
Resistance Mechanisms to HER2-Targeted Therapy in Gastroesophageal Carcinoma: A Systematic Review

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Abstract

The incidence of esophageal and gastric adenocarcinoma is increasing in Western countries. Despite the development of targeted therapies, such as HER2 inhibitors, overall survival remains poor. This can potentially be attributed to a delay in diagnosis or the development of recurrent or metastatic disease after treatment. Resistance to HER2-targeted therapy is a major issue in the treatment of HER2-positive esophageal and gastric malignancies. In this systematic review, we provide an overview of studies investigating different HER2-targeted therapy resistance mechanisms in gastroesophageal carcinoma (GEC). We systematically searched PubMed/MEDLINE, EMBASE, and CENTRAL for eligible studies describing gene expression changes that were associated with drug resistance. In total, 913 records were screened, of which 73 were included, investigating mechanisms conferring resistance to trastuzumab, lapatinib, pertuzumab, afatinib, and trastuzumab emtansine in cell lines, xenograft models, patient tissue samples, and publicly available datasets. We demonstrated that HER2 receptor changes, upregulation of alternative receptors, activation of downstream signaling pathways like PI3K/AKT and MAPK, epithelial-to-mesenchymal transition, acquiring stem cell-like properties, alterations in cell cycle related genes, cellular metabolism, and drug pharmacokinetics could contribute to HER2-targeted therapy resistance. In conclusion, we found that many different mechanisms could contribute to drug resistance in *in vitro* and *in vivo* models of GEC. Despite these preclinical results, evidence for the proposed resistance mechanisms in the clinical setting is lacking. Therefore, further investigation of therapy resistance in GEC patients is essential to overcome resistance.

Laymen's summary

Ieder jaar wordt er bij meer dan één miljoen mensen maag- of slokdarmkanker gediagnostiseerd. Voor beide ziektes zijn er meerdere subtypes beschreven die in verschillende werelddelen voorkomen. In Westerse landen komt vooral het type 'adenocarcinoom' voor, een vorm van kanker bestaande uit klierweefsel. De laatste jaren stijgt de incidentie van maag/slokdarmkanker, maar door late diagnose en beperkte behandelopties zijn de overlevingskansen laag. Tussen de 7 en 43% van de maag/slokdarmkanker patiënten heeft verhoogde expressie van de HER2 receptor, ook wel HER2-positief genoemd. Deze receptor speelt een belangrijke rol in signaal cascades die betrokken zijn bij proliferatie, differentiatie, celdood, adhesie en migratie van cellen. Door verhoogde HER2 expressie, verkrijgen tumorcellen eigenschappen die tumorgroei kunnen bevorderen. Daarom zijn er meerdere medicijnen ontwikkeld die de HER2 receptor en zijn signaal cascades remmen, bijvoorbeeld trastuzumab, pertuzumab en lapatinib. Helaas blijken tumoren resistent te kunnen zijn of worden. In borstkanker is er onderzocht welke mechanismes bijdragen aan resistentie tegen HER2 remmende medicijnen. De resistentie mechanismen in maag/slokdarmkanker zijn echter minder bekend. Daarom hebben wij een systematische review geschreven over alle literatuur die momenteel beschikbaar is over resistentie mechanismen tegen HER2 remmende medicijnen in maag/slokdarmkanker. Op dit moment is het meeste onderzoek uitgevoerd in cellijnen of muismodellen. In de literatuur staat beschreven dat veranderingen van de HER2 receptor zelf (zoals mutaties), verhoogde expressie van andere receptoren en veranderingen in signaal cascades de belangrijkste redenen zijn voor resistentie tegen HER2 remmende medicijnen. Om in de toekomst resistentie tegen HER2 remmende medicijnen te voorkomen en de overlevingskansen van patiënten te verhogen, is er meer onderzoek nodig naar de resistentie mechanismen die actief zijn in maag/slokdarmkanker patiënten tijdens hun behandeling.

Abbreviations

β 2-AR	β 2-adrenergic receptor
AC	Adenocarcinoma
ADC	Antibody-drug conjugate
AKT	Serine/threonine-protein kinase
ARPP-19	cAMP-regulated phosphoprotein 19
CMIP	C-Maf-inducing protein
CSK	C-terminal Src kinase
EAC	Esophageal adenocarcinoma
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EPHA2	Erythropoietin-producing hepatocellular receptor A2
FBXW7	F-box and WD repeat domain-containing 7
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
GAC	Gastric adenocarcinoma
GEC	Gastroesophageal cancer
GSE1	Gse1 coiled-coil protein 1
HER	Human epidermal growth factor receptor
IGF1R	Insulin-like growth factor 1 receptor
MACC1	Metastasis associated with the colon cancer 1
MAPK	Mitogen-activated protein kinase
miR	MicroRNA
MUC	Membrane-type mucin
nCRT	Neoadjuvant chemoradiotherapy
NES1	Normal epithelial cell-specific-1
NRF2	Nuclear factor erythroid 2-related factor 2
OS	Overall survival
PFS	Progression free survival
PI3K	Phosphoinositide-3-kinase
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SFK	Src family kinase
SCC	Squamous cell carcinoma
ST6Gal1	β -galactoside α 2,6-sialyltransferase 1
STAT3	Signal transducer and activator of transcription 3
t-DARPP	Truncated dopamine and cyclic AMP-regulated phosphoprotein of Mr32,000
T-DM1	Trastuzumab emtansine
TGF β	Transforming growth factor β
YB-1	Y-box-binding protein 1

Introduction

Each year, over one million individuals are diagnosed with esophageal or gastric cancer. Both cancer types are major causes of mortality, ranking sixth and third as most common causes of cancer-related deaths worldwide, respectively [1]. The main subtypes from esophageal cancer are squamous cell carcinoma (SCC), mainly located in the upper- or middle part of the esophagus, and adenocarcinoma (AC), situated more distally in the esophagus or at the gastroesophageal junction. Both subtypes are highly variable in geographical region. SCC is predominantly diagnosed in Eastern Asia, and potentially linked to smoking, alcohol consumption, air pollution and diet. On the other hand, the incidence of esophageal AC (EAC) is increasing in Western countries, and could be correlated to an increasing incidence of obesity and is associated with gastroesophageal reflux disease [2, 3]. Contrastingly, almost all gastric malignancies are adenocarcinomas. Whereas eradication of *Helicobacter pylori* infections achieved a decrease in the overall incidence of gastric adenocarcinoma (GAC), the incidence of cardiac gastric cancer remains increasing in the Western world, potentially due to the increase in obesity and reflux disease [4-6]. The prognosis of gastroesophageal cancer (GEC) is dismal. The majority of patients present with advanced disease due to the absence of symptoms at earlier stages [1]. Curative treatment options for these late-stage malignancies are not available, explaining the low 5-year survival rate of <30% [7, 8]. If GEC is diagnosed in earlier stages of disease, curative treatment options are available. These regimens vary around the world, as a consequence of geographical tumor characteristics and local treatment preferences [9]. In Europe, the standard treatment of patients with EAC is neoadjuvant chemoradiotherapy (nCRT) based on carboplatin, paclitaxel, and 41.4 Gray radiotherapy followed by esophagectomy, while patients with GAC receive standard treatment with gastrectomy and perioperative chemotherapy based on docetaxel, oxaliplatin, leucovorin, and fluorouracil [10-12]. Unfortunately, the gain in survival is modest with the current treatment options, especially for esophageal cancer [13, 14]. Moreover, the majority of patients demonstrate progression of disease following curative treatment, potentially due to development of resistance to therapy. Therefore, there is an unmet need to improve current therapies, aiming towards personalized medicine by applying targeted therapies, and to gain understanding in the mechanisms of resistance to treatment.

About 15-43% of the esophageal- and 7-34% of the gastric cancers are found to have overexpression or gene amplification of the human epidermal growth factor receptor 2 (HER2), making HER2 an interesting molecule for targeted therapy [15, 16]. By inhibiting HER2, the outcomes of patients with HER2 amplified or overexpressing breast cancer were significantly improved [17]. HER2 is a transmembrane tyrosine kinase receptor (RTK) and member of the ErbB receptor family, which all play an important role in tumor cell biology. The four ErbB receptors (HER1/epidermal growth factor receptor (EGFR), HER2, HER3, and HER4) are activated by homo- or heterodimerization, causing phosphorylation of the intracellular tyrosine kinase domain. This domain then activates signaling pathways involved in tumor cell proliferation, differentiation, apoptosis, adhesion, and migration. Thus, once HER2 is amplified, its overexpression at the cell surface and subsequent pro-oncogenic signaling could provide beneficial properties for cancerous cells [18, 19]. However, in literature contrasting conclusions have been published on the relation between HER2 overexpression and clinical outcome [20, 21].

In the past decades, several HER2 targeting agents were developed and their potency to improve outcomes of patients with HER2-positive GEC was investigated. In the clinical setting, the majority of

studies investigated the HER2 targeting agents trastuzumab, pertuzumab or lapatinib. The effect of lapatinib, a small molecule inhibitor that binds and prevents the activation of the kinase domains of HER2 and EGFR, has been tested in HER2-positive advanced GEC patients in the TRIO013/LoGiC trial. Unfortunately, no increase in overall survival (OS) was observed when patients were treated with chemotherapy plus lapatinib [22]. Furthermore, In the phase III RTOG 1010 trial the addition of trastuzumab to nCRT for EAC is investigated. Trastuzumab is a human monoclonal antibody that inhibits HER2-driven signaling, prevents cleavage of the HER2 extracellular domain, and induces antibody-dependent cellular cytotoxicity. However, trastuzumab addition did not improve the progression free survival (PFS) and OS of patients with resectable HER2-positive EAC [23]. On the other hand, positive results were obtained in the ToGA trial. Here, the median OS of HER2-positive advanced GEC cancer patients was significantly improved with the addition of trastuzumab to the chemotherapy regimen. These results led to the approval by the US Food and Drug Administration and the implementation of trastuzumab to the standard first-line treatment for patients with HER2-positive advanced GEC. Nevertheless, a considerable number of patients did not respond to trastuzumab treatment [15]. Additionally, several studies evaluated the effect of dual HER2-targeting using trastuzumab and pertuzumab, another monoclonal antibody that inhibits ligand-dependent HER2-HER3 heterodimerization and reduces downstream signaling [24, 25]. In the JACOB trial, no significant improvement was observed when dual-HER2 targeting was applied to treat patients with HER2-positive advanced GEC [24]. Despite promising preclinical results, the addition of pertuzumab to trastuzumab did not improve OS [24]. On the contrary, the addition of trastuzumab and pertuzumab to nCRT for the treatment of curative EAC patients was evaluated in the TRAP trial. First results showed that this regimen is tolerable and preliminary outcomes seem promising, though, a randomized phase III study is required to prove the benefit of this treatment regimen [25]. In addition, in the ongoing INNOVATION trial (ClinicalTrials.gov Identifier: NCT02205047), the addition of trastuzumab and/or pertuzumab to perioperative chemotherapy to treat HER2-positive gastric and gastroesophageal junction cancer is evaluated [26]. Altogether, although the majority of trials demonstrate promising outcomes with HER2 targeting, not all patients gain benefit from these treatment options, potentially due to resistance mechanisms. Some patients exhibit intrinsic resistance, but even patients with initial response to treatment can acquire secondary resistance after a relatively short period of time. Resistance mechanisms to HER2-targeted therapies are mainly defined in breast cancer, and it is unclear if similar mechanisms contribute to resistance of HER2-positive GEC. It has been suggested that mutations, modification, amplification, or upregulation of HER2, other receptor tyrosine kinase receptors, and downstream signaling such as the phosphoinositide-3-kinase (PI3K)/serine/threonine-protein kinase (AKT) and/or mitogen-activated protein kinase (MAPK) pathways contribute to therapy resistance of GEC [27]. Our limited understanding of resistance mechanisms hampers the further development and improvement of HER targeted therapies. For this purpose, we aimed to systematically review the literature regarding resistance mechanisms to HER2-targeted therapy in these malignancies.

Methods

Search Strategy

In line with the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), a systematic search was performed of PubMed/MEDLINE, EMBASE, and CENTRAL from

November 2021 and updated in December 2021 for articles investigating different resistance mechanisms to HER2-targeted therapies in GEC. Both preclinical and clinical articles were included to collect all available data available on anti-HER2 resistance mechanisms in GEC. The search included terms for the type of cancer (gastric or esophageal cancer), HER2 treatment (HER2-neu receptor and anti-HER2 treatments), and resistance mechanisms. The complete search is shown in Appendix 1. Exclusion criteria were articles in non-English/Dutch, review articles, and case reports. The search strategy for PubMed/MEDLINE was rewritten for the search in EMBASE and CENTRAL. Two authors (DB, CS) independently performed the data screening. Disagreements that could not be resolved by discussion, were investigated with a third arbiter (HvL).

Eligibility Criteria

Articles were considered to be suitable for inclusion if the following criteria were met: studies involved gastric or esophageal adenocarcinoma, studies described a resistance mechanism to anti-HER2 therapy, and studies were published in English or Dutch. No selection was made based on the year of publication. Inconsistency between conference abstracts and the final papers were evaluated, and the final papers were included upon availability.

Study Quality and Risk of Bias Assessment

To evaluate the quality of the included studies, an adjusted version of the OHAT risk of bias tool was used [28]. The studies were assessed on reporting bias, information bias based on (statistical) methods and the use of negative controls, selection bias based on cell or mouse population and replicated experiments, incomplete outcome bias, and other bias. The studies were graded 'high risk', 'low risk', or 'unclear risk', for each type of bias (**Supplementary Figure 1**).

In addition, the quality of the proposed resistance mechanism was assessed using predefined criteria: the presence of a control group, the use of >2 methods and models to investigate resistance, identification and analysis of upstream or downstream participants, correlation with resistance outcome, and restoring of sensitivity through removal of the proposed mechanism. Further details can be found in **Supplementary Figure 2**.

Data Extraction and Synthesis

Data from eligible studies were extracted using a predefined extraction sheet, including type of study, type of malignancy, type of resistance, HER2-targeting treatment, the study population (cells, xenograft models, patient tissue, datasets), the control group, the method of creating a resistant model, the method(s) of confirming resistance, and the proposed resistance mechanism. For this review, we categorized proposed resistance mechanisms as follows: changes of the HER2 receptor, alterations in RTK or downstream signaling, epithelial-to-mesenchymal transition (EMT), induction of stemness, cell cycle progression, metabolic reprogramming, and changes in drug pharmacokinetics. The data extraction and quality assessment of included articles were performed by two researchers (DB, CS). Furthermore, each author checked three random studies that were analyzed by the other author. All data were obtained from the published studies or conference abstracts. No meta-analysis was performed since studies presented a large number of heterogeneous outcome measures.

Results

Search

A total of 913 studies were found using our search strategy in the PubMed/MEDLINE, EMBASE, and CENTRAL databases. The search and selection process is illustrated in **Figure 1**. From the identified records, 73 studies were included of which 57 investigated gastric cancer, six investigated esophageal cancer, and 10 investigated both. The majority of the included studies investigated resistance to trastuzumab (n=43), lapatinib (n=14), or a combination (n=4). Other compounds investigated were afatinib (n=4), pertuzumab (n=2), pyrotinib (n=2), and trastuzumab emtansine (T-DM1) (n=5). The most commonly reported causes of HER2-targeted therapy resistance were HER2 receptor changes (n=19), signaling via an alternative receptor (n=24), and aberrant activation of downstream signaling (n=23). Further details on the included studies can be found in **Table 1**.

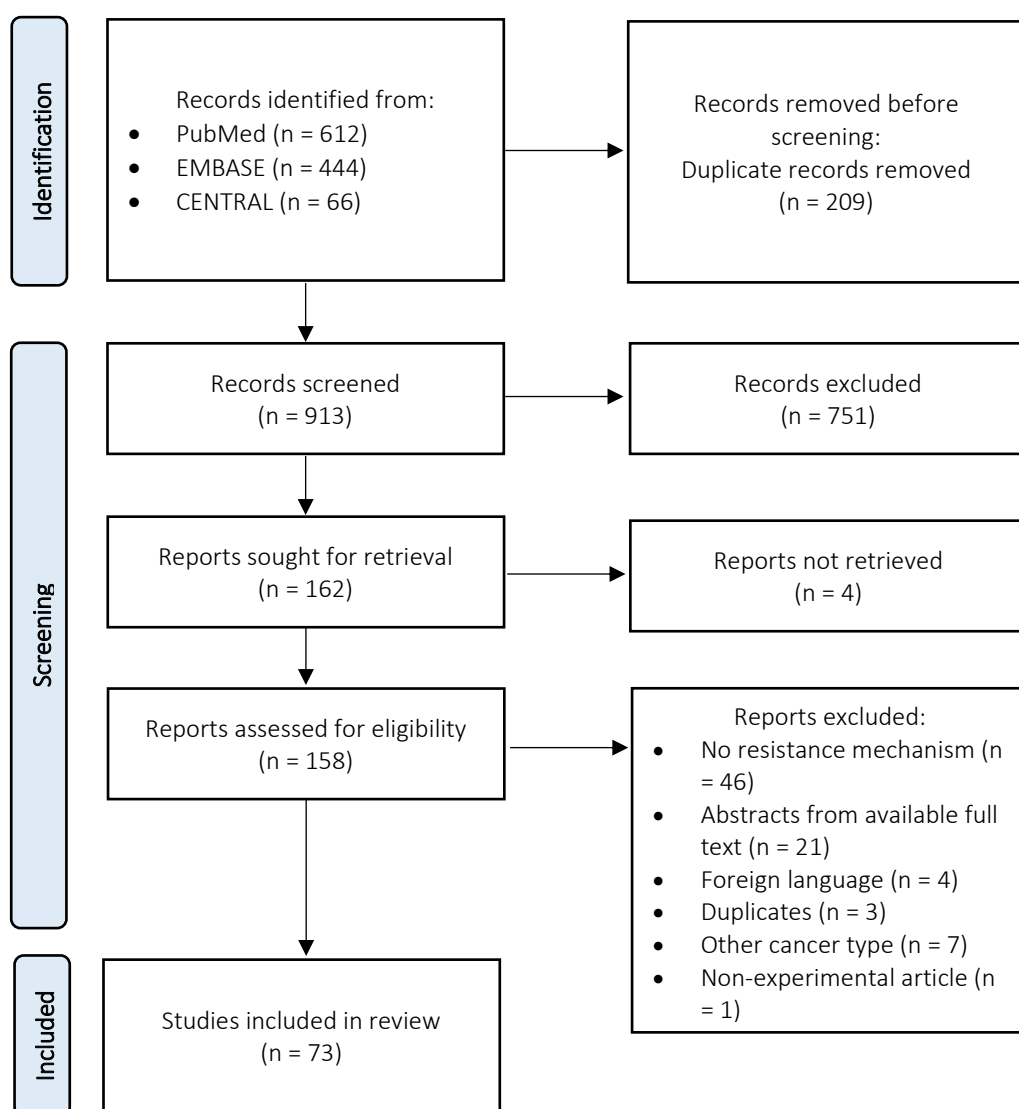


Figure 1. Flow diagram of included studies according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Study Quality and Risk of Bias Assessment

The results from the risk of bias assessment and quality control of resistance mechanisms can be found in **Supplementary Figure 1** and **2**, respectively. Some studies were found to have high selection bias, mainly on base of the use of only one cell line or model. Besides, in some studies, not all data was reported. From the included studies, the resistance mechanisms of 30 studies (41.1%) were considered high quality, 28 studies (38.4%) moderate quality, and 20 studies (20.5%) low quality. The main reason for lower quality of the evidence of the proposed resistance mechanism was the absence of further exploration of upstream or downstream participants in the proposed resistance mechanism, a correlation with resistance outcome, , such as cell viability or apoptosis upon treatment, or restoring compound sensitivity by removal of the mechanism using for instance inhibitors or silencing.

Anti-HER2 agents

Resistance mechanisms to several anti-HER2 agents have been investigated in the included articles. The majority of articles researched resistance to treatment with humanized monoclonal antibodies targeting HER2. Trastuzumab exerts its inhibitory effects through binding of the HER2 extracellular domain IV, thus preventing activation of intracellular signaling [29]. The antibody-drug conjugate (ADC) T-DM1 demonstrated the anti-tumor effects of trastuzumab, as well as DM-1, a cytotoxic anti-microtubule agent, which is released upon degradation of the complex [30]. Pertuzumab also directly binds HER2 at a different epitope than trastuzumab. Thus, pertuzumab prevents dimerization of HER2 with other receptors, particularly the most potent HER2/HER3 dimerization [31]. Other studies investigated resistance to tyrosine kinase inhibitors targeting HER2. Both lapatinib, afatinib, dacomitinib, and pyrotinib irreversibly inhibit the ErbB family, targeting both HER2 and EGFR, whereas AEE788 reversibly binds EGFR and HER2. Mechanisms contributing to resistance to treatment with the above-mentioned anti-HER2 agents can be divided in changes of the HER2 receptor, activation of alternative receptor signaling, activation of downstream signaling, EMT, induction of stemness, cell cycle progression, metabolic reprogramming, and changes in drug pharmacokinetics.

HER2 receptor changes

Nineteen studies observed a correlation between HER2 receptor changes and anti-HER2 therapy resistance in HER2-positive GEC. In eight studies, overexpression, loss, or downregulation of the receptor was investigated [32-39]. Nine studies described impaired HER2-target binding, either due to mutations in HER2 or coverage of the compound-binding domain [40-48]. Additionally, two studies assessed HER2 receptor glycosylation in gastric cancer cells [49, 50].

Table 1. All studies included in this review. Studies are organized per category of resistance mechanism and therefore are mentioned by multiple categories if more than one resistance mechanism was proposed. NA: not applicable. GC: gastric cancer. GEJ: gastroesophageal junction cancer. EC: esophageal cancer. EMT: epithelial-to-mesenchymal transition. PDM: patient derived material.

Author, Year	Study Design	Type of Cancer	Type of Cell Line	Anti-HER2 Agents	Type of Resistance	Factors Involved
<i>Activation of downstream signaling</i>						
Deguchi, 2017	In vitro + in vivo + PDM	GEC	AGS + KATO-II + MKN-1 + MKN-7 + MKN-45 + MKN-74 + NCI-N87 + OE19 + OE33 + SNU-1	Trastuzumab	Acquired	PTEN loss
Eto, 2014	In vitro	GC	NCI-N87 + NUGC4	Trastuzumab	Acquired	PTEN inhibition
Gambardella, 2017b	In vitro	GEC	NCI-N87 + OE19	Lapatinib, Trastuzumab	Acquired	Upregulation of PI3K/AKT and MAPK
Gambardella, 2019	In vitro + in vivo + PDM	GEC	NCI-N87 + OE19	Lapatinib, Trastuzumab	Acquired	Upregulation of PI3K/AKT
Hong, 2014	In vitro	EC	OE19 + OE33	Lapatinib	Acquired	Src activation
Jin, 2017	In vitro + in vivo	GC	NCI-N87 + SNU-216	Trastuzumab	Acquired	Src activation
Kim J, 2014	In vitro + PDM	GEC	ESO26 + OE19 + OE33 + MKN-7 + NCI-N87	Lapatinib, Trastuzumab	Intrinsic	PI3K mutations
Kim, 2013	PDM	GC	NA	Lapatinib, Trastuzumab	Acquired	PTEN loss
Kim, 2017	PDM	GC	NA	Trastuzumab	Acquired	PTEN loss
Liu, 2016	In vitro + in vivo	GC	MKN-45 + NCI-N87 + SGC-7901	Trastuzumab	Acquired	Upregulation of PI3K/AKT
Liu, 2017	In vitro	GC	NCI-N87	Trastuzumab	Acquired	Upregulation of PI3K/AKT
Ma, 2016	In vitro	GC	BGC-823 + HGC-27 + NCI-N87 + SGC-7901	Lapatinib	Intrinsic	JWA -induced MAPK activation
Ning, 2021	In vitro + in vivo	GEC	NCI-N87 + OE19	Lapatinib	Acquired	PTEN loss
Sampera, 2016	In vitro	GC	NCI-N87	Trastuzumab	Acquired	Src activation
Sampera, 2019	In vitro + in vivo	GC	NCI-N87 + OE19	Trastuzumab	Acquired	Src-driven activation of PI3K/AKT and MAPK
Shi, 2013	In vitro + in vivo	GC	BGC-823 + HGC-27 + MGC-803 + NCI-N87	Trastuzumab	Acquired	Upregulation of PI3K/AKT and MAPK
Shi, 2021	In vitro + in vivo + PDM + datasets	GC	NCI-N87 + SNU-216	Trastuzumab	Intrinsic	Shc1-mediated activation of PI3K/AKT and MAPK

Tang, 2017	In vitro + in vivo	GC	BGC-823 + SGC-7901	Trastuzumab	Acquired	Upregulation of PI3K/AKT
Wang, 2019	In vitro + in vivo + PDM	GC	NCI-N87 + SNU-216	Trastuzumab	Intrinsic + Acquired	PI3K mutations
Yang, 2014	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	IL-6/STAT3/Jagged-1/Notch activation
Yokoyama, 2021	In vitro + PDM	GEC	NCI-N87 + OE19	Trastuzumab	Acquired	PTEN loss
Yoshioka, 2019	In vitro + in vivo	GC	NCI-N87 + SNU-216	Afatinib	Acquired	Src activation
Zuo, 2015	in vitro	GC	NCI-N87	Trastuzumab	Acquired	PTEN loss
<i>Alterations in cell adhesion signaling</i>						
Sauveur, 2018	In vitro	GEC	OE19	T-DM1	Acquired	Reduced cell adhesion
Yuan, 2020	In vitro + datasets	GC	SNU-216	Lapatinib	Acquired	Rap1 signaling activation
<i>Alternative receptor signaling</i>						
Chen, 2012	In vitro	GC	NCI-N87 + SNU-16 + SNU-216	Lapatinib	Acquired	MET activation
Chen, 2019	In vitro + in vivo	GC	HGC-27 + MKN-45 + NCI-N87 + NUGC-4 + SNU-216	Afatinib	Acquired	EPHA2 activation
Ebbing, 2016	In vitro + in vivo	EC	OE19 + OE33	Trastuzumab	Acquired	HER3 activation
Gambardella, 2017b	In vitro	GEC	NCI-N87 + OE19	Lapatinib, Trastuzumab	Acquired	FGFR3 upregulation
Guo, 2021	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	FGFR1 and IGF1R upregulation
Hassan, 2019	In vitro + in vivo	EC	ESO51 + OE19 + OE33	Foretinib, Lapatinib	Acquired	MET activation
Kang, 2015	In vitro + in vivo	GC	NCI-N87 + SNU-216	Dacomitinib, Trastuzumab	Acquired	EGFR, HER3, MET, and IGFR activation
Kim HP, 2014	In vitro	GC	SNU-216	Lapatinib	Acquired	HER3 and MET activation
Kim J, 2014	In vitro + PDM	GEC	ESO26 + OE19 + OE33 + MKN-7 + NCI-N87	Lapatinib, Trastuzumab	Intrinsic	EGFR and MET activation
Lee, 2013	In vitro + datasets	GC	SNU-216	Lapatinib	Acquired	MET activation
Li, 2014	In vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	EGFR activation
Park, 2018	In vitro	GC	SNU-216	Lapatinib	Acquired	HER2 and MET downregulation
Piro, 2016	In vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	FGFR3 activation

Sampera, 2019	In vitro + in vivo	GC	NCI-N87 + OE19	Trastuzumab	Acquired	EGFR and HER3/4 activation
Sanchez-Vega, 2019	in vivo + PDM	GEC	NA	Afatinib, Trastuzumab	Acquired	MET activation and EGFR loss
Sato, 2013	In vitro	GC	ESO26 + KYAE-1 + NCI-N87 + OE19 + SK-GT-2	Lapatinib	Acquired	HER3 activation
Shi, 2013	In vitro + in vivo	GC	BGC-823 + HGC-27 + MGC-803 + NCI-N87	Trastuzumab	Acquired	β 2-AR activation
Shi, 2018	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	HER4 activation
Su, 2021	in vitro + in vivo	GC	NCI-N87 + SNU-216	Apatinib, Pyrotinib	Acquired	c-kit activation
Yoshioka, 2019	In vitro + in vivo	GC	NCI-N87 + SNU-216	Afatinib	Acquired	MET activation
Yu Y, 2018	In vitro + PDM	GC	YCC1 + YCC1-F	Lapatinib	Acquired	FGFR2 activation
Zhang, 2014	in vitro + in vivo	GC	NCI-N87 + SNU-216	Lapatinib	Intrinsic	HER3, IGF1R, MET activation
Zheng, 2014	in vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	EGFR/HER2 heterodimerization
Zuo, 2015	in vitro	GC	NCI-N87	Trastuzumab	Acquired	IGF1R activation
<i>Cell cycle progression</i>						
Eto, 2015	In vitro	GC	KATO-III + MKN-45 + NCI-N87 NUGC3 + NUGC4	Trastuzumab	Acquired	Upregulation of cell cycle progression genes
Jin, 2021	In vitro + in vivo	GC	NCI-N87 + SNU-216	Trastuzumab	Acquired	Inhibited DNA damage response
Kim J, 2014	In vitro + PDM	GEC	ESO26 + OE19 + OE33 + MKN-7 + NCI-N87	Lapatinib, Trastuzumab	Intrinsic	Upregulation of cell cycle progression genes
Liu N, 2018	In vitro	GC	SGC-7901	Trastuzumab	Acquired	Stimulated cell cycle progression
Shi, 2021	In vitro + in vivo + PDM + datasets	GC	NCI-N87 + SNU-216	Trastuzumab	Intrinsic	Stimulated cell cycle progression
Shu, 2015	in vivo	GC	GLM1	Trastuzumab	Acquired	Stimulated cell cycle progression
<i>Epithelial-to-mesenchymal transition (EMT)</i>						
Ebbing, 2017	In vitro + in vivo	EC	OE19 + OE33	Trastuzumab, Pertuzumab	Acquired	TGF β -induced EMT
Kim HP, 2014	In vitro	GC	SNU-216	Lapatinib	Acquired	Wnt-induced EMT
Liu W, 2018	In vitro	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	Wnt-induced EMT
Piro, 2016	In vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	FGFR3-mediated EMT

Shi, 2018	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	HER4-YAP1-induced EMT
Yang, 2014	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	Vimentin upregulation
Yang, 2019	datasets	GC	NCI-N87	Trastuzumab	Acquired	Vimentin upregulation
Zhang, 2014	in vitro + in vivo	GC	NCI-N87 + SNU-216	Lapatinib	Intrinsic	Wnt-induced EMT
Zhou, 2018	in vitro	GC	NCI-N87	Trastuzumab	Acquired	TGF β -induced EMT
<i>HER2 receptor changes</i>						
Arienti, 2016	In vitro	GC	AKG + KKP + NCI-N87	Trastuzumab	Acquired	HER2 overexpression
Deng, 2013	In vitro	GC	MKN-45 + NCI-N87	Trastuzumab	Intrinsic	HER2 coverage by mucins
Ding, 2020	datasets	GC	NA	AEE788, Afatinib, Erlotinib, Gefetinib, staurosporine, and TAK285	NA	HER2 mutations
Duarte, 2021	In vitro	GC	MKN-45 + MKN-74 + NCI-N87	Trastuzumab	Acquired	HER2 glycosylation
Hong, 2012	In vitro + in vivo + PDM	EC	OE19 + OE33	Trastuzumab	Acquired	HER2 coverage by t-DARPP
Kashiwada, 2018	PDM	GC	NA	Trastuzumab	Acquired	HER2 loss
Kim, 2017	PDM	GC	NA	Trastuzumab	Acquired	Low HER2 amplification
Li, 2014	In vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	HER2 coverage by mucins
Liu N, 2018	In vitro	GC	SGC-7901	Trastuzumab	Acquired	HER2 glycosylation
Ma, 2016	In vitro	GC	BGC-823 + HGC-27 + NCI-N87 + SGC-7901	Lapatinib	Intrinsic	HER2 downregulation
Piro, 2016	In vitro + in vivo	GC	NCI-N87	Trastuzumab	Acquired	HER2 downregulation
Shi, 2013	In vitro + in vivo	GC	BGC-823 + HGC-27 + MGC-803 + NCI-N87	Trastuzumab	Acquired	HER2 coverage by mucins
Shi, 2018	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	HER2 downregulation
Shibata, 2013	In vitro + PDM	GC	MKN-45 + NCI-N87 + SNU-216	Lapatinib	Acquired	HER2 downregulation
Wang, 2019	In vitro + in vivo + PDM	GC	NCI-N87 + SNU-216	Trastuzumab	Intrinsic + Acquired	HER2 mutations
Yang, 2019	datasets	GC	NCI-N87	Trastuzumab	Acquired	HER2 downregulation

Yu, 2015	In vitro + in vivo + PDM	GC	NIH/3T3	T-DM1, Trastuzumab	Intrinsic	HER2 mutations
Zhang, 2019	in vitro + datasets	GC	NIH/3T3	Pertuzumab	Intrinsic	HER2 mutations
Zhang, 2020	In vitro + PDM	GC	NIH/3T3	Pyrotinib, T-DM1, Trastuzumab	Acquired	HER2 mutations
<i>Metabolism</i>						
Chang, 2020	In vitro	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	GATA6-mediated metabolic reprogramming
Hassan, 2020	In vitro + datasets	EC	OE19	Lapatinib	Acquired	Activation of Warburg effect
Liu, 2016	In vitro + in vivo	GC	MKN-45 + NCI-N87 + SGC-7901	Trastuzumab	Acquired	Activation of Warburg effect
Mori, 2018	In vitro	GC	NCI-N87	Trastuzumab	Acquired	Increase in antioxidant enzymes
Ye, 2018	In vitro	GC	NCI-N87 + SGC-7901	Trastuzumab	Acquired	Autophagy inhibition
<i>microRNA</i>						
Lote, 2020	In vitro	GEC	FLO-1 + NCI-N87	Trastuzumab	Acquired	miR-148a-3p upregulation
<i>Pharmacokinetics</i>						
Gambardella, 2017a	In vitro	GEC	OE19	Lapatinib	Acquired	Upregulation of multidrug resistance associated proteins
Takegawa, 2017	In vitro + in vivo	GC	NCI-N87	T-DM1	Acquired	Enhanced drug efflux
Wang, 2017	In vitro + in vivo	GC	NCI-N87	T-DM1, Trastuzumab	Acquired	Inhibited T-DM1 metabolism
<i>Stemness</i>						
Gao, 2020	In vitro + in vivo + PDM	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	Stem cell like properties
Mori, 2018	In vitro	GC	NCI-N87	Trastuzumab	Acquired	CD44 expression
Wang, 2021	In vitro + PDM	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	GSE1-induced stem cell like properties
Xiang, 2020	In vitro + PDM	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	CMIP-induced SOX2 upregulation
Yang, 2014	In vitro + in vivo	GC	MKN-45 + NCI-N87	Trastuzumab	Acquired	CD44 expression
Yu C, 2018	datasets	GC	NCI-N87	Trastuzumab	Acquired	CD44 expression

Altered HER2 receptor expression

It is suggested that membranous HER2 expression levels affect the binding of targeted therapies and could play a role in drug resistance [21]. Arienti *et al.* reported that increased HER2 expression caused acquired trastuzumab resistance in NCI-N87 cells [32]. On the other hand, in response to trastuzumab or lapatinib treatment, the initially HER2-positive gastric cancer cells NCI-N87, MKN-45, and SNU-216 had downregulated or even absent expression of HER2. Consequently, trastuzumab or lapatinib sensitivity was reduced [33, 35, 39]. More specifically, Shibata *et al.* showed that HER2 expression was downregulated as a consequence of downregulation of its regulator, Y-box-binding protein 1 (YB-1) [35]. These findings were supported by studies from Kashiwada *et al.* and Kim *et al.*, who reported that trastuzumab-treated patients with progressive disease frequently lost HER2 expression, explaining the limited response to anti-HER2 treatment [34, 37]. Furthermore, the loss of HER2 expression could potentially be explained by its degradation in the proteasome [36, 38].

Impaired HER2 receptor binding

Anti-HER2 therapy resistance in gastric cancer could be a consequence of impaired HER2 target binding. Several studies, using patient material or computational analysis, showed that mutations in the HER2 receptor, for instance in the kinase domain, or gene fusions, could confer intrinsic and acquired therapy resistance due to inability of the drug to bind the receptor [44-48]. Additionally, Shi *et al.* and Li *et al.* showed that NCI-N87 cells with acquired trastuzumab resistance have hyperactivation of STAT3 and a consequent increased expression of membrane-type mucin 1 and 4 (MUC1, MUC4) [41, 42]. Deng *et al.* reported the same overexpression of MUC1 in intrinsic trastuzumab-resistant MKN-45 cells [43]. These mucins interfere with the recognition and binding of trastuzumab to HER2, and can also actively maintain HER2 phosphorylation and activation [41-43]. Furthermore, Hong *et al.* reported that overexpression of t-DARPP (truncated dopamine and cyclic AMP-regulated phosphoprotein of Mr32,000) prevented trastuzumab binding to HER2 and subsequent receptor dephosphorylation in OE19 and OE33 cells [40].

HER2 receptor modifications

The HER2 receptor is a known target for β -galactoside α 2,6-sialyltransferase 1 (ST6Gal1) mediated glycosylation and the resulting glycan repertoire is important for regulating receptor function. Duarte *et al.* and Liu *et al.* reported that ST6Gal1 is overexpressed in gastric cancer, stimulating α 2,6- and α 2,3-sialylation of the HER2 receptor [49, 50]. In response to trastuzumab, these modifications reduce the half-life and the stabilization of the HER2 receptor at the membrane, providing acquired resistance to trastuzumab therapy.

Alternative receptor signaling

In response to HER2 inhibition, other RTKs could compensate for this loss by restoring and maintaining activation of downstream pathways. In this section, we particularly focus on different RTK signaling that contributes to resistance. Twenty-four studies investigated different receptors and their relation to therapy resistance. The majority of studies (n=10) demonstrated MET receptor upregulation upon inhibition of the HER2 receptor in resistant models [51-60]. Others described the role of other ErbB receptor family members (n=7) [33, 42, 51, 61-64], fibroblast growth factor receptors (FGFR) (n=4) [39, 65-67], or other receptors (n=6) [41, 57, 66, 68-70].

MET receptor upregulation

As reported by Hassan *et al.*, Kang *et al.*, Chen *et al.*, Lee *et al.*, Zhang *et al.*, Yoshioka *et al.*, Park *et al.*, and Kim *et al.*, MET was upregulated and phosphorylated in cells treated with lapatinib, afatinib, or trastuzumab, or in cells demonstrating acquired resistance to the latter anti-HER2 agents. Thus, MET upregulation contributed to drug resistance by maintaining downstream signaling and stimulating cell cycle progression [53-60]. These results are supported by Sanchez-Vega *et al.* and Kim *et al.*, reporting that MET amplification contributed to intrinsic and acquired trastuzumab resistance in GEC patients [51, 52].

ErbB receptor upregulation

Furthermore, upregulation of the ErbB receptor family member EGFR was associated with trastuzumab resistance in patients [51]. Zheng *et al.* reported that overexpression of EGFR results in increased heterodimerization with HER2, maintaining downstream signaling in a trastuzumab-resistant *in vitro* model [61]. The overexpression of EGFR could be explained by feedback activation via signal transducer and activator of transcription 3 (STAT3) [42]. Moreover, Ebbing *et al.*, Shi *et al.*, Sampera *et al.*, and Sato *et al.* found that HER3 and HER4 were upregulated in acquired trastuzumab and -lapatinib resistant GEC cells [33, 62-64]. More specifically, HER3 upregulation was accompanied by increased levels of its ligand heregulin, which was released from the membrane by matrix metalloprotease ADAM10. The activation of HER3 subsequently led to increased HER2/HER3 heterodimer formation that conferred acquired resistance [62, 63].

FGFR and other receptor upregulation

In endoplasmic reticulum-stressed or lapatinib/trastuzumab-resistant *in vitro* or *in vivo* models, FGFR1, FGFR2, and FGFR3 were upregulated and contributed to acquired drug resistance [39, 65-67]. Interestingly, Guo *et al.* and Yu *et al.* reported that the expression of FGFR1 and FGFR2 was controlled by the expression of microRNAs miR-301a-3p and miR-494. While miR-301a-3p promoted resistance by stimulating FGFR1 expression, miR-494 reversed resistance by downregulating FGFR2 [66, 67]. Other receptors that were found to contribute to intrinsic and acquired anti-HER2 therapy resistance were the insulin-like growth factor 1 receptor (IGF1R), the c-kit receptor, the β 2-adrenergic receptor (β 2-AR), and erythropoietin-producing hepatocellular receptor A2 (EPHA2) [41, 57, 66, 68-70]. Chen *et al.* identified EPHA2 as a new dimerization partner of HER2 that contributed to acquired afatinib resistance [70].

Activation of downstream signaling

Several studies (n=23) investigated alterations in downstream signaling cascades that correlate with HER2-targeted therapy resistance. In 10 studies, the MAPK and PI3K/AKT pathways were held responsible for resistance [38, 41, 45, 52, 71-76]. Furthermore, six studies assessed the loss of phosphatase and tensin homolog (PTEN) expression [37, 68, 77-81], six studies investigated Src kinase signaling [58, 64, 65, 82-84], and one investigated both [77].

PI3K/AKT and MAPK pathway activation

The PI3K/AKT and MAPK signaling pathways are frequently activated in cancers by RTKs such as HER2 and play important roles in cell metabolism, growth, proliferation, differentiation, migration, and survival [85, 86]. Liu *et al.*, Shi *et al.*, Gambardella *et al.*, and Tang *et al.* reported that intrinsic and

acquired trastuzumab resistance could be initiated by increased activation of PI3K/AKT and MAPK signaling pathways, potentially caused by overexpression of the scaffold protein Shc1, nuclear factor erythroid 2-related factor 2 (NRF2), and normal epithelial cell-specific-1 (NES1) protein [71-74]. In addition, Ma *et al.* and Liu *et al.* showed that tumor suppressor JWA and metastasis associated with the colon cancer 1 (MACC1) were both overexpressed in intrinsic and acquired trastuzumab-resistant models and directly activated the PI3K/AKT and MAPK pathways [38, 75]. MAPK signaling was also activated by catecholamine under stress conditions [41]. Moreover, analysis of HER2-positive GEC tissues revealed that activating mutations in PI3K induced intrinsic resistance to lapatinib and trastuzumab and were correlated with worse PFS [45, 52]. Interestingly, Yang *et al.* reported that NCI-N87 and MKN-45 cells with acquired trastuzumab resistance were dependent on IL-6/STAT3/Jagged-1/Notch-mediated survival signaling instead of PI3K/AKT signaling [76].

Loss of PTEN expression

PTEN is a tumor suppressor and negatively regulates the PI3K/AKT and MAPK signaling pathway. Inactivation of PTEN due to genetic alterations or transcriptional modifications lead to major changes in signaling and contribute to treatment efficacy [87]. Ning *et al.*, Zuo *et al.*, and Eto *et al.* reported that mutations or downregulation of PTEN resulted in increased PI3K/AKT and MAPK signaling and acquired resistance to trastuzumab and lapatinib *in vitro* [68, 77, 78]. This is supported by research from Kim *et al.*, Kim *et al.*, Yokoyama *et al.*, and Deguchi *et al.*, who showed that PTEN loss in HER2-positive GEC patients is common (34.5%- 67%) and associated with shorter PFS and OS, and acquired trastuzumab resistance [37, 79-81].

Src kinase activation

Src is a nonreceptor tyrosine kinase and is part of the Src family kinases (SFKs). These kinases interact with other RTKs to activate downstream signaling cascades such as MAPK, PI3K/AKT, and STAT3 pathways [88]. Multiple studies showed that NCI-N87 cells with acquired trastuzumab, lapatinib, or afatinib resistance have increased phosphorylation of Src or YES1, a member of the SFKs. This increased phosphorylation led to increased activation of downstream pathways [58, 64, 65, 82, 83]. Hong *et al.* and Ning *et al.* reported that the Src^{E527K} mutation or mutations in Src inhibitor C-terminal Src kinase (CSK) contributed to increased Src activation, respectively [77, 84].

Epithelial-to-mesenchymal transition

During the developmental regulatory program EMT, cells obtain invasive and migratory properties, and can resist apoptosis, all possibly contributing to drug resistance [89]. The effect of EMT on conferring anti-HER2 drug resistance was investigated by multiple studies [33, 36, 57, 60, 76, 90-92]. Two studies showed that the transforming growth factor β (TGF β) plays an important role in EMT induction in both esophageal and gastric cancer cell lines [90, 91]. In response to trastuzumab treatment, the TGF β signaling pathway was activated in OE19 and OE33 cells and induced EMT, resulting in acquired chemoresistance and increased motility. Interestingly, dual inhibition of HER2 and HER3 using trastuzumab and pertuzumab accelerated EMT induction *in vitro*. Inhibition of the proposed mechanism using trastuzumab, pertuzumab and an TGF β inhibitor reduced EAC patient-derived xenograft tumor growth [90]. In comparison, Zhou *et al.* found that the TGF β /ZEB2 axis induces EMT and confers acquired trastuzumab resistance in NCI-N87 cells, stimulated by downregulation of miR-200c [91]. Furthermore, other studies showed that intrinsic and acquired resistance to lapatinib and trastuzumab could be

explained by activation of the Wnt signaling pathway and subsequent EMT. Inhibition of Wnt signaling reversed the migratory phenotype and restored drug sensitivity [57, 60, 92]. Additionally, Yang *et al.* reported that high vimentin expression, a driver of EMT, correlates with poor overall survival of HER2-positive gastric cancer patients [36].

Cell cycle regulation

Alterations in pathways involved in cell growth and -division could enable cells to maintain proliferative signaling and thus be unresponsive to growth inhibition by chemotherapy [89]. Several studies (n=6) investigated cell cycle regulation and HER2 targeted therapy resistance [50, 52, 72, 93-95]. Shu *et al.* found that trastuzumab resistant GLM-1 cells generated by *in vivo* selection maintained phosphorylation of the retinoblastoma (Rb) protein upon trastuzumab treatment and cell cycle arrest was prevented [93]. Jin *et al.* and Eto *et al.* reported that cell cycle progression in acquired trastuzumab resistant NCI-N87 cells was regulated via multiple pathways [94, 95]. Upregulation of PD-L1 prevented accumulation of DNA damage in cancer cells and stimulated cell cycle progression [94]. Besides, overexpression of miRNA-223 and subsequent downregulation of F-box and WD repeat domain-containing 7 (FBXW7) resulted in upregulation of cell cycle regulators MCL1, c-Myc, and c-Jun [95]. Moreover, Shi *et al.* reported that Shc1-binding protein SHCBP1 was upregulated in gastric cancer and involved in mitotic progression of NCI-N87 and SNU-216 cells. Inhibition of the proposed pathway using silencing RNA or inhibitors restored sensitivity to trastuzumab [72]. Next to destabilizing HER2 expression at the membrane, ST6GAL1-mediated sialylation also reduced trastuzumab-induced cell cycle inhibition [50]. Additionally, genomic profile analysis of 42 HER2-positive GEA samples revealed that multiple cell-cycle related genes such as CCNE1, CCND1, and CDK6 were co-amplified in 17 samples and contributed to intrinsic lapatinib and trastuzumab resistance [52].

Acquiring stemness

There is increasing evidence that cancer cells with stem cell properties are more resistant to chemotherapy or radiotherapy treatments, in part due to self-renewal capacity and tumor cell dormancy [89]. Several studies (n=6) investigated the role of stem cell characteristics in GEA and anti-HER2 drug resistance [76, 96-100]. In trastuzumab resistant NCI-N87 and MKN-45 cells the C-Maf-inducing protein (CMIP) was overexpressed and found to positively regulate SOX2 expression, a stemness transcription factor, promoting acquired resistance to trastuzumab [97]. Similarly, Gse1 coiled-coil protein (GSE1) overexpression was reported to induce acquired resistance to trastuzumab due to promoting stem cell behavior [100]. Furthermore, Gao *et al.*, Mori *et al.*, and Yang *et al.* found that stem cell marker CD44 was upregulated and associated with acquired trastuzumab resistance in NCI-N87 and MKN-45 GC cells [76, 96, 99]. In addition, CD44 and its upstream regulator cAMP-regulated phosphoprotein 19 (*ARPP-19*) were upregulated in HER2-positive gastric cancer tissues with trastuzumab resistance. Increased *ARPP-19* was also linked to acquired trastuzumab resistance in patients and correlated to lower OS [96]. In contrast, Yu *et al.* reported that upregulation of CD44 in trastuzumab resistant GC cells is correlated with favorable OS [98].

Altered pharmacokinetics of HER2-targeting agents

ATP-binding cassette transporters are transmembrane proteins that can facilitate drug transport to protect cells from toxic compounds and contribute to drug resistance [101]. The influence of drug transport on resistance was investigated by three studies [102-104]. Takegawa *et al.* reported that

acquired resistance of NCI-N87 GC cells to T-DM1 was caused by upregulation of drug transporters ABCC2 and ABCG2 and subsequent increased efflux of DM1 [103]. Similarly, Gambardella *et al.* found that acquired lapatinib-resistant OE19 cells have increased expression of ABCC2 [102]. Moreover, Wang *et al.* showed that T-DM1 resistant NCI-N87 cells have decreased V-ATPase activity. This limited the production of the active T-DM1 metabolite lysine-MCC-DM1 and was expected to confer resistance to T-DM1 [104]. Together, these studies illustrate that enhanced transport of antineoplastic agents and reduced drug metabolism could contribute to anti-HER2 drug resistance.

Metabolic reprogramming

Recently, metabolic reprogramming was added to the hallmarks of cancer, as it facilitates constant cell growth and proliferation. Besides, altered metabolism is associated with drug resistance [89]. The effect of reprogramming cellular energy metabolism on anti-HER2 drug resistance has been investigated in five studies [75, 99, 105-107]. Chang *et al.* reported that GATA6-mediated metabolic reprogramming contributed to acquired trastuzumab resistance in NCI-N87 and MKN-45 cells [105, 107]. Moreover, the activation of the Warburg effect was found to be involved in acquired lapatinib resistance in OE19 cells and acquired trastuzumab resistance in NCI-N87 and MKN-45 cells [75, 106]. Also autophagy activation via PI3K/AKT signaling was identified as a mediator of trastuzumab resistance in gastric cancer [107]. Mori *et al.* additionally reported that acquired trastuzumab resistance in NCI-N87 cells could potentially be explained by increased expression of antioxidant enzymes such as glutathione, providing resistance against reactive oxygen species (ROS) accumulation [99].

Alternative mechanisms

Three studies reported resistance mechanisms that could not be linked to the previously discussed categories. Lote *et al.* performed a high-throughput miR-inhibitor screen in NCI-N87 and FLO-1 cells and found that increased levels of miR-148a-3p were involved in acquired trastuzumab resistance. Furthermore, low miR-148a-3p levels were associated with better response to trastuzumab treatment in GEA patients [108]. Yuan *et al.* and Sauveur *et al.* showed that acquired resistance to lapatinib or T-DM1 could be caused by alterations in cell adhesion signaling and consequent increased migration [109, 110]. Sauveur *et al.* additionally reported that altered microtubule dynamics potentially contributed to T-DM1 resistance [110].

Discussion

Although HER2-targeting agents have demonstrated promising outcomes in the treatment of GEC, a considerable number of patients lack durable response to HER2 inhibition. Both intrinsic or acquired resistance to treatment could hamper the efficacy of HER2 targeting in GEC. Nevertheless, the resistance mechanisms to anti-HER2 treatment remain unclear. Herein, we demonstrated several potential mechanisms which could confer treatment resistance, mainly through HER2 receptor mutations inducing impaired ligand binding, activation of alternative receptors, or activation of downstream signaling such as PI3K/AKT and MAPK (**Figure 2**).

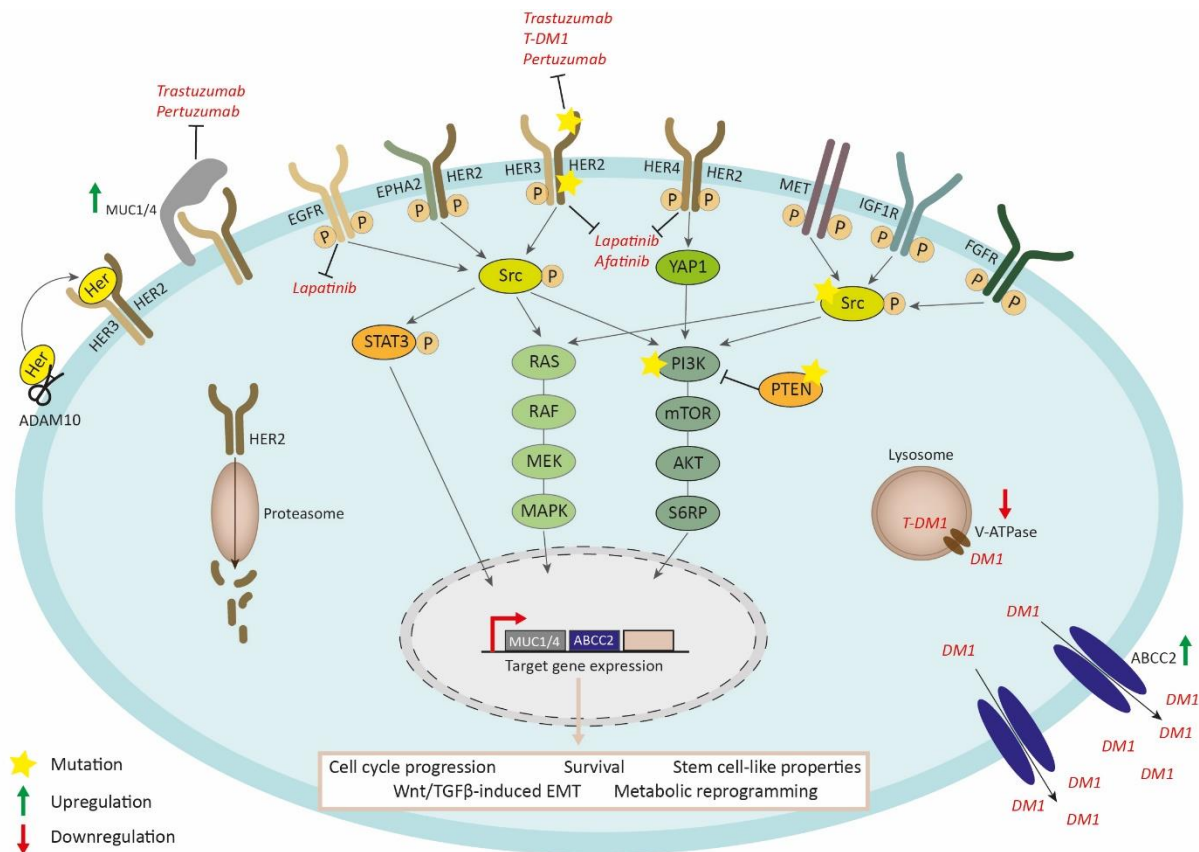


Figure 2. Graphic overview of reported resistance mechanisms against HER2-targeted therapies in GEC. It was suggested that overexpression, downregulation, or degradation of the HER2 receptor could reduce the sensitivity to HER2-targeted therapies, such as trastuzumab, pertuzumab, lapatinib, afatinib, and T-DM1. In response to HER2 downregulation, other receptors (MET, EGFR, HER3, HER4, IGF1R, and FGFR) could be upregulated to activate the PI3K/AKT and MAPK pathways via Src kinase, the main downstream signaling cascades of HER2. Additionally, upregulation of EGFR and subsequent STAT3 activation led to the upregulation of mucins, preventing compound binding to the receptor. Mutations in the receptor's compound binding domain or kinase domain could also impair binding of trastuzumab, pertuzumab, or T-DM1 to HER2. Receptor upregulation was often accompanied by ligand activation, for instance heregulin (Her) and the HER3 receptor. Furthermore, PI3K/AKT and MAPK signaling were activated by overexpression of regulating proteins and mutations in PTEN, PI3K, or Src. Consequently, downstream signaling is maintained, irrespective of receptor activation. Finally, it was reported that induction of EMT by Wnt signaling and TGF β , altered cell cycle regulation, metabolic reprogramming, reduced apoptosis, and decreased production and increased efflux of DM1 contributed to therapy resistance.

Many included articles demonstrated resistance to anti-HER2 treatment based on changes to the HER2 receptor, through different expression levels, impaired receptor binding due to mutations or coverage of the compound binding domain, or receptor modifications. These receptor changes were also proposed in other HER2-positive cancer types such as breast cancer to confer resistance to HER2-targeted therapy [111]. However, some inconsistent conclusions were reported in the included articles. For example, one study reported that resistance was a consequence of HER2 overexpression [32], while the majority of the studies described that loss of HER2 was responsible for resistance [33, 35, 36, 38, 39]. The difference in outcome could be based on different methods used to create a resistant model or variation in the models used. However, the majority of studies demonstrate potential resistance when HER2 expression declines. This is supported by the fact that patients with HER2 3+ (high HER2 overexpression) respond better to treatment with anti-HER2 agents [25, 112-114]. It is also suggested

that GECs demonstrate more heterogeneous expression of HER2 compared to a more homogenous pattern in breast cancer, which could potentially explain less benefit of HER2-targeted therapy in GEC vs breast cancer [115]. As preclinical studies showed loss of HER2 in trastuzumab resistant models after treatment for more than one month, and literature showed that the expression of HER2 could be a dynamic process [21], it could be investigated if treatment efficacy could be improved by changes in treatment timing or dosing.

Furthermore, studies suggested that impaired binding of compounds to HER2 could confer treatment resistance. For instance, Shi *et al.* demonstrated that acquired resistance to trastuzumab was correlated to an increase in MUC1 and MUC4, which could potentially cover the HER2 receptor thus preventing trastuzumab from binding HER2 and exerting inhibitory effects [41]. Besides, other studies demonstrated a correlation between increased MUC expression and worse outcome in patients with gastric cancer and breast cancer [41, 116, 117]. As anti-MUC1 therapies are currently being investigated in the clinical setting, further research could focus on the addition of the latter compound to potentially increase anti-HER2 therapy binding to the HER2 receptor [118]. Additionally, mutations in the compound binding domain of HER2 prevented anti-HER2 drugs to interact with HER2 and consequently limited the anti-tumor effects [40-44].

Other studies described that upregulation of other receptors compensated for HER2 inhibition by maintaining downstream signaling or alternative signaling pathways and thereby contributed to therapy resistance. Upregulation of the MET receptor was reported to contribute to resistance *in vitro* and in the clinic by activating PI3K/AKT and MAPK signaling, and stimulating cell cycle progression [47-56]. Moreover, upregulation of other ErbB family member HER3 contributed to trastuzumab and lapatinib resistance by increased dimerization with HER2 [62, 63]. Previous literature demonstrated synergistic effects of pertuzumab, blocking dimerization of HER2 with HER3, and trastuzumab in both preclinical and clinical studies [25, 119]. However, Ebbing *et al.* reported that the combination of trastuzumab and pertuzumab stimulated TGF β -induced EMT which accelerated drug resistance in surviving cells [90]. This suggests that in response to inhibition of one resistance mechanism, cells could acquire another mechanism to evade treatment-induced toxicity. On the other hand, dual targeting of coexisting resistance mechanisms showed promising results in preclinical models [33, 76]. Altogether, further investigation of treatment regimens and dual blockade is required before this strategy can be applied in the clinic.

PI3K/AKT and MAPK signaling are important pathways contributing to intrinsic or acquired therapy resistance in GEC. Next to activation via different RTKs, these pathways could be activated by upregulation of other proteins such as the tumor suppressor JWA, scaffold protein, Shc1, NRF2, NES1, and MACC1 [38, 72-75]. Furthermore, loss of PTEN expression due to mutations or downregulation affect PI3K and MAPK signaling pathways [68, 77, 78, 80, 81]. Alterations in these cascades were also proposed to contribute therapy resistance in HER2-positive breast cancer [111]. Several studies aimed to circumvent the proposed resistance mechanism by using already existing compounds that target the PI3K/AKT or MAPK pathway, some with promising results [71, 73, 74]. Unfortunately, not enough evidence of efficacy in the clinical setting is available yet. Since PI3K signaling is involved in many essential cellular processes, its inhibition as cancer therapy could have undesired side effects. Therefore, clinical investigation is essential in order to demonstrate potential clinical impact of PI3K/AKT or MAPK pathway inhibition additional to HER2-targeted therapy.

Some studies reported contradictory results, for instance about the role of PI3K pathway mutations in therapy resistance. A retrospective mutation analysis of 29 surgically resected HER2-positive GEC samples showed that PIK3CA mutations were rare (1 sample, 5.6%) and thus were unlikely to contribute to resistance. Importantly, in 11 patients the PI3K mutation status could not be evaluated [81]. However, others showed that PIK3CA mutations were more frequently found (8 samples, 19%; 6 samples, 35.3%) and contributed to drug resistance by activation of the PI3K pathway [45, 52]. These three analyses included a limited number of patients, potentially explaining the contradictory results. Also in other studies small populations of cells were used, often only one resistant clone from one cell line instead of multiple clones and cell lines. Therefore, these studies could exclude the intratumor heterogeneity which is highly present in GEC [21, 120, 121]. Some of these outcome differences are potentially explained by diversity in creating the resistant models. For instance, cells were exposed either to increasing or constant concentrations of drugs, with high variation in the used drugs, drug concentration and exposure time [32, 56, 60, 62, 95, 102, 109]. In some studies, similar resistance mechanisms were reported, despite different methods of creating resistant cells [90, 91, 102, 103]. On the other hand, inconsistent outcomes were reported, despite using similar methods and treatment regimens to create trastuzumab resistant NCI-N87 cells [68, 75, 91]. Thus, these examples suggest that multiple resistance mechanisms can develop independent of treatment in populations of cells.

We performed an extensive blinded systematic search with a high number of inclusions. Moreover, we included all studies assessing resistance mechanism without preselection, thus eliminating potential bias. Besides, we performed extensive quality assessment in order to assess quality of evidence and quality of resistance mechanisms. However, there are some limitations that need to be considered. Firstly, due to the heterogeneity between included studies, which used many different treatment regimens and compounds to create resistant models, no meta-analysis could be performed. Secondly, the majority of the included studies lack clinical impact evidence. Some studies focused on compounds that are rarely used in the clinical setting of GEC [44, 47, 54, 69]. Also, this systematic review included many preclinical studies that reported a resistance mechanism using *in vivo* or *in vitro* models. Consequently, as the majority of the mechanisms are not validated in patient studies, it is not possible yet to predict which resistance mechanisms are more likely to develop in response to particular HER2-targeted therapies in patients.

In summary, multiple different resistance mechanisms are proposed to contribute to HER2-targeted therapy resistance in GEC. While preclinical results are extensive, the frequency and clinical impact of the proposed mechanisms are not fully elucidated yet. Furthermore, coexistence of multiple resistance mechanisms may complicate the targeting of resistant cells. Therefore, more investigation of resistance mechanisms in the clinical setting and subsequent targeting of these mechanisms could provide essential information to improve the outcome of GEC patients.

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	EMBASE
Set	Search
#1	'stomach cancer'/exp OR 'stomach carcinoma'/exp OR 'gastric carcinoma cell line'/exp OR 'stomach tumor'/exp OR 'esophagus cancer'/exp OR 'esophagus carcinoma'/exp OR 'esophageal carcinoma cell line'/exp OR 'esophagus tumor'/exp OR 'esophageal adenocarcinoma'/exp OR 'esophageal adenocarcinoma cell line'/exp OR 'esophageal squamous cell carcinoma'/exp OR 'esophageal squamous cell carcinoma cell line'/exp OR 'stomach adenocarcinoma'/exp OR 'gastric adenocarcinoma cell line'/exp OR 'gastric cancer cell line'/exp OR 'stomach cancer':ab,ti OR 'stomach tumor':ab,ti OR 'gastric cancer cell line':ab,ti OR 'stomach carcinoma':ab,ti OR 'gastric carcinoma cell line':ab,ti OR 'stomach adenocarcinoma':ab,ti OR 'gastric adenocarcinoma cell line':ab,ti OR 'esophagus tumor':ab,ti OR 'esophagus cancer':ab,ti OR 'esophageal cancer cell line':ab,ti OR 'esophagus carcinoma':ab,ti OR 'esophageal carcinoma cell line':ab,ti OR 'esophageal adenocarcinoma':ab,ti OR 'esophageal adenocarcinoma cell line':ab,ti OR 'esophageal squamous cell carcinoma':ab,ti OR 'esophageal squamous cell carcinoma cell line':ab,ti
#2	'her2 gene'/exp OR 'her2 protein human'/exp OR 'her3 gene'/exp OR 'her3 protein'/exp OR 'human epidermal growth factor receptor 2'/exp OR 'human epidermal growth factor 2'/exp OR 'human epidermal growth factor receptor 2 gene'/exp OR 'human epidermal growth factor receptor 3'/exp OR 'erb2 protein human'/exp OR 'erb2 gene'/exp OR 'erb2 protein mouse'/exp OR 'erb3 gene'/exp OR 'erb3 protein human'/exp OR 'neuregulin 1beta'/exp OR 'neuregulin 1 gene'/exp OR 'neuregulin 1 beta'/exp OR 'trastuzumab'/exp OR 'trastuzumab emtansine'/exp OR 'trastuzumab deruxtecan'/exp OR 'pertuzumab'/exp OR 'lapatinib'/exp OR 'dacomitinib'/exp OR 'epidermal growth factor receptor 2'/exp OR 'antineoplastic drug':ab,ti OR 'human epidermal growth factor receptor 2':ab,ti OR 'human epidermal growth factor 2':ab,ti OR 'human epidermal growth factor receptor 3':ab,ti OR 'human epidermal growth factor receptor 2 gene':ab,ti OR 'human epidermal growth factor receptor 2 positive gastric cancer':ab,ti OR 'her2 gene':ab,ti OR 'her2 protein human':ab,ti OR 'her3 gene':ab,ti OR 'her 3 protein':ab,ti OR 'erb2 protein human':ab,ti OR 'erb2 gene':ab,ti OR 'erb2 protein mouse':ab,ti OR 'erb3 gene':ab,ti OR 'epidermal growth factor receptor 3':ab,ti OR 'erb3 protein human':ab,ti OR 'neuregulin 1beta':ab,ti OR 'neuregulin 1 gene':ab,ti OR 'neuregulin 1 beta':ab,ti OR 'trastuzumab':ab,ti OR 'trastuzumab emtansine':ab,ti OR 'trastuzumab deruxtecan':ab,ti OR 'pertuzumab':ab,ti OR 'lapatinib':ab,ti OR 'dacomitinib':ab,ti
#3	'drug resistance'/exp OR 'multidrug resistance'/exp OR 'resistance'/exp OR 'therapy resistance'/exp OR 'drug resistance':ab,ti OR 'multidrug resistance':ab,ti OR 'therapy resistance':ab,ti OR resistance:ab,ti
#4	#1 AND #2 AND #3
#5	#4 AND [embase]/lim NOT (([embase]/lim AND [medline])/lim)

	CENTRAL
Set	Search
#1	MeSH descriptor: [Esophageal Neoplasms] explode all trees
#2	MeSH descriptor: [Stomach Neoplasms] explode all trees
#3	((esophag* NEXT (cancer* or neoplas* or tumor* or tumour* or malig* or carcino* or adeno*)) or (oesophag* NEXT (cancer* or neoplas* or tumor* or tumour* or malig* or carcino* or adeno*)) or (gastric NEXT (cancer* or neoplas* or tumor* or tumour* or malig* or carcino* or adeno*)) or (stomach NEXT (cancer* or neoplas* or tumor* or tumour* or malig* or carcino* or adeno*)) or (gastroesophag* or gastroesophag* NEXT (cancer* or neoplas* or tumor* or tumour* or malig* or carcino* or adeno*)) or gastric cancer cell line* or esophageal cancer cell line* or gastric cancer adenocarcinoma cell line* or esophageal cancer adenocarcinoma cell line* or oesophageal cancer cell line* or oesophageal adenocarcinoma cell line* or gastric carcinoma cell line* or esophageal carcinoma cell line* or oesophageal carcinoma cell line*):ti,ab,kw
#4	#1 OR #2 OR #3
#5	MeSH descriptor: [Genes, erbB-2] explode all trees

#6	MeSH descriptor: [Receptor, ErbB-2] explode all trees
#7	MeSH descriptor: [Receptor, ErbB-3] explode all trees
#8	((epidermal growth factor receptor 2 or human epidermal growth factor receptor 2 or her-2 or her-2 neu or her-2/neu or erbb2 or neuregulin receptor or cerbb2 or neu receptor or trastuzumab or pertuzumab or lapatinib)):ti,ab,kw OR ((epidermal growth factor receptor 3 or human epidermal growth factor receptor 3 or her-3 or erbb3 or cerbb3 or pertuzumab or dacomitinib or trastuzumab-emtansine or trastuzumab deruxtecan)):ti,ab,kw
#9	#5 OR #6 OR #7 OR #8
#10	MeSH descriptor: [Drug Resistance] explode all trees
#11	(acquired resistanc* or intrinsic resistanc* or resistanc* or antineoplastic drug resistanc* or drug resistanc* or therapy resistanc* or block* or intransigenc*):ti,ab,kw
#12	#10 OR #11
#13	#4 AND #9 AND #12

Appendix 2: Supplementary Figures

Supplementary Figure 1. Quality and Risk of Bias assessment using an adjusted version of the OHAT risk of bias tool. '+' indicates low risk of bias, '?' indicates unclear risk of bias, and '-' indicates high risk of bias.

	Reporting bias	Information bias	Selection bias	Incomplete outcome bias	Other bias
Arienti, 2016	?	-	-	+	?
Chang, 2020	+	?	?	+	+
Chen, 2012	+	-	?	+	+
Chen, 2019	+	+	?	-	+
Deguchi, 2017	+	+	?	?	+
Deng, 2013	-	-	-	?	?
Ding, 2020	+	?	+	+	+
Duarte, 2021	+	?	+	+	+
Ebbing, 2016	+	+	?	+	+
Ebbing, 2017	+	+	?	+	+
Eto, 2014	+	?	+	-	+
Eto, 2015	?	?	?	-	+
Gambardella, 2017a	+	?	-	?	?
Gambardella, 2017b	+	?	?	?	?
Gambardella, 2019	+	+	?	+	+
Gao, 2020	?	+	?	?	+
Guo, 2021	+	+	+	?	?
Hassan, 2019	?	?	?	?	?
Hassan, 2020	?	?	?	?	?
Hong, 2012	+	?	+	+	+
Hong, 2014	+	+	-	?	+
Jin, 2017	?	+	-	-	?
Jin, 2021	+	?	-	?	+
Kang, 2015	?	?	?	?	?
Kashiwada, 2018	?	?	+	?	?
Kim, 2013	?	?	?	?	?
Kim HP, 2014	+	?	-	?	?
Kim J, 2014	+	?	+	+	+
Kim, 2017	+	+	+	+	+
Lee, 2013	+	+	?	+	+
Li, 2014	+	+	+	+	+
Liu, 2016	+	+	?	+	+
Liu, 2017	+	?	-	+	?
Liu N, 2018	+	?	-	?	?
Liu W, 2018	+	+	?	+	+
Lote, 2020	?	?	+	?	?
Ma, 2016	+	+	+	+	+
Mori, 2018	?	?	?	?	?
Ning, 2021	+	+	?	+	+
Park, 2018	+	+	+	+	+
Piro, 2016	+	+	+	-	+
Sampera, 2016	?	?	?	?	?
Sampera, 2019	+	+	+	+	+
Sanchez-Vega, 2019	+	-	?	+	+
Sato, 2013	-	-	+	+	?
Sauveur, 2018	+	+	+	+	+
Shi, 2013	+	+	-	+	+
Shi, 2018	+	+	+	+	+
Shi, 2021	+	+	+	+	+
Shibata, 2013	+	?	+	?	+
Shu, 2015	?	?	?	?	?
Su, 2021	+	+	?	+	+
Takegawa, 2017	-	+	?	+	+
Tang, 2017	+	+	+	+	+
Wang, 2017	+	+	?	+	+
Wang, 2019	+	-	+	+	?
Wang, 2021	+	+	+	+	+
Xiang, 2020	+	+	+	+	+
Yang, 2014	+	?	+	+	+
Yang, 2019	-	+	?	+	?
Ye, 2018	-	+	+	+	+
Yokoyama, 2021	+	-	+	+	+
Yoshioka, 2019	+	+	?	+	+
Yu, 2015	-	+	?	+	+
Yu C, 2018	+	-	+	+	+
Yu Y, 2018	-	+	?	+	+
Yuan, 2020	+	-	?	+	+
Zhang, 2014	+	+	?	+	+
Zhang, 2019	+	?	?	?	+
Zhang, 2020	+	-	?	+	+
Zheng, 2014	+	?	?	+	+
Zhou, 2018	+	+	-	?	+
Zuo, 2015	+	+	-	?	+

Supplementary Figure 2. *Quality assessment of resistance mechanisms. Quality of the proposed mechanisms were evaluated using the following criteria: the presence of a control group, the use of >2 methods to investigate resistance (e.g. immunohistochemistry, ELISA, qualitative polymerase chain reaction, western blot), the use of >2 models to investigate resistance (e.g. datasets, cell lines, resistant clones, xenografts, patient-derived materials), identification and analysis of upstream or downstream participants, correlation with resistance outcome (e.g. viability or apoptosis upon treatment, IC50, OS/PFS, reduction of tumor growth), and restoring of sensitivity through removal of the proposed mechanism. For each criterion, the studies were scored '+', '-', or '?'. For '+' 1 point was given, and for '-' or '?' no points were given. A score of ≤ 3 indicates low quality, a score of 4-5 indicates moderate quality, and a score of 6 indicates high quality.*

	Presence of controls	two/more methods	two/more models	Analysis of up/downstream participants	correlation with resistance outcome	Restoring sensitivity	Final assessment
Arienti, 2016	+	+	+	-	-	-	Low
Chang, 2020	+	+	+	-	-	-	Low
Chen, 2012	+	+	+	+	+	+	High
Chen, 2019	+	+	+	+	+	+	High
Deguchi, 2017	+	+	+	+	+	-	Moderate
Deng, 2013	+	+	+	-	-	-	Low
Ding, 2020	+	+	-	-	-	-	Low
Duarte, 2021	+	+	+	-	+	-	Moderate
Ebbing, 2016	+	+	+	+	+	+	High
Ebbing, 2017	+	+	+	+	+	+	High
Eto, 2014	+	+	+	+	+	+	High
Eto, 2015	+	+	+	+	+	+	High
Gambardella, 2017a	+	+	-	-	-	-	Low
Gambardella, 2017b	?	+	+	+	-	-	Low
Gambardella, 2019	+	+	+	+	+	+	High
Gao, 2020	+	+	+	+	+	+	High
Guo, 2021	+	+	+	+	+	+	High
Hassan, 2019	+	+	+	-	-	+	Moderate
Hassan, 2020	+	+	-	-	-	-	Low
Hong, 2012	+	+	+	+	+	+	High
Hong, 2014	+	+	+	+	+	+	High
Jin, 2017	+	+	+	-	+	+	Moderate
Jin, 2021	+	+	+	+	+	+	High
Kang, 2015	+	+	+	-	-	-	Low
Kashiwada, 2018	-	?	-	+	-	-	Low
Kim, 2013	?	?	-	+	+	-	Low
Kim HP, 2014	+	+	+	+	+	+	High
Kim J, 2014	+	+	+	+	+	+	High
Kim, 2017	+	+	-	+	+	-	Moderate
Lee, 2013	+	+	+	+	-	+	Moderate
Li, 2014	+	+	+	+	+	+	High
Liu, 2016	+	+	+	+	+	+	High
Liu, 2017	+	+	-	+	+	+	Moderate
Liu N, 2018	+	+	-	+	+	-	Moderate
Liu W, 2018	+	+	+	+	+	+	High
Lote, 2020	+	+	+	-	-	-	Low
Ma, 2016	+	+	+	+	+	+	High

	Presence of controls	two/more methods	two/more models	Analysis of up/downstream participants	correlation with resistance outcome	restoring sensitivity	Final assessment
Mori, 2018	?	+	-	+	?	+	Low
Ning, 2021	+	+	+	+	+	+	High
Park, 2018	+	+	+	+	+	+	High
Piro, 2016	+	+	+	+	+	+	High
Sampera, 2016	+	+	+	+	-	+	Moderate
Sampera, 2019	+	+	+	+	+	+	High
Sanchez-Vega, 2019	+	+	+	-	+	-	Moderate
Sato, 2013	+	+	+	-	+	+	Moderate
Sauveur, 2018	+	+	+	-	-	+	Moderate
Shi, 2013	+	+	+	+	+	+	High
Shi, 2018	+	+	+	+	+	+	High
Shi, 2021	+	+	+	+	+	+	High
Shibata, 2013	+	+	+	+	+	+	High
Shu, 2015	+	+	-	+	+	+	Moderate
Su, 2021	+	+	+	-	+	+	Moderate
Takegawa, 2017	+	+	+	-	+	+	Moderate
Tang, 2017	+	+	+	+	+	+	High
Wang, 2017	+	+	+	+	+	+	High
Wang, 2019	+	+	+	-	+	+	Moderate
Wang, 2021	+	+	+	-	+	+	Moderate
Xiang, 2020	+	+	+	-	+	-	Moderate
Yang, 2014	+	+	+	+	+	+	High
Yang, 2019	+	-	+	-	+	-	Low
Ye, 2018	+	+	-	+	+	+	Moderate
Yokoyama, 2021	+	+	+	-	+	+	Moderate
Yoshioka, 2019	+	+	+	-	+	+	Moderate
Yu, 2015	+	+	+	-	+	-	Moderate
Yu C, 2018	+	-	+	-	+	-	Low
Yu Y, 2018	+	-	+	-	+	+	Moderate
Yuan, 2020	+	+	+	-	+	+	Moderate
Zhang, 2014	+	+	+	+	+	+	High
Zhang, 2019	+	+	+	-	-	-	Low
Zhang, 2020	+	+	+	-	+	+	Moderate
Zheng, 2014	+	+	+	-	+	+	Moderate
Zhou, 2018	+	+	-	+	+	+	Moderate
Zuo, 2015	+	+	-	+	+	+	Moderate