Chapter 17. The EGFR oncogene story 220724ds3

Drugs Against Cancer: Stories of discovery and the quest for a cure.

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CHAPTER 17

The EGFR Oncogene story.

Prolog: the ErbB oncogene story

In the 1970's, researchers had been investigating certain viruses common in birds. These "avian erythroblastosis retroviruses" had an RNA genome and a "reverse transcriptase" that copied its RNA into DNA, which then became incorporated into the host's DNA, from which it was recopied into viral RNA for the next cycle of virus production.

When injected into susceptible chickens, the viruses caused an overproduction of red blood cells (erythroblastosis), but unexpectedly sometimes also produced cancers (sarcomas). Tracking down the cause of the cancers, investigators found, in the RNA of the cancer-producing retroviruses, a nucleotide sequence that they thought to be the culprit and surmised it to be an "oncogene" –- a gene that caused the cancers. They dubbed the oncogene *erb* for erythroblastosis; there were two types: *ErbA* and *ErbB*.

Amazingly, genes with nucleotide sequence similarities to *ErbB* were found in the genomes of vertebrate animals from fish to humans; moreover, those oncogenic sequences in the *ErbB* gene resembled sequences found in the human epidermal growth factor receptor gene (*EGFR*) (Downward et al., 1984; Saule et al., 1981). Incredibly, the *erb* oncogene in an avian retrovirus had nucleotide sequence similarity to a normal human gene! It was the epidermal growth factor receptor (*EGFR*) gene, which was found to become an oncogene when mutated or amplified -- which is the topic of this Chapter.

That summarizes a complicated and confusing history of experiments. However, it led to a dual terminology that one had to become accustomed to, because EGFR was discovered by a totally independent route -- which is the topic of the next sections.

For future reference, the terminology for the 4 members of the EGFR family is as follows: ErbB1=EGFR=HER1; ErbB2=HER2; ErbB3=HER3; ErbB4=HER4. (HER stands for "human epidermal growth factor receptor.") The HER terminology is specific for the human genes, whereas the ErbB and EGFR terms are used more generally.

Receptor tyrosine kinases (RTK's): how signals are transmitted from outside to inside the cell.

I'll begin with an overview of how RTK's work by transmitting signals from outside to inside the cell.

Chapter 14 told how specific inhibitors of the ABL tyrosine kinase provided effective treatment for chronic myelogenous leukemia (CML) patients. That story was a striking example of how a specific molecular abnormality in a certain type of cancer led to a cancer cure.

There are many tyrosine kinases that transmit signals to the cell nucleus. There are two general types: (1) receptor tyrosine kinases (RTK's) that transmit signals from outside the cell -- the EGFR family are among these; and (2) non-receptor kinases that may roam the cytoplasm and enter the nucleus to affect gene expression; ABL is one of these.

To transmit signals from outside to inside the cell, the receptor part of the RTK molecule sticks out of the cell, ready to bind a signaling molecule, such as a "growth factor", that may be drifting about in the exterior. Most cell types will grow and divide only in the presence of appropriate growth factor molecules. The receptor part of the RTK molecule that binds the growth factor outside the cell connects to a narrow piece that passes through the plasma membrane and, in turn, connects to a large part that is inside the cell. That is the business part: triggered by the extracellular domain of the RTK, the intracellular domain of the RTK engages in a complicated set of interactions inside the cell that stimulate the growth and division of the cell.

I go on now to tell the story of how the first RTK was discovered. The story begins however with the discovery of a signaling molecule, a small protein called epidermal growth factor (EGF) that was later found to bind to the exterior or extracellular part of a membrane protein that became known as epidermal growth factor receptor (EGFR).

Discovery of epidermal growth factor (EGF).

One the most important developments in cancer biology and therapeutics was the discovery of the growth factors that were found to bind to the extracellular domain of the epidermal growth factor receptor. The story began in the early 1960's, when Stanley Cohen (Figure 17.1) at Vanderbilt University in Nashville, Tennessee isolated a small protein that

stimulated the proliferation of skin cells (Cohen, 1965) (Figure 17.2). That small protein was later to be called epidermal growth factor, EGF.

Figure 17.1. Stanley Cohen (1922-2020) was awarded the Nobel Prize in Physiology and Medicine, along with Rita Levi-Montalcini, in 1986 for the discovery of epidermal growth factor and the purification of nerve growth factor. A son of Jewish immigrants, Cohen was born in Brooklyn, New York and received his bachelor's degree from Brooklyn College in 1943. He carried out his major research at Vanderbilt University in Nashville, Tennessee from 1959 until his retirement in 1999. He contributed much to the story told in the early part of this chapter.

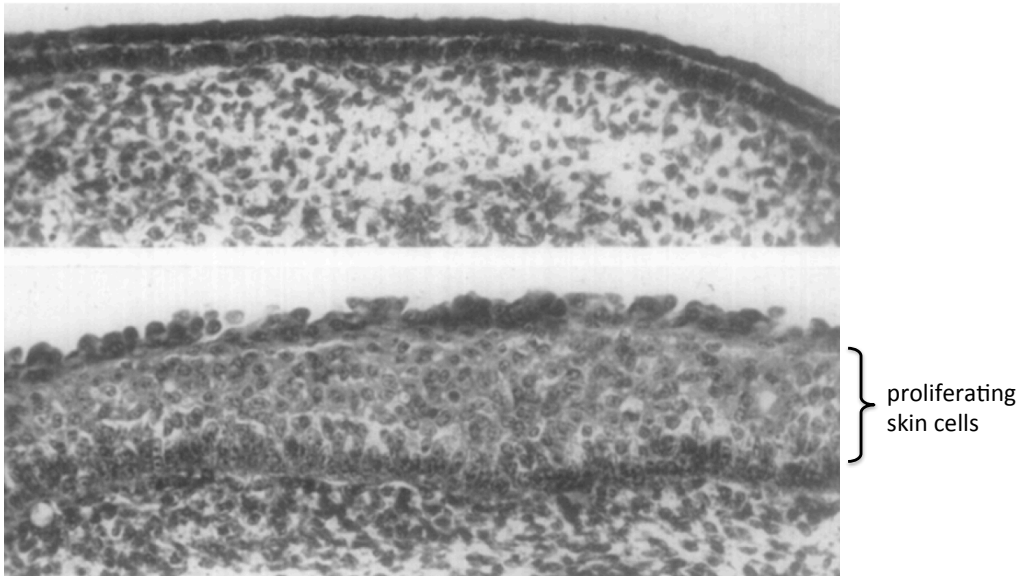

Figure 17.2. Cross-sections of skin, showing one of the first demonstrations of the growth-promoting effect of a small protein that was later named epidermal growth factor (EGF). This experiment, carried out by Stanley Cohen in 1965, shows how EGF causes skin cells to proliferate (bottom), compared with the cells of skin cultured in the absence of EGF (top). The skin was dissected from chick embryos and grown in a solution containing essential ingredients with (bottom) or without (top) EGF (Cohen, 1965).

In the early part of the work, Cohen collaborated with Rita Levi-Montalcini, who had devised a method to grow skin from chick embryos in a culture medium, where it could be studied under well-defined conditions. They shared the Nobel Prize in Physiology and Medicine in 1986 for the discovery of epidermal growth factor and the isolation of nerve growth factor.

Here I cannot resist recounting, even though embarrassing to me, my one-time encounter with Rita Levi-Montalcini, as it shows her delightful and indomitable personality (she lived to be 103). We were seated next to each other at dinner at a conference and had just begun to chat, when she asked me right-out whether I knew who she was and about her work. Perhaps more indignant than amused by my nonplussed expression, she took some time before admitting at last that she was in fact the famous discoverer of nerve growth factor -- which was an area of research about which I was at the time unfortunately abysmally ignorant.

To continue: Stanley Cohen's small protein, later dubbed "epidermal growth factor" (EGF), was first purified from the salivary glands of mice -- Cohen and Carpenter isolated the human version in 1975; the two EGF's, mouse and human, had very similar physical and biological properties -- the same growth-promoting effects on cells (Cohen and Carpenter, 1975). EGF stimulated the proliferation, not only of skin epidermal cells, but also of cells from many epithelial tissues and epithelial cancers. (An epithelium consists of one or more layers of cells that separate the outside from the inside of a tissue. Most cancers develop from epithelial cells.)

Chemical structure of EGF.

The first step in analyzing the chemical structure of EGF was to determine its sequence of amino acids. The molecule was found to consist of a chain of 53 amino acids, with three crosslinks (Figure 17.3). Each crosslink connected two cysteine (CYS) amino acids located at different places in the chain. (Cysteines have a sulfur atom at the end; two sulfur atoms can bind to each other to form a “disulfide” crosslink that connects between different parts of the amino acid chain. This, by the way, can only happen outside the cell, because the "reducing" conditions inside cells keep the sulfur atoms from binding to each other. Growth factors are located outside the cell, where disulfide bonds are stable.)

The next step was to determine the 3-dimensional structure. That is important, because the EGF molecule must have the right shape to fit into a receptor site and to exert its effects.

The structure was determined by x-ray crystallography in 2001 (Lu et al., 2001), and is shown in Figure 17.4. The EGF amino acid chain is not long enough to maintain a 3-dimensional structure all by itself (it wiggles around too much); the three disulfide crosslinks help to keep its shape.

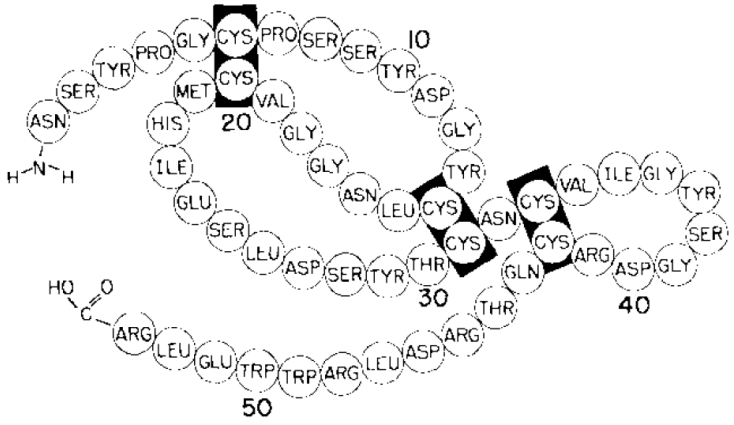

Figure 17.3. The amino acid chain structure of epidermal growth factor (EGF), showing the 3 cysteine (CYS) pairs that hold different parts of the chain together (Carpenter and Cohen, 1979). The 3-dimensional structure of the amino acid chain is shown in Figure 17.4.

Figure 17.4. The 3-dimensional structure of human epidermal growth factor (EGF) protein (Lu et al., 2001). The shape of the structure is held securely in place by the three Cys-Cys crosslinks (shown in yellow – shown also in Figure 17.3). (The fat green arrows show the direction of the amino acid chain from the N-terminus to the C-terminus.)

How does EGF trigger a growth signal to be sent from outside to inside the cell?

A puzzle: EGF was found to transmit a growth signal to the cell nucleus -- but how was the signal transmitted, since EGF is located outside the cell and cannot get in? Somehow, EGF transmits its signal right through the intact cell surface membrane without the EGF molecule itself, or any part of it, going through.

The first clue to how that happens came from a finding by Hollenberg and Cuatrecasas, who, in 1973, demonstrated that EGF binds to specific receptors on the cell surface (Hollenberg and Cuatrecasas, 1973). Using radioactively-tagged EGF, Stanley Cohen and his colleagues determined that there was a limited number of such receptor sites on the surface of various types of cells (Carpenter and Cohen, 1979). To give an idea of numbers, there are typically about 70,000 EGF receptor sites on the surface of a cell -- which is not very many, considering how tiny the molecules are, compared to the size of a cell. Figure 17.5 showed that EGF molecules were bound to the surface of cells; to make the molecules visible, a fluorescent chemical group was attached to the EGF, which made is glow under ultraviolet light. The receptors to which EGF bound on the cell surface were later found to be the extracellular part the epidermal growth factor receptor (EGFR), whose story follows.

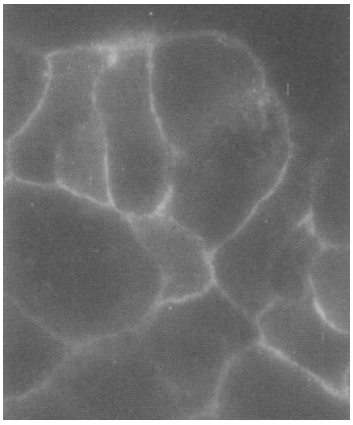

Figure 17.5. Epidermal growth factor (EGF) molecules bound to the cell surface. A fluorescent tag was attached to the EGF molecules to make them glow when viewed with a fluorescence microscope. When mixed with cells, the fluorescence-labeled EGF was seen bound to the surface of the cells, as shown here by the bright edges where the surfaces of adjacent cells meet. This experiment was carried out in Stanley Cohen's laboratory and published in 1978 (Haigler et al., 1978).

Discovery of the EGF receptor (EGFR).

One of the first clues to the existence of receptors for epidermal growth factor and how the receptor transmits a signal from outside to inside the cell came from experiments

conducted by Stanley Cohen and his colleagues in 1980 (Cohen et al., 1980). Using isolated cell membranes, they found that epidermal growth factor (EGF) bound to the membranes and caused a large protein in the membranes to become phosphorylated (*i.e.,* EGF caused phosphate groups to be added to a large membrane-associated protein). The large protein turned out to be the receptor for EGF (EGFR). EGFR was estimated to be about 150,000 Daltons in molecular weight, compared to only about 6,000 Daltons for EGF.

The discovery of EGFR was a major breakthrough for cancer therapy. Excessive function of EGFR was later found to send abnormally strong signals that push cells to divide excessively and without control. This was found to be an important contributory cause of about 30% of all cancers (malignant tumors arising from epithelia).

Quite remarkably, the cancer cells often became addicted to the high EGFR activity: when EGFR activity was inhibited by means of a drug, the cancer cells tended to die! However, much first had to be discovered about EGFR and how it worked.

By 1987, Yosef Yarden and Joseph Schlessinger together with other researchers purified EGFR and determined that the molecule consists of 3 parts: an extra-cellular domain that binds EGF, a trans-membrane domain, and an intra-cellular domain that has tyrosine kinase activity (Yarden and Schlessinger, 1987a, b). They also showed that the active unit consisted of two EGFR molecules bound together, and that each member of the pair phosphorylated the other; the binding of EGF caused the pairing and stimulated each to phosphorylate the other.

The magic of how EGFR transmits signals from outside to inside the cell.

EGFR was found to be one of a great number of receptor tyrosine kinases (RTKs) that function to pass extracellular signals of various kinds from outside the cell to molecules inside the cell; the signals then pass to intracellular protein molecules to eventually reach the cell nucleus, where the signals affect which genes will be expressed. The RTKs passed nothing physical through the surface membrane of the cell, only information – but how could they do that?

The epidermal growth factor receptors (EGFR–family) were the first and most intensively studied receptor tyrosine kinases and were found to have major roles in cancer cause and treatment (Endres et al., 2014; Lemmon et al., 2014). The EGFR family has four members, Erb1, Erb2, Erb3, and Erb4; the human versions are designated HER1, HER2, HER3, and HER4. The first member to be discovered, EGFR, has the alternative names Erb1 and HER1.

It seemed magical that binding to something outside the cell caused something to happen inside the cell without any substance moving through the cell surface membrane. The details of how that is accomplished took much time and effort to be revealed, and there is much to tell about how it works and about drugs designed to block those actions – and how those discoveries were made.

Here is how it works:

The binding of an EGF to an EGFR causes two EGFR molecules to bind to each other to form a homodimer ("dimer" = "two-part"; "homo" indicates that the 2 parts are the same). If different members of the EGFR family bind to each other, they form a "heterodimer" ("hetero" meaning "different"). The four EGFR family members can bind to each other in all possible binary combinations to form homodimers and heterodimers. A heterodimer particularly important for human cancer was found to be the EGFR-HER2 pair, more generally known as the ErbB1-ErbB2 pair.

The binding of EGF causes two EGFR-family members to come together to form a homo- or hetero-dimer. The dimer formation then causes the two EGFR-family molecules to change shape in such a way that it enables the intracellular parts of the two molecules of the dimer to add phosphate groups to each other at certain of their tyrosine amino acids. This happens because the intracellular part of each EGFR-family molecule has tyrosine kinase enzyme activity.

The phosphate groups at specific places on the intracellular domains then bind particular molecules inside the cell (helped by phosphate's negative charge). That leads to several reaction paths, one of the most important being a chain of reactions that proceeds by way of one or another member of the RAS-family (which is the subject of the next chapter). How this amazing signal transmission is accomplished is diagrammed in Figure 17.6 – for the case where EGFR (ErbB1) interacts with HER2 (ErbB2).

The signaling process is also portrayed as a whimsical story in Textbox 1. The ErbB2/HER2 pairing with ErbB1/EGFR or other ErbB's is especially relevant to cancers of breast and ovary, where the cancer cells produce too much ErbB2/HER2, and where drugs were being developed to inhibit its tyrosine-phosphorylation activities (Fabi et al., 2014).

ErbB2/HER2 is unique among the 4 family members in that its extracellular domain lacks the ability to bind any growth factor; it is activated when it binds to growth factor-activated ErbB1/EGFR); the details are described in Textbox 1, wherein I have taken some liberties to lighten the complexities that are diagrammed in Figures 17.6 and 17.7 and described in the legends.

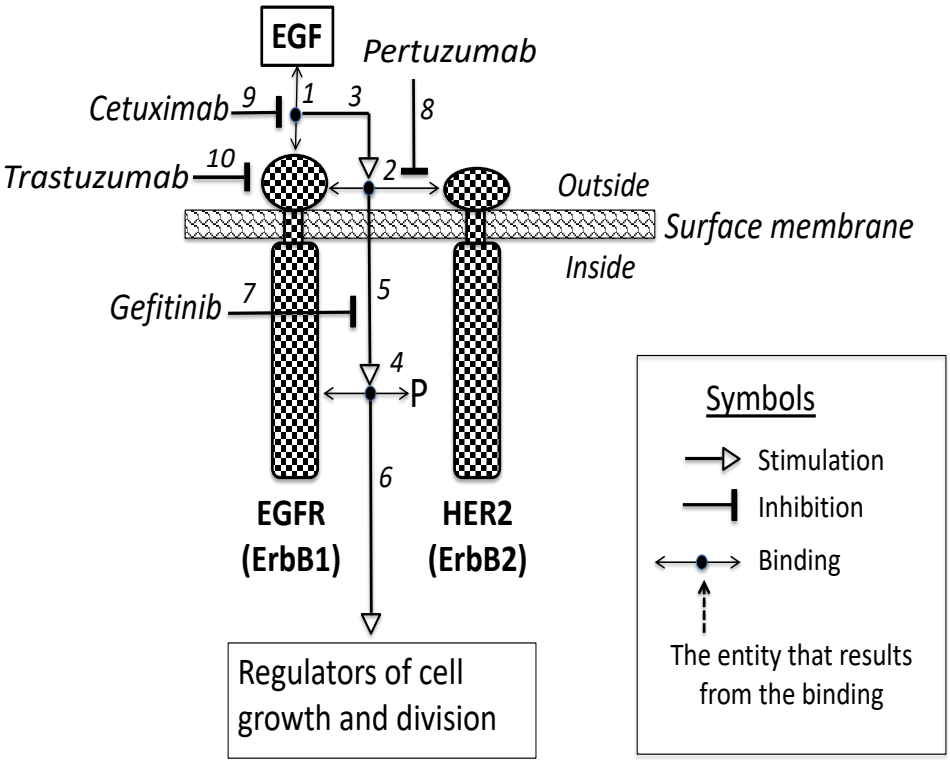

Figure 17.6. Molecular interaction map showing how epidermal growth factor (EGF) binds epidermal growth factor receptor EGFR, allowing it to bind HER2, resulting in a growth signal sent into the cell. The steps are as follows: [*1*] EGF binds EGFR outside the cell; [*2, 3*] the EGF binding allows EGFR to bind HER2; [*4, 5*] the EGFR and HER2 then phosphorylate each other (only one phosphorylation of EGFR is shown; actually, many sites on both EGFR and HER2 become phosphorylated; [*6*] the phosphorylated EGFR-HER2 heterodimer then sends signals to the cell nucleus via complicated steps that are not shown in this diagram. [*7*] Drugs, such as gefitinib, inhibit the kinase domains of the ErbB's, thereby inhibiting the phosphorylations. The actions of three monoclonal antibodies are shown: [*8*] pertuzumab inhibits the ErbB's from binding to each other; [*9*] cetuximab may prevent the binding of a growth factor; and [*10*] trastuzumab inhibits multiple functions of the extracellular domain. (A note about the notation: the small, filled circles on the interaction lines represent the product of the interaction. For example, small, filled circles represent EGF-bound EGFR in step [*1*] and phosphorylated EGFR in step [*5*]). The molecular interaction map notation is fully described in (Kohn et al., 2006).

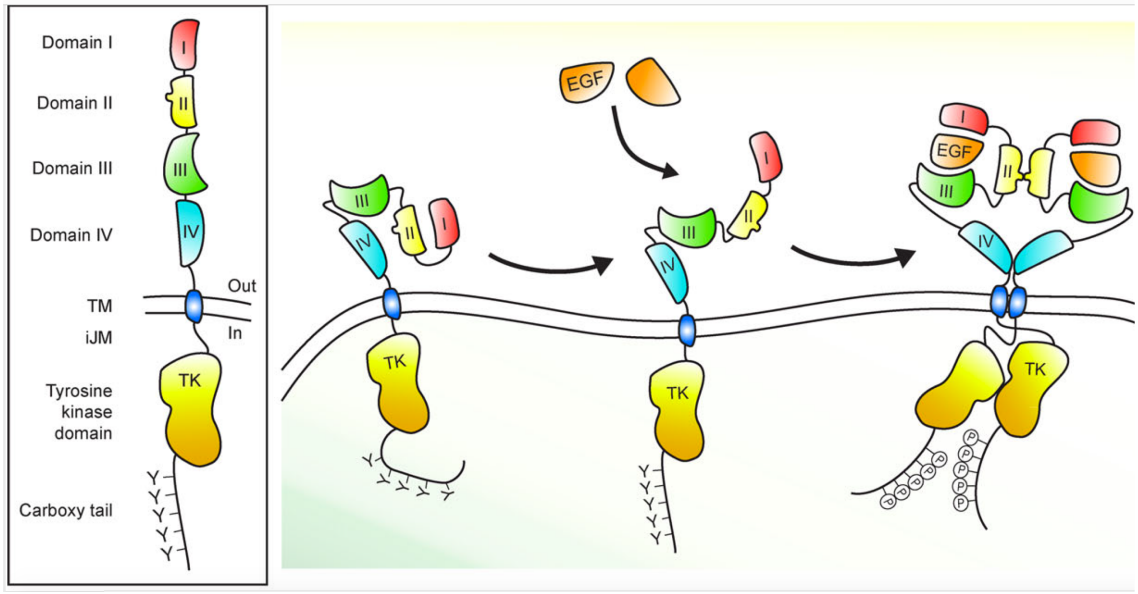

Figure 17.7. Another depiction of how a receptor tyrosine kinase (RTK) transmits a signal across the cell surface membrane, showing some recently discovered details (Sigismund et al., 2018). (*Permission needed.*) In the case shown, the 2 RTK's are of the same type, forming, for example, an EGFR homodimer. Upon binding of a growth factor, such as EGF, the 4 extracellular domains of the RTK (shown in different colors) rearrange in a manner that allows 2 RTK molecules to bind to each other (via subdomain II). This brings together their tyrosine kinase domains (TK), allowing each of them to phosphorylate several tyrosines (Y's) on the intracellular tail of the other. The resulting phosphorylated tyrosines (encircled P's) then bind intracellular molecules that convey signals for cell division or other cell functions (not shown).

In summary up to this point, the vast realm of receptor tyrosine kinases (RTK's) tells cells what to do in response to a wide variety of signals from outside the cell. They are amazingly well-designed molecular machines that transmit information from outside to inside the cell without transmitting anything material. The four members of the epidermal growth factor receptor (EGFR) family are particularly important in cancer. They pair up in all pairs to form homodimers and heterodimers, each combination having its own characteristics and functions. HER2 (the human version of the ErbB2 gene) was found to be an important driver of some breast cancers.

I selected the EGFR-ErbB2 pair for illustration in Figure 17.6, because, aside from being medically important, it gives a simpler diagram. The diagram uses just four symbols, which are defined in the Figure: stimulation, inhibition, binding, and entity that is the product of a binding (such as a dimer or a phosphorylated molecule).

To summarize how it works (also explained in the Figure 17.6 legend):

The part that is outside (the receptor domain) binds a growth factor molecule -- small protein molecules floating around between the cells. For example, EGFR can bind

epidermal growth factor (EGF) [*1*] (the numbers in brackets correspond to the numbers next to the interactions in Figure 17.6). There are several other growth factors that EGFR can bind, but EGF is the most common. After binding EGF, EGFR can go on to bind HER2 [*2, 3*] (or another member of the EGFR-family). HER2 is the only member of the EGFR-family that lacks the ability to bind growth factors from outside of the cell. However, when bound to EGFR, HER2 phosphorylates and activates the EGFR to which it is bound. When EGFR and HER2 are bound together, the shapes of the two molecules change in a way that brings their intra-cellular domains together, which allows the tyrosine kinase domain of one member of the pair to add phosphate groups to the intracellular part of the other member of the pair [*4, 5*]. (Although Figure 17.6 shows only one phosphate added to EGFR, several phosphates actually are added to both EGFR and HER2.)

The anticancer drug, gefitinib, inhibits these phosphorylations [*7*]. The phosphorylations require ATP, and each kinase domain has a cleft where the ATP binds to do its work. It is in this cleft where gefitinib binds and prevents ATP from coming in. That is how gefitinib inhibits the EGFR receptors.

The phosphorylated sites on EGFR and HER2 then bind and activate a host of different molecules in the cytoplasm that convey a cascade of signals into the depths of the cell to get it ready to divide [*6*]. Even though these down-stream signals are conveyed by many different molecules interacting in a complex network, the signals are transmitted remarkably quickly, mostly within a fraction of a minute (Reddy et al., 2016). Anti-cancer drugs such as gefitinib (also known as Iressa) inhibit the tyrosine kinase activities, so that the phosphorylation process is blocked [*7*]. Also depicted are the actions of three monoclonal antibodies that were later developed as promising anti-cancer drugs [*8*], [*9*], and [*10*]. The elegant beauty of how receptor tyrosine kinases work their magic is described also, and in livelier fashion, in Textbox1.

Textbox 1

A Tale of Two ErbB's.

ErbB1 (another name for EGFR) is floating within the membrane at the surface of a cell. He is hoping for a signal that he could send to his people down in the innards of cell to tell them it's ok to get the cell ready to divide. He has a head that sticks out from the cell surface, a thin neck that passes through the membrane and connects to a big body he has inside the cell. The signal he is waiting for is brought by a small molecule called EGF that floats around outside the cell. Various tissue cells make EGF, but only in small amounts, so that cells having EGFR-family receptors on their surface will divide only occasionally. If he is lucky enough to catch one of these rare EGF's, he binds it tightly [*1*] (the numbers in brackets refer to reaction steps in Figure 17.6). This causes his head to change shape, which makes him ready for an encounter with one of his potential mates, such as ErbB2/HER2. Now ErbB2 is very much like ErbB1, except that her head is smaller, which actually makes her smarter, because now she doesn't have to bother with any signal

molecule from the outside. She just looks for an ErbB1 that already has an EGF stuck to his head and therefore is ready to mate without further ado [*2, 3*]. The rest of the story is pretty dull. The two of them phosphorylate each other [*4, 5*] and create lots of children in the form of information that they spill into the depths of cell [*6*]. How that information is processed inside the cell is rather complicated, and only those who absolutely need those details bother learning them (Roskoski, 2016).

How do receptor tyrosine kinases (RTKs) stimulate cells to become cancerous, and could they be targeted for therapy?

To manage the great variety of signals arriving from outside, a cell has many types of receptor tyrosine kinases (RTKs) on its surface, each of which activates molecules that transmit a signal from outside to inside the cell. As of 2016, there were 58 known receptor tyrosine kinases, as well as 32 non-receptor tyrosine kinases, most of which transmitted a signal from the inner side of the cell surface to the nucleus (Roskoski, 2016). Many of those signals stimulated the cell to grow and divide, and several of them were implicated in cancer causation.

For RTKs of the EGFR-family, four processes were identified that direct cells on a path to cancer: (1) mutation of the RTK that causes it to emit strong persistent signals to genes in the cell nucleus, stimulating genes that promote cell growth and division; (2) increase in the number RTK gene copies, thereby producing a strong chorus of such growth and cell division signals; (3) reduced destruction of RTK molecules, thereby allowing the newly synthesized RTKs to accumulate excessively in the cell surface membrane; (4) excessive activity of one the molecules in the pathway that conveys the signals from the RTKs to the cell nucleus. Efforts were made to block each of those steps with drugs or antibodies. Blocking the action at any of those steps often caused cancer cells, not only to stop dividing, but to die. It was as if the cancer cells had become addicted to increased levels of RTK activity. Particular attention was given to the EGFR family of RTKs.

Mutations of the epidermal growth factor (EGFR) gene.

If EGFR emits growth-promoting signals without control, it pushes cells to divide excessively. Such "over-expression" of EGFR was found to be a major factor promoting some cancers to develop and progress. One of the reasons for the excessive signaling, particularly in lung cancers, was found to be a mutation in the EGFR gene, for example a mutation that causes amino acid number 790 in the EGFR protein to be changed from threonine to methionine. This T790M mutation caused EGFR to send growth-promoting signals into the cells, even when there was no signal from the outside, e.g., even in the absence of binding of a growth factor, such as EGF.

Many different mutations were mapped in the *EGFR* gene in different cancers (Figure 17.8) (Sigismund et al., 2018). Several of those mutations made EGFR active even without binding a growth factor and therefore made the mutated EGFR an oncogene: an uncontrolled gene that continually sends growth signals to the cell nucleus pushing the cell to divide excessively.

Would inhibiting EGFR's tyrosine kinase stop the excessive cell division in cancers? That thought stimulated a search for drugs that inhibit EGFR's tyrosine kinase.

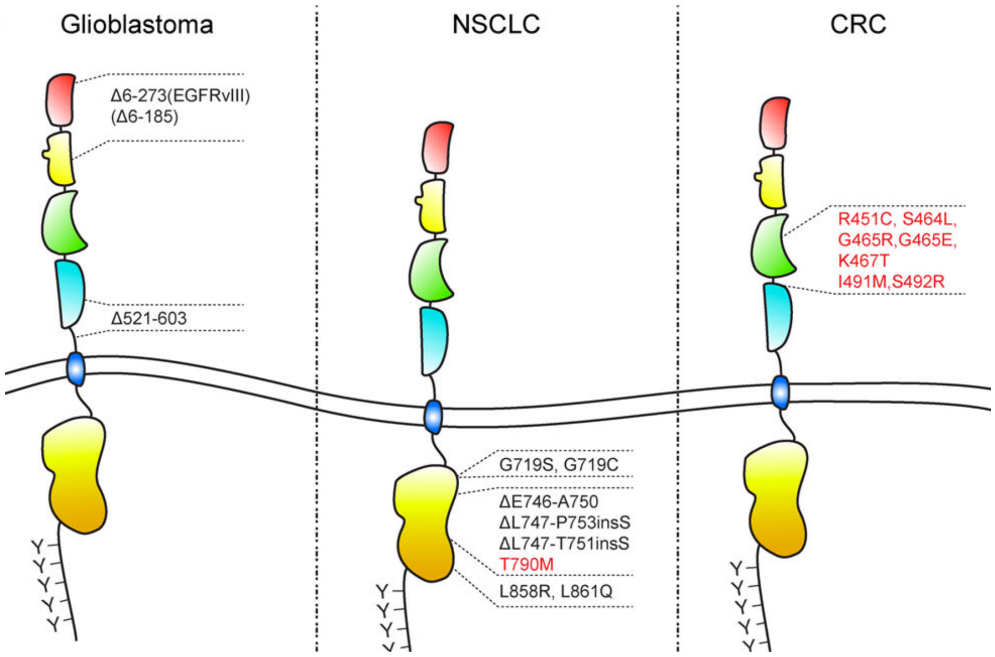

Figure 17.8. Common sites of mutation of EGFR gene in cancers of brain (glioblastoma), lung (NSCLC), and colon (CRC). (Δ6-273 means deletion of amino acids 6 through 273; G719S means mutation that replaces glycine at position 719 with serine, using single-letter amino acid symbols: R=arginine, E=glutamic acid, Q=glutamine, Y=tyrosine.) Mutations indicated in red made the mutated EGFR resistant to inhibitor drugs (Sigismund et al., 2018). (Why is it that different cancers are associated with different mutation sites?)

Finding inhibitors of EGFR's tyrosine kinase.

The focus on EGFR began in 1985 when Towia Libermann, Joseph Schlessinger and their coworker reported that the brain tumors of some patients had too many copies of the EGFR gene in their cancer cells (Libermann et al., 1985). Such amplification of the EGFR genes was found in about 40% of malignant brain tumors. The search for EGFR tyrosine kinase inhibitors then began in 1987 with the work of Gazit, Yaish, and Levitsky (Gazit et al., 1991; Gazit et al., 1989). The first inhibitors they found however were not specific for EGFR but inhibited the tyrosine kinase of many RTK types.

By 2006, it was clear that mutations, as well as amplifications, of the EGFR gene occurred in malignant brain tumors; investigators at Dana Farber Institute and Harvard Medical School found that 18 of 132 patients had missense mutations in their tumor's EGFR protein (missense mutations cause an improper amino acid to replace the normal one in the protein) (Lee et al., 2006). The mutations discovered at that time were in the part of the gene that codes for the extracellular domain of the EGFR protein. Cultured brain tumor cell lines bearing such mutations had increased growth capabilities and their growth was later found to be retarded by EGFR-inhibiting drugs. This strengthened researchers' conviction that such drugs could be clinically useful.

The search for inhibitors of EGFR was driven, first of all, by evidence that cancers that expressed high levels of EGFR's tyrosine kinase tended to grow faster and metastasize more frequently. Such cancers thus were more malignant and patient survival was poor. Secondly, most critical normal tissues had no obvious need for EGFR, suggesting that EGFR-inhibitors would not cause major toxicity. Moreover, methods were available to determine the degree of expression of EGFR in the cancer of a particular patient. Finally, EGFR inhibitors were expected to be especially effective in cancers that produced both EGF (the ligand) and EGFR (the receptor for the ligand); the stimulator (EGF) and the effector upon which the stimulator acts (EGFR) would then be produced by the very same tumor: the EGF would be produced in the very vicinity where the EGFR receptors are located on the tumor cells (Arteaga, 2003). That circumstance is known as "autocrine" if the same cell produces both ligand and receptor. Or "paracrine" if ligand and receptor are produced by different cells in the same tumor. In either case, the tumor would self-stimulate and could grow without control.

It turned out, however, that most *EGFR* mutations in human cancers inactivated the tyrosine kinase – so, using a tyrosine kinase inhibitor was pointless and useless in those cases. Most of the cancers, however, did not have *EGFR* mutations, but rather had an *amplified EGFR* gene, and they did respond to the tyrosine kinase inhibitors (Tsao et al., 2005).

Regardless of whether the cause was mutation or gene amplification, excessive growth signals would be sent to the cancer cell nucleus. The path to the nucleus went by way of signaling molecules that could bear mutations causing effects similar to EGFR family mutations per se. Drugs that specifically inhibited this signaling pathway could cause the cancer cells to die, because those cells would have become addicted to the presence of the strong growth signals. An example was a mutation of *BRAF*, which was one of molecules in the signaling chain from EGFR to the nucleus; the mutation made BRAF abnormally active and uncontrolled: it sent growth-signal barrages to the nucleus, even without stimulation from EGFR. This was the cause of about half of melanoma cases, and these melanomas responded well, albeit only for several months, to BRAF inhibitors. This story is the subject of Chapter 19.

EGFR and its 3 close relatives were the first receptor tyrosine kinases to be studied intensively (Carpenter, 1987; Yarden and Ullrich, 1988). At that time, over-expression of EGFR was already suspected to be a driver of the malignant cell division process in cancer.

The EGFR family of receptor tyrosine kinases were also among the first for which inhibitor drugs were developed and approved by the U.S. Food and Drug Administration for cancer treatment. After imatinib/Gleevec (an inhibitor of the non-receptor tyrosine kinase BCR-ABL approved in 2001, see Chapter 14), the next to be approved, in 2003, was gefitinib/Iressa (Figure 17.9). Over the next 13 years, more than 20 tyrosine kinase inhibitors were approved, burgeoning an extremely active area of anticancer drug development (Roskoski, 2016).

Thus: EGFR (Epidermal Growth Factor Receptor) and their family members are receptor tyrosine kinases: they are located in the surface membrane with an extracellular part that binds (is a receptor for) small regulatory molecules, such as epidermal growth factor (EGF); they have an intracellular part that has tyrosine kinase activity that stimulates certain proteins to signal cell division. HER2 is similar to EGFR, except that it lacks an extracellular growth factor receptor (Figure 17.6 and Textbox 1).

HER2 and breast cancer.

The importance of the *HER2* gene in breast cancer became apparent when it was found that patients whose cancer cells had an excess number of *HER2* genes had a relatively poor prognosis (Slamon et al., 1987). The *HER2* gene was found to be amplified in some cases of breast, ovarian, and occasionally other types of cancer. A search therefore began for specific inhibitors of the HER2 protein's function. Two kinds of HER2-inhibitors were developed: monoclonal antibodies that bound to the extracellular part of the HER2 molecule and drugs that bound and blocked HER2's tyrosine kinase activity in the intracellular part of the molecule. The cancers were, not only driven by abnormally high expression of the *HER2* gene, but became dependent on (addicted to) that high degree of expression, such that inhibition of the HER2 protein caused the cancer cells to die.

How inhibitors block EGFR.

Crystallographic analysis of EGFR's protein structure showed how these drugs bound in a cleft at the active site of EGFR's tyrosine kinase domain, as shown in Figure 17.10. The tyrosine kinase reaction was found to take place within this cleft where ATP had to be present to contribute the phosphate group that the enzyme pushed onto the tyrosines of the EGFR protein. A drug molecule that entered and bound in the cleft would prevent ATP from entering, which is how those drugs blocked EGFR's function.

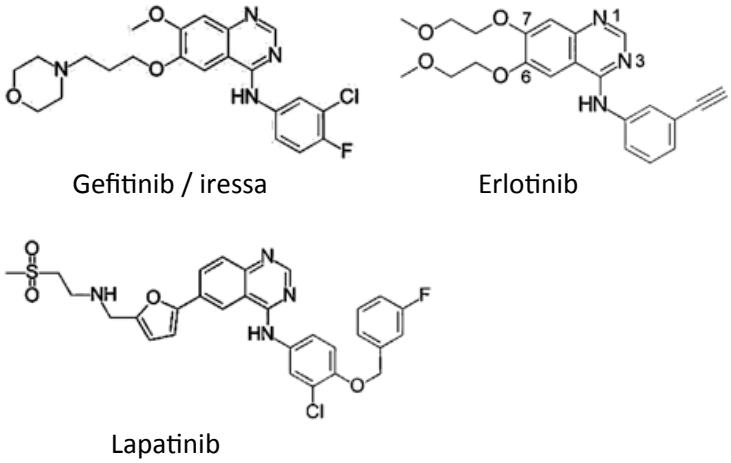

Figure 17.9. Three inhibitors EGFR's tyrosine kinase approved for cancer treatment by the U.S. Food and Drug Administration (FDA). Gefitinib, approved in 2003 for treatment of lung cancer, inhibited platelet-derived growth factor (PDGF) as well as EGFR. Erlotinib, approved in 2004 for treatment of lung and pancreas cancers, was a more specific inhibitor of EGFR; Lapatinib, approved in 2007 for treatment of breast cancer, inhibited both EGFR and HER2 (Roskoski, 2016).

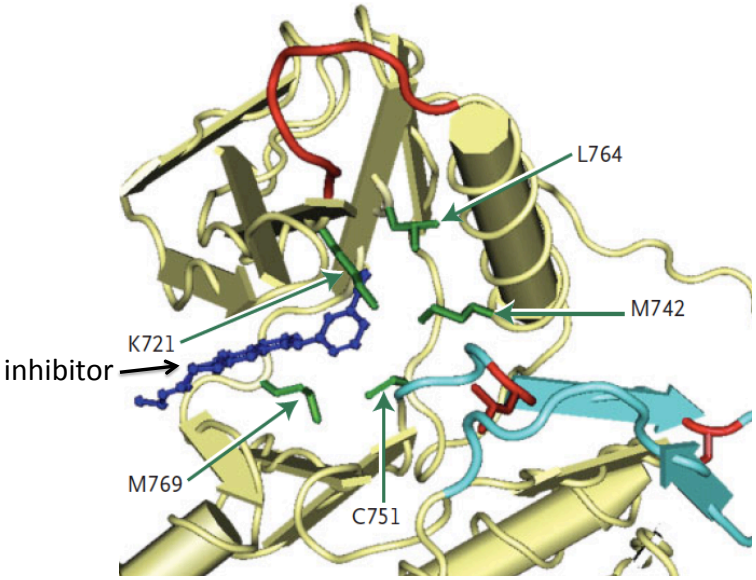

Figure 17.10. The active site of epidermal growth factor receptors, where phosphorylation reactions take place. Inhibitors, such as gefitinib bind in the cleft where ATP normally binds. The ATP molecule contributes the phosphate group used in the phosphorylation reactions. Some of the amino acids involved in catalyzing the phosphorylation reaction are labeled (Lynch et al., 2004). The cysteine (C) and the two methionines (M) each contribute a sulfur atom to the reaction, and the lysine (K) contributes a positive charge that stabilizes the ATP or inhibitor in the cleft.

How EGFR inhibitors kill cancer cells.

After the discovery of the first of those drugs, it seemed that the excessively dividing cells driven by an overactive EGFR oncogene often became addicted and dependent on the oncogene's tyrosine kinase overactivity. Indeed, the tyrosine kinase inhibitor drugs caused the addicted cells to suicide ("apoptosis"). It was as if the cells could not tolerate interference with their addiction.

Thus, tyrosine kinase inhibitor drugs became useful treatment for cancers that were driven by an overactive gene of the EGFR family. The response to a given inhibitor however depended on the type of mutation in the particular cancer it was intended to treat; some mutations even made cells resistant to the drugs (shown in red in Figure 17.8). It turned out, however, that overactive EGFR was not very often due to *EGFR* mutation and was more often caused by an abnormally high number (amplification) of normal *EGFR* genes, and that those were the cancers that responded to the tyrosine kinase inhibitors.

Amplified EGFR genes and response to EGFR inhibitors.

Increased gene copy number (gene amplification) had been observed in cancers that acquired *resistance* to antifolate drugs due to amplification the dihydrofolate reductase gene (Chapter 5). In the same vein, some cancers had amplified *EGFR* genes that were *drivers* of the malignancies, and those cancers were found to respond to EGFR inhibitors.

Early studies revealed that malignant brain tumors (glioblastomas) often had an amplified EGFR gene as the probable cause of unusually high levels of EGFR protein that seemed to drive that cancer. The first report came in 1985 from Libermann and coworkers, who observed that 3 out of the 12 glioblastomas that they studied had more than 20 copies of the *EGFR* gene in their DNA and an increased amount of the EGFR protein in their cancer's cells (Libermann et al., 1985).

Then, in 1996, Sauter and coworkers used a more precise technique to gauge gene amplification, which enabled them to count the number of *EGFR* genes per chromosome in a cell. They found *EGFR* amplified in about 40% of glioblastomas, although the number of amplified genes varied among the cells of a given tumor (Sauter et al., 1996). As we now understand it, only the EGFR-overexpressing cells would be EGFR-dependent and killed by an EGFR inhibitor. Some cells in the same tumor may have little or no EGFR-amplification, and those cells would survive and regrow the tumor. The newly grown tumor would then be resistant to the drug. That may be an important reason for the usually brief response of most cancers to EGFR inhibitors -- which gave impetus to studies to figuring out how to get around this difficulty.

EGFR inhibitors and treatment of lung cancer.

Lung cancers were a notoriously difficult problem. Cisplatin and docetaxel improved survival a little, but after that there was no further therapy available – until, in 2003, early clinical trials tested tyrosine kinase inhibitors in lung cancer patients whose previous chemotherapy had failed. Both gefitinib and erlotinib seemed to improve the survival of these patients. In 2005, a larger randomized double-blind study was reported of advanced lung cancer patients whose previous chemotherapy had failed (Shepherd et al., 2005). The study showed that erlotinib improved the survival of some of the patients. However, the clearest measure of response was the length of time that the treatment held the cancer in check, before it resumed growing (Figure 17.11). The graph indicated that about half of the patients did not respond to erlotinib. But the remaining half had a clear response: erlotinib extended the length of time before those cancers progressed. It may be that the responding patients were those whose cancers had an amplified *EGFR* gene -- but that possibility was not tested in this study. That idea – that only lung cancers that had amplified *EGFR* genes responded to erlotinib – was supported by further studies from the laboratory of Frances A. Shepherd at the University of Toronto, Canada (Figures 17.12 and 17.13) (Tsao et al., 2005).

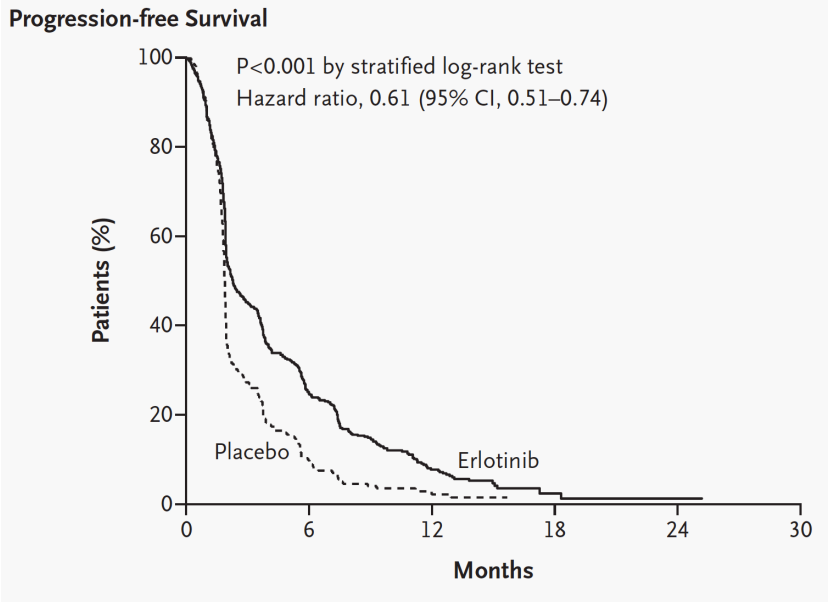

Figure 17.11. Progression-free survival of advanced lung cancer patients who were randomized as to whether or not they received erlotinib treatment. The patients had received previous chemotherapy, which was not (or no longer was) effective. The graph shows that some of these patients responded to erlotinib: the drug increased the length of time before their tumors progressed (Shepherd et al., 2005). The upper part of the graph shows that about half the patients did not respond at all, while the lower part of the graph shows that the remaining half of the patients had a clear response.

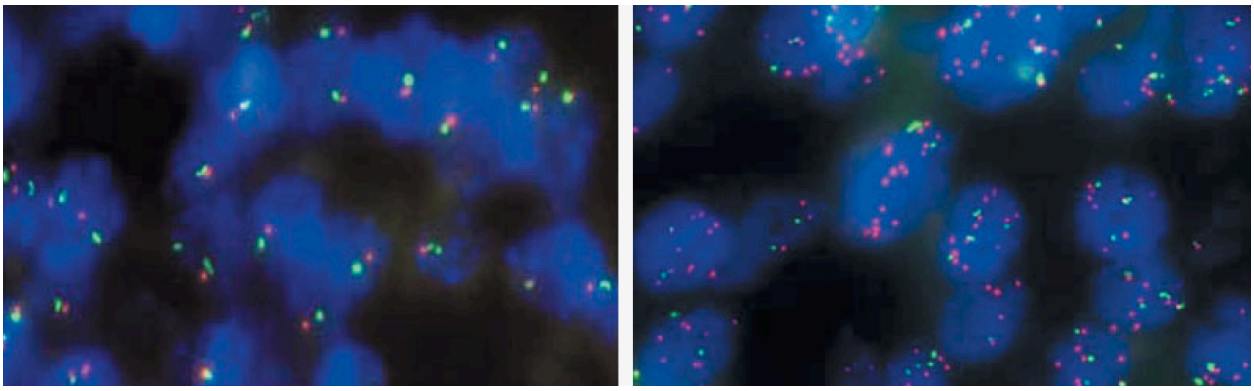

Figure 17.12. Increased number of copies (amplification) of the *EGFR* gene in cells of a lung cancer (*right*), compared with a lung cancer that lacked the gene amplification (*left*). This experiment used a double-staining method: a red spot shows where a DNA sequence of the *EGFR* gene is located, a green spot shows the centromere of chromosome 7, which is the chromosome in which *EGFR* is located. The cells of the cancer without the *EGFR* amplification show a single *EGFR* gene near the centromere of its host chromosome (*left*), while the cancer with the gene amplification shows an excessive number of *EGFR* genes (*right*). Some of cells had many *EGFR* copies (red spots) while some had relatively few, indicating that the number of copies varied among the cells of the same tumor (Tsao et al., 2005).

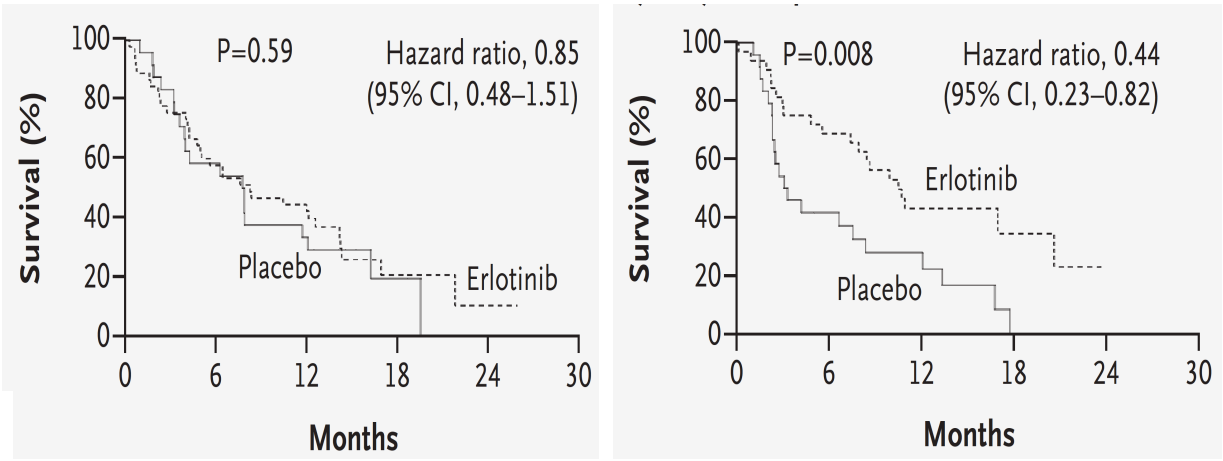

Figure 17.13. An early clinical trial, reported in 2005, showed that the tyrosine kinase inhibitor, erlotinib, increased the survival of lung cancer patients whose cancers had amplified *EGFR* genes (*right*), but had no effect on the survival of the patients whose cancers lacked the amplification (*left*) (Tsao et al., 2005).

How HER2 genes were discovered to drive the malignancy of many cancers.

It had already been found out by 1995 that the cancer cells of advanced breast cancer patients often overexpressed either EGFR or of its relative, HER2, and that those patients had a relatively poor prognosis (Earp et al., 1995). Then by 2002, when the molecular structure of EGFR's tyrosine kinase domain had been determined, it became possible to design drug molecules that would bind to a pocket in the protein structure where the enzyme action occurs and thereby inhibit it (Figure 17.10). As already mentioned, the first inhibitors to be developed were designed to bind in a pocket in the tyrosine kinase domain of both EGFR and HER where ATP normally binds and where the enzyme action takes place (Figure 17.10). The first such inhibitor to become a useful anticancer drug was gefitinib (Iressa) (Figure 17.9) (Wakeling et al., 2002).

Here is how the discovery of HER2 came about: In 1984, Alan Schechter, Robert Weinberg and their colleagues discovered an oncogene in a rat brain cancer called neuroglioblastoma. Because of where it was found, they called the new oncogene *neu* (Schechter et al., 1985; Schechter et al., 1984). (How they discovered oncogenes was related in Chapter 15.) They observed that DNA sequences of their *neu* gene were similar to those found in the previously discovered *erbB* oncogene of avian erythroblastosis virus. The *neu* gene therefore acquired the name *erbB2* – because the original *erbB* gene had been found to be similar to *EGFR*, which then became *erbB1*. The two genes, *EGFR/ErbB1* and *neu/ErbB2/HER2* were found to be closely related and they became the first of the 4 members of the EGFR family. The HER designation was for the human versions of those epidermal growth factor receptors. Despite this confusion of names, the prominence of the *HER2* gene in human cancer perhaps merits its ownership of 3 names: *neu, erbB2, and HER2*.

Then in 1987, Dennis Slamon and his collaborators at the UCLA and University of Texas Medical Center reported that patients with breast cancers that had an abnormally high number of *HER2* genes often had a relatively short survival time (Slamon et al., 1987) (Figure 17.14). They found that approximately 25% of breast cancers had an amplified number of *HER2* genes (Slamon et al., 2001). These cancers appeared to be driven by the increased number of *HER2* genes in the cell. But the HER2-positive breast cancers did respond (albeit only transiently) to inhibitors of the tyrosine kinase function of HER2.

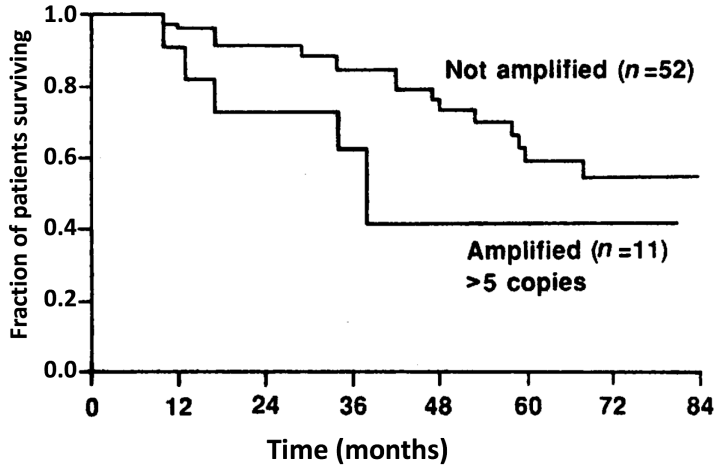

Figure 17.14. Amplification of the HER2 gene (more than 5 copies of the gene per cancer cell) impaired the survival of breast cancer patients (among patients who had positive lymph nodes for cancer) (Slamon et al., 1987). This was among the first findings to show the importance of the *HER2* gene in breast cancer.

Antibodies that inhibit EGFR and/or HER2.

Instead of targeting the tyrosine kinase activity of EGFR or HER2, another idea was to create antibodies that would bind to the extracellular domains of those receptor tyrosine kinases (RTKs). The idea was that the antibodies would block the binding of growth factors, such as EGF, to the extracellular domains or prevent the extracellular domains of two RTKs from coming together to form an active dimer (dimer formation and its consequences are diagrammed in Figures 17.6 and 17.7). Either of those inhibitions would prevent signaling to the nucleus to initiate DNA replication and cell division. Antibodies cannot penetrate directly through the cell surface membrane, but they could bind and inhibit the extracellular domains without entering the cell.

In the early 1990s, John Mendelsohn and his coworkers at the Memorial Sloan-Kettering Cancer Center in New York developed monoclonal antibodies targeted to EGFR's extracellular domain. One of their antibodies (MAb528) inhibited the binding of growth factor EGF to the extracellular domain of EGFR and inhibited the growth of a human cancer in immune-deficient mice (Figure 17.15) (Baselga et al., 1993). They grew the tumor from cells of a human cancer cell line, which they injected into immune-deficient mice (that did not reject the cells of another species). When the tumor-bearing mice were treated with both MAb528 and doxorubicin, the growth of the tumor was completely inhibited, whereas each of those treatments by themselves only slowed the tumor growth to some degree (Figure 17.15) (Baselga et al., 1993).

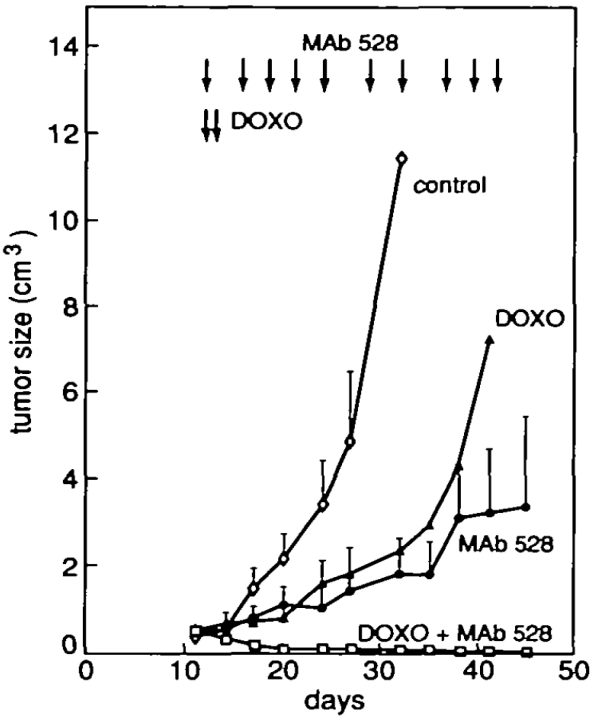

Figure 17.15. One of the first monoclonal antibodies (MAb528) that targeted EGFR's extracellular domain was shown in this experiment to inhibit the growth of a cancer. Combining the antibody with doxorubicin (DOX) completely inhibited the growth of a human tumor in immune-deficient mice. MAb528 bound the extracellular domain of EGFR and blocked the binding of growth factor EGF, thereby inhibiting the activation of EGFR's tyrosine kinase domain. MAb528 and doxorubicin each slowed the growth of the tumor compared with the growth of the tumor in untreated control mice. The combination of both treatments, however, completely inhibited the growth of the tumor. The arrows at the top show when the treatments were administered (Baselga et al., 1993).

In 1989, researchers at Genentech Inc in San Francisco, California had reported the effects of several monoclonal antibodies directed against the extracellular domain of HER2. They found that one of their antibodies, which they called 4D5, had the hoped-for specific action against HER2-overexpressing breast cancer cells. After confirming their antibody's ability to bind HER2, they found that the antibody, unlike other antibodies they had prepared, did in fact inhibit the growth of a HER2-overexpressing human breast cancer cell line (adenocarcinoma SK-BR-3), but had little or no effect on several other cell lines (Hudziak et al., 1989).

Their 4D5 antibody, however had a drawback: it was a mouse protein, which would likely be rejected by the human immune system. They therefore engineered a hybrid antibody that retained only the part of the mouse antibody that recognized the HER2 domain and replaced the rest of the mouse antibody with corresponding human antibody parts. The resulting humanized antibody became known as trastuzumab or Herceptin, and became a

useful addition to cancer treatment (Carter et al., 1992). When combined with standard chemotherapy, trastuzumab improved the prognosis of those breast cancer patients whose tumors produced high amounts of HER2 (Figure 17.16 and 17.17) (Moasser, 2007a, b; Romond et al., 2005; Stebbing et al., 2000).

These successes by scientists at Memorial Sloan-Kettering Cancer Center in New York and Genentech Inc in San Francisco opened a new era of cancer treatment using EGFR- and HER2-directed antibodies.

Genentech then developed another HER2-directed antibody called 2C4, which prevented the dimer formation between EGFR and HER2 (Franklin et al., 2004). They engineered a humanized version of the antibody which became known as pertuzumab (Adams et al., 2006). Pertuzumab differed from trastuzumab by targeting subdomain II rather than subdomain IV of the extracellular domains of EGFR and HER2 (Figures 17.18 and 17.7). Subdomain II is the part of EGFR and HER2 that allows dimer formation between them, which has to happen before they can phosphorylate each other and send signals downstream; pertuzumab prevents that from happening (Figure 17.18).

Did pertuzumab have a role in the treatment of cancer? In a phase II clinical trial, pertuzumab gave promising results in the treatment of advanced ovarian cancer. Ovarian cancer frequently had active, albeit not overexpressed, HER2. Although there were no complete remissions in these patients, who had previously been extensively treated with chemotherapy, there were several partial remission or stable disease where the cancer was held in check for several months (Gordon et al., 2006).

Although the early tests of pertuzumab failed to confer enthusiasm among clinicians, preclinical studies indicated that pertuzumab increased the effectiveness of trastuzumab, when the two antibodies were used together. The two antibodies differed in where on HER2's extracellular domain they bound and the manner in which they blocked HER2's function (Figure 17.18). The effect of the two antibodies used together was substantially greater than the effect of each of them by itself: they were synergistic.

The synergism was first demonstrated in cultures of HER2-overexpressing breast cancer cells, conducted by researchers at M.D. Anderson Cancer Center in Houston, Texas. They showed that the two antibodies acted synergistically to kill the cancer cells (Nahta et al., 2004) (Figure 17.9).

A large randomized clinical trial of previously untreated metastatic HER2-overexpressing breast cancer patients was reported in January 2012 by a large international group of clinical investigators led by Jose Baselga of Massachusetts General Hospital and Harvard Medical School (Baselga et al., 2012). They randomly assigned patients to be treated with trastuzumab plus docetaxel with or without the addition of pertuzumab. The results showed definitively that the addition of pertuzumab improved progression-free survival of the patients (Figure 17.19). The trastuzumab-docetaxel-pertuzumab triplet extended the time by a median of six months before the cancer progressed. Although that was far from a cure, it indicated a possible path toward that goal.

By 2012, several clinical trials indicated that pertuzumab by itself did little to benefit patients with HER2-positive metastatic breast cancer. However, when combined with trastuzumab plus docetaxel, the three-drug combination gave favorable responses. That same year, the FDA approved the three-drug combination for the treatment of metastatic breast cancer (Hubalek et al., 2012; Keating, 2012; Traynor, 2012).

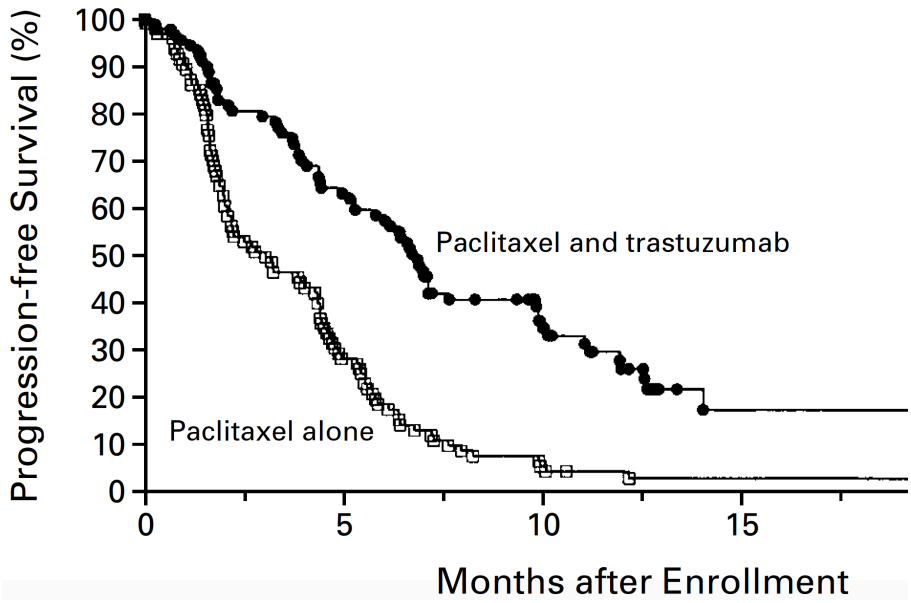

Figure 17.16. Women with *HER2*-amplified breast cancer that had already metastasized were helped by adding trastuzumab (Herceptin) to their chemotherapy. In this randomized clinical trial, adding trastuzumab to paclitaxel lengthened the time that the cancer remained dormant and did not progress, compared with treatment with paclitaxel alone (Slamon et al., 2001). Other studies showed that trastuzumab also increased the progression-free time when added to other chemotherapy drugs.

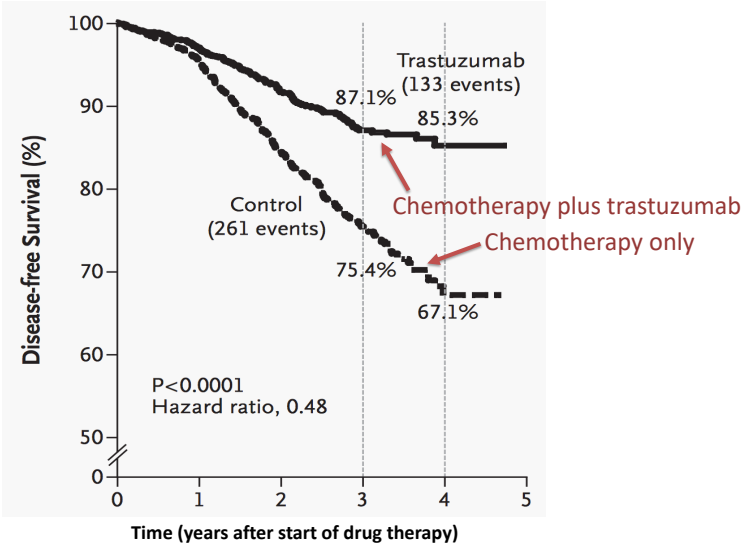

Figure 17.17. Trastuzumab (Herceptin) increased the length of time before the cancer progressed in breast cancer patients whose cancer had had amplified *HER2* genes and no evidence of distant metastases. All patients had surgery to remove their primary tumors followed by combination chemotherapy with or without trastuzumab; the control group received chemotherapy only (Romond et al., 2005).

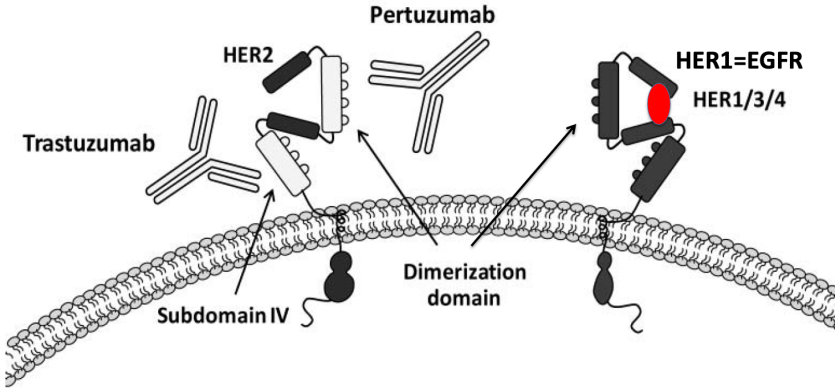

Figure 17.18. The antibodies trastuzumab and pertuzumab bind to different subdomains of HER2 (structure on the left; Figure 17.7 shows the subdomain structure.) (Harbeck et al., 2013). Trastuzumab binds to subdomain IV and thereby inhibits HER2's ability to emit growth signals. Pertuzumab also inhibits HER2 from emitting signals but does so by binding to subdomain II and thereby blocking HER2 dimerization and ability to bind growth factors. Trastuzumab and pertuzumab thus act in complementary fashion to block HER2 function. EGFR (HER1) must bind a growth factor, such as EGF (red oval) to achieve the correct conformation of the subdomains to permit dimer formation by way of subdomain II. However, HER2 already has the correct subdomain II conformation for dimer formation and does not have to bind growth factor.

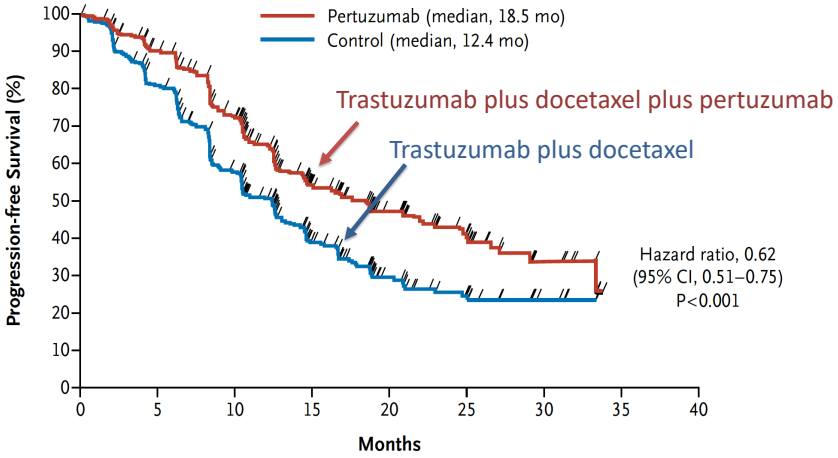

Figure 17.19. Trastuzumab plus docetaxel had previously been shown to extend the progression-free survival of patients who had metastatic HER2-overexpressing breast cancers. This random clinical trial showed that adding pertuzumab to the therapy further extended the median progression-free survival of the patients for an additional 6 months (Baselga et al., 2012).

How do cancer cells become resistant to those antibodies?

Cancer cells were remarkably capable of becoming resistant, not only to all kinds of chemotherapy but to antibody agents as well. Although trastuzumab and pertuzumab were known to bind to HER2, the effects of that binding on the molecular and physiologic events in the cell remained enigmatic. The more researchers investigated, the more consequences of the antibody treatments they discovered. Of the multitude of effects that were discovered, it remained uncertain which of them were relevant to the resistance. Our state of knowledge about that in 2018 was summarized by (Derakhshani et al., 2020).

Yet another therapeutic antibody.

A humanized monoclonal antibody, cetuximab, was developed that targeted the extracellular domain of *epidermal growth factor* (EGFR) rather than of HER2. It improved the therapy of *colon* cancer and was approved in 2004 for treatment of EGFR-expressing colon cancer. Like trastuzumab and pertuzumab, cetuximab was made up of a combination of human and murine parts; only the antigen-recognition parts were non-human (Figure 17.20) (Brand et al., 2011). Since most of the molecule was human, it was hoped that the human immune system would not react against it.

Among several early clinical trials of cetuximab, one that is notable was a collaboration of Canadian and Australian clinical research groups in 2007 that reported that, among

patients with advanced colon cancer whose cancers expressed EGFR, about half responded to cetuximab (even after chemotherapy had failed) (Jonker et al., 2007).

Particularly interesting was the effect of cetuximab on the time until progression of the tumor (Figure 17.21). Its response curve was remarkably similar to that of the earlier study by (Shepherd et al., 2005) of the response of lung cancer patients to erlotinib (Figure 17.11). Even though the two studies differed in the type of cancer studied (lung versus colon cancer) and in the type of EGFR-targeted drug used (cetuximab versus erlotinib), the progression-free survival curves were amazingly similar. Both curves suggest that only about half the patients responded, as if there were two distinct groups of patients in each of the two studies. The patients whose cancers responded may have been those whose cancers had amplified *EGFR* genes, but that possibility was not investigated in these studies.

A caveat that should be mentioned here is that targeting EGFR with either tyrosine kinase inhibitor or antibody was ineffective if the patient's cancer had an activating mutation in KRAS (the most important member of the RAS family). That is because KRAS is downstream from EGFR: EGFR activates KRAS -- but, if KRAS was already activated by a mutation, it did no good to inhibit EGFR, because KRAS would drive the malignancy regardless (Li et al., 2015). KRAS is a topic in the next chapter.

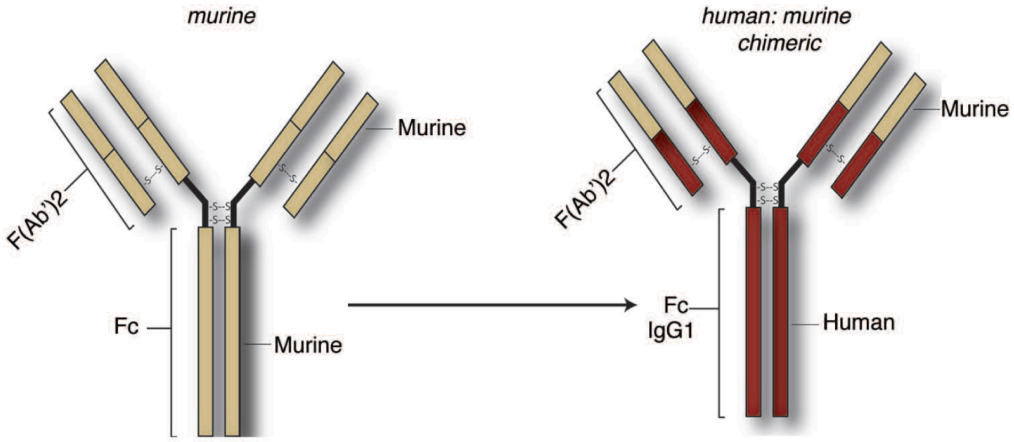

Figure 17.20. Development of the EGFR-targeted antibody, cetuximab. First, several antibodies against EGFR were generated in mice (left), and the most promising one of them was tested, but ran into the problem that patient's immune system reacted against this mouse protein. Most of the mouse protein was therefore replaced by means of genetic engineering with the corresponding human sections (right, red parts). The only murine part that was retained was the part that recognized the EGFR (Brand et al., 2011).

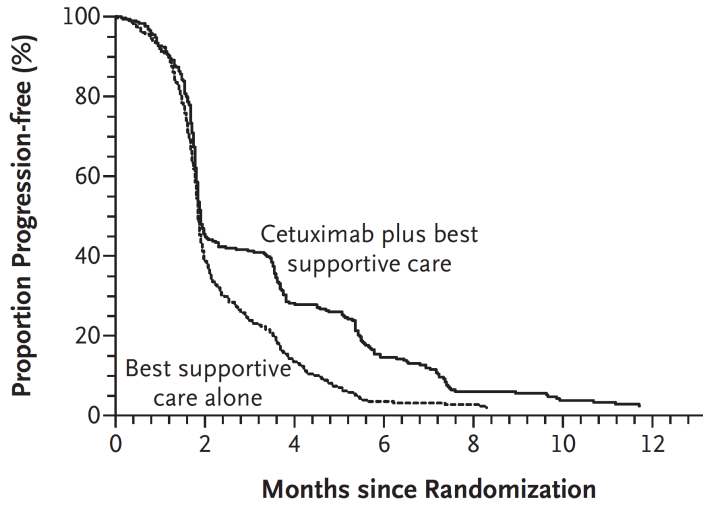

Figure 17.21. Response of patients with advanced colon cancer to cetuximab. The patients selected for this study had cancers that failed to respond or no longer responded to chemotherapy and whose cancers expressed EGFR (Jonker et al., 2007). The curves indicate that about 40% of the patients responded to cetuximab, whereas 60%, whose disease progressed within 2 months, did not respond at all.

Recycling and destruction of EGFR proteins.

Although antibodies cannot penetrate through the cell surface membrane, they can be taken up into the cytoplasm when bound to a receptor, such as EGFR or HER2, by a process known as endocytosis: the cell surface membrane together with attached EGFR-bound antibody is engulfed into vesicles. The antibodies are then located within the vesicles in the cytoplasm and are subject to processes that recycle EGFR (Figure 17.22).

As EGFR molecules are made and sent to the surface membrane, they couldn't accumulate there indefinitely. A solution to this problem was found in 1998 by Yosef Yarden and his colleagues (Lenferink et al., 1998; Levkowitz et al., 1998; Waterman et al., 1999) at the Weizmann Institute in Israel. They found that EGFR molecules are sucked back from the cell surface membrane within vesicles that move into the cytoplasm. How that happens and what then happens to EGFR in the vesicles is pictured in Figure 17.22 as conceptualized by Sigismund et al (Sigismund et al., 2018). What ensues is a kind of choreography of vesicles, which can move and merge in various ways. The EGFR molecule then has one of two possible fates. It can be destroyed in special vesicles (lysosomes) that contain digestive enzymes. Or it can be recycled as the vesicle in which it resides merges with the surface membrane. All that is pictured in Figure 17.22.

The balance between the two pathways that lead to these alternative fates affects how many EGFR molecules would be on the surface at any one time, thereby affecting the strength of the EGFR signaling. It seemed that control of the balance between those pathways would be one way the cell could regulate EGFR activity. It would also affect the amount EGFR-bound antibody displayed on the cell surface.

A beautiful example of control of how much EGFR is displayed on the cell surface was the effect of growth factor EGF. When there was little or no EGF available, the amount of EGFR on the cell surface accumulated and made the cell sensitive to detecting the rare EGF molecules. If EGF became abundant, there was the danger of EGFR overactivity. The cell could solve this dilemma by simply sucking EGF-bound EGFR out of the surface membrane 10-times more quickly than unbound EGFR.

Excessive expression of EGFR would push cells toward becoming cancerous. The most common way that happened was by mutation of EGFR or one of its family members. But another way would be for the EGFR destruction machinery to be defective. In 2001, Waterman and Yarden suggested that it might be possible to develop drugs to inhibit the recycling pathway, thereby perhaps enhancing the destruction pathway or to develop antibodies that, after binding to the EGFR extracellular domain, would enhance the uptake of the EGFR-antibody complex into cytoplasmic vesicles headed for destruction (Waterman and Yarden, 2001). Cancer cells that had become addicted to a high level of EGFR might then die for lack of it. They proposed that this might be a way to treat overactive HER2 in aggressive forms of breast, ovary, and lung cancers. Evidence supporting that idea was reported in 2009 by Tsipi Ben-Kasus, Yoseph Yarden, Michael Sela and their colleagues at the Weitzman Institute, Jerusalem. They found that certain monoclonal antibodies increased the rate of removal of HER2 from the surface membrane into endosomes (Ben-Kasus et al., 2009). They accomplished this by means of a pair of monoclonal antibodies that attached to different parts of the HER2 molecule, thereby producing a large complex with a strong tendency to be sucked into endosomes (Marmor and Yarden, 2004; Mellman and Yarden, 2013; Mosesson et al., 2008).

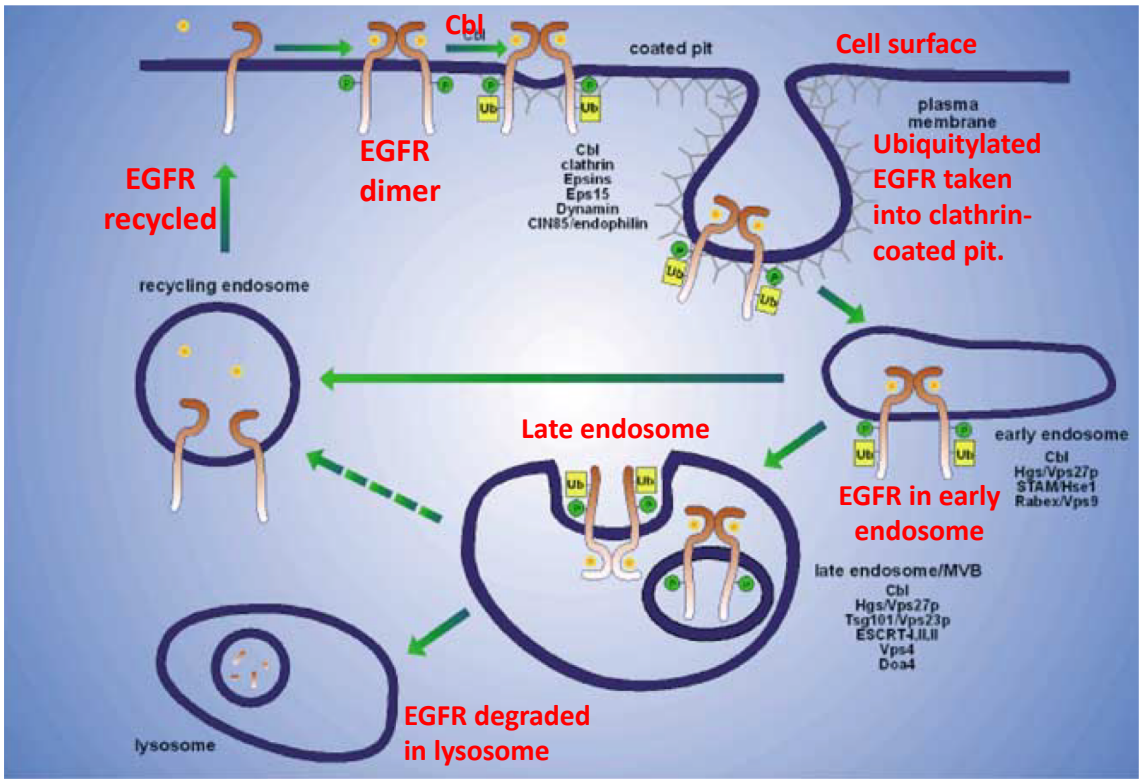

Figure 17.22. EGFR degradation and recycling by way of vesicles (endosomes and lysosomes) in the cytoplasm. The cycle begins with an EGFR homodimer in the cell surface membrane (top, left); in the first step, the enzyme Cbl adds ubiquitin (Ub) molecules to the EGFR. That causes the EGFR to be taken into pits in the membrane, which is brought about by clathrin molecules that coat the cytoplasmic side of the membrane. The pits then close to form vesicles, called endosomes. The EGFR is then transferred, either to lysosomes, where it is degraded, or to other endosomes that recycle the EGFR to the cell surface membrane. From (Marmor and Yarden, 2004) with labels in red added.

Combining an EGFR antibody and a cytotoxic drug in the same molecule.

In 2008, researchers at Genentech Inc. reported their development of an antibody-drug conjugate that takes advantage of the ability of cells to slurp from the cell surface EGFR-bound antibodies. Their new antibody-drug conjugate carried with it into the cytoplasm a toxic drug that killed or disabled the cell. Several antibody-drug conjugates targeted against various types of cancer cells had previously been developed, but Genentech's trastuzumab-emtansine or trastuzumab-DM1, specifically targeted HER2-expressing cancers, including about one quarter of breast cancers. The conjugate consisted of the HER2-targeted antibody, trastuzumab (Herceptin), chemically linked to the cell-killing drug maytansine, which bound and inhibited the function of the cell's microtubules (Chapter 12) (Figure 17.23) (Lewis Phillips et al., 2008).

The idea was that the trastuzumab part of the conjugate would bind exclusively to HER2 on the cell surface and would carry toxic maytansine into the cancer cell, whereupon the cancer cell would die. That scheme was targeted against cancers that had an amplified *HER2* gene, such as HER2-positive breast cancers. Critical normal tissue cells presumably would be spared due to their having few, if any, HER2 molecules on their surface.

In their studies in 2008, the researchers reported evidence that the trastuzumab-emtansine conjugate indeed was effective against human HER2-overexpressing breast cancers grown as xenografts in immune-deficient mice, and moreover that it was more effective than trastuzumab by itself (Figure 17.24) (Lewis Phillips et al., 2008).

But how effective would trastuzumab-emtansine be against HER2-overexpressing breast cancers in patients? In 2012, an international group of researchers randomly assigned 991 patients to treatment with trastuzumab-emtansine or to the standard therapy of a combination of lapatinib plus capecitabine (Verma et al., 2012). Lapatinib inhibited the tyrosine-kinase activities of HER2 and EGFR, and capecitabine was metabolized in the cell to release 5-fluorouracil (Chapter 6). The selected patients were in an advanced stage of their disease and had already been treated with trastuzumab plus a taxane (a microtubule inhibitor; Chapter 12). Thus, the test was whether trastuzumab and a microtubule inhibitor (maytansine) as a conjugate (trastuzumab-emtansine) would be effective after tastuzumab and a microtubule inhibitor given separately had failed. Analysis of the results showed that the patients did indeed respond to the trastuzumab-emtansine conjugate (Figure 17.25). Although the response was significantly better than a standard therapy ($p<0.001$), the addition of only 3 months to the time before the cancer progressed remained a bleak outlook for the patients. The clinical activity of trastuzumab emtansine was limited by the development of resistance. Although several possible causes of resistance were suggested, which of them might be relevant remained uncertain (Hunter et al., 2020) (Garcia-Alonso et al., 2020).

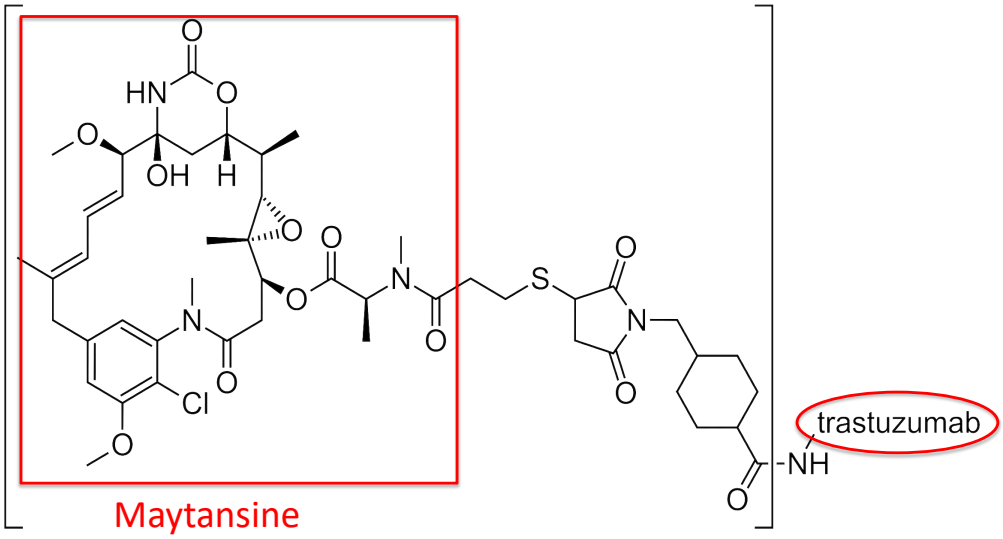

Figure 17.23. Genetec's antibody-toxin conjugate drug, named trastuzumab-emtansine or trastuzumab-DM1 (abbreviated T-DM1) consisted of trastuzumab connected by way of a linker to the microtubule blocker maytansine. The conjugate drug targeted HER2-positive cancers, particularly breast cancers that had amplified *HER2* genes (Lewis Phillips et al., 2008). The trastuzumab (Herceptin) part of the conjugate molecule was an antibody whose structure was similar to the structure shown on the left side of Figure 17.18. It bound to HER2 on the surface of HER2-positive cancer cells, which took up the conjugate drug and delivered it into the cytoplasm. The toxin part of the conjugate, maytansine, then bound and inactivated the cell's microtubules. The linker between the trastuzumab and the maytansine was stable and did not release free maytansine. From (Lewis Phillips et al., 2008) modified in red to show the parts of the molecule.

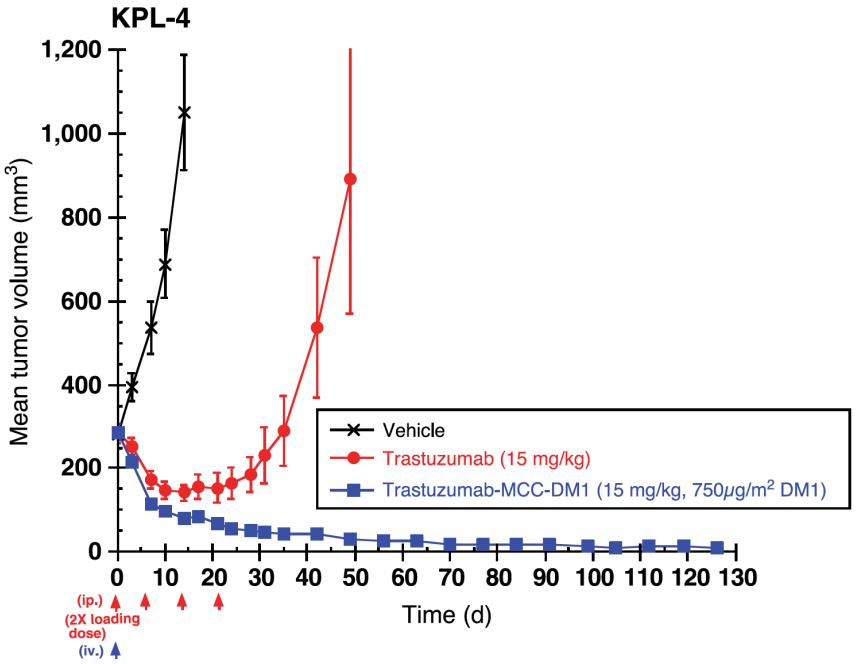

Figure 17.24. The antibody-drug conjugate trastuzumab-DM1 (trastuzumab emtansine) cured human HER2-overexpressing cancers implanted as xenografts in immune deficient mice (blue squares) (Lewis Phillips et al., 2008). Trastuzumab by itself transiently inhibited the tumors, which however recovered and grew exponentially (red circles). In untreated mice, the tumors grew rapidly without delay (black x).

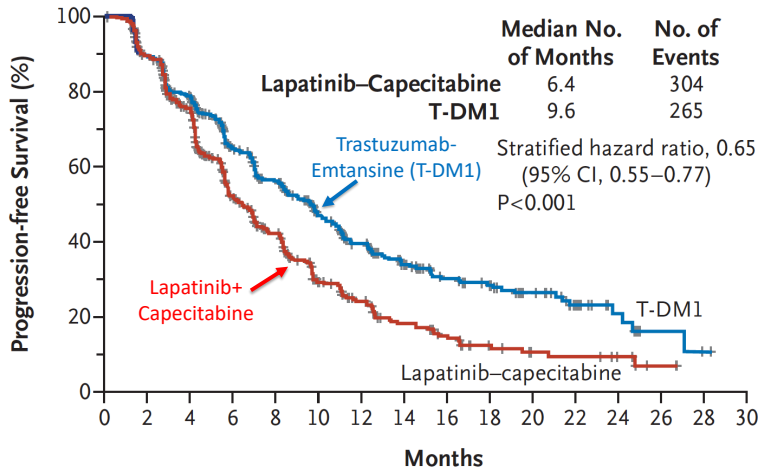

Figure 17.25. The trastuzumab-emtansine (T-DM1) drug conjugate of trastuzumab linked to maytansine was better than standard chemotherapy with lapatinib plus capecitabine in prolonging the time before the cancers progressed (9.6 versus 6.4 months). The patients had advanced HER2-overexpressing breast cancers who did not or no longer responded to trastuzumab and a taxane given separately ((Verma et al., 2012) with colored labels added).

Another type of HER2-targeted antibody-toxin conjugate, trastuzumab-deruxtican, was made in 2016 by researchers in Japan. This conjugate consisted of trastuzumab linked to a camptothecin-related inhibitor of topoisomerase I (Chapter 11). The HER2 humanized antibody (trastuzumab) was linked to a camptothecin-related topoisomerase I inhibitor (deruxtecan) by way of a peptide chain (five amino acids) that would be cleaved by a protease in the cytoplasm to release free deruxtecan (Figure 17.26) (Ogitani et al., 2016a; Ogitani et al., 2016b).

The free deruxtecan has no electric charge and therefore could penetrate the blood-brain barrier. This is important, because patients with advanced HER2-positive breast cancer often had brain metastases. Moreover, the released deruxtecan would perhaps have a relatively high concentration within the bulk of the tumor and would kill also the fraction of cancer cells that lacked high HER2 expression (a so-called bystander effect). That theoretically would confer an advantage, because HER2-positive breast cancers often consisted of cells that expressed HER2 at various levels. Some of the malignant cells had relatively low levels of HER2 expression, and the released deruxtecan could kill these bystander cells.

In 2019, researchers in Japan reported a non-randomized phase I study of trastuzumab-deruxtican in advanced HER2-positive breast cancer patients who had previous treatment with trastuzumab-emtansine. They found significant benefit of trastuzumab-deruxtican in the patients beyond the benefit of the trastuzumab-emtansine treatment, and they concluded that the new drug should go on to phase 2 and phase 3 studies (Tamura et al., 2019). In December 2019, this new antibody-drug conjugate received approval for treatment of HER2-expressing metastatic breast cancers that had failed previous HER2-directed treatments (Keam, 2020).

Deruxtican by itself has interesting potential as a drug. It is similar to the commonly used topoisomerase I blocker topotecan (see Figure 11.11 in Chapter 11), the essential difference being that topotecan has a positive charge, whereas deruxtican is uncharged. Therefore, deruxtican may pass through membranes and enter the brain, whereas topotecan may not be able to do so. Moreover, deruxtican could enter cells more easily. However, as of April 2020, I could not find any reports of deruxtican as a drug in its own right.

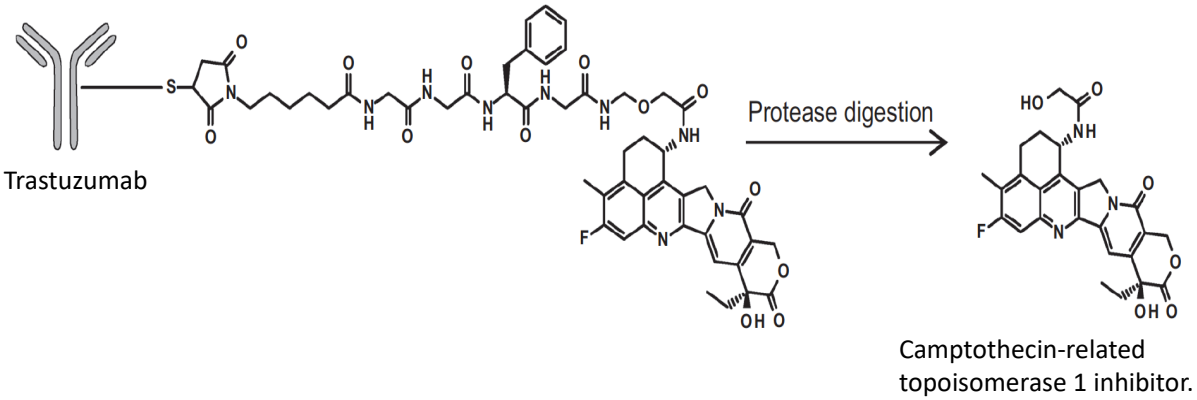

Figure 17.26. A HER2-targeted antibody-toxin conjugate, trastuzumab-deruxtecan, linked trastuzumab to a camptothecin-related topoisomerase I inhibitor (deruxtecan). The linker consisted of a chain of five amino acids that would be digested by enzymes in the cytoplasm to release free deruxtecan (*right*) (Ogitani et al., 2016a).

Targeting EGFR exon 20 insertion mutations in lung cancers.

Non-small cell lung cancer (NSCLC) accounts for 80% to 85% of all lung cancers. Approximately 2% to 3% of those patients have *EGFR* exon 20 insertion mutations that promote rapid cancer cell growth and spread (Figure 17.27). Lung cancer was the leading cause of cancer mortality worldwide. Hence the 2% to 3% still added up to a substantial number of people. These mutations have not responded to current EGFR-targeted drugs, but today (May 21, 2021) the FDA gave accelerated approval to amivantamab, a monoclonal antibody targeted against those EGFR exon 20 mutations. Patients with lung cancers driven by that mutation will now for the first time have targeted therapy available.

The accelerated approval of amivantamab was based on a clinical trial of 81 patients with lung cancers with EGFR exon 20 insertion mutations whose cancers were progressing despite treatment with platinum drugs (Chapter 3). 40% of the patients then responded to amivantamab with a median response duration of 11 months. This was impressive progress, yet less than half the patients responded to the drug and their responses usually lasted less than a year.

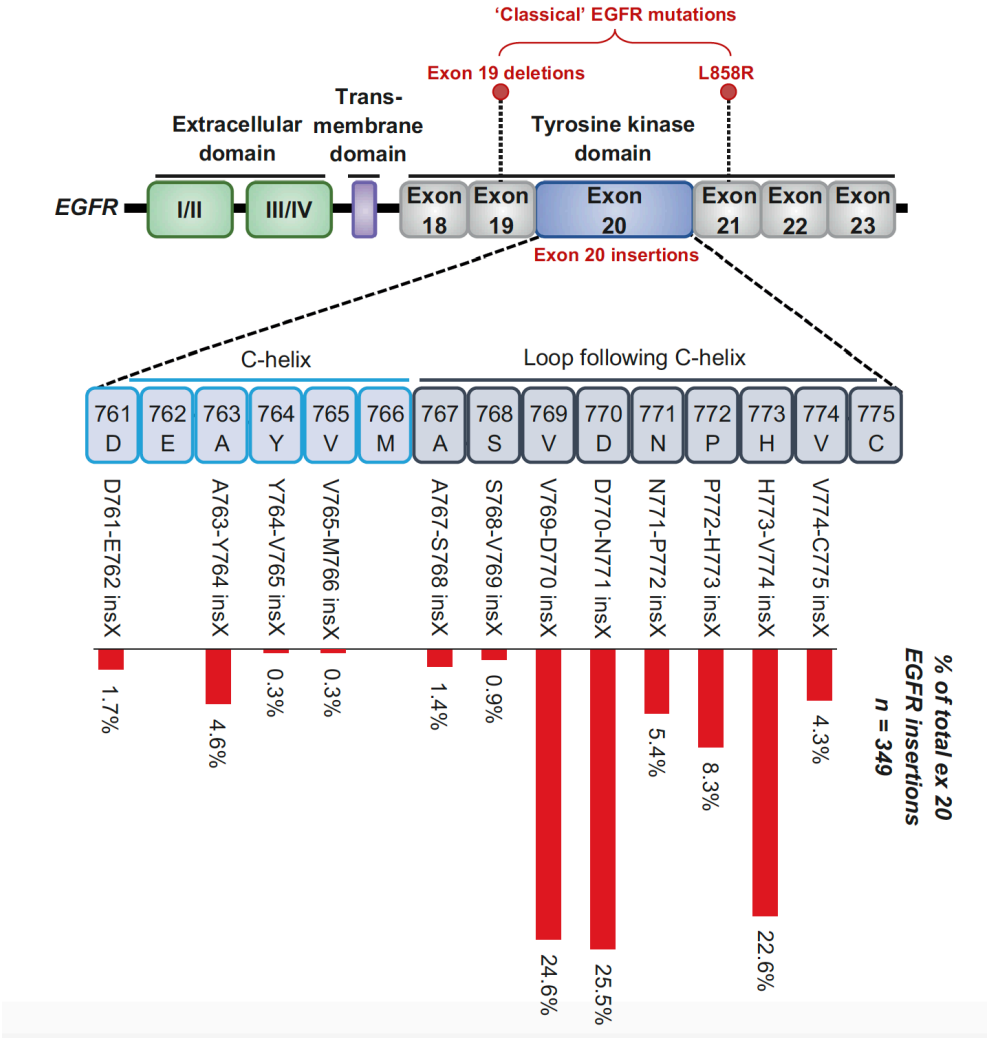

Figure 17.27. Insertion mutations in EGFR exon 20, which is within the kinase domain in the intracellular region of the EGFR protein (Vyse and Huang, 2019). Most of the insertion mutations were found to be at three sites in the amino acid chain. For example, 24% of the insertions were between amino acids 769 and 770 and 25% were between amino acids 770 and 771.

Synopsis

It was a long road from the discovery epidermal growth factor (EGF) by Stanley Cohen in 1965 to the development of targeted drugs and antibodies that improved the prognosis of patients with metastatic cancers of breast, lung, and others, as of the time of this writing in April 2020. A large number of tyrosine kinase inhibitor drugs were synthesized targeted to the enzyme activity of the epidermal growth factor receptor (EGFR) family, some of which became clinically effective. Another class of EGFR-targeted agents were antibodies targeted to extracellular domains of EGFR or its relative, HER2. Improved outcomes were achieved by combining the inhibitors and antibodies in various ways, including combinations of

antibody and drug within the same molecule. Useful and promising clinical results were achieved against some of the common metastatic cancers, but it remained far from a cure of any of them. These achievements were made possible by much basic research that revealed molecular mechanisms of how mutation and amplification of the *EGFR* or *HER2* genes drive the cancer process and how treatment could take advantage of the molecular vulnerabilities of those cancers. It was a long, complex, and fascinating story that still remains in progress. The recent developments of EGFR-targeted drugs and antibodies shows the lightning speed of current progress in biomedical research, while there evidently remains a long way to go.

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