done its job to trigger the downstream signaling. Once internalized by endocytosis, the receptors would either be recycled back to the cell membrane or be degraded by fusion with lysosomes mediated by ubiquitination (50,53). Along with persistent positive activation, impaired downregulation might take part in the oncogenicity of G719X mutants as well.

Few studies focused on negative regulation. Chen *et al* discovered that the G719S mutants were refractory to ubiquitination and had more sustained tyrosine phosphorylation than wild-type (53). A similar phenomenon was observed in Del19 and L858R mutants (61). Furthermore, internalization was also found to be impaired in Del19 mutants (61).

Structure determination. The determination of kinetic and binding parameters provided clues to help us understand the mechanisms of the pathogenic and pharmacological effects of the mutation. Nonetheless, to further elucidate why the mutation affects oncogenicity and sensitivity requires determination of the structures of mutated EGFRs and EGFR-TKI complexes.

Yun *et al* determined the 3D structures of the G719S-AMPPNP complex by crystal diffraction (60). The comparison of the structures between G719S and wild-type EGFR indicated that the G719S mutation destabilized the inactive conformation and thus promoted the active conformation of the kinase (60). This explained the oncogenic potential of G719S mutants on the basis of structures.

The inactive conformation of EGFR requires the C-helix to be rotated outward to be displaced from the active site. The glycine residue at position 719 interacts with several hydrophobic residues flanking L858, including F723, L747 and L862. Packing together with the N-terminal portion of the activation loop, the hydrophobic cluster changed into a helical turn, causing the C-helix to rotate outward by steric hindrance. Therefore, any substitution of a glycine residue at position 719 would sabotage the stable hydrophobic interactions, which are essential for the receptor to adopt the inactive conformation (60).

Furthermore, they also elucidated the binding mode of the G719S-gefitinib complex. Unfortunately, they were unable to resolve the differences in the binding modes among G719S-gefitinib, L858R-gefitinib and wt-gefitinib complexes, thus failing to explain the different binding affinities of various EGFRs and the distinct sensitivities to TKIs (61).

Another structural study of G719X was performed by Doss *et al* in 2014, with computational structure simulation, a new approach to study the structures of proteins. They simulated the real-time conformational alterations in G719S mutants to discover that the G719S mutation increased the distance between residues L718 and G796, forming a wider opening for TKIs to get into the ATP-binding pocket than wild-type, indicating that this mutation should respond to TKIs (62). However, they did not include Del19 or L858R mutants in their study.

Cell viability. Constitutive kinase activity of G719X mutants will persistently activate downstream signaling, resulting in

EGFR-signaling-pathway-mediated cell proliferation in a ligand-independent manner. Will we observe uncontrolled cell proliferation when the G719X mutation is introduced into certain cell lines? And to what extent can TKI inhibit cell proliferation?

Elevated cell viability in G719S transformed NIH-3T3 and Ba/F3 cell lines were observed in several studies, but it was lower than that of Del19 and L858R mutants (51,52,56). Moreover, it can be abrogated by gefitinib, yet is somehow more resistant than L858R (52). To be more quantitative, the 50% inhibiting concentration (IC_{50}) of gefitinib in various mutants was further measured in series of studies. The IC50 of gefitinib in G719S mutants was between that of gefitinib in wild-type cells and Del19/L858R mutants, implying an intermediate sensitivity of G719S (16,52,54,55,57,63).

The inhibition of cell viability by TKIs was observed, but the mechanisms for the abrogation remained to be elucidated. Jiang *et al* focused on the impact on the cell cycle of the mutant cells resulting from TKI treatment. Using FACS and immunoblotting, they found that gefitinib induced cell cycle arrest in the G_1 phase by downregulating the level of D-type cyclins and CDK4 in G719S mutant Ba/F3 cell lines (54). However, they detected no apoptotic cells in G719S mutants (54), while apoptosis induced by gefitinib was observed in L858R mutants (48,64).

In conclusion, TKIs inhibit the proliferation of transformed cells to various extents in different mutants. The transformed cells with the G719S mutation exhibited intermediate sensitivity to TKI inhibition compared with Del19 and L858R mutants.

Animal models. The cell culture experiments are still too far from the authentic situation, thus it is necessary to investigate the role of the G719X mutation in animal models and to determine whether TKI treatment would be effective in animals with tumors driven by the G719X mutation.

Greulich *et al* injected the G719S transformed cells into immuno-compromised mice and observed tumorigenesis (52). Moreover, tumor size varied among the different mutation groups. The average diameter of tumors in the G719S group was half of that in the L858R group (52). They confirmed the oncogenic potential of the G719S mutation in an animal model; however, their experiment might be more complete if they took the survival condition of the inoculated animals and TKI treatment into account.

Summary of basic studies. The laboratory studies of G719X are summarized in Table III, with a brief overview of the results and the corresponding methods of the experiments. The high level of consistency in the results of the laboratory studies including protein function experiments, cell viability experiments and animal experiments further authenticate the oncogenicity and sensitivity of the G719X mutation. However, we still need a better understanding of the structure leading to intermediate sensitivity to further complete this research system.

3. A comprehensive model for studying uncommon mutations

Based on the reviewed studies of the G719X mutation in EGFR in NSCLC, we propose a systematic model to investigate the pathogenic and pharmacological characteristics of an uncommon mutation at both the laboratory and clinical levels (Fig. 3). Due to the small number of patients with uncommon mutations, it is hardly possible to enroll enough patients to carry out RCTs, which would provide the most convincing evidence for the clinical features of a particular uncommon mutation. For this reason, we need a comprehensive experimental system.

The laboratory experiments are comprised of studies of protein structure, studies of functional alterations, cell viability assays and animal experiments, providing an understanding of the features of the mutations from the level of molecules to cells and then to animal models, microcosmically to macroscopically. In terms of clinical studies, although it is not feasible to perform RCTs, prospective observational studies might be a possible way to enroll more patients. On the other hand, summarizing cases retrospectively enables us to obtain the average response rates and survival data from a relatively large sample. The results would be more reliable if we enrolled adequate studies of low heterogeneity to conduct meta-analyses.

This model was organized logically. Structure determination elucidates the mechanisms of the functional alterations and pharmacologic effects of TKIs, which are further verified in cells and animals, and even in patients in clinical settings, thus providing sufficient evidence to determine the oncogenicity and sensitivity to TKI of uncommon mutations in EGFR in NSCLC.

4. Discussion

Basic studies of G719X are far from enough. Laboratory studies of G719X have included almost all processes in the EGFR signaling pathway; however, they are still far from being complete or satisfactory.

Dimerization of G719S differs from that of Del19 and L858R mutants in terms of the dependence on its ligand EGF, indicating a lower activating level of G719S. This may explain its lower oncogenicity. However, it remains to be investigated whether mutations alter the mode of dimerization and how TKI will affect ligand binding and dimerization.

Regarding negative regulation, the effects of TKI on impaired negative regulation were not explored. If TKI treatment could reverse the impairment of negative regulation by the G719S mutation, this could be another explanation of the sensitivity to TKIs. However, we found no studies regarding the internalization or recycling of G719S mutated EGFR. In addition, the negative regulation of a pathogenic mutation might be a new target for drug development.

For structural studies, apart from real 3D structure determination by crystal diffraction, computational structure studies are another more economical and efficient way to study the structures of proteins and the effects of mutations on protein structure. Computational structure studies can be used to determine the structures of mutants using real-time computation and can simulate the conformational alterations caused by specific mutations without having to purify the mutated proteins and obtain crystals. However, neither type of study explained the lower sensitivity of G719X compared to Del19 and L858R mutants.

Table III. Summary of laboratory studies of the G719X mutation in EGFR.

Research type	Conclusion	Main method	Ref.
Protein structure	Elucidated the mechanism of constitutive kinase activity of G719S.	Crystal diffraction	60
	Determined the binding mode of TKI-G719S complex: same as wt and L858R.	Crystal diffraction	60
	Computational structural studies revealed that G719 caused TKI to move closer to the binding site and TKI easier to get into the ATP-binding pocket.	Molecular dynamic simulation	62
Protein function			
Ligand binding and dimerization	Basically the same between G719S and wt, while EGF-independent dimerization found in Del19 and L858R.	^{125}I-labelled EGF binding assay	51
Kinetics	Compare the catalytic activity among EGFRs: L858R>G719S>wt.	Continuous colorimetric assay and fluorescence-quenching assay	60
Affinity	Compare the binding affinity for TKI versus ATP to EGFRs: L858R>G719S>wt.	Continuous colorimetric assay	60
Kinase activity	Confirmed constitutive kinase activity in autophosphorylation and downstream signaling: Wt<G719X<L858R/Del19.	Immunoblotting	52,54,55
		Western blotting	51,53,56
		Immunofluorescence staining	57
TKI inhibition	Determined IC_{50} of TKI able to block constitutive kinase activity of mutants: wt>G719X>L858R/Del19	Immunoblotting	54
		Western blotting	53
		BRET assay	58
		Continuous colorimetric assay	59
		YFP-EGFR-ICD relocation assay	57
Negative regulation	Negative regulation of kinase activity is impaired in G719S.	Western blotting	53
Cell proliferation	Confirmed the transforming potential of G719S mutants.	Colony formation assay	52
		^{3}H thymidine incorporation assay	51
		eGFP+ cell FACS	56
	G719X transformed cells showed intermediate sensitivity to TKI.	Colony formation assay	52,56
		Cell viability assay by trypan/MTT/MTS staining	54,55,56,63
		Colorimetric assay	16
	G719X transformed cells show strong sensitivity to TKI.	Cell viability assay by MTT staining	53
	TKI induced cell cycle arrest in G719S transformed cells.	FACS and immunoblotting	54
Animal model	Injection of G719S transformed cells cause tumorigenesis.	Nude mice injection	52

wt, wild-type.

The structural analyses by either crystal diffraction or computational structural studies provide a structural basis for oncogenicity and sensitivity to TKIs. Moreover, fully understanding the structures of mutants and the binding features of drug-mutant complexes will help us to predict the sensitivity of a mutation to a particular potential targeted drug, and our ultimate purpose is to design a tailored targeted drug for people with specific gene mutation-related diseases using computational biology and bioinformatics. These are important goals of precision medicine.

Cell viabilities are determined by both cell proliferation and apoptosis. Tumorigenesis and response to TKIs

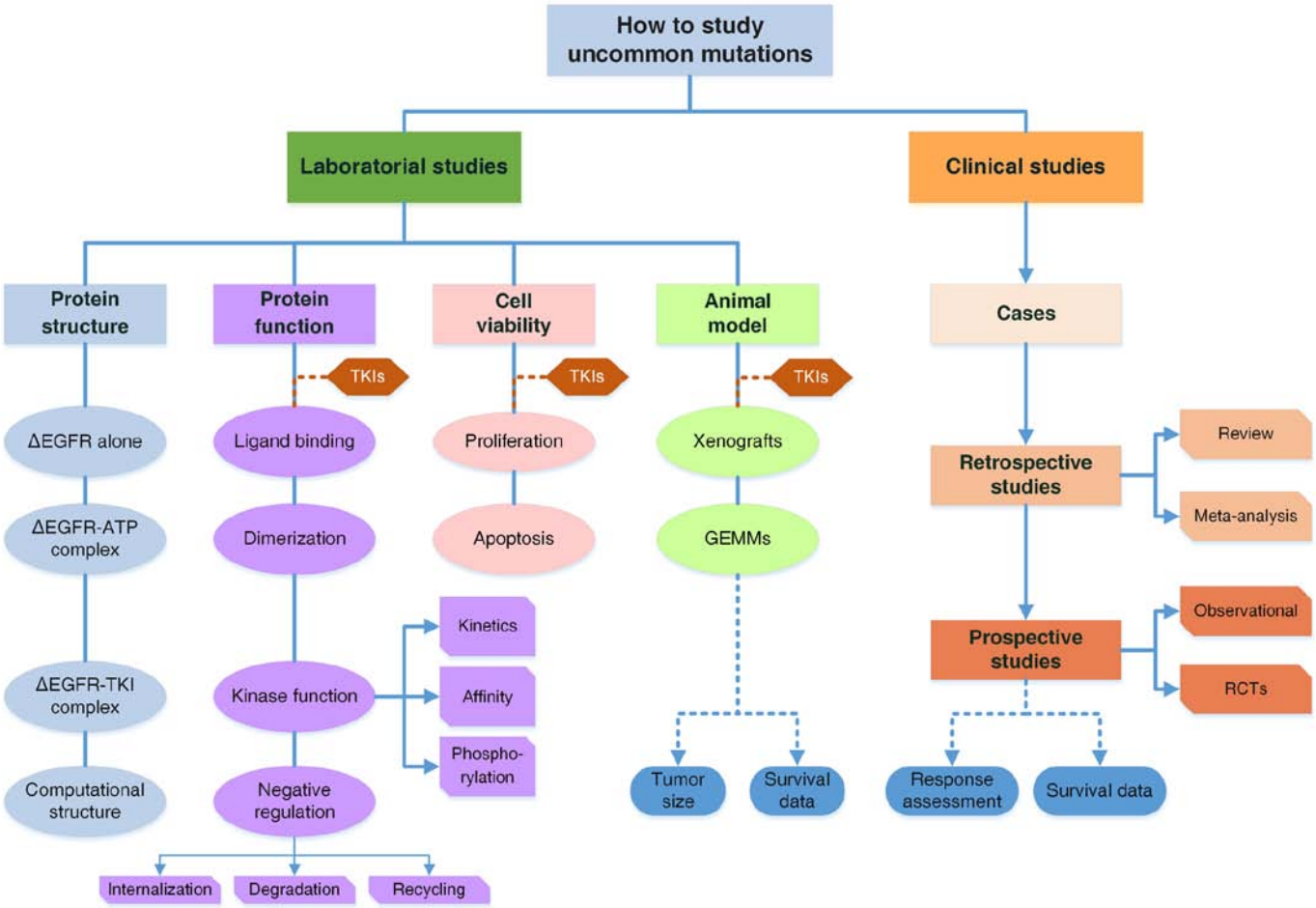

Figure 3. A comprehensive model to study uncommon mutations in EGFR. The system is comprised of both basic and clinical studies. Clinical studies include three parts: Case reports and series; Retrospective studies are reviews and meta-analyses to combine the data of RR and survival of patients with the G719X mutation; Prospective studies of observational ones or randomized controlled trials. There are two major issues in clinical studies: response to TKIs and survival data of patients. Laboratory studies are organized from mutant protein structures to functional changes, cell viability and animal models. Structural analyses by crystal diffraction or computational simulation determine the structures of mutant EGFR, EGFR-ATP complexes and EGFR-TKI complexes, to elucidate functional changes. Functional studies are categorized into four parts from the perspective of EGFR activation, including ligand biding, dimerization, kinase activity and downregulation, while the latter two are further subdivided as illustrated. For cell viability, the two aspects of proliferation and apoptosis are considered. Animal models involve GEMMs and tumor-cell inoculated xenografts including PDX models, which are discussed in terms of tumor sizes and animal survival. TKI treatment is introduced into all experiments on three levels. With basic and clinical studies integrated together, a complete evidence system is formed to draw conclusions regarding the pathogenic and pharmacological properties of uncommon mutations with high reliability. ΔEGFR, mutant EGFR.

could also be studied according to these two measures. Proliferation mainly refers to experiments concerning the cell cycle, and apoptosis is basically about the caspase-mediated apoptotic pathway. The studies of proliferation are relatively clear; however, few studies focused on apoptosis in G719X mutants. The L858R mutation was found to enable cells to escape from apoptosis (48), yet no similar studies on G719X were found. Furthermore, G719X and L858R mutants showed distinct responses to TKIs in terms of apoptosis (48,54,64), providing a possible mechanism for the difference in their sensitivities. Unlike common mutations, there are no cell lines bearing a single G719X mutation that are established for research on cell viability, while plenty of cell lines harboring Del19 or L858R mutations are available to be used directly, such as H3255 and PC-9 (65-68). This might be the reason for the scarcity of relative studies of the G719X mutation concerning cell viability. Establishing cell lines harboring uncommon mutations would provide a foundation for studying these mutations.

For xenograft-type models, apart from these transformed cell-inoculated animals, the patient-derived xenograft (PDX) model, also known as 'Mouse Avatars', is a new type of animal model used to study neoplasms (69). The PDX model has demonstrated substantial clinical potential in predicting drug sensitivity, including sensitivity to TKI targeted therapies. As the tumor sample is obtained from the actual patient, it is the closest representation of the individual's authentic situation.

Another type of animal model is genetically engineered mouse model (GEMM). Distinct from xenografts, they are generated using genetic engineering techniques to harbor specific mutations for driver mutations. To the best of our knowledge, there are no GEMMs for the G719X mutation in EGFR available for basic or clinical studies. Gazdar *et al* discussed the comparison between these two types (70).

Is G719X a TKI-sensitive mutation? The NCCN guidelines for NSCLC first mentioned G719X as significantly associated with response to TKIs in 2011 (version 2.2011) and

then referred to G719X as a drug-sensitive mutation in 2012 (version 2.2012), mainly based on laboratory studies of Greulich *et al* (52) and clinical studies of Lynch *et al* and Han *et al* (23,24), which each reported a single case of G719X with partial response to gefitinib. The newest NCCN guidelines for NSCLC (version 4.2016) (71) remained almost the same. In a word, the G719X mutation in EGFR is basically considered to be sensitive according to the NCCN guidelines.

Nevertheless, based on subsequent clinical studies involving more cases and a series of laboratory experiments, we have drawn the conclusion that it would be more accurate to define the G719X mutation as intermediately sensitive to first-generation TKIs. The high heterogeneity of NSCLC might explain the ambiguity of the G719X mutation in terms of sensitivity to TKIs.

Moreover, we still need to integrate some deeper and broader studies of G719X into the model to form a complete evidence system in order to be more confident about its sensitivity. Based on the research model, the following issues remain to be investigated. In the laboratory, the conformational differences between complexes of TKIs with various mutants requires more analysis to explain the differences in sensitivity between mutants on the basis of 3D structure. The influences of the G719X mutation and TKI treatment on negative regulation are far from clear. In terms of cell viability, more studies are required to determine whether G719X mutants could escape from apoptosis such as L858R mutants. In terms of animal experiments, we still need more data regarding tumor sizes and survival data from TKI treatment on animals with tumors driven by the G719X mutation or in PDX models from patients with the G719X mutation. Still, there is an urgent need to establish cell lines harboring the G719X mutation. At the clinical level, the major problem and difficulty lies in the sample sizes of the corresponding studies. It would be feasible to systematically review the clinical data in large-scale trial projects to retrieve information about responses and survival of patients with the G719X mutation (72). Based on these data, we could further conduct a meta-analysis on the sensitivity of the G719X mutation.

Complex mutations. Another interesting phenomenon to be noted is that G719X often occurs along with other mutations in EGFR, especially with S768I and L861Q with frequencies of 24.5 and 8.2% in all G719X mutations (15). They were also found to co-exist with mutations in other genes, for instance KRAS, BRAF and PIK3CA (15). It is referred to as complex mutation. Other uncommon mutations also tend to occur in the form of complex mutations. A possible explanation is that these uncommon mutations harbor inadequate tumor-driving ability and, therefore, must co-occur with another mutation to initiate tumorigenesis. Compared with the G719X single mutation, complex mutations are scarcer and their sensitivities to TKI are more obscure. Our research model provides practical approaches for studying these complex mutations.

Second generation EGFR-TKIs and G719X. New generations of EGFR-TKIs are developed to improve selectivity and efficacy and, thus, to reduce toxicity. First generation-TKIs (1G-TKIs) refer to reversible TKIs such as gefitinib, erlotinib and icotinib, while afatinib, dacomtinib and neratinib are categorized as irreversible 2G-TKIs (73,74). 3G-TKIs, including AZD-9291 (Osimertinib) and CO-1686 (75), are highly specific irreversible inhibitors for mutated receptors only, which are primarily used to target the secondary resistant mutation T790M (75,76). The G719X mutation showed intermediate sensitivity to 1G-TKIs; however, it was notable that 2G-TKIs demonstrated markedly high efficacy in patients with the G719X mutation. Although neratinib showed barely satisfactory efficacy in treating NSCLC patients in its phase 2 clinical trial (77), it exhibited great potential in patients with the G719X mutation. Three out of four patients with the G719X mutation exhibited a partial response with the tumor shrinking more than 50% in diameter, and one exhibited stable disease, indicating an RR of 75% and a DCR (disease control rate) of 100% (77). In addition, Yang *et al* reviewed patients with the G719X mutation who were treated with afatinib in the LUX-LUNG trial series and found that the overall response rate was 77.8% (14/18) (78). In laboratory studies, the G719A mutation exhibited higher sensitivity than Del19 in terms of both kinase activity and cell viability (16).

Although 2G-TKIs are not widely used clinically due to their high toxicity, some studies did explore their potential in treating patients with mutations in exon 18, especially the G719X mutation. These findings also shed light on TKI selection in patients with uncommon mutations. Although some types of TKIs failed to treat NSCLC patients with common sensitive mutations, they might have great potential against uncommon mutations.

Innovations and limitations. We systematically reviewed studies of G719X from both laboratory and clinical settings, and we sorted out the history of these studies. The basic studies are summarized regarding conclusions and corresponding experimental techniques. In terms of the clinical studies, we summarized 22 studies (18,23-43) investigating the response of the G719X mutation and determined an average response rate based on a relatively larger sample size. To the best of our knowledge, this is one of the most comprehensive systematic reviews of the G719X mutation in EGFR in NSCLC. Furthermore, the comprehensive research model we proposed provides researchers with a practical approach to determine the clinical significance of uncommon mutations of NSCLC or other diseases associated with gene mutation.

Nevertheless, there are some limitations of this review. Although we have included considerable studies of G719X in our review, it is inevitable that we may still have omitted some important studies. Moreover, due to the heterogeneity of the studies that we enrolled to calculate the average response rate, there might be a bias in the result. The heterogeneities of the studies mainly consist of demographic features, stage and histological classification of the tumor, different treatment lines and inconsistency in the criteria for response assessment. Additionally, the exclusion of 3 studies of which the original data were inaccessible in our calculation might also cause bias (47,79,80). Although we combined several studies to increase the sample size, it was still far from enough to draw a convincing conclusion on the sensitivity. By properly combining the response and survival data extracted from several large-scale trial projects, we can obtain more accurate and persuasive results.

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