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Review

Prospect for Anti-Her2 Receptor Therapy in Breast Cancer

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Abstract. *The carcinogenic process is characterized, in part, by the dysfunction of cellular communication pathways, such as the one involving HER2. HER2 is a member of the EGF receptor family, which participates in cell growth and proliferation. HER2 may be overexpressed in 15 to 30% of breast cancer cases and is associated with poor prognosis, shortened overall survival and shorter time to disease progression. Furthermore, an increasing number of studies have demonstrated the relevance of HER2 serum concentrations (sHER2, extracellular domain released into the blood by proteolysis) as a predictive marker of resistance to chemotherapy in HER2-overexpressing metastatic breast cancer. The determination of HER2 overexpression/ amplification in the diagnosis of relapse of breast cancer is currently a routine procedure. Immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) techniques, used to detect HER2 expression in the tumor, are improving constantly and other parallel techniques such as chromogenic in situ hybridization (CISH) are emerging. sHER2 concentrations can be measured using ELISA techniques, which can be automated. All of these procedures still need to be standardized. The discovery of a monoclonal antibody (4D5) that can inhibit the growth and proliferation of cells overexpressing HER2 led to the development of trastuzumab. Like 4D5, trastuzumab recognizes an epitope on the extracellular domain of HER2. Moreover, trastuzumab is also able to stimulate antibody-dependent cellular toxicity (ADCC). It is administered alone or in combination (with navelbine, taxol, carboplatin, etc.) in patients with metastatic breast cancer overexpressing HER2. Other active antibodies have since been discovered, as well as other specific molecules, such as tyrosine kinase inhibitors which will undoubtedly find a place in the therapeutic arsenal used in breast cancer, especially to avoid resistance to treatment.*

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Carcinogenesis is characterized by the proliferation and growth of cells that develop in an anarchical way, to the detriment of the surrounding cells. A disturbance of the cell machinery gives these cells an abnormal phenotype: loss of contact inhibition, secretion of specific proteins, immortalization, loss of differentiation, *etc.* This alteration in the normal cell phenotype is caused by the mutation or modification of the molecules that participate in cell growth and division: proto-oncogenes, growth factors, growth factor receptors, signal transducing and effector molecules of the intracellular signaling pathways, and molecules regulating the cell cycle (kinase proteins, cyclins). Just as the organism attempts to curb tumor development (suppressor genes, immune response to the tumor), therapies have been tested which aimed at destroying or limiting tumors. In parallel with surgery, the initial chemotherapies and radiotherapy, along with their various side-effects, were for a long time the only effective weapons against cancer. Subsequently, molecules with greater specificity were developed: hormonotherapy in breast cancer and, more recently, immunotherapy and anti-angiogenic molecules. Other strategies, such as cell and gene therapy, have also made their appearance. The discovery in the early 1980s of the v-erbB2 oncogene and then of c-erbB2/*neu* and its transcript (a membrane receptor, HER2), found to be involved in breast cancer, led to the development of a HER2-specific antibody therapy. The HER2 onco-receptor, derived from EGFR (epidermal growth factor receptor, HER1), would appear to play a role in a certain number of tumor development processes. Research on this receptor is now providing us with more information about the chromosome location of the oncogene, its protein structure

and functional characteristics, the nature of its involvement in tumor development, *in vivo* and *in vitro* detection of the protein and/or the oncogene and the interest of blocking it with targeted therapies (antibodies, kinase inhibitors).

In this review, these different aspects are examined, in the particular context of breast cancer, concluding with the prospects offered by anti-HER2 receptor therapy in the treatment of this disease.

The c-erbB2/*neu* Oncogene, or HER2/*neu*, and the HER2 Receptor

It was in the early 1980s that the *neu* cellular oncogene or c-erbB-2 was discovered. The oncogene was first identified as *neu* for neuroblastomas/glioblastomas in a line of rats in which a gestating female had been treated with ethylnitrosourea (1, 2)), and for its homology with the viral oncogene v-erbB (avian erythroblastosis virus) and its close relationship with the epidermal growth factor receptor (EGFR or c-erbB-1) gene (3, 4). Amplification of this oncogene appeared to be directly involved in a certain number of tumor processes in humans, particularly in breast cancer. The identification of the gene product, an 185,000 Da glycoprotein (p185), confirmed its tyrosine-kinase enzymatic activity (5), and the similarity of certain protein motifs to those of EGFR (6,7), forming the start of a family of receptors, the HER receptors (for human EGF receptor), 4 members of which have been identified to date: HER1 (or EGFR), HER2, HER3 and HER4. The gene can thus be more simply referred to as HER2/*neu*, the receptor as HER2 and the protein itself as $p185^{HER2}$. The HER-2/*neu* gene is located on the long arm of chromosome 17 (17q21) (6) and encodes the $p185^{HER2}$ protein comprised of 1255 amino acids distributed between the extracellular domain (632 amino acids), the transmembrane domain (22 amino acids) and the intracellular domain (580 amino acids) of the receptor (8). The extracellular domain, made up of 4 sub-domains (I to IV), enables ligand binding and receptor dimerization, while the intracellular domain possesses tyrosine kinase activity (phosphorylation/ dephosphorylation of transmembrane proteins or intracellular intermediates) necessary for the transduction of intracellular signals. The cellular signal then causes the transcription of one or several genes involved in the proliferation, growth, survival, motility and adhesion of cells (9). Signal transduction is only possible through ligand binding and dimerization, either with the same receptor (HER2-HER2: homodimers) or with another receptor of the same family (HER2-HER1, HER2-HER3, HER2-HER4: heterodimers). Among the potential ligands are EGF, amphiregulin, TGFα (for HER1), beta-cellulin (for HER1 and HER4) and heregulin (for HER3 and HER4) (Figure 1). The receptors are most often heterodimers since HER2 does not bind ligands and its preferred dimerization partner is the HER3 receptor (8,10). The signaling pathways involved when the HER receptor dimers are activated vary: the MAP kinase pathway, the PI3K/Akt pathway, the phospholipase Cγ pathway and the janus kinase pathway. They are responsible for cell proliferation, survival and growth processes (9). Heterodimers containing HER2 have been found to be more stable, to have a low level of dissociation and internalization and to generate stronger signals than other heterodimers (tighter ligand binding) (8, 9) justifying, in part, their involvement in certain tumor processes. As a transmembrane protein, the HER2 receptor may undergo proteolysis, a physiological process, resulting in cleavage between a 97 to 115 kDa extracellular domain, called p105 or sHER2 (soluble HER2), which is released in the extracellular sector, and the rest of the 95 kDa protein, called p95, which remains embedded in the plasma membrane. The p95 portion may be associated with increased tyrosine kinase activity and, thus, with a greater signaling potential (11, 12).

However, such cell anomalies do not escape the vigilance of the organism and it has been shown that a HER2-specific immune response can be developed (13, 14). A cellular response (CD4, CD8 and NK T cells as well as macrophages and polynuclear neutrophils) and a protective humoral response (anti-HER2 antibodies) (15) together attempt to maintain the integrity of the organism by destroying the tumor. This demonstrates the immunogenic nature of the HER2 receptor, which can be targeted by antibodies directed against certain of its epitopes. *A priori*, such targeting opens up possibilities of detection by measuring the expression of the receptor at the cell surface (with or without detection of gene amplification), or of its soluble part in the serum, as well as treatment possibilities, both of these aspects being closely linked.

Expression and Prognosis in Breast Cancer

The HER2 receptor is normally present at the surface of the cells of numerous tissues including the gastro-intestinal, respiratory and urogenital epithelium, and skin, mammary, placental and cardiac cells (16). In the presence of a tumor, the number of receptors can increase 40-fold (overexpression), going from 50,000 HER2 receptors per cell to over 2,000,000 (17). HER overexpression is involved in a number of cancers, including breast, ovarian, stomach, lung, prostate and colon cancer (18).

In the case of breast cancer, overexpression was found in 15 to 30% of cases (19), depending on the histological type (10,18,20). It was believed that there was an excellent concordance between the HER2 status (overexpression or amplification) of the primitive tumor and metastatic lesions (21). However, in their study, Meng *et al.* found contradictory results (22). Overexpression is associated essentially with amplification of the HER2/*neu* proto-oncogene, while cases

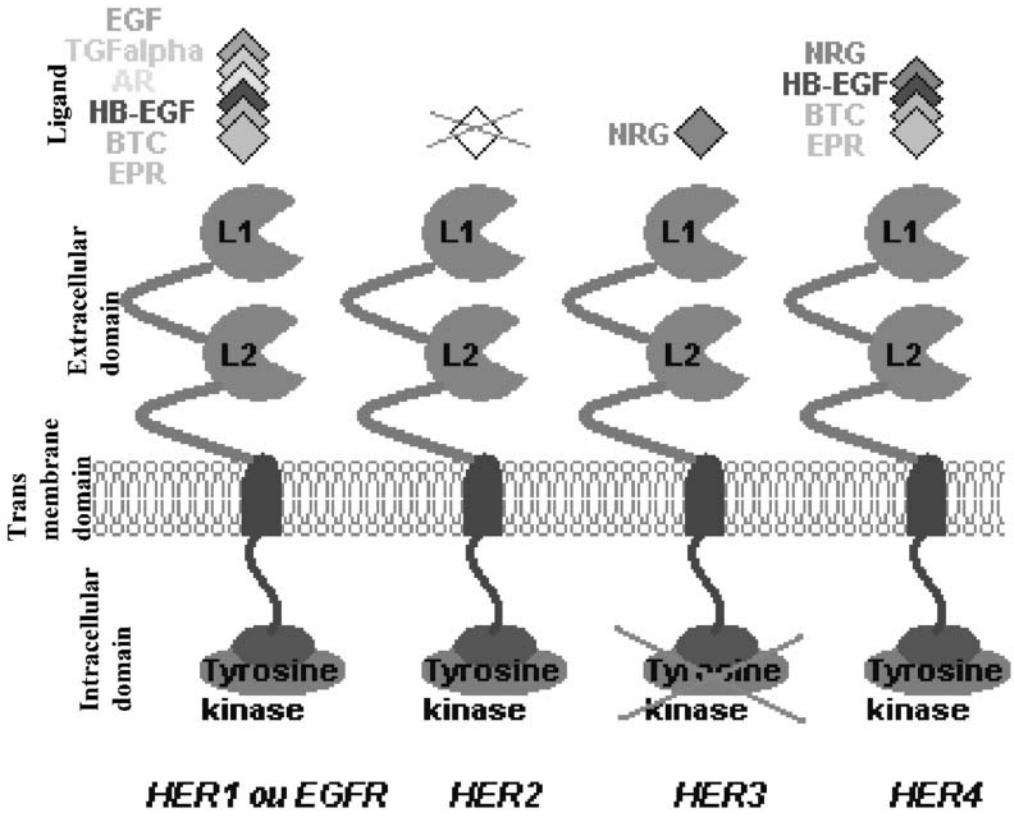

Figure 1. *Structure of 4 receptors of the HER family and their respective ligands. HER2 has no known ligand and HER3 has no tyrosine kinase activity. AR = amphiregulin, BTC = betacellulin, EGF = epidermal growth factor, EPR = epiregulin, HB-EGF = heparin binding EGF, NRG = neuregulin or heregulin, TGF = tumor growth factor. L1 and L2: ligand binding domains.*

where overexpression occurs without amplification can be explained by transcriptional or post-transcriptional deregulation (10). In these oncogenic conditions, not only do heterodimers form more easily, but HER2-HER2 homodimers also form spontaneously. Overall, these dimers can generate an even more potent signal. This results in deregulation of the cell cycle, promoting abnormal cell growth and proliferation and escape from apoptosis (10).

In the first studies on the HER2/*neu* oncogene and the HER2 receptor, a correlation was found between their presence and the development of various tumors, including gastro-intestinal, lung and genito-urinary tumors. However, most studies on the predictive and prognostic impact of HER2 have concerned breast cancer. From 1987, when Slamon *et al.* found HER2/*neu* gene amplification to have a predictive value in overall survival and time before relapse (23), investigations linking HER2 (amplification and/or overexpression) to the progression of breast cancer have generally identified it as a factor of poor prognosis. The lack of agreement found in the first studies subsequently gave way to more uniform results (24), thanks essentially to better standardization of the detection techniques, which had been the stumbling block of studies carried out on the subject. HER2/*neu* oncogene amplification has been found to be predictive of disease progression, of association with lymph node metastases (N) and recurrence (24). When measured by FISH (fluorescence *in situ* hybridization), amplification was also found to be predictive of short- and long-term outcome in N- carcinomas (25), of association with early recurrence/recurrence at any time/disease-related death for N- carcinomas treated initially by surgery (24) and was a predictive factor independent of tumor size/grade and hormone receptor status (HR) (24, 25). HER2 receptor overexpression has been found to be predictive of overall survival and disease-free survival, as well as of progression in early-stage or advanced breast cancer and has been correlated with high tumor grade and hormone receptor negative status (HR–) (24). Eighty-one studies on the subject (conducted between 1987 and 2003) found correlation between HER2 and breast cancer progression (Table I). These studies cover over 27,000 cases and demonstrate the pertinence of the results (26). Thus, 90% of the studies, covering 93% of the patients, showed that HER2 amplification or overexpression predicted the progression of

Table I. *Percentage of studies and cases, showing a correlation between HER2 and breast cancer progression on univariate or multivariate analysis (after Ross JS et al., 2003).*

Correlation	No. of studies (%)	No. of cases (%)
All cases		
No correlation	8 (10%)	1972 (7%)
Univariate significance only	21 (26%)	5766 (21%)
Independent on multivariate analysis	52 (64%)	19424 (72%)
Total	81 (100%)	27162 (100%)
Cases without correlation		
Detection of overexpression (IHC and ELISA)	5 (63%)	1195 (61%)
Detection of amplification	3 (38%)	777 (39%)
Total	8 (100%)	1972 (100%)
Cases with univariate significance only		
Detection of overexpression (IHC and ELISA)	14 (67%)	4551 (79%)
Detection of amplification	7 (33%)	1215 (21%)
Total	21 (100%)	5766 (100%)
Cases with multivariate significance		
Detection of overexpression (IHC and ELISA)	38 (73%)	13634 (70%)
Detection of amplification	14 (27%)	5790 (30%)
Total	52 (100%)	27162 (100%)

the breast cancer, on both univariate and multivariate analysis, confirming the key role of this gene in the outcome of the disease.

In addition to its value as a prognostic indicator of disease progression, HER2 detection has also proved of value as a predictor of the effectiveness of antitumor therapy and predictive of poorer response to chemotherapy and hormonal therapy, despite some contradictory results (26). Furthermore, HER2 overexpression is predictive of the effectiveness of treatment by the monoclonal antibody Herceptin® (trastuzumab), which specifically targets the HER2 receptor. A score of "3+" in the assay to determine HER2 overexpression is thus required for a patient to qualify for trastuzumab therapy.

Interest of Detecting p105 (extracellular domain of $p185^{HER2}$) in Serum or Plasma

Twenty to 40% of metastatic breast cancers are characterized by high levels of sHER2 (>15 $ng.mL^{-1}$) (27) and a good correlation (84%) has been found between HER2 3+ overexpression and high sHER2 levels (28). However, there are still cases of high sHER2 without overexpression and *vice versa*, possibly due to variations in the cleavage of the receptor releasing sHER2 (29), or to a difference in expression between the time sHER2 is detected on the tumor and the moment it is measured in the blood (30), or even to changes in overexpression during the course of the disease (22). When HER2 was used as a tumor marker, a good correlation was found between high sHER2 levels and rate of recurrence, metastases and shortened survival (26). Finally, increasing interest is being shown in sHER2 as a predictive factor in treatment response, in the case of classic therapy and, above all, targeted therapy (essentially trastuzumab). Elevated sHER2 levels have been associated with poorer response to hormonal therapy (in particular aromatase inhibitors) (28) and chemotherapy (particularly combined paclitaxel–doxorubicin therapy) (30). In patients treated with trastuzumab, a relationship was found between elevated sHER2 levels (>500 $ng.mL^{-1}$), excessively low trastuzumab plasma concentrations and the absence of response to trastuzumab when used alone (31). Furthermore, the response to trastuzumab administered in combination (docetaxel) was found to be better in patients with high initial sHER2 levels than in those who initially had low levels (32). In addition, from the 8th day of treatment (samples taken weekly), a decrease in sHER2 levels was observed in patients responding to therapy, but not in patients with progressive disease. sHER2 kinetics would thus appear to be a predictive factor for disease progression in patients undergoing trastuzumab therapy, as early as the 15th day of treatment (33).

Only the ELISA (enzyme linked immuno sorbent assay), test-marketed by Bayer Diagnostics®, is authorized by the FDA (Food and Drug Administration) "for follow-up and monitoring of patients with metastatic breast cancer". In addition to the uses described above, the test can be used to determine sub-groups of patients among HER2 3+ IHC patients for whom trastuzumab would be more effective (29). Finally, it might also identify effective antitumor therapies other than trastuzumab, starting from the principle that $p185^{HER2}$ is released after tumor lysis (34).

Methods of Detecting the HER2/*neu* Gene or the HER2 Protein

To date, a wide variety of methods have been used to detect – in the broad sense of the term – the HER2 receptor and the HER2/*neu* oncogene. The initial sample might be tumor tissue, possibly paraffin-embedded, but biological fluids can also be used, the obvious choices being serum or plasma. *In vivo* detection techniques have also been developed. Detection focuses on a number of different aspects: detection of overexpression, using immunohistochemical (IHC) or ELISA techniques on tumor tissue (primitive tumor or metastases); detection of the extracellular domain of the protein in serum or plasma using automated or manual ELISA techniques; and, finally, *in vivo* detection using immuno-radiotracing.

Detection of p185HER2 protein. IHC is used to detect overexpression of the HER2 receptor on the cell membrane using either monoclonal antibodies (as in the CTA - Clinical Trial Assay – and the Pathway™ detection kit), or polyclonal antibodies (as in the HercepTest™ detection kit) (20, 26). Revelation is performed through an immunoenzyme reaction whereby a chromogene is transformed into a colored molecule. Overexpression is positive when it reaches "3+" (35). ELISA is used to measure the quantity of $p185^{HER2}$ protein present in the cytosol of the malignant cells (35) or the quantity of circulating sHER2 receptor (in fact the extracellular domain released in the serum by proteolytic cleavage) (36). A capture monoclonal antibody and a detection monoclonal antibody are used in a sandwich assay with $p185^{HER2}$ or sHER2, the reaction being revealed by coupling with a specific enzyme (streptavidin peroxidase) and, as previously, a chromogene substrate is transformed into a colored molecule. IHC detection is read using a fluorescence microscope, while protein levels detected in ELISA are measured by spectrophotometry. Finally, magnetic resonance imaging (MRI), an *in vivo* technique, can be used to detect tumors overexpressing HER2 through to a contrast agent specific to an anti-HER2 antibody administered to the patient (37).

Detection of HER2/neu gene. Today, the most commonly used technique for detecting the HER2/*neu* gene is FISH, performed on tumor tissue. This technique uses a DNA probe complementary to the HER2/*neu* gene sequence identified by a fluorescent ligand and counterstaining. The samples are observed with a fluorescence microscope. Amplification is established if the ratio of the number of HER2/*neu* signals to the number of chromosome 17 signals is more than or equal to 2 (20). Another emerging method using a hybridization probe is chromogenic *in situ* hybridization (CISH). The principle is the same as in FISH, but the fluorescent ligand is replaced by a chromogene. The samples are viewed using a conventional microscope and the results are expressed in the same way (26). Other methods include the RT-PCR technique, in which there is growing interest, and the Southern blot technique, which was used in the first studies but has since been abandoned (26). IHC and FISH are the two techniques used to determine the HER2 status of a mammary tumor. IHC is the approved method for identifying patients eligible for Herceptin® treatment in the metastatic stage of their cancer. Only the HercepTest™ kit from DAKO and Pathway™ from VENTANA are authorized by the FDA for this test (20, 26). FISH is the method generally used to interpret doubtful IHC cases (cases of overexpression with a score of "2+") (20). The HER2 FISH PharmDx™ kit from DAKO is authorized in Europe for this purpose.

A good correlation has been found between overexpression results determined by IHC and the amplification determined by FISH (over 90% concordance). The limiting factors are undoubtedly cost (ratio of 1 to 3 between an "authorized" IHC and a FISH assay) and experience in performing the tests (20). In parallel, it is important to consider the variability affecting IHC results, due to differing sensitivity of the antibodies used, different methods of fixing tumor tissue samples and variations in interpretation of the test (38).

Therapeutic Targeting of HER2

Since HER2 overexpression by breast cancer cells is quite specific, therapies directed specifically against the receptor have rapidly gained recognition as effective weapons against tumor growth, replacing or, better still, complementing existing non-specific therapies. The immunogenic character of HER2 and its tyrosine-kinase function have led to the development of a whole range of molecules and antibodies. Some have completed phase III trials and demonstrated their efficacy, while others are offering new solutions, in particular to the problems of resistance, and are still being tested.

Trastuzumab. Anti-HER2 antibody therapy was started in the mid-1980s with what was to become trastuzumab (Herceptin®): the murine monoclonal antibody 4D5, which demonstrated its ability to inhibit tumor growth *in vitro* and *in vivo*. Tests in humans started after a humanized form of this immunoglobulin G targeted against the extracellular domain of $p185^{HER2}$ was developed with the aim of suppressing or minimizing the formation of HAMA (human anti-mouse antibodies) in patients receiving the antibody and also inducing antibody-dependent cellular cytotoxicity (ADCC) by immunity effector cells (39, 40). Although the precise mechanism of action of the antibody is not fully understood, it is known that it affects signal transduction and signal effector proteins and acts through cellular or non-cellular cytotoxicity. Trastuzumab thus accelerates destruction through endocytosis of the HER2 receptor, reduces the formation of heterodimers containing HER2 and inhibits its cleavage. It causes induction of $p27^{kip1}$ and p130 kinase inhibitors and initiates cell cycle arrest during the G-phase. It also inhibits angiogenesis and metastatic dissemination. Finally, as well as inducing ADCC, it activates complement and sensitizes tumor cells to the cytotoxicity of TNFα (41, 42). In metastatic breast cancer, the only disease for which trastuzumab has been granted a Product Marketing Authorization (trials are in progress to validate its use in neoadjuvant therapy), these aspects contribute to the therapeutic efficacy of the antibody, prolonging survival (and improving quality of life) in those patients treated. Because of the specificity of the antibody, adverse effects are minimized, except for cardiac toxicity (congestive heart failure), the mechanisms of which remain to be elucidated (43). Because of this risk, the patient's cardiac

function must be monitored regularly by measuring the left ventricular ejection fraction (LVEF). If this value falls below 50%, treatment may have to be suspended, this being a contra-indication in all cases when it is combined with cardiotoxic drugs (44).

The therapeutic efficacy of trastuzumab has been observed, not only when it is administered singly but also, and above all, when it is combined with other anticancer agents. *In vitro*, on mammary cancer cells overexpressing HER2 (principally SK-BR-3 cells), and *in vivo*, on xenografted mice, a synergic effect has been observed between trastuzumab and doxorubicin, paclitaxel, cyclophosphamide, methotrexate, etoposide and vinblastine. Nowadays, it is used essentially in combination with cisplatin (45), paclitaxel (44, 46), docetaxel (47, 48), vinorelbine (49) and gemcitabine (50, 51). Despite the targeted nature of trastuzumab therapy, studies have shown that only one-third of the selected patients with metastatic breast cancer (*i.e.* overexpressing HER2) that undergo trastuzumab-based therapy respond to the treatment, revealing the existence of resistance mechanisms that have yet to be determined. A number of possibilities have been suggested to explain this resistance: responsibility of IGF-1 receptor (IGF-1R) activation pathways (52), a possible drop in intracellular $p27^{kip1}$ concentrations (53) or simultaneous overexpression of EGFR (54). Other possible mechanisms are the expression of truncated HER2 receptors that no longer bind trastuzumab, mutation of signal transduction molecules or signal effector molecules downstream of HER2, or even diminished immune function in the advanced stages of breast cancer (55). However, it is more likely that such resistance is multifactorial and involves activation pathways other than those dependent on HER2.

Trastuzumab can also serve as a "vector" for cytotoxins that cannot be administered alone because they are too toxic (radio-isotopes, toxins, *etc.*). Studies have been carried out with immunoconjugates, such as trastuzumab-geldanamycin (a molecule that inhibits the Hsp90 chaperone protein which is essential for cellular functions), demonstrating their ability to enhance trastuzumab activity (56).

Other anti-HER2 Antibodies

Other antibodies targeted against HER2 have been developed since the conception of trastuzumab, offering many solutions for inhibiting tumor growth or fighting against trastuzumab resistance. For example, pertuzumab (57, 58), developed by Roche under the name Omnitarg®, is an antibody targeted against a different epitope from the one recognized by trastuzumab. It is currently being tested in phase II clinical trials. The antibody causes steric hindrance and inhibits HER dimerization, potentiating the action of trastuzumab on mammary cells overexpressing HER2. Since the large size of the anti-HER2 antibodies can hinder their access to the tumor (40), research is being carried out on smaller molecules such as fusion proteins. These proteins, composed of a fragment of HER2-specific antibody (for example a single-chain antibody) and IL-2, retain the specificity of the corresponding monoclonal antibody with the advantage of being smaller and, therefore, having better pharmacokinetic properties (59). These fusion proteins also facilitate cell-mediated cytotoxicity as a way of inducing lysis of tumor cells (60), as in the case of bispecific antibodies (combination of two F(ab') fragments, one HER2-specific and the other specific to the cytotoxic immune cell) (61).

Non-antibody Anti-HER2 Therapies

In the context of immune response by the host against cells overexpressing HER2, vaccines provoking a humoral response following injection of HER2 antigenic motifs are currently being developed (62). Though perhaps less HER2-specific, the tyrosine kinase inhibitor emodin was shown to suppress the tyrosine kinase activity of breast cancer cells overexpressing HER2 grafted on athymic mice, and to sensitize these cells to the action of paclitaxel (63).

Finally, bearing witness to the many paths that have been explored to arrest the tumoral activity of the overexpressed HER2 receptor, the synergistic effects of antisense therapy with oligodeoxynucleotides (ODN) and conventional chemotherapy agents have been demonstrated (64).

Conclusion

The use of trastuzumab to target the HER2 receptor has already been considered a paradigm in breast cancer treatment and in the treatment of cancers in general: the specificity of one for the other, overexpressed in a non-negligible proportion of breast cancer cases, would seem to augur well for the future. The reality is slightly different, but the enthusiasm remains, and with reason. It was believed that the ultimate weapon had been found; in fact, it is only a partial solution. In the future, oncologists treating breast cancer overexpressing HER2 will most certainly use combinations of different antibodies or therapies with more varied targets, in order to overcome the problem of emerging resistance mechanisms, a reminder of the ability of cancer cells to adapt. Biologists will be able to provide oncologists with measurements needed to distinguish patients responding to specific therapy from those who are not responding. Finally, with advances in oncogenetics, the targets of future therapies will be identified with greater precision.

Measurements of circulating HER2 receptor concentrations provide the opportunity for monitoring the response to anti-HER2 antibody therapy (trastuzumab) in breast cancer. These levels could also be valuable in detecting early escape from this treatment. Knowledge of circulating

HER2 receptor concentrations can be beneficial not only in terms of the quality of patient follow-up, but also in reducing the costs related to the use of expensive drugs.

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