Table 3 Association between PTEN LOH and HER2 overexpression

Variables	*n*	PTEN		*P*-value
		ROH ($n = 100$)	LOH ($n = 31$)	
HER2 overexpression				
Negative	94	80 (85.1)	14 (14.9)	<0.001
Positive	37	20 (54.1)	17 (45.9)	

Table 4 Expression of pAkt and PR by status of PTEN LOH and HER2 overexpression

PTEN LOH/ HER2 overexpression	*n*	pAkt		PR	
		Negative ($n = 83$)	Positive ($n = 48$)	Negative ($n = 73$)	Positive ($n = 58$)
ROH/Negative	80	62 (77.5)	18 (22.5)	33 (41.3)	47 (58.7)
ROH/Positive	20	9 (45.0)	11 (55.0)	14 (70.0)	6 (30.0)
LOH/Negative	14	6 (42.9)	8 (57.1)	10 (71.4)	4 (28.6)
LOH/Positive	17	6 (35.3)	11 (64.7)	16 (94.1)	1 (5.9)

Association between the coexistence of PTEN LOH and HER2 overexpression and either Akt activation or PR expression

Since PTEN LOH and HER2 overexpression were unexpectedly found to be positively associated, we investigated the influence of coexistence of PTEN LOH and HER2 overexpression on Akt activation. Expression levels of pAkt and PR by status of PTEN LOH and HER2 overexpression are shown in Table 4. In order to elucidate the association between Akt activation and the coexistence of PTEN LOH and HER2 overexpression, the multivariate exact logistic regression analysis was performed in consideration with the small sample size. As shown in Table 5(a), odds ratios of PTEN LOH and HER2 overexpression for Akt activation indicate that both were significantly positively associated with pAkt expression level (odds ratio are 2.87 and 2.79 with $P = 0.0222$ and $P = 0.0384$, respectively). Akt was much more activated when both PTEN LOH and HER2 overexpression coexisted (odds ratio; 7.990) (Table 5(b)). In addition, the association between PR expression and the coexistence of PTEN LOH and HER2 overexpression was analyzed with the same method. Odds ratios of PTEN LOH and HER2 overexpression for PR expression were 0.271 and 0.241 respectively, and these factors were also significantly associated with the loss of the PR expression ($P = 0.0090$, 0.0104, Table 6(a)). Intriguingly, PR expression was quite low in cases with both PTEN LOH and HER2 overexpression (odds ratio; 0.065, Table 6(b)). These results suggest that the coexistence of PTEN LOH and HER2 overexpression is considered to enhance Akt activation and thereafter lead to a loss of PR expression.

Association between Akt activation and PR negative expression in the ER-positive cases

From a previous study, activation of PI3K/Akt pathway is considered one of the factors that inhibit PR expression [27]. The mechanisms of negative regulation of PR expression in ER-positive cases are considered very important, thus we analyzed the relationship between Akt activation and PR expression in the ER-positive cases. As shown in Table 7, positivity of PR expression was lower in the pAkt-positive cases, although it was not statistically significant. In addition, we also investigated the influence of the coexistence of PTEN LOH and HER2 overexpression for PR expression in the ER-positive cases by the multivariate exact logistic regression analysis. PR

Table 5 Association between pAkt expression and coexistence of PTEN LOH and HER2 overexpression

(a) Multivariate analysis by the exact logistic regression for pAkt expression

	Odds Ratio	95% CI of Odds Ratio	*P*-value
HER2 overexpression	2.868	(1.149, 7.242)	=0.0222
PTEN LOH	2.786	(1.051, 7.511)	=0.0384

(b) Odds ratios for pAkt expression influenced by coexistence of PTEN LOH and HER2 overexpression

PTEN LOH/ HER2 overexpression	Odds ratio for pAkt expression
ROH/Negative	1.000
ROH/Positive	2.868
LOH/Negative	2.786
LOH/Positive	7.990

Table 6 Association between PR expression and coexistence of PTEN LOH and HER2 overexpression

(a) Multivariate analysis by the exact logistic regression for PR expression			
	Odds Ratio	95% CI of Odds Ratio	*P*-value
HER2 overexpression	0.271	(0.088, 0.749)	=0.0090
PTEN LOH	0.241	(0.064, 0.750)	=0.0104

(b) Odds ratios for PR expression influenced by coexistence of PTEN LOH and HER2 overexpression	
PTEN LOH/ HER2 overexpression	Odds Ratio for PR expression
ROH/Negative	1.000
ROH/Positive	0.271
LOH/Negative	0.241
LOH/Positive	0.065

expression by status of PTEN LOH and HER2 overexpression in ER-positive cases was shown in Table 8. The odds ratios of HER2 overexpression and PTEN LOH are both estimated less than 1, giving suggestions of those factors influencing the negative PR expression (Table 9). The statistical tests for their associations did not exhibit significant *P*-values because of, presumably, inflations of the false negative rates due to the small sample sizes (Table 9). Although we could not reveal the association between the coexistence of PTEN LOH and HER2 overexpression and PR expression in the ER-positive cases in the sense of the statistical significance in this study, further analyses with larger sample size are expected to present the significant association.

Discussion

As far as we know, this is the first report to show a positive correlation between Akt activation with both LOH at the PTEN locus and HER2 overexpression. In addition, we also demonstrated that a coexistence of LOH at the PTEN locus and HER2 overexpression enhances the Akt activation and thus also induces a loss of PR expression, even in ER-positive breast carcinomas.

Akt/PKB is a serine/threonine protein kinase, which is a crucial regulator of widely divergent cellular processes, including apoptosis, proliferation, differentiation, and metabolism [1]. A disruption of normal Akt/PKB signaling frequently occurs in several human cancers, and this enzyme appears to play an important role in cancer progression and cell survival [1]. Akt is activated by a variety of stimuli, through growth factor receptors such as HER2, in PI3K-dependent manner.

Table 7 Association between pAkt and PR expression in ER-positive cases

Variables	*n*	pAkt		*P*-value
		Negative (*n* = 57)	Positive (*n* = 26)	
PR				
Negative	31	18 (58.1)	13 (41.9)	=0.1431
Positive	52	39 (75.0)	13 (25.0)	

Table 8 PR expression by status of PTEN LOH and HER2 overexpression in ER-positive cases

PTEN LOH/ HER2 overexpression	*n*	PR	
		Negative (*n* = 31)	Positive (*n* = 52)
ROH/Negative	65	21 (32.3)	44 (67.7)
ROH/Positive	6	1 (16.7)	5 (83.3)
LOH/Negative	6	3 (50.0)	3 (50.0)
LOH/Positive	6	6 (100.0)	0 (0)

Table 9 Association between PR expression and coexistence of PTEN LOH and HER2 overexpression in ER-positive cases—Multivariate analysis by the exact logistic regression for PR expression

	Odds Ratio	95% CI of Odds Ratio	*P*-value
HER2 overexpression	0.709	(0.135, 4.076)	=0.8944
PTEN LOH	0.186	(0.027, 0.929)	=0.0385

Another major mechanism of Akt activation is a loss of the PTEN function [1, 2].

In this study, we showed that Akt activation was significantly associated with HER2 overexpression (Table 2). This result was expected from previous studies [9–11].

A loss of the PTEN function due to PTEN mutation, LOH at the PTEN gene locus, and epigenetic down-regulation of PTEN expression have been reported in nearly 50% of breast cancers and in many other cancer types [12, 15]. In terms of structural alteration, mutation of the PTEN gene is rare in breast carcinomas. On the other hand, LOH at 10q23 (PTEN gene locus) is observed in approximately 30%–40% of all sporadic breast cancers [17–19, 37]. In many previous studies, however, an analysis of LOH was performed with conventional assays for microsatellite alterations using radiolabeled polymerase chain reaction (PCR) and X-ray films or similar methods [17–19, 37]. The sensitivity and accuracy of LOH detection is limited by these assays, and consequently, LOH may often not be interpreted accurately. New techniques to label nucleic acids with fluorescent dye compounds have evolved, and a combination of fluorescent labeling and laser scanning as in automated DNA sequencers, is now used in a wide variety of nucleic acid analyses. In such systems, the sensitivity and accuracy of quantitative detection have improved. Indeed, the use of an automated sequencer for microsatellite analyses is now increasing. We have developed a new fluorescence system to analyze microsatellite alterations, and thus assayed various kinds of malignancies [30, 31, 38–41]. To analyze LOH more precisely, we employed this method for the LOH assay [32]. LOH at the PTEN locus was observed in 31 of the 131 informative cases (23.7%). The rate is lower than that described in previous

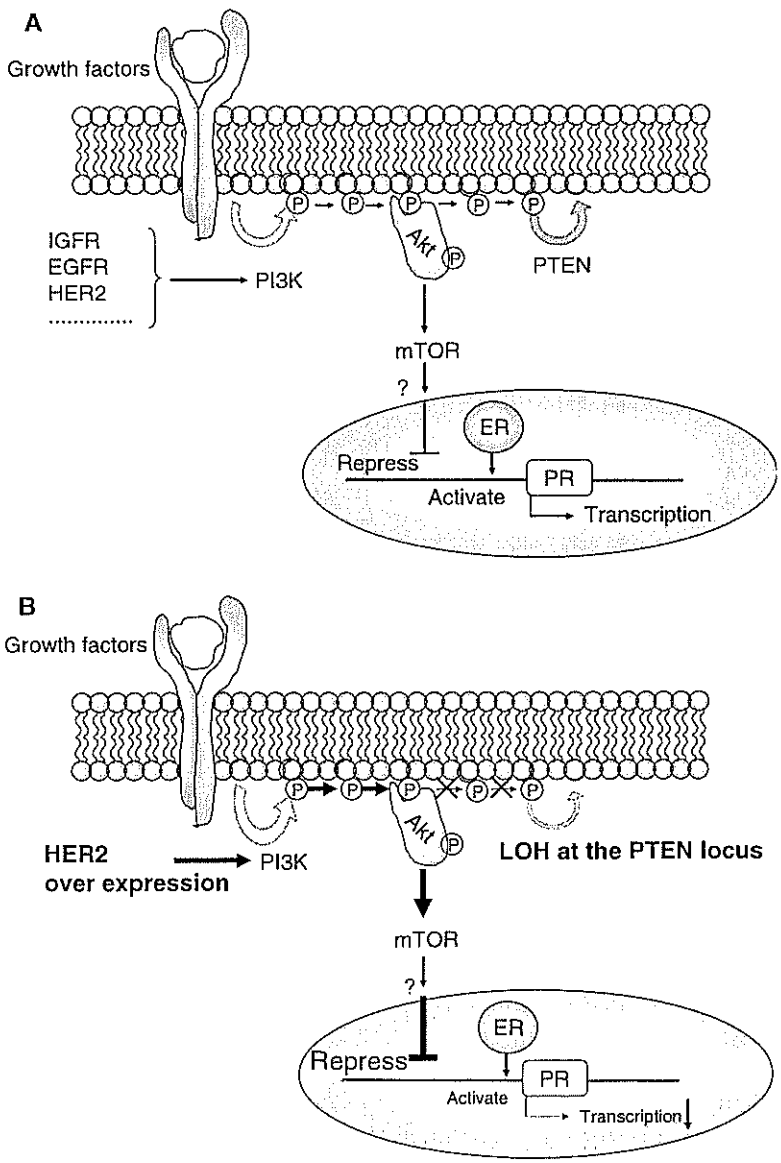

Fig. 2 The proposed mechanism for the coexistence of PTEN LOH and HER2 on PR expression. Both the HER2 overexpression and PTEN LOH can activate Akt. Activated Akt represses the ER-dependent PR transcription, thus leading to a loss of PR expression (**A**) Normal condition (**B**) Coexistence of PTEN LOH and HER2 downregulates PR expression via Akt activation

reports. However, we think that our data are more precise, because we employed a more accurate assay and analyzed more cases than in previous studies.

A reduction of PTEN protein expression is associated with Akt activation [21, 23], however, there have been no reports to indicate the relationship between LOH of the PTEN locus and the activation of Akt. We chose our system for LOH analysis to investigate the aberration of PTEN function since it is more difficult to assess a diminished expression of PTEN protein using immunohistochemistry. We evaluated Akt activation by immunohistochemical staining of phosphorylated Akt as previously reported [10]. LOH of the PTEN locus was thus found to be significantly associated with Akt activation (Table 2). PTEN LOH is therefore considered to diminish the PTEN function, thereby inducing Akt activation.

We unexpectedly found a positive correlation between PTEN LOH and HER2 overexpression (Table 3), although the mechanism for this observation is still unclear. We speculated that Akt might be more activated in the cases with both PTEN LOH and HER2 overexpression. As shown in Table 5, the expression of pAkt was significantly associated with the coexistence of PTEN LOH and HER2 overexpression.

We found that Akt activation is inversely correlated with PR expression (Table 2). In addition, the coexistence of PTEN LOH and HER2 overexpression also was found to enhance a loss of PR expression (Table 6). This association was even observed in ER-positive patients, although the statistical tests for their associations did not exhibit significant *P*-values because of, presumably, inflations of the false negative rates due to the small sample sizes (Tables 7–9). It has recently been reported that the PR expression is inhibited in breast cancer cells via the PI3K/Akt pathway, not mediated via a reduction of ER levels or activity, in an in vitro study [27]. This finding may support our results, although additional studies are required to fully elucidate its mechanism. The possible mechanism for these effects is illustrated in Fig. 2.

In conclusion, we herein showed that the Akt activation to be significantly associated with HER2 overexpression or LOH at the PTEN gene locus, and a reduced expression of PR. The incidence of Akt activation and a reduced PR expression was significant when both LOH at the PTEN locus and HER2 overexpression coexisted. Our results suggest that dysregulated HER2/Akt/PTEN in breast carcinoma may therefore lead to loss of PR expression, and thus resulting in a poor response to endocrine therapy, however, further studies are required in order to elucidate this mechanism more in detail.

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REVIEW ARTICLE

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Methodological aspects of current problems in target-based anticancer drug development

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Abstract Differently from the conventional antineoplastic agents, target-based drugs are designed a priori, based on our knowledge of various physiological molecules that has been obtained by the development of molecular biology. This "Copernican revolution" in drug development may imply a paradigm shift in this field. However, contrary to the initial expectations, many drugs developed by this approach are now faced with difficulties, mainly because of the fundamental and theoretical limits of this approach. All of the physiological functions are not always known in all target molecules. In low-molecular-weight drugs, i.e., "inhibitors," targets disperse, due to the structural similarities in physiological molecules. This double-faced "out-of-focusing" causes many problems in various steps of drug development, drug design, clinical trials, and administration to patients. Many drugs are now being abandoned because of unexpectedly lower response rates or unforeseeable adverse effects, and the variety of the drugs exhibits a kaleidoscopic appearance. The double-faced "out-of-focusing" derives from the methodological limits in molecular biology, i.e., elementalism, and limits in our techniques for drug development. To overcome these currently inevitable limits, it appears essential to elucidate the specific changes in target molecules that chiefly promote tumor growth and, consequently, strongly predict response to the administered drugs. Precise and efficient detection of responder populations is the key to the development and establishment of target-based anticancer therapies.

Key words Target-based anticancer agents · Imatinib · Gefitinib · Trastuzumab

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Introduction – from discovery to design: a "Copernican revolution" in drug development?

In the past decade, a new approach for cancer treatment has emerged. In contrast to conventional drug development, this new approach, now widely referred to as "target-based" therapies, employs drugs that have been designed to work on a single molecule functioning in the body. Thus far, drugs have been discovered by the screening and chemical modification of naturally occurring compounds, according to biological, i.e., phenomenological, activities. Target-based drugs, on the other hand, are designed a priori, based on the knowledge of each physiological molecule that has been obtained by the development of molecular biology. This dramatic change in methodology may imply a "Copernican revolution" in drug development and a paradigm shift in this field. However, contrary to the initial expectations, many drugs that have been developed by this approach are now confronted with difficulties. Although target-based drugs are defined as those designed to target a single molecule in cells, they are, in fact, developed by the screening and chemical modification of known inhibitors or newly synthesized compounds, as are classical drugs. The exceptions are recombinant protein drugs and antibody drugs. The latter, in particular, have become possible and available because of the target-based approach. Low-molecular-weight drugs, i.e., "inhibitors", predominate in this field, and a limited number of antibody drugs are now available or being developed. Although some recombinant protein drugs have been developed, none are now regarded as promising. This discrepancy between the ideal and the real in the techniques used for drug development underlies the currently inevitable limits of the target-based approach.

Furthermore, our knowledge obtained by molecular biology and biochemistry is not always complete. Using only these theoretically "elementalistic" methodologies, it appears difficult to fully understand cells or organisms, which may form "complex" systems. This elementalistic tendency in the methodologies underlying target-based drug development also leads to its fundamental and theoretical limits. In this article, we discuss the methodological aspects of current problems in target-based anticancer therapies.

Current status of target-based anticancer drug development

In Table 1, we have summarized studies of target-based anticancer drugs reported at the American Society for Clinical Oncology (ASCO) annual meetings from 2002 to 2005, and several intriguing tendencies can be seen in this field. First, growth factors and their receptors predominate as the target molecules. Particularly, higher priorities appear to be given to inhibitors of tyrosine kinase receptors, except for the first tyrosine kinase inhibitor, imatinib, which counteracts the disease-specific fusion gene product including the nuclear tyrosine kinase, c-ABL. Second, low-molecular-weight compounds referred to as inhibitors are predominant. There are no recombinant protein drugs in the Table, with the exception of angiostatin and endostatin, which were developed, but have now been abandoned. Only two antibody drugs, bevacizumab and cetuximab, are consistently being studied, although there are some antibody drugs that are now regarded as established target-based anticancer agents. However, more importantly, the most remarkable tendency in this field is that drugs have a rapid turnover, with the life span of many drugs being short. In Table 1, there are only four drugs that have been consistently developed during this 4-year period. Although the cost of development of each target-based drug is vast, many drugs are being abandoned for various reasons, mainly the unexpectedly lower response rates and unforeseeable adverse effects. For the more efficient and effective development of target-based anticancer agents, it appears to be important to discuss carefully these negative aspects and their causes in the development of this category of drugs. We discuss these problems below.

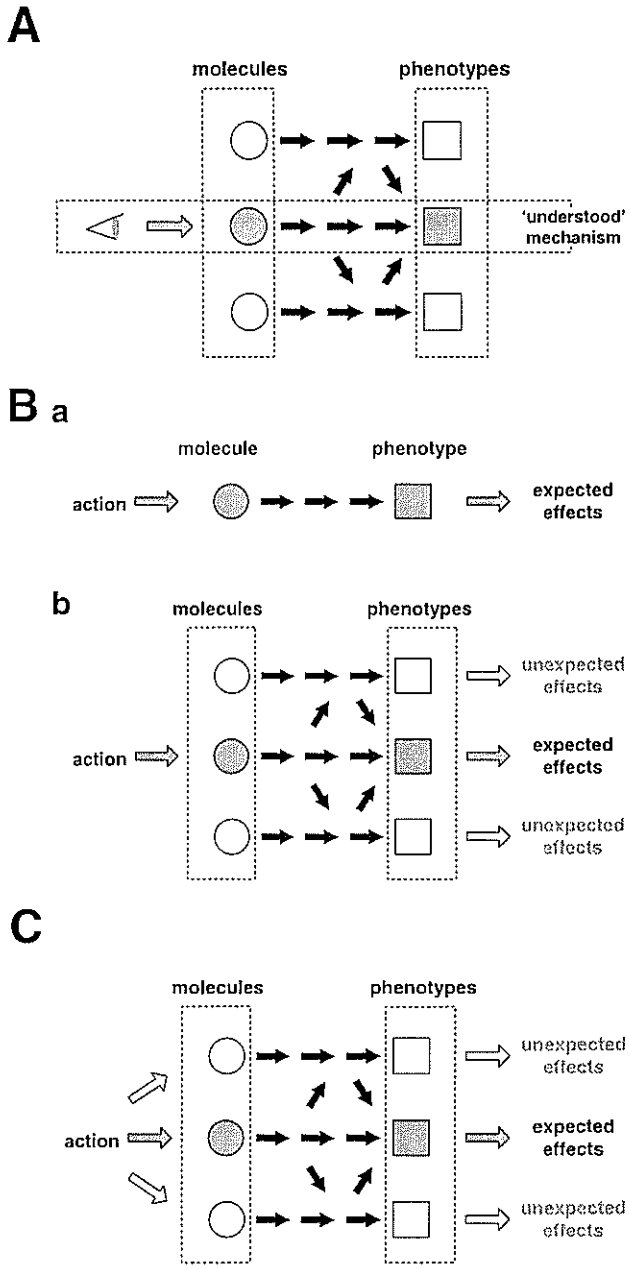

Fig. 1A–C. Schematic representations of the relationships among drugs, target molecules, phenotypes, and effects. **A** Our viewpoint; **B** recombinant protein/antibody drugs; **C** low-molecular-weight drugs

Problem I: mechanisms of action and drug design

Target-based anticancer agents can be classified into two categories: (a) recombinant proteins/antibodies and (b) low-molecular-weight compounds. In drugs in the former category, mechanisms of action appear simple and unequivocal (Fig. 1B*a*). However, these agents test our real and net knowledge of the physiological functions of the molecules in question. Angiostatin and endostatin are examples of proteins that were regarded as candidates in this category. Development of these recombinant protein drugs unexpectedly faced a deadlock, since the initial experimental results could not be reproduced in several independent experimental systems. Apart from the lack of reproducibility associated with technical problems, this symbolic example indicates the fundamental and theoretical limits of target-based drug development. In fact, all of the physiological functions are not always known in all the molecules regarded as a targets (Fig. 1A). As another example, some matrix metalloprotease (MMP) inhibitors were regarded as promising candidates as neo-

vascularization inhibitors, although they had been developed as inhibitors of invasive tumor growth. This example also reflects the fundamental and theoretical limits of target-based approaches.

It is evident that molecular biology and biochemistry largely underlie target-based drug development, because this approach address a single molecule that functions in the body of an organism. However, as is widely recognized, molecular biology and biochemistry are theoretically elementalistic methodologies. Cells are "complex" systems in which the relationship between a part and the whole is not simple, as it is in a clock or a car. From these molecular biology and biochemistry approaches only, it is sometimes difficult to understand the real relationship between a part and the whole, i.e., the functions of physiological molecules and cellular phenotypes or phenomena in the whole body of an organism. Nevertheless, molecular biology and biochemistry are essential for molecule-based drug design. Attention must be paid to the "understood" functions of physiological molecules. Before designing target-based drugs, it appears to be important to verify objectively all of our knowledge of the physiological functions of the molecule in question. Thus, target-based approaches involve fundamental and theoretical limits.

In drugs classified as low-molecular-weight compounds, this problem becomes more critical (Fig. 1C). As mentioned above, many anti-receptor tyrosine kinase inhibitors are now being developed. However, this category, tyrosine kinase is comprised of vast numbers of diverse molecules. In fact, receptor tyrosine kinases can be classified into four subtypes, according to structural similarity, and the structures of the functional domains exhibiting tyrosine kinase activity are very similar in each subtype.[1] It is now known that an agent which was initially designed to target a single tyrosine kinase molecule can exhibit its inhibitory effect on other tyrosine kinase molecules, due to structural similarity in the kinase domains. A typical example is imatinib (STI571; Gleevec). Imatinib was originally designed to inhibit tyrosine kinase activity in the products of the *BCR-ABL* fusion gene that is a hallmark of chronic myeloblastic leukemia (CML).[2] The tyrosine kinase activity in BCR-ABL fusion proteins is derived from the unique tyrosine kinase protein which functions in nuclei, c-ABL. Because there is a similarity between the kinase domain structure of c-ABL and those of other tyrosine kinases, such as platelet-derived growth factor receptors (PDGFR) and c-KIT, it has been demonstrated that imatinib also inhibits the kinase activity in these other receptor tyrosine kinases.[3] Indeed, imatinib has been tested to see whether it exhibits growth inhibitory effects on gastrointestinal stromal tumors (GIST) that overexpress c-KIT tyrosine kinase,[4] and it is now the first-choice drug for the treatment of this neoplastic disease.

Similarly, SU5416[5] and SU6668[6] were initially developed as inhibitors of vascular endothelial growth factor receptor (VEGFR), Flt-1 (VEGFR-1), and Flk-1 (VEGFR-2). These drugs have also been proven to inhibit other tyrosine kinase activities, including PDGFR, fibroblast growth factor receptor (FGFR), and c-KIT.[7] Thus, in the category of low-molecular-weight compounds referred to as "inhibitors," molecular targets inevitably disperse, due to the structural similarities in the functional domains between the initially targeted molecules and related proteins. In CML, in which tumor growth depends exclusively on the activity of a single, unique, i.e., disease-specific, molecule, BCR-ABL fusion protein, this problem does not emerge. However, this case is exceptional. The *BCR-ABL* fusion gene is found in leukemia cells in almost 100% of CML patients. On the other hand, in almost all other malignancies, the contribution of a targeted molecule to tumor growth is not always exclusive, and is sometimes marginal, and the extent of contribution varies widely between individuals. This problem leads directly to other problems concerning, for example, clinical trials, criteria for administration, and adverse effects, which are discussed below.

Problem II: clinical trials

In clinical trials in general, inflation of the false-negative rate, in other words, decrease in the statistical power, influences the results greatly, particularly when the fraction of the drug-sensitive population in a trial is small compared to the whole patient population.[8,9] The sample size, i.e., the number of patients, in clinical trials is generally determined based on the expected difference of treatment effects between the experimental and control arms, with given α and β levels. According to calculation by the logrank test, when one requires 80% statistical power to detect an improvement in 1-year survival from 10% to 20% with a two-sided 0.05 level of significance in a placebo-controlled parallel group trial, 127 patients need to be randomized to each treatment arm. This estimation is implicitly based on the assumption that the drug should be uniformly effective in all patients. However, if the drug has an effect only in a limited fraction of the whole patient population ("responder fraction") and the rest of the population has no benefit from the treatment, the statistical power decreases dramatically. Figure 2 shows the relationship between the responder fraction and the statistical power. In the case that the responder fraction is 30%, the false-negative rate reaches 50%, which implies that studies are judged as negative with a one-in-two probability, despite the fact that the treatment itself has a clinical benefit. Needless to say, in order to judge this case to be positive with a higher probability, larger sample sizes are required. For positive results, the sample size is inversely proportional to the responder fraction.

When the dependence of tumor growth on a target molecule is not exclusive and, consequently, the sensitivity to a drug targeting the molecule varies depending on the individual, the responder fraction in the patient population is limited, and studies trying to demonstrate a clinical benefit of the treatment face difficulties. This problem may partly explain the rapid turnover and the short life spans of target-based anticancer drugs.

Table 1. Clinical trials on molecular target-based drugs reported at ASCO meetings 2002–2005

Code name	INN	Commercial name	Targeted Molecule	2002	2003	2004	2005
1. Growth factor antibody							
anti-VEGF	Bevacizumab	Avastin	VEGF Ab	CR,R(I-II)	CR(cIII), B(cIII), Pa(cII), Pr(cII), Lu(cII)	Hp(sIII), Pa(cII), R(cII), B(cI), CR(cII)	B(cIII, cII), Lu(cIII/III, cII), CR(cIII, cII), G(cII), Hp(sII,cII), Pa(cII), R(cII), Ov(cII,sII)
VEGF Trap			VEGF1,2 Ab		–(sI)		
2. Growth factor receptor antibody							
IMC-1C11			VEGFR2 Ab	CR(I)			
anti-VEGFR1		Angiozyme	VEGFR1 Ab	–(I)			
ABX-ECF			EGFR Ab	R(I-II)	CR(sII)		
EMD72000	Matuzmab		EGFR Ab	ST(I)	–(sI)	CR(sI), HN(sI), Pa(cI)	
IMC-C225	Cetuximab	Erbitux	EGFR Ab	HN, Lu, CR(II-III)	–(sI/II), CR(cII), Lu(cII)	HN(cIII), Lu(cII), CR(cII), CR(cIII)	CR(cIII,cII),Lu(sII)
rhuMAb2C4	Pertuzumab		Her2 Ab		–(sI)		
3. Growth factor inhibitor							
ABT-526			(VEGF,bFGF,IL-8,HGF) Ihb			My(cII)	
VEGF Trap			VEGF Ihb			–(sI)	
VEGF-antisense	Veglin		VEGF Ihb			–(sI), Hm(sI)	
ABT-510			(VEGF,bFGF,IL-8,HGF) Ihb			–(cI)	S(sII),R(sII)
4. Tyrosine Kinase Receptor Inhibitor							
PKI166/CGP75166			TK(EGFR,erbB2) Ihb	–(I)			
SU6668			TK(VEGFR) Ihb	–(I)			
CP-547632			TK(VEGFR) Ihb	–(I)		Lu(cI)	
STI571	Imatinib	Gleevec	TK(BCR-ABL) Ihb	CML,GIST(I-III)	R(sII), Pr(sII)		
ZD1839	Gefitinib	Iressa	TK(EGFR) Ihb	Lu,R,HN,CR(I-II)	B(sII), R(sII)		
SU5416	Semaxanib		TK(VEGFR) Ihb	AML,Lu,R,Pr(I-II)	M(sII)		
OSI-774	Erlotinib	Tarceva	TK(EGFR) Ihb	–,Lu(I-III)	B(sII/cI),CR(sII/cI),Lu(sII/cI)	Lu(cIII), Lu(sII), Hp(sII), R(sII), CR(cII), B(cI), Pa(cI), GI(sII)	Pa(cIII), Pa(cII), Hp(sII), Lu(sII), B(sII) –(cI)
CI-1033	Canertinib		TK(pan-erbB) Ihb	–(I)	–(sI)	Ov(sII), –(sI)	
PTK787/ZK222584	Vatalanib		TK(VEGFR1,2,3) Ihb	GI,–(I)	GI(sI),CR(sI,cI),AML(sI, cI)	CR(cII)	CR(cIII,cI,cII), Hp(sI), Ov(cI)
ZD6474			TK(VEGFR,EGFR) Ihb	–(I)	–(sI)	Lu(cII)	Lu(cII)
EKB-569			TK(EGFR,Her2) Ihb		Pa(cI)		
CEP-701			TK(FLT3) Ihb		AML(sII)		
BAY43-9006	Sorafenib		TK(Raf Kinase,VEGFR,PDGFR) Ihb		–(sI, cI)	R(sII), Pa(cI/II), –(cI)	R(sIII,sII), HN(sII), Lu(cI), M(cI,sI/II), –(sI)
GW572016	Lapatinib		TK(ErbB1,ErbB2) Ihb		–(sI)	B(cII), –(sI), –(cI)	B(sII,cI), Lu(sII), –(cI)
CP-724714			TK(ErbB2)			B(sI)	
AZD2171			TK(VEGFR) Ihb			–(sI)	–(sI),Pr(sI)
GW786034			TK(VEGFR1,2,3) Ihb			–(sI)	–(sI)
AMG706			TK(VEGFR1,2,3,PDGFR,KIT,RET) Ihb				–(sI)
AEE788			TK(EGFR,Her2,VEGFR) Ihb				–(sI),GI(sI)
CHIR-258			TK(VEGFR,FLT3,EGFR,KIT) Ihb				–(sI)
BIBF1120			TK(VEGFR,PDGFR,KIT,FGFR) Ihb				–(sI)
BMS-354825	Dasatinib		TK(SRC,BCR-ABL,PDGFR,KIT) Ihb				–(sI),GIST(sI)

Table 1. Continued

Code name	INN	Commercial name	Targeted Molecule	2002	2003	2004	2005
5. Inhibitors of downstream signal transduction molecules							
R115777		Zarnestra	Farnesyl transferase Ihb	CR,Pa,Lu,B,Pr(II-III)			
SCH66336	Lonafarnib	Sarasar	Farnesyl transferase Ihb	–(I)			
LY317615			Protein Kinase Cb Ihb	–(I)			
CI-1040			MEK Ihb	–(I)	Lu(sII), B(sII), CR(sII), Pa(sII)		–(sI)
CCI-779	Temsirolimus		mTOR Ihb		R(cI)	R(cI), B(cII)	Lu(cII), B(cII)
RAD001	Everolimus		mTOR Ihb			GIST(cI/II)	–(sI)
AP23573			mTOR Ihb			–(sI)	S(sII), Hm(sII)
PD0325901			MEK Ihb				–(sI/II)
CP-751821			IGF-1R Ihb				My(sI)
6. Inhibitors of cell cycle regulatory proteins							
HMR1275	Alvocidib		CDK Ihb	Lu,CR, –(I)	Lu(cI)		
BMS-387032			CDK2/Cyclin E Ihb		–(sI)		
7. Inhibitors of proteasome							
PS-341	Bortezomib	Velcade	Proteasome Ihb	My, –(I-II)	Lu(sII),B(cI),Hm(cI),Ov(cI)	My(cIII), CR(cII), Hp(sI/II), Lu(cII), Lu(sII), Lu(cI), B(sII), Pr(cI/II)	
8. Inhibitors of chromatin regulator molecules							
CI-994			Histon deacetylase Ihb	Lu,Pa, –(I-II)	Lu(cII)		
MS-275			Histon deacetylase Ihb		–(sI)		
9. Inhibitors of Secretory Proteins etc							
BB-2516	Marimastat		MMP Ihb	CR(II)			
AG3340	Prinomastat		MMP Ihb	Lu,Es(II-III)			
CP-471358			MMP Ihb	–(I)			
rh-angiostatin			HmRcmb angiostatin	–(I)			
rh-endostatin			HmRcmb endostatin	–(I)			
10. Others							
TLK286			Glutathione-S-transferase P1 Ihb		Lu(sII),Ov(sII)		
Celecoxib	Celecoxib	Celebrex	COX-2 Ihb		B(sII-III),Ut(sII),Lu(cII)		
Rituximab	Rituximab	Rituxan	CD20 Ab			Ly(sIII)	

cI,cII,cIII, phase I, II, III trials using combination treatment regimen; sI,sII,sIII, phase I, II, III trials using single treatment regimen; B, breast cancer; CR, colorectal cancer; Es, esophagus cancer; Gl, glioma; Hm, hematological malignancies; HN, head & neck cancer; Hp, hepatic cancer; Lu, lung cancer; Ly,lymphoma; Ml, melanoma; My, myeloma; Ov, ovarian cancer; Pa, pancreatic cancer; Pr, prostate cancer; R, renal cancer; S, sarcoma; Ut, uterine; –, unspecified; Ab, antibody; Ihb, inhibitor; TK, tyrosine kinase; CDK, cycline dependent kinase; MMP, matrix metalloproteinase; HmRcmb, human recombinant

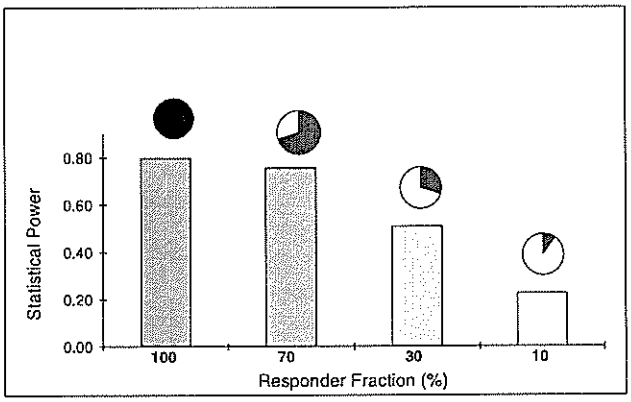

Fig. 2. Relationship between the responder fraction and statistical power: a hypothetical calculation. The 1-year survival rates for the experimental and control arms are assumed to be 20% and 10%, respectively. Exponential distributions are assumed for both treatment arms. Two-year accrual and 1-year follow-up periods are assumed

Problem III: criteria for administration

The problems concerning criteria for drug administration are essentially the same as those discussed above. In the section above, "Problem I: mechanisms of action and drug design," it has been made clear that the significance of target molecules in the pathological state in question, i.e., tumor growth, should be fully understood and established. It is now widely accepted that genomic instability underlies tumorigenesis in various neoplasms. Genomic instability comprises the "mutator phenotype," in which mutation rates in the genome are markedly elevated and mutation occurs in various genes, and the "chromosomal instability," which causes diverse abnormalities in chromosomal number and structure. These structural alterations of the genome frequently lead to the deregulated expression of various genes. Therefore, particularly in cancer, found genetic changes, either in the structure or in the expression status, do not necessarily imply that tumor growth depends on these changes. As discussed above, the fusion gene derived from an abnormal chromosome translocation, *BCR-ABL*, is found in almost 100% of CML patients, and the growth of CML cells is entirely dependent on this chimera gene. Indeed, the regulated expression of BCR-ABL causes leukemia in a model system using transgenic animals.[10] Thus, the rationale for the administration of a tyrosine kinase inhibitor, imatinib, to patients with CML is unquestionable. Indeed, it is known that imatinib treatment of CML shows high response rates. On the other hand, when imatinib is administered to patients with gastrointestinal stromal tumors (GISTs) that express a growth factor tyrosine kinase receptor, c-KIT, are the circumstances the same?

Almost all GISTs express c-KIT. The problem is that c-*kit* mutations in regions including exon 11, which code the transmembrane domain, are reported in GIST.[11] Inheritance of these mutations is known to cause a familial predisposition to GIST.[12] These c-*kit* mutations may alter the tyrosine kinase activity in c-KIT proteins. In sporadic cases of GIST, the frequency of these c-*kit* mutations is reported to be lower than 70%.[7] It may be important to discriminate GISTs depending on abnormally elevated tyrosine kinase activity due to c-*kit* mutations from GISTs that simply express wild-type c-KIT molecules. In fact, it has been reported that clinical outcomes in GIST patients differ widely depending on the mutation status of the c-*kit* gene,[13] which strongly suggests that tumor growth in GISTs with c-*kit* mutations, particularly mutations in exon 11, is highly dependent on elevated tyrosine kinase activity in mutated c-KIT molecules. However, at present, the rationale for the administration of imatinib to patients with GIST is based on the immunohistochemical confirmation of simple c-KIT expression in tumor cells. Interestingly, it was reported at the ASCO 2003 meeting that other neoplasms expressing c-KIT did not respond to imatinib treatment.

There is a similar problem in target-based therapies for non-small-cell lung cancer (NSCLC). Gefitinib (ZD1839; Iressa;) and erlotinib (OSI-774; Tarceva) inhibit the tyrosine kinase activity in epidermal growth factor receptor 1 (EGFR1), which is frequently expressed in various cancers, including NSCLC. These target-based drugs were initially intended to be used for all patients with tumors expressing EGFR. However, it was reported that tumors with *EGFR1* mutations, particularly a 15-bp inframe deletion in exon 19, were more sensitive to gefitinib than those without the mutations.[14,15] However, Hirsch and colleagues[16] reported that *EGFR1* amplification (to be precise, multiplicity in the copy number due to chromosome 7 polysomy or aneuploidy) was more closely related to gefitinib/erlotinib sensitivity. Although several comparative studies have been done,[16–19] there is still a controversy (Table 2). At present, there seems to be a consensus that tumors harboring *EGFR1* mutations are relatively more sensitive to gefitinib/erlotinib, and that tumors with these mutations frequently carry *EGFR1* amplification. The problem is that the tyrosine kinase activity in mutant EGFR1 has not been biochemically determined. Mutant EGFR1 may be less active, and cancer cells may try to compensate for insufficient tyrosine kinase activity to promote cell growth with an increase in the gene copy number, particularly when cells have chromosomal instability. Gefitinib/erlotinib may be more effective in such tumor cells. This may be one possible explanation for the linkage. Needless to say, some tumors may have only one copy of the mutated *EGFR1* gene, and other tumors may carry several copies of wild-type *EGFR1* as a simple reflection of aneuploidy, because they are not dependent on its tyrosine kinase activity. The most important information is whether or not the tumor growth depends on EGFR1 tyrosine kinase activities. The rationales for the administration of these target-based drugs should be based on this information. In anti-EGFR therapies in NSCLC, clinical testing to determine the *EGFR1* gene structures may be regarded as routine in the near future.

There is a similar, but more serious problem with trastuzumab (Herceptin). Trastuzumab is the first humanized monoclonal antibody drug that has been developed as

Table 2. EGFR status and clinical outcomes in patients treated with EGFR tyrosine kinase inhibitors

Authors (year)	Gene copy number			Gene mutation			Protein expression			Reference
	Method	Correlation with response	Correlation with survival	Method/axon	Correlation with response	Correlation with survival	Antibody	Correlation with response	Correlation with survival	
Cappuzzo (2005)	FISH	Yes	Yes	Sequencing /18,19,21	Yes	No	Clone 31G7 monoclonal Ab	Yes	Yes	16
Tsao (2005)	FISH	Yes	Yes	Sequencing /18–21	Yes	No	OakoEGFR PharmOx kite	No	Yes	18
Takano (2005)	Quantitative real-time PCR	Yes	No	Sequencing /18–21	Yes	Yes	Not done	–	–	19
Bell (2005)	Quantitative real-time PCR	No	No	Sequencing /18–21	Yes	No	Not done	–	–	17

FISH, fluorescent in situ hybridization; PCR, polymerase chain reaction; EGFR, epidermal growth factor receptor

a target-based anticancer agent. It reacts with an EGFR family member, ErbB-2/HER2/NEU receptor tyrosine kinase. As with imatinib therapy for GIST, the rationales for the administration of trastuzumab are currently based on the immunohistochemical grading of the HER2/NEU expression level in tumor cells. Efforts have been made to achieve accuracy and reproducibility in the immunohistochemical assays for HER2/NEU expression. However, it is known that response rates are not different between patients with tumors that have different grades of HER2/NEU expression.[20] As in the case of *EGFRI* in NSCLC, the existence of c-*erbB-2*/c-*neu* gene amplification makes this problem more complicated. It has been reported that response rates for trastuzumab treatment are relatively higher in tumors with c-*erbB-2*/c-*neu* gene amplification than in those without this gene amplification.[21] On the other hand, Baselga et al.[20] reported that response rates and time to progression were not different between HER2/NEU-overexpressing tumors and tunors with c-*erbB-2*/c-*neu* gene amplification. Fluorescent in situ hybridization (FISH) performed on tissue specimens is currently used to detect c-*erbB-2*/c-*neu* gene amplification. Interestingly, it is known that tumors with c-*erbB-2*/c-*neu* gene amplification, confirmed by FISH, do not necessarily overexpress HER2/NEU. Apart from technical problems in performing tissue FISH, these observations suggest that the relationship between the expression level of a given gene and changes in its structure, either mutation or amplification, is not simple as has been expected.

What does tumor growth depend on? The answer to this question is the only touchstone with which we can rationalize the administration of target-based anti-cancer drugs. Some tumors may be dependent on target molecules, but others may not. Some genetic changes may reflect the dependence of tumor growth on the elevated activity of target molecules, but others may not. Comprehensive and thorough studies to elucidate the complex relationships among the structures, expression levels, and functions of the gene in question are required. Once these relationships are elucidated, it will be possible to establish a system for accurate and, consequently, efficient clinical testing to support target-based anticancer therapies. However, there seems to be easiness in our approaches to clinical testing for target-based therapies. Needless to say, cost-effectiveness must be also considered. DNA sequencing, which has recently become markedly easier than it used to be, is still expensive and not available at all medical facilities, while immunohistochemistry is relatively inexpensive and widely available. However, once the specific genetic changes on which tumor growth highly depends are found, it may be possible to develop a new technique that is specialized for detecting those changes and, is, consequently, efficient and inexpensive. The development of commercialized custom testing may also improve cost-effectiveness in clinical testing for target-based anticancer therapies.

Problem IV: adverse effects

As stated earlier, all of the physiological functions are not always known in all the molecules regarded as targets. In the category of low-molecular-weight compounds, "inhibitors," molecular targets are disperse, due to the structural similarities in the functional domains between the initially targeted molecules and related proteins. This double-faced out-of-focusing sometimes causes unexpected adverse effects of target-based anticancer agents, markedly delaying clinical trial steps and sometimes leading to the abandonment of development of the agent. This problem may partly explain the kaleidoscopic changes in the variety of anticancer drugs in development, as discussed above (see Table 1). A symbolic example is an MMP inhibitor, marimastat, which was initially developed as an inhibitor of invasive tumor growth. The MMP family regulates physiological cell traffic in fibrous tissue matrix, particularly in inflammation, immune response, bone/cartilage regeneration, and vascularization, as well as regulating pathological processes such as tumor invasion and metastasis. As a result, severe arthralgia and bone pain were frequently observed in patients who received marimastat.[22,23] This unforeseeable (but, in a sense, foreseeable) adverse effect finally led to suspension of the development of this drug. In the case of gefitinib, its crucial side effect, pulmonary fibrosis, raised a social problem, and finally led to the suspension of governmental approval of this drug in the United States.

Problem V: verification processes

When a new therapeutic approach has been introduced, it appears to be important to examine the currently available clinical results retrospectively, in order to establish its significance. The first-generation target-based anticancer drugs, i.e., imatinib, gefitinib, and trastuzumab, are now regarded as established. As shown by the data in Table 1, it is clear that combined therapies using these drugs and conventional antineoplastic agents are now frequently being tested. However, with these first-generation drugs, the overall response rates are not necessarily as high as initially expected. Comprehensive and thorough studies of the gene structure and the expression status, using clinically obtained materials, e.g., tumor tissue specimens, may elucidate specific changes in target molecules which chiefly promote tumor growth and, consequently, strongly predict response to the administered drugs. In fact, such studies have already been carried out in NSCLC patients treated with gefitinib/erlotinib.[16–19] However, the conclusions of the studies differ widely (Table 2). Needless to say, careful processing of the statistical data is essential. In addition, it is also essential to elucidate the qualitative (not biological but biochemical, i.e., enzymological) differences among mutated EGFR1 proteins and wild-type ones, which have not, thus far, been addressed. The confusion in this field may be partly due to a lack of this information. Such basic studies are also required to be carried out in parallel with the re-examination of the clinical data. Similar problems have also been raised for imatinib, particularly for GIST, and trastuzumab.

All of the methodological problems discussed above converge on the following two problems: (1) information about the target molecules is never complete (Fig. 1A), and (2) drugs never target only the target molecules (Fig. 1B,C). The former problem derives from the methodological limits of molecular biology and biochemistry, i.e., elementalism, and the latter from the methodological limits in our techniques for drug development. These limits are currently inevitable. However, elaborative and careful verification of the clinically obtained data, if supported by precise and sensitive analyses, may provide us with important information concerning criteria for drug administration and, consequently, improve the efficacy of treatment. Precise and efficient detection of responder populations is the key to the development and establishment of target-based anticancer therapies.

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Preclinical study

Aberrant hypermethylation of the promoter region of the *CHFR* gene is rare in primary breast cancer

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Key words: aberrant hypermethylation, breast cancer, CFHR, methylation-specific PCR, taxane

Summary

Taxanes are among the most active agents and they are now known to be an indispensable component in chemotherapy for breast cancer. However, some patients are resistant to taxanes and the identification of the molecular characteristics that can predict the sensitivity to taxanes would be useful in selecting the most appropriate patients to receive taxane therapy. Taxanes are antimicrotubular agents that promote microtubular assembly and stabilize microtubules by preventing depolymerization. They interfere with normal mitotic transition and causes cell cycle arrest in the G2-M metaphase. *CHFR* (checkpoint with forkhead-associated and ring finger) is a recently identified gene, which functions as an important checkpoint protein early in G2-M transition. Its activation delays the cell cycle in prophase and promotes cell survival in response to the mitotic stress induced by either nocodazole or taxane. CHFR is frequently downregulated in human cancers, mostly owing to the hypermethylation of its promoter region. CHFR downregulation has been found in primary cancers or in the established tumor cells of various origins, such as the lung, colon, esophagus, and stomach. The aberrant hypermethylation of *CHFR* promoter appears to be a good molecular marker to predict sensitivity to taxanes in gastric, lung, and colon cancer. A downregulation of CHFR was observed in breast cancer cells, however, no apparent promoter hypermethylation has yet been reported. In addition, an alteration of the CHFR expression or aberrant promoter hypermethylation in primary breast cancer has not been fully investigated. In this study, we examined the methylation status of the promoter region of *CHFR* gene in 110 primary breast cancers. We observed the hypermethylation of the *CHFR* promoter region in only one case (0.9%). We herein show that the aberrant hypermethylation of this region is quite a rare event in primary breast carcinoma.

Introduction

Taxanes, such as paclitaxel and docetaxel, are among the most active agents and they are known to be an indispensable component in chemotherapy for breast cancer. In addition to the regimens for metastatic breast cancer, their incorporation into regimens for early breast cancer is increasing in both neoadjuvant and adjuvant settings in the clinical treatment of breast cancer [1,2]. However, some patients are resistant to taxanes and the predictive factors regarding the sensitivity to taxane have not yet been well defined. The identification of the molecular characteristics to predict the sensitivity or resistance to taxane would be useful for selecting the most appropriate patients to receive taxane therapy.

So far, several studies have tried to identify the predictive factors that can help to determine sensitivity to taxane. Several mechanisms have been attributed to resistance to taxane; namely, the up-regulation of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL [3], the up-regulation of membrane transporters [4], mutations in β-tubulin [5] and the up-regulation of HER2 [6]. However, these factors have not yet been established as predictive factors for taxane sensitivity in clinical use.

Taxanes are antimicrotubular agents that promote a microtubular assembly from tubulin dimers and stabilize microtubules by preventing depolymerization, thereby interfering with normal mitotic transition. Because the microtubule dynamics are particularly critical during mitosis, the disruption of the microtubule dynamics by taxanes causes the cell cycle progression of cells to be arrested in the G2-M metaphase [7]. Due to this mechanism, the factors related to the spindle assembly checkpoint, such as Mad2, BubR1 or p34cdc2,

have been reported to play an important role in taxane sensitivity [8,9]. However, mutations of these mitotic spindle checkpoint genes, including hsMAD2, hBUB1 and hBUB3, are rare [10–13].

CHFR (checkpoint with forkhead-associated and ring finger) is a recently identified gene, which is localized to chromosome 12q24.33 [14]. CHFR functions as an important checkpoint protein early in the G2/M transition and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to the mitotic stress induced by nocodazole or paclitaxel [14]. In addition, CHFR promotes cell survival in response to mitotic stress. CHFR is ubiquitously expressed in normal tissues, however, it is frequently downregulated in human cancers, mostly due to the hypermethylation of its CpG island in the promoter region [15–22]. CHFR downregulation has been found in primary lung, colon, esophagus, nasopharyngeal and gastric carcinomas and tumor cells of lung, colon, esophageal, brain, bone, gastric, nasopharyngeal and hematopoietic origin [14–22]. The aberrant hypermethylation of the *CHFR* promoter has been reported to be a good molecular marker for predicting sensitivity to microtubule inhibitors such as docetaxel and paclitaxel in colon, lung and gastric cancer [16,18,19]. In terms of breast cancer, the downregulation of CHFR was observed in breast cancer cells, however, no apparent promoter hypermethylation has not been reported [15,16]. In addition, the alteration of the CHFR expression or aberrant promoter hypermethylation in primary breast cancer has not yet been fully investigated. These facts prompted us to further investigate whether alterations of CHFR occur in primary breast cancer. In this study, we examined the methylation status of the promoter region of the *CHFR* gene in 110 primary breast cancers. We observed the hypermethylation of *CHFR* promoter region in only one case (0.9%). We herein show that the aberrant hypermethylation of this region is quite a rare incidence in primary breast carcinoma.

Materials and methods

Specimens and extraction of genomic DNA

One hundred and ten primary breast carcinomas and paired normal tissue specimens were obtained from Japanese patients who underwent surgery at Department of Surgery and Science, Kyushu University Hospital, from 1994 to 2002. Informed consent was obtained from all patients prior to tissue acquisition. Immediately after resection, the specimens were placed in liquid nitrogen and then were used for analyses of genomic DNA. The remaining tissue specimens were routinely processed for histopathological analyses by histopathological specialists in our hospital. The histopathological diagnosis was determined according to the criteria of the Japanese Breast Cancer Society [23].

Frozen tissue specimens were broken up in liquid nitrogen and lysed in digestion buffer (10 mM Tris–Cl; pH 8.0, 0.1 M EDTA; pH 8.0, 0.5% SDS, 20 μg/ml pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then was dissolved in 1 × TE (10 mM Tris–Cl; pH 7.5, 1 mM EDTA).

Methylation analysis

Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit™ (ZYMO RESEARCH, Orange CA, USA), which integrates DNA denaturation and bisulfite conversion processes into a single step followed by rapid in-column desulphonation and DNA clean-up, according to the manufacturer's instructions. Methylation-specific PCR (MSP) was carried out with the following oligonucleotide primers, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite conversion as described above. Methylated DNA-specific primers were MF1 (forward; 5′-ATATAATATGG CGTCGATC) and MR1 (reverse; 5′-TCAACTAATCC GCGAAACG). Unmethylated DNA-specific primers were UF1 (forward; 5′-ATATAATATGGTGTTG-ATT) and UR1 (reverse; 5′-TCAACTAATCCACAAA ACA) [18]. PCR amplification consisted of 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min (MF1 and MR1); and 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min (UF1 and UR1). The resultant PCR products were separated on 2% agarose gel. CpGenome™ Universal Methylated DNA (CHEMICON INTERNATIONAL, Temecula, CA, USA), which is enzymatically methylated human male genomic DNA, was used as a positive control for methylation specific PCR. Purified genomic DNA isolated from the human placenta (BioChain Institute Inc. Hayward CA, USA) was used as a negative control for non-mehylated DNA. All analyses included positive and negative controls were performed at least twice.

Results

Identification of aberrant hypermethylation of the promoter region of the CHFR gene

We investigated whether the aberrant promoter hypermethylation of the *CHFR* gene was present in primary breast cancer specimens based on methylation-specific PCR (MSP). We investigated the specimens of 110 primary breast cancer cases in this study. We analyzed the methylation status of the promoter region of the *CHFR* gene, not only in genomic DNA from cancer tissue specimens, but in the genomic DNA from paired normal tissue specimens for all cases. Almost all cases were

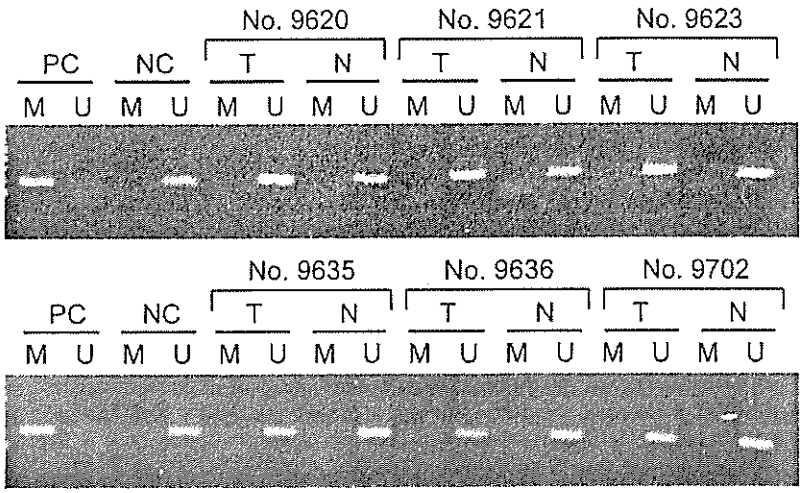

Figure 1. Methylation of the promoter region of the *CHFR* gene analyzed by MSP in primary breast cancers. No *CHFR* methylation was present in these cases. The U lane represents the amplification of unmethylated alleles, while the M lanes represents that of methylated alleles. CpGenome™ Universal Methylated DNA, which is enzymatically methylated human male genomic DNA, was used as a positive control (PC) and purified genomic DNA isolated form human placenta was used as a negative control (NC) as non-mehylated DNA. T: tumor, N: normal.

negative for aberrant promoter hypermethylation of the *CHFR* promoter (Figure 1). The aberrant promoter hypermethylation was found in only one case of breast cancer that we analyzed (0.9%) (Figure 2). This patient had undergone surgery for colonic carcinoma 1 year before the breast cancer, and she died due to cholangiocarcicona 1 year after the breast surgery. In addition, her siblings also had colorectal carcinoma and many episodes of other cancers, including laryngeal or uterine carcinomas, had occurred in this pedigree. She seems to belong the hereditary non-polyposis colorectal carcinoma (HNPCC) family. We had already analyzed macrosatellite alterations in this case. Intriguingly, this case showed drastic macrosatellite instability as previously reported [24].

As a result, the aberrant hypermethylation of the *CHFR* promoter was found to be quite a rare event in primary breast carcinomas.

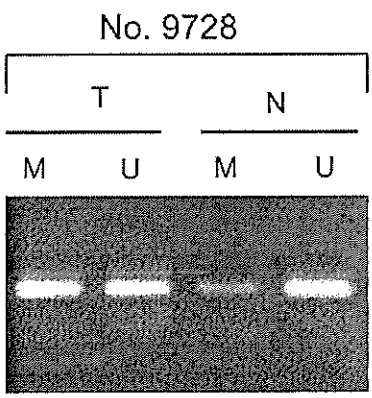

Figure 2. A positive case showing the methylation of the promoter region in the *CHFR* gene in primary breast cancer. *CHFR* methylation was observed in only this one case out of the 110 cases of primary breast cancer that we investigated. PC: positive control, NC: negative control, T: tumor, N: normal.

Discussion

CHFR encodes a protein with FHA and RING finger domains that plays a role in the mitotic checkpoint pathway which regulates the transition from prophase to prometaphase [14]. While normal human cells that express CHFR show a delayed start of mitosis in the presence of mitotic stress induced by microtubule-disrupting agents such as nocodazole and paclitaxel, cancer cell lines lacking CHFR enter metaphase prematurely [14,19,22]. The epigenetic inactivation of *CHFR* is frequently observed in human tumors [22], and the methylation of CpG islands in promoter region seems to be correlated with *CHFR* silencing in several human cancer cell lines and primary tumors, although the incidence varies among those malignancies (Table 1). The incidence of the aberrant hypermethylation of the promoter region of *CHFR* gene was 0–100% in established cancer cell lines and 10–60% in primary cancers [15,16,18–20,25] (Table 1). However, the aberrant hypermethylation of the promoter region of *CHFR* gene has not yet been reported in primary breast cancer. Previous reports have suggested that the aberrant hypermethylation of *CHFR* promoter could be utilized as a predictive factor of the sensitivity to taxanes in the colon, lung and gastric cancer [16,18,19]. Erson and Petty reported that a low CHFR expression was associated with high mitotic indices in response to nocodazole treatment in the breast cancer cell lines, and that the transfection of CHFR in one of these cancer cell lines lowered the mitotic indices after nocodazole treatment [26]. These findings encouraged us to investigate the methylation status of the *CHFR* gene in primary breast cancer, in which therapy taxanes are among the most important agents. As far as we know, this is the first report to investigate the aberrant hypermethylation

Table 1. Reported incidence regarding the aberrant hypermethylation of the promoter region in the *CHFR* gene

Organ	Incidence	Reference
Cell lines		
Lung	25% (4/16)	[18]
Colon	80% (4/5)	[16]
Colon	43% (9/21)	[15]
Esophagus	0% (0/2)	[16]
Esophagus	27% (4/15)	[20]
Stomach	20% (4/20)	[19]
Stomach	20% (2/10)	[28]
Stomach	67% (8/12)	[29]
Brain	100% (2/2)	[16]
Bone	100% (2/2)	[16]
Leukemia	50% (1/2)	[16]
Prostate	0% (0/1)	[16]
Breast	0% (0/2)	[16]
Breast	0% (0/19)	[15]
Primary cancer		
Lung	19% (7/37)	[18]
Lung	10% (2//20)	[16]
Colon	37% (11/30)	[16]
Colon	36% (8/22)	[15]
Esophagus	16% (7/43)	[20]
Nasopharynx	61% (22 /36)	[25]
Esophagus	16% (7/43)	[20]
Stomach	39% (24/61)	[19]
Stomach	35% (25/71)	[28]
Stomach	44% (19/43)	[29]
Breast	0.9% (1/110)	This study

of the *CHFR* gene promoter in primary breast carcinomas.

We utilized specimens from 110 cases of primary breast carcinomas, including both tumor tissue and paired normal tissue in all cases in this study. This number of the cases is considered to be sufficient to understand the tendency of the methylation status of the *CHFR* promoter compared with previous reports dealing with other cancers. We observed an aberrant hypermethylation of the promoter region of *CHFR* gene in only one case (0.9%). As a result, the aberrant hypermethylation was thus found to be quite a rare event in primary breast cancer. We also evaluated the methylation status of the *CHFR* promoter in primary gastric cancer using the same method. The incidence of the aberrant promoter hypermethylation was thus found to be 33% (21 of 63 cases) in primary gastric carcinomas (Figure 3). This result is consistent with the findings of the previous reports and it suggests that our method was both appropriate and accurate.

Intriguingly only the one case that revealed the hypermethylation of the *CHFR* promoter region showed the microsatellite instability (MIN) phenotype. This case revealed MIN at all five loci examined and the clinical features of this case thus suggested that this case could be included in the high-risk group for cancers and it may

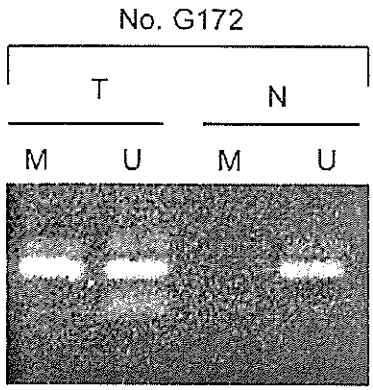

Figure 3. Represenative positive case of the methylation of the promoter region of the *CHFR* gene in primary gastric cancer. *CHFR* methylation was observed in 33% (21/63) of primary gastric cancers. PC: positive control, NC: negative control, T: tumor, N: normal.

be categorized into HNPCC as previously reported [24]. Recent reports suggest the MIN phenotype to be associated with the hypermethylation of the *CHFR* promoter [15,27]. These reports also support our findings.

In conclusion, the aberrant hypermethylation of the promoter region in the *CHFR* gene is a rare alteration in primary breast cancer, and the methylation status of the *CHFR* gene cannot be used as predictive factor of taxane sensitivity in primary breast cancer.

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REVIEW ARTICLE

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Trastuzumab and breast cancer: developments and current status

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Abstract The emergence of trastuzumab has drastically changed therapy for breast cancer. Trastuzumab (Herceptin; Genentech) is a recombinant humanized monoclonal antibody that targets an epitope in the extracellular domain of the human epidermal growth factor receptor 2 (HER2) protein. HER2 is a member of a family of four transmembrane receptor tyrosine kinases that regulate cell growth, survival, and differentiation via multiple signal transduction pathways. Overexpression of HER2 or amplification of the *HER2* gene occurs in 20%–30% of human breast cancers. Preclinical models have demonstrated that this antibody has significant antitumor activity as a single agent, and it also has a synergy with certain chemotherapeutic drugs. Phase II and III clinical trials performed in women with metastatic breast cancers that overexpress HER2 have shown trastuzumab to have clinical activity when used as monotherapy, while also improving survival when used as a first-line therapy in combination with chemotherapy. At present, clinical investigations are focusing attention on the efficacy of trastuzumab in both the adjuvant and neoadjuvant setting, as well as in the metastatic setting. In this review, we describe the developments and current status of trastuzumab-based treatment for breast cancer.

Key words Trastuzumab · HER2 · Breast cancer · Molecular-targeted therapy

Introduction

The human epidermal growth factor receptor 2 (*HER2*) gene encodes a 185-kd transmembrane receptor tyrosine kinase which plays an important role in cell growth, differentiation, and survival.[1] Overexpression of the HER2 protein, amplification of the *HER2* gene, or both, occur in about 20% to 30% of all human breast cancers, and these phenomena are also associated with a poor prognosis or aggressive behavior of the tumor,[2,3] and with the relative resistance to some types of cytotoxic and endocrine therapy.[4–7] Since HER2 was discovered in the late 1980s, many researchers have conducted investigations to generate treatments targeting this receptor.

Trastuzumab (Herceptin; Genentech, South San Francisco, CA, USA) is a humanized monoclonal antibody which recognizes the extracellular domain (ECD) of HER2. Trastuzumab has been shown to benefit patients with HER2-positive metastatic breast cancer, alone [8] or in combination with chemotherapy.[9] In this review, we describe the development and current status of trastuzumab-based treatment for breast cancer.

Trastuzumab

Several murine monoclonal antibodies against the extracellular domain (ECD) of the HER2 protein have been found to inhibit the proliferation of human cancer cells that overexpress HER2, both in vitro and in vivo.[10–12] Trastuzumab is a recombinant monoclonal antibody that has been humanized to minimize the immunogenicity associated with murine monoclonal antibodies, while maximizing the potential for recruiting endogenous immune effector cells.[13] To date, trastuzumab is the only HER2-targeted therapy approved by the United States Food and Drug Administration (FDA) for the treatment of breast cancer.

Mechanisms of trastuzumab action

The mechanisms by which trastuzumab induces the regression of tumors with HER2 overexpression have not yet

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