Table 2 Prediction of participation or declining to trials

	Univariate analysis[a]		Multivariate analysis[b]	
	Odds ratio (95% CI)	***P*-value**	**Odds ratio (95% CI)**	***P*-value**
Gender (male *vs* female)	1.008 (0.586–1.733)	0.977	0.646 (0.300–1.391)	0.264
Age (<60 *vs* ⩾60)	0.735 (0.432–1.250)	0.254	0.701 (0.376–1.310)	0.266
Smoking history (+ *vs* −)	1.394 (0.815–2.386)	0.225	2.538 (1.162–5.541)	0.019
Clinical stage (III *vs* IV)	0.608 (0.339–1.089)	0.093	0.681 (0.346–1.340)	0.266
PS (0 *vs* 1)	1.398 (0.792–2.467)	0.247	0.785 (0.396–1.554)	0.487
Physicians (A–E)		<0.001		<0.001

Abbreviations: NP = non-participant; P = participant; PS = performance status; ROD = rate of declining. [a]By Pearson's χ^2-test. [b]By logistic regression analysis.

Table 3 Number of courses of the first-line chemotherapy

	Clinical trial 1		Clinical trial 2		
	Participants	**Non-participants**	**Participants**	**Non-participants**	***P*-value**
	100	16	96	57	
First-line cycles					
1	10 (10%)	4 (25%)	6 (12%)	4 (9%)	0.418[a]
2	18 (18%)	4 (25%)	8 (16%)	12 (27%)	
3	37 (37%)	7 (44%)	5 (10%)	9 (20%)	
⩾4	35 (35%)	1 (6%)	30 (61%)	20 (44%)	
Gefitinib median duration (day)			73	99	0.118[b]
Range			13–752	34–1065	
IQR			29–204	38.5–512	

Abbreviation: IQR = interquartile range. [a]By Pearson's χ^2-test. [b]By log rank test.

smoking history, tumour histology, clinical stage or PS was observed (Table 2). There were, however, large differences in the rates of decline among the attending physicians who informed the patients about the trials and asked them to participate ($P<0.001$).

The treatment regimens for those who declined participation in the clinical trials were as follows. The majority of those who declined participation in Trial 1 selected one of the four platinum-based combination regimens presented in the trial: cisplatin–irinotecan 4, cisplatin–vinorelbine 3, cisplatin–gemcitabine 1, carboplatin–paclitaxel 4. Three patients in Trial 1 desired to have no more active treatments and opted for supportive care only, but later received active treatment at their referred hospitals. The detail of their therapy is unknown.

The majority of those who declined participation in Trial 2 selected carboplatin-based combination chemotherapy: carboplatin–paclitaxel 34 and carboplatin–gemcitabine 11, there by reflecting the shift to carboplatin for advanced NSCLC in Japan at the time of Trial 2, on the basis of the reports on the activity of the carboplatin-based regimens (Kelly *et al*, 2001; Schiller *et al*, 2002; Ohe *et al*, 2007). Twelve patients (21%) selected gefitinib as first-line chemotherapy.

Survival was analysed for all of the 196 participants and 76 of the non-participants. Post-therapy was analysed for all of the 196 participants and 73 of the non-participants, who were treated at our centre. There was one possible treatment-related death due to perforation of the colon during gefitinib treatment in Trial 2. No other toxic deaths were observed among either participants or non-participants. More participants of both the clinical trials were given four cycles or more of the first-line chemotherapy, probably reflecting protocol regulations (Table 3).

Table 4 summarises the treatment after the initial therapy. There were no significant differences between participants and non-participants in the number of chemotherapy regimens. Six (8%) of those who declined participation in the trial later participated in early-phase clinical trials of experimental therapies.

Table 4 Treatment after the first-line chemotherapy

	Participants 196 (%)	**Non-participants 73 (%)**	***P*-value[a]**
Chemotherapy regimen			
0[b]	26	40	0.108
1	38	26	
2	22	25	
3	9	8	
>4	5	1	
Radiotherapy	49	34	0.031
Pleural or pericardial drainage	10	5	0.227
Operation on metastatic brain tumors	1	3	0.122
Early-phase trials	13	8	0.300

[a]By Pearson's χ^2-test. [b]Patients received first-line chemotherapy only.

We have observed no clinically relevant differences in the clinical outcomes between participants and non-participants (Table 5). Clinical response to the initial therapy was analysed for all of the 196 participants and 73 of the non-participants, excluding three patients who were not treated at our institute. The response rate was 30.6% in participants and 34.2% in non-participants ($P=0.325$). The median follow-up time at our centre was 388 days for participants and 406 days for non-participants, which was not statistically different.

The OS was not different between participants and non-participants (Table 5 and Figure 1), with a hazard ratio of participants *vs* non-participants of 0.998 (95% confidence interval: 0.76–1.32). No significant difference in OS was observed either in Trial 1 (Figure 2) or in Trial 2 (Figure 3).

Table 5 Clinical outcomes

	Clinical trial 1		Clinical trial 2		Total		
	Participants	**Non-participants**	**Participants**	**Non-participants**	**Participants**	**Non-participants**	***P*-value**
Response rate (%)[a]	29 (29/100)	12.5 (2/16)	32.3 (31/96)	40 (23/57)	30.6 (60/196)	34.2 (25/73)	0.569[b]
Median follow-up time (day)	329	339	493	444	388	406	0.846[c]
Range	45–2704	1–2176	36–2036	22–1688	36–2704	1–2176	
IQR	177–665	59–582	213–861	175–658	197–742	146–604	
Median survival time (day)	416	408	573	519	489	461	0.987[c]
Range	34–2704	53–2380	40–2036	35–1688	34–2704	35–2380	
IQR	264–815	140–698	251–938	276–1012	259–863	229–774	
1-year survival (%)	56.0	63.2	65.6	64.9	60.7	64.5	0.567[b]
2-year survival (%)	29.4	21.1	38.5	29.8	33.9	27.6	0.379[b]

Abbreviation: IQR = interquartile range. [a]Excluding three patients who did not receive active treatment at our center. [b]By Pearson's χ^2-test. [c]By log rank test.

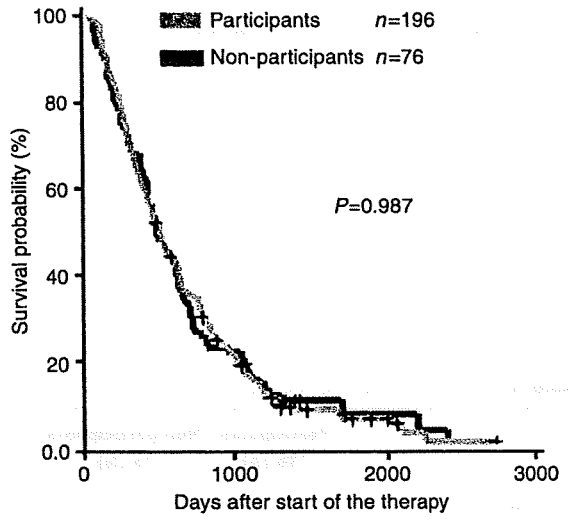

Figure 1 Overall survival of those who declined to participate in randomised trials (blue line, $n = 76$) as compared with the participants (pink line, $n = 196$). No significant difference can be observed.

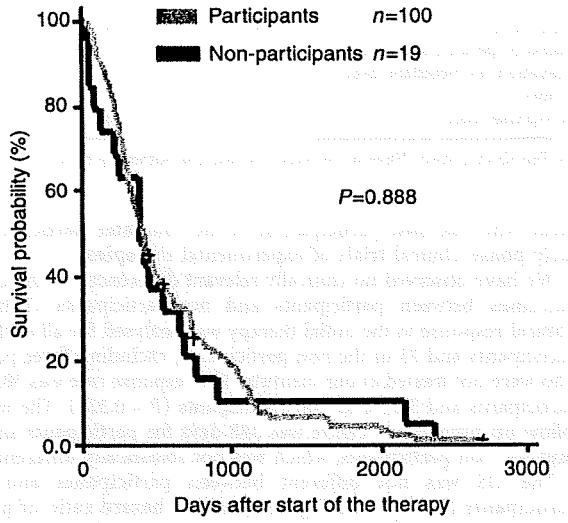

Figure 2 Overall survival of those who declined to participate in Trial 1 (blue line, $n = 19$) as compared with the participants (pink line, $n = 100$). No significant difference can be observed.

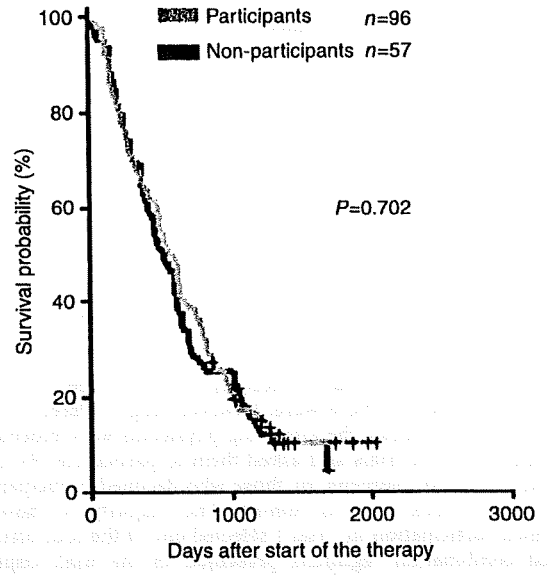

Figure 3 Overall survival of those who declined to participate in Trial 2 (blue line, $n = 57$) as compared with the participants (pink line, $n = 96$). No significant difference can be observed.

With the Cox proportional hazards model adjusted for gender, age, smoking history, clinical stage and PS, the hazard ratio of participants *vs* non-participants was 0.965 (95% confidence interval: 0.73–1.28, $P = 0.805$). Among the patient characteristics, PS was the only significant factor associated with OS in multivariate analysis ($P = 0.006$, by Cox proportional model).

DISCUSSION

It has been argued that trial participants have better outcomes than those who are not enroled in clinical trials. Several investigations have reported a favourable overall trend with trial entry (Braunholtz *et al*, 2001; Peppercorn *et al*, 2004; West *et al*, 2005). This 'trial effect' could derive from several factors, such as protocol effect (the way treatments are delivered), care effect (extra care related to data gathering), Hawthorne effect (changes in doctor or patient behaviour on the basis of the knowledge that they are under observation) or placebo effect (psychologically mediated benefits) (Braunholtz *et al*, 2001; Peppercorn *et al*, 2004).

In majority of the reports comparing outcomes between participants and non-participants of clinical trials, however, the

non-participant 'controls' were chosen from differently pooled database, which could include baseline imbalances between groups and hindsight bias (Davis *et al*, 1985; Braunholtz *et al*, 2001; Peppercorn *et al*, 2004). In this study, we compared the characteristics and outcomes of those who met the eligibility criteria but declined to participate in randomised trials, and instead chose to receive standard therapy. We thus aimed at excluding confounding factors as much as possible.

On the other hand, physician triage is pointed out to be one of the barriers to cancer clinical trial accrual (Lara *et al*, 2001; Corrie *et al*, 2003; Go *et al*, 2006; Ho *et al*, 2006). We excluded the barrier by making it a rule to offer clinical trials to every patient with advanced NSCLC who satisfied the eligibility criteria.

The response rate, MST, 1-year and 2-year survival rates were all similar in both groups. We have to admit that response evaluation might not be as strict in off-protocol therapy. However, the hazard ratio for the OS was very close to 1. Although the confidence interval of 0.73 to 1.28 could not rule out the existence of clinically important difference in the treatment effect, it could not by any means be taken as a clinically relevant prognostic factor. We thus believe this confidence interval of the adjusted hazard ratio, 0.73–1.28, was narrow enough to justify the conclusion that the clinical outcomes of trial participants and non-participants were not different in our study. The differences in the number of cycles of chemotherapy given to participants and non-participants may suggest the so-called protocol effect (Braunholtz *et al*, 2001; Peppercorn *et al*, 2004), in which explicit careful description of treatment regimens could lead to improvement of outcomes. On the other hand, there clearly existed no 'care effect' representing the differences in incidental aspects of treatment or care between participants and non-participants, which the protocol may require, such as extra follow-up or extra nursing care (Braunholtz *et al*, 2001; Peppercorn *et al*, 2004). In our cases, the same treatment teams took charge of and followed both groups of patients in the same manner, and found no differences in the post-treatment characteristics or follow-up periods. Thus, our first finding was that the clinical trials themselves seemed to have no influence on the outcomes or pattern of care of the patients.

The second finding was that we could not find any demographic characteristics to influence the patients' willingness to participate in clinical trials. Taken together with the first finding, both the characteristics and outcomes of the non-participants were very similar to the participants. This would imply that the participants ably represented the whole patient population of the disease status who met the eligibility criteria, and that conclusions from the clinical trials could be generalised.

Our study, however, could only show the similarity in the prognosis of the participants and non-participants, and, unlike an earlier report (Link *et al*, 1986), not that of the treatment effect itself. This could not be evaluated because there were no significant differences in the clinical effect between the arms in both Trial 1 and Trial 2. If newer, much more effective experimental treatment were presented in the trials, the outcome could be better in trial participants, which was the case in the adjuvant chemotherapy trial for osteosarcoma (Link *et al*, 1986). In that report, eligible patients who declined randomisation, but were given adjuvant chemotherapy, also had better outcomes. Therefore, a very effective treatment could lead to a better outcome both on and off trial. Ideally, strict comparison of the effects of the study participation itself would require randomised design of the trial participation (Braunholtz *et al*, 2001; Peppercorn *et al*, 2004), which is almost impossible to conduct.

Thirdly, the declining rate seemed to be influenced by the trial design. Trial 1 was the comparison of four similar platinum-doublet regimens. On the other hand, Trial 2 was the comparison of two arms with sequentially different types of chemotherapy. In general, people might have the impression that injection therapy would be more effective, and less convenient, than oral administration. It is easy to understand that more patients felt difficulty in accepting the randomisation of different types of therapy, such as Trial 2 (Schmoor *et al*, 1996; Jenkins and Fallowfield, 2000).

The declining rate also seemed to be greatly affected by the attending physician. The attending physician with longer experience as a thoracic oncologist tended to have lower rate of declination. Even though we do not have records on who actually informed the participants regarding the trial, residents or trainees under Physician A seemed to have had more chance to lead the consultation, which might have affected the rate of declination. Trust in the doctor is one of the most important reasons for agreeing to enter an RCT, whereas it has also been cited as the main reason for declining to participate (Jenkins and Fallowfield, 2000; Ellis *et al*, 2001; Stryker *et al*, 2006). Patients prefer the doctor to make the treatment decisions rather than to be randomised. A recent report emphasises the influence of physicians' clinical communication on patients' decision-making on participation in clinical trials (Albrecht *et al*, 2008). Improving communication and more interventions by clinical research coordinators and other medical staff members in all eligible patients may improve the accrual rate (Fallowfield *et al*, 1998; Wright *et al*, 2004; Stryker *et al*, 2006).

Finally, it was interesting to find that 8% of those who declined the RCTs participated in early-phase trials during follow-up. It is possible that the lack of effective therapies had changed their recognition of clinical trials. However, it might support the psychological states of patients as reported in earlier studies (Jenkins and Fallowfield, 2000; Ellis *et al*, 2001; Wright *et al*, 2004); patients expect experimental therapies to give them improved effectiveness but with fear of uncertainty. They are reported to have negative opinions regarding the principle of randomisation. Better understanding of the patients' decision-making process and the factors influencing their psychological states may lead to improvement in RCT accrual.

Our study has several limitations. One is that it was conducted at a single academic institution; the situation might well have been different in others or when the research was performed on a multi-institution basis. The second is that we analysed data from only two trials and could not definitely conclude that a trial design would affect the patient accrual. Third, we have no data on the reasons for patient participation. That information would be definitely useful for analysing factors for consent or declining to participate, and would help to improve the accrual rate. Further research is required.

In conclusion, there was no evidence of any difference in the response rates and survival times between participants and non-participants. The declining rate of clinical trials was influenced by the referring physicians and trial designs. Further analysis of the decision-making process of those offered trials is warranted, for it may improve patient accrual to RCTs.

REFERENCES

Albrecht TL, Eggly SS, Gleason MEJ, Harper FWK, Foster TS, Peterson AM, Orom H, Penner LA, Ruckdeschel JC (2008) Influence of clinical communication on patients' decision making on participation in clinical trials. *J Clin Oncol* 26: 2666–2673

Braunholtz DA, Edwards SJL, Lilford RJ (2001) Are randomized clinical trials good for us (in the short term)? Evidence for a 'trial effect'. *J Clin Epidemiol* 54: 217–224

Burgers JA, Arance A, Ashcroft L, Hodgetts J, Lomax L, Thatcher N (2002) Identical chemotherapy schedules given on and off trial protocol in small cell lung cancer response and survival results. *Br J Cancer* 87: 562–566

Corrie P, Shaw J, Harris R (2003) Rate limiting factors in recruitment of patients to clinical trials in cancer research: descriptive study. *BMJ* 327: 320–321

Davis S, Wright P, Schulman SF, Hill LD, Pinkham RD, Johnson LP, Jones TW, Kellogg HB, Radke HM, Sikkema WW, Jolly PC, Hammar SP (1985) Participants in prospective, randomized clinical trials for resected non-small cell lung cancer have improved survival compared with nonparticipants in such trials. *Cancer* **56**: 1710–1718

Ellis PM, Butow PN, Tattersall MHN, Dunn SM, Houssami N (2001) Randomized clinical trials in oncology: understanding and attitudes predict willingness to participate. *J Clin Oncol* **19**: 3554–3561

Fallowfield LJ, Jenkins V, Brennan C, Sawtell M, Moynihan C, Souhami RL (1998) Attitudes of patients to randomised clinical trials of cancer therapy. *Eur J Cancer* **34**: 1554–1559

Go RS, Frisby KA, Lee JA, Mathiason MA, Meyer CM, Ostern JL, Walther SM, Schroeder JE, Meyer LA, Umberger KE (2006) Clinical trial accrual among new cancer patients at a community-based cancer center. *Cancer* **106**: 426–433

Ho J, Pond GR, Newman C, Maclean M, Chen EX, Oza AM, Siu LL (2006) Barriers in phase I cancer clinical trials referrals and enrollment: five-year experience at the Princess Margaret Hospital. *BMC Cancer* **6**: 263

Jenkins V, Fallowfield L (2000) Reasons for accepting or declining to participate in randomized clinical trials for cancer therapy. *Br J Cancer* **82**: 1783–1788

Kelly K, Crowley J, Bunn PA, Presant CA, Grevstad PK, Moinpour CM, Ramsey SD, Wozniak AJ, Weiss GR, Moore DF, Israel VK, Livingston RB, Gandara DR (2001) Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non-small cell lung cancer: a Southwest Oncology Group Trial. *J Clin Oncol* **19**: 3210–3218

Lara PN, Higdon R, Lim N, Kwan K, Tanaka M, Lau DHM, Wun T, Welborn J, Meyers FJ, Christensen S, O'Donnell R, Richman C, Scudder SA, Tuscano J, Gandara DR, Lam KS (2001) Prospective evaluation of cancer clinical trial accrual patterns: identifying potential barriers to enrollment. *J Clin Oncol* **19**: 1728–1733

Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, Pritchard J, Malpas JS, Baker AR, Kirkpatrick JA, Ayala AG, Shuster JJ, Abelson HT, Simone JV, Vietti TJ (1986) The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. *N Engl J Med* **314**: 1600–1606

Madsen SM, Holm S, Davidsen B, Munkholm P, Schlichting P, Riis P (2000) Ethical aspects of clinical trials: the attitudes of participants in two non-cancer trials. *J Intern Med* **248**: 463–474

Madsen SM, Mirza MR, Holm S, Hilsted KL, Kampmann K, Riis P (2002) Attitudes towards clinical research amongst participants and nonparticipants. *J Intern Med* **251**: 156–168

Nokihara H, Ohe Y, Yamada K, Kawaishi M, Kato T, Yamamoto N, Sekine I, Kunitoh H, Saijo N, Tamura T (2008) Randomized phase II study of sequential carboplatin/paclitaxel (CP) and gefitinib (G) in chemotherapy-naïve patients with advanced non-small-cell lung cancer (NSCLC): final results. *J Clin Oncol* **26**: 441s (Suppl; abstr 8069)

Ohe Y, Ohashi Y, Kubota K, Tamura T, Nakagawa K, Negoro S, Nishiwaki Y, Saijo N, Ariyoshi Y, Fukuoka M (2007) Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* **18**: 317–323

Peppercorn JM, Weeks JC, Cook EF, Joffe S (2004) Comparison of outcomes in cancer patients treated within and outside clinical trials: conceptual framework and structured review. *Lancet* **363**: 263–270

Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* **346**: 92–98

Schmoor C, Olschewski M, Schumacher M (1996) Randomized and non-randomized patients in clinical trials: experiences with comprehensive cohort studies. *Stat Med* **15**: 263–271

Stryker JE, Wray RJ, Emmons KM, Winer E, Demetri G (2006) Understanding the decisions of cancer clinical trial participants to enter research studies: factors associated with informed consent, patient satisfaction, and decisional regret. *Patient Educ Couns* **63**: 104–109

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Glabbeke MV, Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* **92**: 205–216

West J, Wright J, Tuffnell D, Jankowicz D, West R (2005) Do clinical trials improve quality of care? A comparison of clinical processes and outcomes in patients in a clinical trial and similar patients outside a trial where both groups are managed according to a strict protocol. *Qual Saf Health Care* **14**: 175–178

Wright JR, Whelan TJ, Schiff S, Dubois S, Crooks D, Haines PT, DeRosa D, Roberts RS, Gafni A, Pritchard K, Levine MN (2004) Why cancer patients enter randomized clinical trials: exploring the factors that influence their decision. *J Clin Oncol* **22**: 4312–4318

Differences in the Quality of Information on the Internet about Lung Cancer between the United States and Japan

Yasushi Goto, MD, Ikuo Sekine, MD, PhD,* Hiroshi Sekiguchi, MD,† Kazuhiko Yamada, MD,* Hiroshi Nokihara, MD,* Noboru Yamamoto, MD,* Hideo Kunitoh, MD,* Yuichiro Ohe, MD,* and Tomohide Tamura, MD**

Introduction: Quality of information available over the Internet has been a cause for concern. Our goal was to evaluate the quality of information available on lung cancer in the United States and Japan and assess the differences between the two.

Methods: We conducted a prospective, observational Web review by searching the word "lung cancer" in Japanese and English, using Google Japan (Google-J), Google United States (Google-U), and Yahoo Japan (Yahoo-J). The first 50 Web sites displayed were evaluated from the ethical perspective and for the validity of the information. The administrator of each Web site was also investigated.

Results: Ethical policies were generally well described in the Web sites displayed by Google-U but less well so in the sites displayed by Google-J and Yahoo-J. The differences in the validity of the information available was more striking, in that 80% of the Web sites generated by Google-U described the most appropriate treatment methods, whereas less than 50% of the Web sites displayed by Google-J and Yahoo-J recommended the standard therapy, and more than 10% advertised alternative therapy. Nonprofit organizations and public institutions were the primary Web site administrators in the United States, whereas commercial or personal Web sites were more frequent in Japan.

Conclusion: Differences in the quality of information on lung cancer available over the Internet were apparent between Japan and the United States. The reasons for such differences might be tracked to the administrators of the Web sites. Nonprofit organizations and public institutions are the up-and-coming Web site administrators for relaying reliable medical information.

Key Words: Internet, Information quality, Lung cancer.

(*J Thorac Oncol.* 2009;4: 829–833)

*Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan; and †Department of Pulmonary and Critical Care Medicine, Mayo Clinic Minnesota, Rochester, Minnesota.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Yasushi Goto, MD, Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan. E-mail: ygoto-tky@umin.net

ISSN: 1556-0864/09/0407-0829

The Internet has given rise to an information revolution of unprecedented magnitude. Whereas the Internet has great potential in marshaling the large volume of health information resources available, it is becoming increasingly difficult to discern which of the resources are reliable and accurate or appropriate for the users.[1–6] This issue has become a cause for great concern, especially in the field of oncology, and many studies have evaluated the pros and cons of obtaining information from the Internet.[2–6] Meanwhile, the medical community is being increasingly faced with patients asking us about the medical information available on the Internet. We can no longer neglect the public importance of the information available and have to use it effectively for patients to better understand their disease.

Although one of the main characteristics of the Internet is its worldwide accessibility, differences in language use around the world serve as a bottleneck for collecting information from the Internet. The estimated number of people using the Internet is about the same in the United States and Japan (70 and 67%,[7,8] respectively), and 80% of patients obtain health information via the Internet in the United States.[9] Until now, most studies that have evaluated the quality of the health care information available over the Internet are from the English-speaking community, and very few studies have been conducted in relation to information available in Japanese.[10,11] Furthermore, only a limited number of studies evaluating the differences in the quality of information available between two languages have been published,[12] and no such study comparing such information in the English and Japanese languages has been published.

Our goal was to imitate the search for medical information by the general population in Japan and United States and to evaluate the differences in the process between the two countries. We also investigated the administrators of the Web sites and attempted to identify any correlation existing between the Web site administrators and the quality of information available on the Internet. We focused on information available on lung cancer, which is the leading cause of cancer-related death in both the Unites States and Japan.[13,14] Because search engines are the leading tools to obtain any kind of information, whether general or medical, on the Internet,[15] we used Google and Yahoo, which are the two most commonly used search engines for Web search in both the United States and Japan.

METHODS

Web Site Search

We conducted a prospective, observational Web review by performing keyword searches using Google in both Japanese and English, and Yahoo in Japanese. Japanese searches were conducted by author YG in Japan (Tokyo) on May 29, 2007, and the English search was conducted by author HS in the United States (New York) on May 25, 2007. We used "Hai-gan (both letters in Chinese characters)," "Hai (Chinese character)-gan (hiragana)," and "Hai (Chinese character)-gan (katakana)," for the Japanese search, and "lung cancer" and "lung carcinoma" for the English search. The search word that resulted in the largest number of search results was chosen for the subsequent study.

The first 50 Web sites displayed by Google and Yahoo in Japanese, and Google in English, excluding the advertisement area, were used for further evaluation. Web sites that were inaccessible, not designed to provide health information (i.e., news and advertisement of books), or displayed for the second (or more) time were excluded from the subsequent evaluation. Samples from the Yahoo in English were supplemented to compare the search utility on January 21, 2009.

Site Characteristics

Author YG evaluated the Web sites within a week of the original search. We evaluated the Web sites based on criteria known as the "JAMA" benchmark[16]: display of authorship (authors and contributors, their affiliations, and relevant credentials), attribution (references and sources for all content and all relevant copyright information), disclosure (Web site ownership, sponsorship, advertising, commercial funding arrangements or support, or potential conflicts of interest), and currency (dates on which the contents were posted and updated). We considered each criterion as fulfilled when it was fully displayed. For further evaluation, we focused on the description about the treatment of advanced non-small lung cancer. To our knowledge, there is no established tool-based instrument to evaluate the information available on cancer treatment. Therefore, we classified the information into three categories: acceptable (description of systematic reviews, such as guidelines from authorized facilities,[17–20] links to systematic reviews, or abstracts of systematic reviews), unacceptable (recommendation of alternative medicine or a generally unapproved treatment), and inevaluable (lack of adequate description). The administrators of the Web sites were classified into five categories: nonprofit organization (NPO) or public institution, medical institution, commercial (for specific treatments), personal (pages made by patients or their families), and others.

Analysis

Descriptive statistics were used to determine the numbers and percentages related to the characteristics of the Web sites. To compare the differences between two countries in view of user experience and search utility, Web sites displayed in Google-U was compared with that of Yahoo-J and Google-J, respectively. The χ^2 test or Fisher's exact test was used as appropriate.

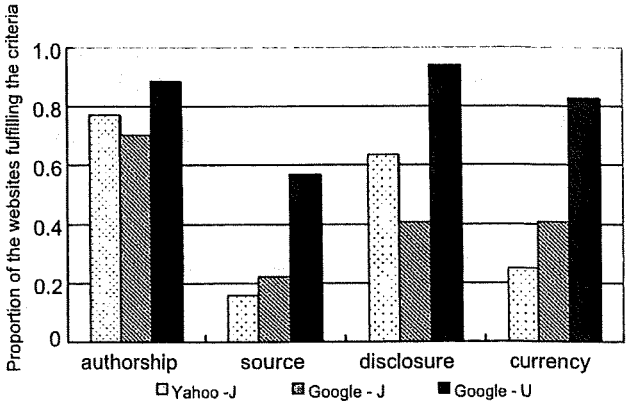

FIGURE 1. JAMA benchmark: Description of the JAMA benchmark[16] is listed by the search engines; display of authorship (authors and contributors, their affiliations, and relevant credentials); attribution (references and sources for all content, and all relevant copyright information); disclosure (Web site ownership, sponsorship, advertising, commercial funding arrangements or support, or potential conflicts of interest); and currency (dates on which the contents were posted and updated).

RESULTS

Differences by Notation

In Google Japan, search using the word "Hai-gan (both letters in Chinese characters)" resulted in a display of approximately 7.7 million Web sites, and in Google United States, search using the phrase "lung cancer" threw up approximately 52 million Web sites. These notations were, therefore, used for the subsequent evaluation. After excluding Web sites that were inaccessible, were not designed to provide health information, or ranked for the second (or more) time in each search, 44, 27, 39, and 35 Web sites displayed by Yahoo Japan (Yahoo-J), Google Japan (Google-J), Yahoo United States (Yahoo-U), and Google United States (Google-U), respectively, were evaluated for further study.

Web Site Characteristics

Figure 1 summarizes the quality of the Web sites that satisfied the criteria of the JAMA benchmark. Authorship was displayed in more than 70% of the Web sites displayed by the three searches: 31 in Google-U (88.6%), 34 in Yahoo-J (70.3%, $p = 0.243$), and 19 in Google-J (88.6%, $p = 0.106$). Attribution of the content was found in 20 (57.1%) of the Web sites in Google-U, and 7 (15.9%, $p < 0.001$) and 6 (22.2%, $p = 0.009$) of the Web sites in Yahoo-J and Google-J, respectively. Twenty-eight (63.6%, $p = 0.001$) Web sites in Yahoo-J, 11 (40.7%, $p < 0.001$) in Google-J, and 33 (94.2%) in Google-U made the disclosure. Display of currency was found in 29 (82.9%) sites in Google-U, but in less than 50% of the Web sites in the Japanese searches; 11 (25.0%, $p < 0.001$) in Yahoo-J and 11 (40.7%, $p = 0.001$) in Google-J.

Quality of Description of the Treatment

Evaluation of the treatment description for advanced non-small cell lung cancer is summarized in Figure 2. The

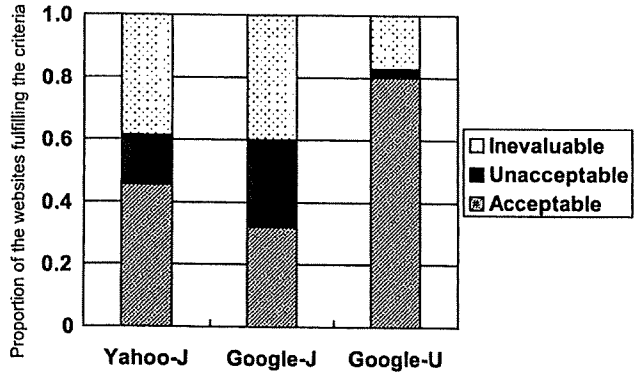

FIGURE 2. Evaluations of the treatment description in the Web sites: The treatment description is classified into three categories: acceptable (description of the systematic review such as guidelines from authorized facilities[17–20]; links to systematic reviews; abstracts of systematic reviews), unacceptable (recommendation of alternative medicine or a generally unapproved treatment), and invaluable (lack of description).

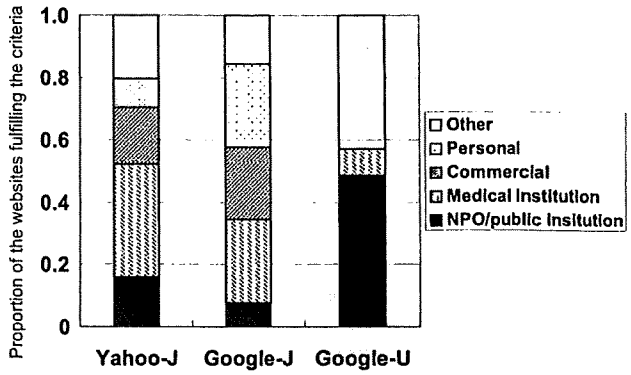

FIGURE 3. Administrators of the Web sites: Administrators were classified into five categories: NPO (nonprofit organization) or public institution, medical institution, commercial (for the specific treatments), personal (pages made by patients or their families), and others.

TABLE 1. Correlation of Sites Between the Top 50 Google and Yahoo, and the Rate of Reliable Sites in Each Engine

	United States	Japan
Correlation of titles in top 50 site of Google and Yahoo	11	10
Percentage of reliable sites in top 50 (%)		
Google	80.0	29.6
Yahoo	71.8[a]	45.5

Correlation of titles in both engines was almost the same in both countries. Proportions of reliable sites were comparable in countries but were not in search engines.

[a] Accessed and evaluated on January 21, 2009.

description was acceptable in 28 (80.0%) of the Web sites generated by Google-U, as these sites described chemotherapy as the standard treatment for advanced lung cancer. Only one site recommended alternative medicine. In Web sites ranked by Yahoo-J and Google-J, standard therapy was only described in 20 (45.5%, $p < 0.001$) and 10 (37.0%, $p < 0.001$) sites, respectively, whereas 7 (15.9%, $p = 0.070$) and 7 (25.9%, $p = 0.017$) sites, respectively, recommended alternative medicine. Table 1 summarizes the quality of the Web sites displayed in Yahoo and Google by both countries. Proportions of reliable sites were comparable in countries but were not in search engines.

Administrators of the Web sites

The administrators of the Web sites are shown in Figure 3. In Google-U, the administrators of 16 (45.7%) Web sites were NPO or public institution, whereas only 7 (15.9%, $p = 0.006$) and 2 (7.4%, $p = 0.001$), respectively, in Yahoo-J and Google-J were managed by them. Commercial site for specific treatments was not displayed in Google-U but was displayed in 8 (18.2%, $p = 0.007$) and 6 (22.2%, $p = 0.005$) Web sites in Yahoo-J and Google-J, respectively. Web sites administered personally by the patients themselves or their families were also not found among the Web site displayed in Google-U, whereas 4 (9.1%, $p = 0.125$) sites in Yahoo-J and 7 (25.9%, $p = 0.002$) sites in Google-J were personally managed.

Administrators and Quality of the Contents of the Web Sites

Table 2 shows the correlation between the Web site administrator and the quality of the contents of the sites. Ten sites generated by both Google-J and Yahoo-J were integrated. There was no site from NPO or public institution category, either Japanese or English, which provided misleading information. Most of the unacceptable sites were managed by commercial or personal sites, neither of which was found in the English-language sites.

DISCUSSION

By comparing the differences of quality of cancer information on the Internet between the different languages, we, for the first time, evaluated the correlation between the Web site administrator and the quality of the medical information in the Web sites. Furthermore, it is one of the few studies to evaluate the information on lung cancer available on the Internet.[15] We also showed that the Web sites displayed in the United States provide information of much higher quality than those displayed by Japanese Web sites, with regard to lung cancer treatment, and this may be related to the quality of the administrators of the displayed Web sites.

It is generally a difficult task to make people access reliable Web sites that would provide the precise information that they are looking for. Regulating access to only trustworthy Web sites that provide useful information is extremely difficult, because a global rule is a necessary step toward controlling the content of the worldwide Web sites. There are also no confirmed tools for weighting the information on the Internet in any field, including medicine. In this chaotic scenario, search engines such as Google and Yahoo have come up with a solution by developing an algorithm to rank the sites. Nowadays, their value is well established in the

TABLE 2. Correlation Between the Quality of the Web site Administrators and the Quality of the Information

	NPO Public Institution	Med Institution	Commercial	Personal	Other	Total
Japanese						
Acceptable	6	10	0	1	5	22
Unacceptable	0	0	10	7	2	19
Inevaluable	2	10	1	1	6	20
Total	8	20	11	9	13	61
English						
Acceptable	15	3	0	0	10	28
Unacceptable	0	0	0	0	1	1
Inevaluable	2	0	0	0	4	6
Total	17	3	0	0	15	35

Ten sites generated by both Google-J and Yahoo-J were integrated. No site from the NPO or public institution category provided misleading information in either the Japanese or the English search. Commercial administrators recommending specific treatments and personal sites accounted entirely for the sites providing unacceptable information.

Internet, and people are generally using this tool for searching medical and other information. Even though there is a concern that the order in which the sites are placed by these tools is not entirely appropriate for the field of medicine,[3,21,22] the high frequency at which these are used has made it meaningless to say that they pose a problem in one-particular field. Therefore, what we must consider now is how to provide reliable information using these tools.

Why is misleading and nonreliable information provided on the Internet? One key characteristic of the Internet is the interaction between the provider and the consumer (in the medical field, patient). Web sites that are not accessed frequently will be ranked lower in the search engine system. Therefore, when discussing the results of Web sites ranked by the search engine, we should consider it from both the standpoint of the provider and the consumer. People access the Internet by requesting the information they want. Many cancer patients suffer from an incurable disease and look for a ray of hope in the Internet. This situation is most advantageous to the information senders. They can promote their treatment as the treatment that would bring about the miraculous cure that the patients are seeking. In this study, most of the sources recommending alternative or unapproved drugs were from commercial and personal sites. Information on medical subjects should be correct and be of assistance to the users to help them better understand their disease. People should be protected from disruptive information. Creating confusion in the minds of people by providing misleading information for profit to the administrator is a vexing situation.

One of the interesting findings in this study was that the correlation between the quality of the Web site administrator and the quality of the contents of the site was seen not only for sites providing misleading information but also for those providing reliable information. At present, there are two major administrators providing reliable information, namely, medical institutions and specialized organizations for information administered by patient advocate NPO or public institution. However, the type of information provided differed between the two types of administrators. In general, each medical institution provides reliable messages but not review articles, whereas the patient advocate group NPO and public institution provide a path to the review articles. This is not surprising because the aims of providing information are different between the two types of administrators. For each medical institution, the goal is to display the treatment that they are interested in, and describing the entire medical consensus is outside their reach. Therefore, sites specialized in providing information are the ones that can be most expected to provide general information. Differences in the number of reliable sites between the languages in this study may be because of the difference in the number of such organizations between the countries. The number of public institution sites may depend on the countries in which each language is spoken in, and the growth in the number of patient advocate NPO may depend on the social system or the differences in culture. However, it is noteworthy that patient advocate NPO can play a major role in providing reliable health information.

There were several limitations in this study. One is that we evaluated sites only from Yahoo Japan and Google Japan, and Google United States. We chose Google United States as the reference, because most previous studies on the Internet have been conducted in the United States, and Google is the most popular search engine in the United States.[23] In Japan, Yahoo ranks first as the most frequently used search engine, followed next by Google,[24] which is the reason we selected these two as the representative search engines for our search of Web sites in Japanese. Although this approach may limit evaluation of the overall Internet situation in the two countries, we believe that this was the closest way to reproduce the way people browse the Internet. Another concern is the number of sites generated by these tools. The total number of Web sites displayed by our search using the keywords differs between the two languages and maybe attributable to the differences in the quality of the administrators. Google-U generated approximately seven times as many Web sites as Google-J. This discrepancy could be because of the difference in the number of people using the two languages. However, we only evaluated the top 50 sites, which is far short of the total number of sites displayed but may already

be too much for anyone seeking any type of information. Because the ranking system has prevailed, the quality of the highest ranked Web sites and not the total number of sites displayed is important to the user. Lastly, another important problem is whether people in the United States and Japan desire the same answers from the Internet. In general, search engines attempt to rank the Web sites sought by the users. If these differed between countries, the ranking would also reflect these differences. Differences in the social backgrounds of the populations in the two countries were confounding factors in this study. However, no studies evaluating the topic from this perspective have been conducted. These are topics of interest that need further investigation.

In this era of abundance of information, it is absolutely essential for people to make their choices based on the quality. As medical professionals, we have the responsibility of providing appropriate information to people who are unaware and anxious about their future. In the new era of the Internet technology, facilitating easy access to reliable information, and providing reliable information is important. This study may facilitate an understanding of the actual status of dispersal of information and pave the way for discussing methods to achieve better accessibility to high-quality health information.

REFERENCES

1. Wilson P. How to find the good and avoid the bad or ugly: a short guide to tools for rating quality of health information on the internet. *BMJ* 2002;324:598–602.
2. Bichakjian CK, Schwartz JL, Wang TS, Hall JM, Johnson TM, Biermann JS. Melanoma information on the internet: often incomplete—a public health opportunity? *J Clin Oncol* 2002;20:134–141.
3. Meric F, Bernstam EV, Mirza NQ, et al. Breast cancer on the world wide web: cross sectional survey of quality of information and popularity of websites. *BMJ* 2002;324:577–581.
4. Helft PR, Hlubocky F, Daugherty CK. American oncologists' views of internet use by cancer patients: a mail survey of American Society of Clinical Oncology members. *J Clin Oncol* 2003;21:942–947.
5. Trumbo CW. Cancer information on the world wide Web: gross characteristics. *J Natl Cancer Inst* 2004;96:332–333.
6. Helft PR, Eckles RE, Johnson-Calley CS, Daugherty CK. Use of the internet to obtain cancer information among cancer patients at an urban county hospital. *J Clin Oncol* 2005;23:4954–4962.
7. Miniwatts Marketing Group. Internet Usage and Population in North America, November 30, 2007. Available at: http://www.internetworldstats.com/stats14.htm, Accessed on August 1, 2008. (Accessed at: http://www.internetworldstats.com/stats14.htm).
8. Miniwatts Marketing Group. Asia Marketing Research, Internet Usage, Population Statistics and Information. July 5, 2008. Available at: http://www.internetworldstats.com/asia.htm#jp, Accessed on August 1, 2008.
9. Most internet users start at a search engine when looking for health information online. Very few check the source and date of the information they find. October 29, 2006. Available at: http://www.pewinternet.org/pdfs/PIP_Online_Health_2006.pdf. Accessed on August 1, 2008. (Accessed at: http://www.pewinternet.org/pdfs/PIP_Online_Health_2006.pdf).
10. Nemoto K, Tachikawa H, Sodeyama N, et al. Quality of Internet information referring to mental health and mental disorders in Japan. *Psychiatry Clin Neurosci* 2007;61:243–248.
11. Tatsumi H, Mitani H, Haruki Y, Ogushi Y. Internet medical usage in Japan: current situation and issues. *J Med Inter Res* 2001;3:E12.
12. Berland GK, Elliott MN, Morales LS, et al. Health information on the Internet: accessibility, quality, and readability in English and Spanish. *JAMA* 2001;285:2612–2621.
13. Minino AM, Heron MP, Murphy SL, Kochanek KD. Deaths: final data for 2004. *Natl Vital Stat Rep* 2007;55:1–119.
14. Kato H, Sobue T, Katanoda K. Cancer Statistics in Japan. November 11, 2006. Available at: http://ganjoho.ncc.go.jp/public/statistics/backnumber/odjrh3000000o8is-att/preface.pdf. Accessed August 1, 2008.
15. Linssen C, Schook RM, The AM, Lammers E, Festen J, Postmus PE. A web site on lung cancer: who are the users and what are they looking for? *J Thorac Oncol* 2007;2:813–818.
16. Silberg WM, Lundberg GD, Musacchio RA. Assessing, controlling, and assuring the quality of medical information on the Internet: Caveant lector et viewor—Let the reader and viewer beware. *JAMA* 1997;277:1244–1245.
17. Pfister DG, Johnson DH, Azzoli CG, et al. American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. *J Clin Oncol* 2004;22:330–353.
18. Socinski MA, Morris DE, Masters GA, Lilenbaum R. Chemotherapeutic management of stage IV non-small cell lung cancer. *Chest* 2003;123:226S–243S.
19. The Lung Cancer Disease Site Group. Lung Cancer Evidence-based Series (EBS) and Practice Guidelines (PG). May 7, 2008. Available at: http://www.cancercare.on.ca/pdf/pebc7-2f.pdf, Accessed on August 1, 2008.
20. National Institute for Clinical Excellence. The Diagnosis and Treatment of Lung Cancer. February, 2005. Available at: http://www.nice.org.uk/nicemedia/pdf/cg024fullguideline.pdf. Accessed on August 1, 2008.
21. Griffiths KM, Christensen H. Quality of web based information on treatment of depression: cross sectional survey. *BMJ* 2000;321:1511–1515.
22. Borges AAH, Cervi PM, Arcaya MLTAd, Guardado G, Rabaza AR, Sosa AJ. Rate of compliance with the HON code of conduct versus number of inbound links as quality markers of pediatric web sites. In MEDNET2001;2001, Udine, Italy: Technology and Healthcare, IOS Press Amsterdam, 2001.
23. comScore. Core Search Report September, 2007. Available at: http://www.comscore.com/press/release.asp?press=1805. Accessed on August 1, 2008.
24. comScore. Top Japanese Web Rankings for September, 2007. Available at: http://www.comscore.com/press/release.asp?press=1838, Accessed on August 1, 2008.

Int J Clin Oncol (2009) 14:112–119
DOI 10.1007/s10147-008-0813-z

REVIEW ARTICLE

Ikuo Sekine · Chikako Shimizu · Kazuto Nishio
Nagahiro Saijo · Tomohide Tamura

A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in patients with breast cancer

Received: February 15, 2008 / Accepted: June 24, 2008

Abstract
Background. We aimed to identify, through a review of the literature, candidate genes for a prospective predictive chemosensitivity test in patients with breast cancer.
Methods. Papers demonstrating an association between gene alterations in tumor tissue and clinical chemosensitivity in breast cancer patients were selected by Medline searches. We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and CIs were estimated using the DerSimonian-Laird method.
Results. A total of 18 genes were evaluated for association with clinical chemosensitivity in 6378 patients registered in 69 studies. The median (range) number of patients in each study was 73 (29–319). Overexpression of *ABCB1* (P-glycoprotein) was associated with poor responses to first-line chemotherapy (combined OR [CI], 0.16 [0.05–0.59]; n = 322). Overexpression and amplification of *TOP2A* (topoisomerase II-alfa) were more frequently observed in patients who responded to first-line chemotherapy (combined OR [CI], 2.73 [1.02–7.27]; n = 323). Overexpression of *ERBB2* (c-erbB2) was associated with favorable responses in patients treated with both first-line anthracycline-based chemotherapy and second-line taxane-based chemotherapy (combined ORs [CIs], 1.60 [1.19–2.17]; n = 1807 and 2.24 [1.06–4.74]; n = 259, respectively). *BCL2* overexpression was associated with resistance to first-line chemotherapy (combined OR [CI], 0.44 [0.21–0.91]; n = 816).
Conclusion. *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for future clinical trials of predictive chemosensitivity tests in patients with breast cancer.

Key words Chemotherapy · Sensitivity · Drug resistance · Breast cancer · Gene alterations

I. Sekine (✉) · T. Tamura
Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan
Tel. +81-3-3542-2511; Fax +81-3-3542-3815
e-mail: isekine@ncc.go.jp

C. Shimizu
Division of Medical Oncology, National Cancer Center Hospital, Tokyo, Japan

K. Nishio
Department of Genome Biology, Kinki University School of Medicine, Osaka, Japan

N. Saijo
Division of Internal Medicine, National Cancer Center Hospital East, Kashiwa, Japan

Introduction

Breast cancer remains a major medical problem in women in spite of dramatic advances in the past three decades in the understanding of the biologic and clinical nature of the disease. About 1% to 5% of patients with breast cancer have distant metastasis at the time of initial diagnosis and 20% to 30% of patients develop systemic recurrence after surgery for local disease.[1] Chemotherapy for these patients, however, has limited efficacy, such that clinical objective response rates to standard chemotherapy regimens are 20%–40% at most, and such that patients with distant metastases rarely live long.[1] In addition, 40% to 80% of patients with breast cancer who undergo surgical resection receive adjuvant chemotherapy without its efficacy ever being monitored.

Tumor response to chemotherapy varies from one patient to another. Thus, it would be extremely useful to know ahead of time which patients have tumors that would respond to chemotherapeutic agents and also which tumors would be resistant to such therapy. For this purpose, cell culture-based chemosensitivity tests have been developed for more than 20 years, but they are not widely accepted because of technical problems, including the large amount of surgical material required, a low success rate for primary

culture, the time-consuming nature of the technique, and a poor correlation with the clinical response.[2,3] To overcome these obstacles, DNA, RNA, and protein-based chemosensitivity tests have been tried, but it remains unknown which gene alteration is well predictive of the clinical drug response. In our previous studies, 80 in vitro chemosensitivity-associated genes were identified in the medical literature,[4] and the association between alterations of these genes and clinical drug responses in lung cancer patients was described.[5] The purpose of this study was to find candidate genes to develop clinically useful chemosensitivity tests for patients with breast cancer.

Materials and methods

We identified 80 in vitro chemosensitivity-associated genes that met the following definition in the medical literature: (1) their alteration could be identified in human drug-induced resistant solid tumor cell lines; (2) their transfection induced drug resistance; or (3) their downregulation increased drug sensitivity. The genes included transporters: *ABCA2*, *ABCB1*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCG2*, *MVP*, *ATP7A*, *ATP7B*, *SLC29A1*, *SLC28A1*, and *SLC19A1*; drug targets: *TUBB*, *TUBB4*, *TUBA*, *TYMS*, *TOP1*, *TOP2A*, *TOP2B*, and DHFR; target-associated proteins: *MAP4*, *MAP7*, *STMN1*, *KIF5B*, *HSPA5*, *PSMD14*, and *FPGS*; intracellular detoxifiers: *GSTP1*, *GPX*, *GCLC*, *GGT2*, *MT*, *RRM2*, and *AKR1B1*; DNA damage recognition and repair proteins: *HMGB1*, *HMGB2*, *ERCC1*, *XPA*, *XPD*, *MSH2*, *MLH1*, *PMS2*, *APEX1*, *MGMT*, *BRCA1*, and *GLO1*; cell-cycle regulators: *RB1*, *GML*, *CDKN1A*, *CCND1*, *CDKN2A*, and *CDKN1B*; mitogenic signal regulators: *ERBB2*, *EGFR*, *KRAS2*, *HRAS*, and *RAF1*; survival signal regulators: *AKT1* and *AKT2*; integrins: *ITGB1*; transcription factors: *JUN*, *FOS*, *MYC*, and *NFKB1*; and apoptosis regulators: *TP53*, *MDM2*, *TP73*, *BCL2*, *BCL2L1*, *MCL1*, *BAX*, *BIRC4*, *BIRC5*, *TNFRSF6*, *CASP3*, *CASP8*, and *HSPB1*.[4] Papers describing an association between the alteration of the gene and clinical drug response in patients with breast cancer were identified by extensive Medline searches using the name of the gene as a key word. Papers in which the association was evaluated in 25 or more patients were included in this study.

We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and CIs were estimated using the DerSimonian-Laird method, as previously described.[5] The formula used for the combined OR and that for 95% CI were as follows:

$$\text{Combined OR} = \exp\{\Sigma(\text{weight}_i \cdot \ln\text{OR}_i)/\Sigma\text{weight}_i\}$$

$$95\%\ \text{CI of combined OR} = \exp\{\ln\text{combined OR} \pm 1.96\ (1/\Sigma\text{weight}_i)^{1/2}\}$$

where weight_i is the weight for each study determined by variance of the study, and OR is the OR of each study.

Results

Clinical drug responses were evaluated in 18 genes from 69 studies, which included a median of 73 patients (range, 29–319 patients) per study to give a total of 6378 patients. The methods used to identify the gene alteration were immunohistochemical protein expression analysis (n = 52), protein activity analysis using tritium-release assay (n = 1), polymerase chain reaction (PCR)-based mRNA expression analysis (n = 8), PCR-based mutation analysis (n = 3), and gene amplification analysis using fluorescence in situ hybridization or chromogenic in situ hybridization (n = 5). The gene alteration was associated with the clinical response in 25 of the 69 (36%) studies.

High expression of *ABCB1* was associated with a poor response to first-line chemotherapy in three of five studies, and the combined OR (CI) in a total of 322 patients was 0.16 (0.05–0.59). Other transporter expressions were not associated with chemotherapy responses (Table 1). Study results showing associations between drug target alterations and clinical responses were promising. The alteration of *TYMS* (thymidylate synthetase), *TUBB* (beta-tubulin class I), and *TUBB4* (beta-tubulin class III) was associated with chemosensitivity, although there was only one study for each gene. The overexpression and amplification of *TOP2A* (topoisomerase II-alfa) were more frequently observed in patients who responded to first-line chemotherapy in four out of five studies with a combined OR (CI) of 2.73 (1.02–7.274) in a total of 323 patients (Table 2). The high expression of the DNA repair gene *BRCA1* (Breast cancer 1) was associated with chemosensitivity in one study (Table 3). The overexpression of *ERBB2* (c-erbB2, Her2, or neu) was associated with favorable responses in patients treated with first-line anthracycline-based chemotherapy, and the combined OR (CI) was 1.60 (1.19–2.17) in a total of 1807 patients (Table 4). This was also true among patients treated with second-line chemotherapy containing taxanes (combined OR [CI], 2.24 [1.06–4.74]; n = 259; Table 5). *TP53* mutations were not associated with clinical drug responses (combined OR [CI],1.09 [0.73–1.62]; n = 1588; Table 6), whereas *BCL2* overexpression was associated with resistance to first-line chemotherapy (combined OR [CI], 0.44 [0.21–0.91]; n = 816; Tables 7 and 8).

Discussion

Association between a gene alteration and clinical chemosensitivity was evaluated in 18 of the 80 in vitro chemosensitivity-associated genes in patients with breast cancer. Among them, *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for further studies.

ABCB1 has been extensively studied as a major cellular mechanism of multidrug resistance,[6] but there has been no firm evidence that the expression of this transporter in tumor cells has been associated with a poor response to cytotoxic chemotherapy in patients with breast cancer. A

Table 1. Expression of transporter proteins and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
ABCB1						
Ro[19] (1990, USA)	CPA, DOX, VCR	IHC	Low	20	95	0.08
			High	20	60	(0.01–0.71)
Veneroni[20] (1994, Italy)	DOX ± VCR	IHC	Low	21	86	0.02
			High	18	11	(0.0–0.14)
Chevillard[21] (1996, France)	CPA, DOX, 5-FU	IHC	Low	36	50	0.75
			High	7	43	(0.15–3.84)
Bottini[22] (2000, Italy)	CPA, MTX, 5-FU, or EPI	IHC	Low	99	28[a]	0.51
			High	42	17[a]	(0.20–1.27)
Burger[23 b] (2003, Netherlands)	CPA, MTX, 5-FU, or	RT-PCR	Low	47	68	0.09
	CPA, DOX or EPI, 5-FU		High	12	17	(0.02–0.48)
Combined odds ratio (95% CI) for ABCB1 (n = 322): 0.16 (0.05–0.59)						
ABCC1 (Multidrug resistance-associated protein 1; MRP1)						
Burger[23 b] (2003, Netherlands)	CPA, MTX, 5-FU, or	RT-PCR	Low	30	60	0.82
	CPA, DOX or EPI, 5-FU		High	29	55	(0.29–2.31)
ABCC2 (Multidrug resistance-associated protein 1; MRP2)						
Burger[23 b] (2003, Netherlands)	CPA, MTX, 5-FU, or	RT-PCR	Low	28	64	0.48
	CPA, DOX or EPI, 5-FU		High	28	46	(0.16–1.41)
ABCG2 (Breast cancer resistance protein; BCRP)						
Burger[23 b] (2003, Netherlands)	CPA, MTX, 5-FU, or	RT-PCR	Low	42	64	0.39
	CPA, DOX or EPI, 5-FU		High	17	41	(0.12–1.23)
MVP (major vault protein, lung resistance-related protein)						
Burger[23 b] (2003, Netherlands)	CPA, MTX, 5-FU, or	RT-PCR	Low	37	65	0.45
	CPA, DOX or EPI, 5-FU		High	22	45	(0.15–1.33)

RR, response rate. Drugs: CPA, cyclophosphamide; DOX, doxorubicin; EPI, epirubicin; 5-FU, 5-fluorouracil; MTX, methotrexate; VCR, vincristine. Methods: IHC, immunohistochemical analysis; RT-PCR, reverse transcriptase-polymerase chain reaction

[a] Complete response rate (%)

[b] In this study 20% of patients had received adjuvant chemotherapy

Table 2. Drug targets, intracellular detoxifier, and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
TYMS (thymidylate synthetase)						
Foekens[24] (2001, Netherlands)	5-FU- based	TRA	Low expression	13	8	12.0
			High expression	108	50	(1.51–95.5)
TUBB (beta-tubulin class I)						
Hasegawa[25] (2003, Japan)	DTX	Real-time	Low expression	19	63	0.25
		PCR	High expression	20	30	(0.07–0.95)
TUBB4 (beta-tubulin class III)						
Hasegawa[25] (2003, Japan)	DTX	Real-time	Low expression	19	68	0.15
		PCR	High expression	20	25	(0.04–0.62)
TOP2A (topoisomerase II-alfa)						
Jarvinen[26] (1998, Finland)	EPI	IHC	Low expression	31	58	0.61
			High expression	24	46	(0.21–1.79)
Coon[27] (2002, USA)	Anthracycline-based	IHC	Low expression	26	77	2.40
			High expression	9	89	(0.25–23.2)
MacGrogan[28] (2003, France)	EPI, MTX, VCR	IHC	Low expression	68	32	2.88
			High expression	57	58	(1.38–5.97)
Martin-Richard[29] (2004, Spain)	CPA, DOX, 5-F-U or CPA, EPI, 5-FU	IHC	Low expression	25	24	5.28
			High expression	16	63	(1.35–20.7)
Park[30] (2003, Korea)	DOX	CISH	Normal	48	54	15.2
			Amplified	19	95	(1.88–123)
Combined odds ratio (95% CI) for TOP2A (n = 323): 2.73 (1.027–7.27)						
GSTP1 (glutathione S-transferase pi)						
Wright[31] (1992, UK)	MIT	IHC	Low expression	30	37	1.22
			High expression	29	41	(0.43–3.48)

Drugs: DTX, docetaxel; MTX, methotrexate; MIT, mitoxantrone: CISH, chromogenic in situ hybridization; TRA, tritium-release assay

previous meta-analysis, summarizing the data of 115 patients published between 1990 and 1996, showed only a marginal association between *ABCB1* expression in tumor tissue before treatment and failure of response (relative risk, 1.47; 95% CI, 0.94–2.29; $P = 0.088$).[7] The present study included recent studies with a total of 322 patients, and showed that the expression of *ABCB1* was significantly associated with a poor drug response. Key anticancer agents in the

Table 3. DNA repair gene, cell-cycle regulator and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
BRCA1 (Breast cancer 1)						
Egawa[32] (2003, Japan)	CPA, EPI	Real-time PCR	Low	25	32	4.01
			High	26	65	(1.25–12.9)
CCND1 (cyclin D1)						
Bonnefoi[33] (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Low	126	22[a]	2.02
			High	52	37[a]	(1.00–4.07)

[a] Complete response rate (%)

Table 4. *ERBB2* (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to first-line anthracycline-based chemotherapy

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
Niskanen[b,34] (1997, Finland)	CPA, EPI, 5-FU	IHC	Low expression	89	33	2.07
			High expression	14	50	(0.66–6.45)
Rozan[35] (1998, France)	CPA, DOX, 5-FU	IHC	Low expression	131	21	1.62
			High expression	36	31	(0.71–3.69)
Jarvinen[26] (1998, Finland)	EPI	IHC	Low expression	36	64	0.26
			High expression	19	32	(0.08–0.85)
Vincent-Salomon[36] (2000, France)	CPA, DOX, 5-FU	IHC	Low expression	36	78	0.57
			High expression	18	67	(0.16–2.01)
Geisler[37] (2001, Norway)	DOX	IHC	Low expression	72	37	1.17
			High expression	17	41	(0.40–3.43)
Coon[27] (2002, USA)	Anthracycline-based	IHC	Low expression	20	70	2.79
			High expression	15	87	(0.47–16.4)
MacGrogan[28] (2003, France)	EPI, MTX, VCR	IHC	Low expression	102	40	1.82
			High expression	20	55	(0.69–4.78)
Bonnefoi[33] (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Low expression	132	24[a]	1.61
			High expression	47	34[a]	(0.78–3.32)
Zhang[38] (2003, USA)	CPA, DOX, 5-FU	IHC	Low expression	69	78	3.61
			High expression	28	93	(0.77–17.0)
Martin-Richard[29] (2004, Spain)	CPA, DOX, 5-FU or CPA, EPI, 5-FU	IHC	Low expression	30	37	1.44
			High expression	11	45	(0.35–5.84)
Burcombe[39] (2005, UK)	Anthracycline-based	IHC	Low expression	84	71	1.87
			High expression	34	82	(0.69–5.08)
Prisack[40] (2005, Germany)	CPA, EPI	IHC	Low expression	257	10[a]	2.13
			High expression	62	19[a]	(1.01–4.51)
Manna Edel[41] (2006, Brazil)	Anthracycline-based	IHC	Low expression	86	63	1.11
			High expression	23	65	(0.42–2.91)
Park[30] (2003, Korea)	DOX	CISH	Normal	36	47	7.54
			Amplified	31	87	(2.19–26.0)
Konecny[c,42] (2004, USA)	CPA, EPI	FISH	Normal	88	33	1.80
			Amplified	49	46	(0.88–3.68)
Bozzetti[43] (2006, Belgium)	Anthracycline-based	FISH	Normal	86	62	1.63
			Amplified	29	72	(0.65–4.11)
Combined odds ratio (95% CI) for *ERBB2* (anthracyclines; $n = 1807$): 1.60 (1.19–2.17)						

FISH, fluorescence in situ hybridization
[a] Pathological complete response rate
[b,c] In these studies, 15% and 40%, respectively, of patients had received adjuvant chemotherapy

Table 5. ERBB2 (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to second-line taxanes

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
Taxanes						
Baselga[44] (1997, USA)	DTX or PTX	IHC	Low expression	76	65	3.40
			High expression	46	36	(1.58–7.33)
Sjostrom[45] (2002, Finland)	DTX	IHC	Low expression	36	53	1.02
			High expression	30	53	(0.39–2.70)
Di Leo[46] (2004, Europe)	DTX	FISH	Normal	50	40	3.00
			Amplified	21	67	(1.03–8.74)
Combined odds ratio (95% CI) for *ERBB2* (taxanes, $n = 259$): 2.24 (1.06–4.74)						

DTX, docetaxel; PTX, paclitaxel

Table 6. Tumor protein *TP53* (p53) mutation and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Mutation	No. of pts	RR (%)	Odds ratio (95% CI)
Niskanen[c 34] (1997, Finland)	CPA, EPI, 5-FU	IHC	Normal	86	37	0.52
			Mutated	17	24	(0.16–1.73)
Frassoldati[47] (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Normal	26	42	0.68
			Mutated	3	33	(0.05–8.50)
Bonetti[d 48] (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Normal	21	30	0.94
			Mutated	22	27	(0.25–3.56)
Rozan[35] (1998, France)	CPA, DOX, 5-FU	IHC	Normal	97	22	1.25
			Mutated	70	26	(0.61–2.58)
Jarvinen[26] (1998, Finland)	EPI	IHC	Normal	37	57	0.61
			Mutated	18	44	(0.20–1.90)
Colleoni[49] (1999, Italy)	CPA, DOX or VNR, 5-FU	IHC	Normal	59	53	5.42
			Mutated	14	86	(1.11–26.4)
Bottini[22] (2000, Italy)	CPA, MTX, 5-FU or EPI	IHC	Normal	111	72	1.16
			Mutated	32	75	(0.47–2.86)
Kandioler-Eckersberger[50] (2000, Austria)	CPA, EPI, 5-FU	IHC	Normal	20	85	0.01
			Mutated	15	7	(0.00–0.13)
Kandioler-Eckersberger[50] (2000, Austria)	PTX	IHC	Normal	20	35	3.71
			Mutated	12	67	(0.82–16.8)
Bonnefoi[33] (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Normal	126	29[a]	0.73
			Mutated	53	23[a]	(0.35–1.55)
MacGrogan[28] (2003, France)	EPI, MTX, VCR	IHC	Normal	89	40	2.38
			Mutated	34	62	(1.06–5.35)
Rahko[c 51] (2003, Finland)	Anthracycline-based	IHC	Normal	15	33	0.73
			Mutated	15	27	(0.15–3.49)
Ogston[52] (2004, UK)	CPA, DOX, VCR	IHC	Normal	65	52[b]	1.25
			Mutated	38	59[b]	(0.56–2.81)
Prisack[40] (2005, Germany)	CPA, EPI	IHC	Normal	269	11[a]	2.12
			Mutated	38	21[a]	(0.89–5.06)
Berns[53] (2000, Netherlands)	CPA, DOX, 5-FU or CPA, MTX, 5-FU	sequencing	Normal	16	63	0.34
			Mutated	25	36	(0.09–1.24)
Geisler[37] (2001, Norway)	DOX	TTGE, sequencing	Normal	64	36	1.31
			Mutated	26	42	(0.52–3.32)
Geisler[54] (2003, Norway)	MMC, 5-FU	TTGE, sequencing	Normal	17	41	0.55
			Mutated	18	28	(0.13–2.26)
Combined odds ratio (95% CI) for *TP53* ($n = 1588$): 1.09 (0.73–1.62)						

Drugs: MMC, mitomycin C; VNR, vinorelbine. Method: TTGE, temporal temperature gel electrophoresis
[a] Pathological complete response rate
[b] Good pathological response rate
[c,d] In these studies, 15% and 30%, respectively, of patients had received adjuvant chemotherapy

Table 7. *BCL2* (B-cell CLL/lymphoma 2) and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
Frassoldati[47] (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Low	19	47	0.48
			High	10	30	(0.09–2.42)
Bonetti[c 48] (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Low	32	44	0.19
			High	23	13	(0.05–0.78)
Colleoni[49] (1999, Italy)	CPA, DOX or VNR, 5-FU	IHC	Low	27	52	1.58
			High	46	63	(0.60–4.15)
Bottini[22] (2000, Italy)	CPA, MTX, 5-FU or EPI	IHC	Low	48	71	1.15
			High	95	74	(0.53–2.49)
Geisler[37] (2001, Norway)	DOX	IHC	Low	46	37	1.12
			High	43	40	(0.47–2.62)
Ogston[52] (2004, UK)	CPA, DOX, VCR	IHC	Low	55	71[b]	0.22
			High	48	25[b]	(0.10–0.52)
Buchholz[55] (2005, USA)	CPA, DOX, 5-FU	IHC	Low	33	27[a]	0.11
			High	49	4[a]	(0.02–0.57)
Prisack[40] (2005, Germany)	CPA, EPI	IHC	Low	118	25[a]	0.16
			High	124	5[a]	(0.06–0.42)
Combined odds ratio (95% CI) for *BCL2* ($n = 816$): 0.44 (0.21–0.91)						

[a] Pathological complete response rate
[b] Good pathological response rate
[c] In this study, 30% of patients had received adjuvant chemotherapy

Table 8. Other apoptosis regulators and clinical response to chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
BCL2L1 (Bcl2-like 1, Bcl-xL)						
Sjostrom[56] (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	59	36	1.32
			High	64	42	(0.64–2.73)
BAX (Bcl2-associated X protein)						
Krajewski[57] (1995, Finland)	CPA, EPI, 5-FU (first-line)	IHC	Low	39	21	2.84
			High	65	43	(1.13–7.13)
Sjostrom[56] (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	59	39	1.03
			High	53	39	(0.48–2.20)
Buchholz[55] (2005, USA)	CPA, DOX, 5-FU (first-line)	IHC	Low	12	58[a]	0.04
			High	69	6[a]	(0.01–0.20)
TNFRSF6 (tumor necrosis factor receptor superfamily, member 6, FAS, CD95)						
Sjostrom[56] (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	53	42	0.83
			High	70	37	(0.40–1.73)

[a]Pathological complete response rate

treatment of breast cancer, such as anthracyclines, vinca alkaloids, and taxanes, are substrates of ABCB1 protein, and its expression must therefore be an important determinant for chemosensitivity. The association between the expression and clinical drug responses of other transporters is also worth evaluating, although no statistically significant association has been obtained due to the too-small sample size.

Qualitative and quantitative alterations of the drug's target are another important mechanism involved in classical drug resistance. DNA topoisomerase II enzymes pass one double-stranded DNA segment through a transient, enzyme-mediated break in another strand to relax a highly twisted superhelical DNA.[8] One isoform of these enzymes, TOP2A, is the target of most active anticancer agents, including anthracyclines, because its expression levels are tightly linked to the proliferative state of the cell, and are higher in tumor tissue than in adjacent normal tissue.[8] Although there have been many attempts to correlate *TOP2A* status with anthracycline efficacy in breast cancer patients, the results have been controversial.[9] The present study showed that *TOP2A* gene amplification and protein overexpression were associated with a higher response rate in a total of 323 patients. *TYMS* and beta-tubulins are also important targets for fluoropyrimidines and taxanes, respectively. Further studies are needed before the association can be definitively established between alteration of these gene expressions and clinical chemotherapy responses.

ERBB2 is a member of the human epidermal growth factor receptor family, which plays an important role in regulating cell growth, survival, adhesion, migration, and differentiation, by forming heterodimers within the family. The ERBB2 receptor is the most potent oncoprotein, and amplification and overexpression of *ERBB2*, noted in about 30% of breast cancers, are associated with a poor prognosis.[10,11] The predictive value of *ERBB2* overexpression for poor responses to endocrine therapy and trastuzumab therapy has been well documented, but the association between *ERBB2* status and chemosensitivity remains controversial.[11,12] This issue has been evaluated mainly in the adjuvant setting after surgery, and the association between *ERBB2* status and difference in progression-free survival can therefore be attributable to the overall prognosis as well as the efficacy of chemotherapy. The *ERBB2* status and responses to chemotherapy in patients with locally advanced or the metastatic breast cancer have been evaluated in small studies. Few studies, however, showed any significant difference in the response rates between ERBB2-normal and ERBB2-overexpressed patients.[12] The present study showed that patients with overexpression or amplification of *ERBB2* responded significantly better to anthracycline-based chemotherapy than patients with a normal *ERBB2* status. This was explained by the correlation between the expressions of the *ERBB2* and *TOP2A* genes; high expression of the *TOP2A* gene was detected in 30%–60% of breast cancer tissue with *ERBB2* overexpression, while it was detected in only 5%–10% of breast cancer tissue without *ERBB2* overexpression. The mechanism of this correlation remains unclear. The *ERBB2* and *TOP2A* genes were previously thought to be coamplified, because both the genes are located on chromosome 17q12-21. Recent studies, however, showed that when these genes were amplified, they were located in different amplicons. In other studies, the number of copies of the *ERBB2* and *TOP2A* genes were not identical.[13] The present study also showed that the overexpression or amplification of *ERBB2* was significantly associated with better responses to taxanes. Other genetic events on the 17q12-21 and other chromosomal regions that occur when *ERBB2* is amplified may be involved in its mechanisms.[14]

TP53 preserves genome integrity as the "guardian of the genome" in response to various cellular stresses by invoking cell-cycle arrest and allowing the repair system to eliminate mutations, or by inducing apoptosis when the correct DNA repair is not accomplished.[15] Because most chemotherapeutic agents induce apoptosis through either DNA damage or microtubule disruption, the *TP53* status may affect the sensitivity of tumor cells against these agents. Animal and in vitro studies, however, failed to show general trends of associations between *TP53* status and drug sensitivity.[15,16] The present study also showed inconsistent results in clinical studies. This is probably because only *TP53* gene mutations and mutated TP53 protein accumulation have been

examined, but many mechanisms regulating TP53 protein activity have never been evaluated, which include post-translational modification and interaction with other upstream and downstream molecules.[15]

The Bcl-2 family of proteins plays a central role in regulating apoptosis by balancing expression between pro- and anti-apoptotic family members. Cytotoxic stimuli that promote apoptosis, including DNA damage or microtubule disruption by chemotherapy, can be prevented by *BCL2* expression. An in vitro study consistently showed that overexpression of *BCL2* increased the resistance of MCF-7 cells to doxorubicin, and this resistance was positively correlated with *BCL2* expression levels of individual MCF/BCL2 clones.[17] In clinical studies, however, the association between the expression of *BCL2* and chemosensitivity was not conclusive, mostly due to the small sample size of each study. The present study showed that patients with *BCL2*-positive breast cancer were twice as likely to be resistant to chemotherapy.

The methodological limitations of studies on the association between gene alterations and clinical drug sensitivity are summarized as follows: (1) all the studies were retrospective subgroup analyses; (2) the endpoint of these studies was the response rate in the metastatic or neoadjuvant setting, which is not as objective an endpoint as survival; (3) the sample size of these studies was relatively small; and (4) the majority of the studies assessed the alterations by immunohistochemistry using monoclonal antibodies, but no international standard criteria of positivity and negativity have been defined.[18] In addition, the present study had major problems, such as large heterogeneity among studies; publication bias; and a selection bias, in that studies with incomplete information were excluded from this study. In spite of these limitations, the exploratory analyses in this study will help select genes for future confirmatory studies of molecular markers associated with the clinical response to cytotoxic chemotherapy.

In conclusion, *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for future clinical trials of predictive chemosensitivity tests in patients with breast cancer.

Conflict of Interest

The authors indicate no potential conflicts of interest.

Acknowledgments We thank Yuko Yabe and Mika Nagai for their invaluable assistance in the collection and arrangement of the large number of papers. This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan.

References

1. Ellis MJHD, Lippman ME (2003) Treatment of metastatic breast cancer. In: Harris JRLM, Morrow M, Osborne CK (eds) Diseases of the breast, third edn. Lippincott Williams & Wilkins, Philadelphia, pp 1101–1159
2. Cortazar P, Johnson BE (1999) Review of the efficacy of individualized chemotherapy selected by in vitro drug sensitivity testing for patients with cancer. J Clin Oncol 17:1625–1631
3. Fruehauf JP, Alberts DS (2003) Assay-assisted treatment selection for women with breast or ovarian cancer. Recent Results Cancer Res 161:126–145
4. Sekine I, Minna JD, Nishio K, et al. (2007) Genes regulating the sensitivity of solid tumor cell lines to cytotoxic agents: a literature review. Jpn J Clin Oncol 37:329–336
5. Sekine I, Minna JD, Nishio K, et al. (2006) A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in patients with lung cancer. J Thorac Oncol 1:31–37
6. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2:48–58
7. Trock BJ, Leonessa F, Clarke R (1997) Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst 89:917–931
8. Kellner U, Sehested M, Jensen PB, et al. (2002) Culprit and victim – DNA topoisomerase II. Lancet Oncol 3:235–243
9. Di Leo A, Isola J (2003) Topoisomerase II alpha as a marker predicting the efficacy of anthracyclines in breast cancer: are we at the end of the beginning? Clin Breast Cancer 4:179–186
10. Zhou BP, Hung MC (2003) Dysregulation of cellular signaling by HER2/neu in breast cancer. Semin Oncol 30:38–48
11. Ross JS, Fletcher JA, Bloom KJ, et al. (2003) HER-2/neu testing in breast cancer. Am J Clin Pathol 120 (Suppl):S53–71
12. Yamauchi H, Stearns V, Hayes DF (2001) When is a tumor marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. J Clin Oncol 19:2334–2356
13. Mano MS, Rosa DD, De Azambuja E, et al. (2007) The 17q12-q21 amplicon: Her2 and topoisomerase-II alpha and their importance to the biology of solid tumours. Cancer Treat Rev 33:64–77
14. Arriola E, Marchio C, Tan DS, et al. (2008) Genomic analysis of the HER2/TOP2A amplicon in breast cancer and breast cancer cell lines. Lab Invest 88:491–503
15. Lacroix M, Toillon RA, Leclercq G (2006) p53 and breast cancer, an update. Endocr Relat Cancer 13:293–325
16. Cimoli G, Malacarne D, Ponassi R, et al. (2004) Meta-analysis of the role of p53 status in isogenic systems tested for sensitivity to cytotoxic antineoplastic drugs. Biochim Biophys Acta 1705:103–120
17. Davis JM, Navolanic PM, Weinstein-Oppenheimer CR, et al. (2003) Raf-1 and Bcl-2 induce distinct and common pathways that contribute to breast cancer drug resistance. Clin Cancer Res 9:1161–1170
18. Hamilton A, Piccart M (2000) The contribution of molecular markers to the prediction of response in the treatment of breast cancer: a review of the literature on HER-2, p53 and BCL-2. Ann Oncol 11:647–663
19. Ro J, Sahin A, Ro JY, et al. (1990) Immunohistochemical analysis of P-glycoprotein expression correlated with chemotherapy resistance in locally advanced breast cancer. Hum Pathol 21:787–791
20. Veneroni S, Zaffaroni N, Daidone MG, et al. (1994) Expression of P-glycoprotein and in vitro or in vivo resistance to doxorubicin and cisplatin in breast and ovarian cancers. Eur J Cancer 30A:1002–1007
21. Chevillard S, Pouillart P, Beldjord C, et al. (1996) Sequential assessment of multidrug resistance phenotype and measurement of S-phase fraction as predictive markers of breast cancer response to neoadjuvant chemotherapy. Cancer 77:292–300
22. Bottini A, Berruti A, Bersiga A, et al. (2000) p53 but not bcl-2 immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients. Clin Cancer Res 6:2751–2758
23. Burger H, Foekens JA, Look MP, et al. (2003) RNA expression of breast cancer resistance protein, lung resistance-related protein, multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response. Clin Cancer Res 9:827–836
24. Foekens JA, Romain S, Look MP, et al. (2001) Thymidine kinase and thymidylate synthase in advanced breast cancer: response to tamoxifen and chemotherapy. Cancer Res 61:1421–1425
25. Hasegawa S, Miyoshi Y, Egawa C, et al. (2003) Prediction of response to docetaxel by quantitative analysis of class I and III

beta-tubulin isotype mRNA expression in human breast cancers. Clin Cancer Res 9:2992–2997
26. Jarvinen TA, Holli K, Kuukasjarvi T, et al. (1998) Predictive value of topoisomerase II alpha and other prognostic factors for epirubicin chemotherapy in advanced breast cancer. Br J Cancer 77:2267–2273
27. Coon JS, Marcus E, Gupta-Burt S, et al. (2002) Amplification and overexpression of topoisomerase II alpha predict response to anthracycline-based therapy in locally advanced breast cancer. Clin Cancer Res 8:1061–1067
28. MacGrogan G, Rudolph P, Mascarel Id I, et al. (2003) DNA topoisomerase II alpha expression and the response to primary chemotherapy in breast cancer. Br J Cancer 89:666–671
29. Martin-Richard M, Munoz M, Albanell J, et al. (2004) Serial topoisomerase II expression in primary breast cancer and response to neoadjuvant anthracycline-based chemotherapy. Oncology 66:388–394
30. Park K, Kim J, Lim S, et al. (2003) Topoisomerase II-alpha (topoII) and HER2 amplification in breast cancers and response to preoperative doxorubicin chemotherapy. Eur J Cancer 39:631–634
31. Wright C, Cairns J, Cantwell BJ, et al. (1992) Response to mitoxantrone in advanced breast cancer: correlation with expression of c-erbB-2 protein and glutathione S-transferases. Br J Cancer 65:271–274
32. Egawa C, Motomura K, Miyoshi Y, et al. (2003) Increased expression of BRCA1 mRNA predicts favorable response to anthracycline-containing chemotherapy in breast cancers. Breast Cancer Res Treat 78:45–50
33. Bonnefoi H, Diebold-Berger S, Therasse P, et al. (2003) Locally advanced/inflammatory breast cancers treated with intensive epirubicin-based neoadjuvant chemotherapy: are there molecular markers in the primary tumour that predict for 5-year clinical outcome? Ann Oncol 14:406–413
34. Niskanen E, Blomqvist C, Franssila K, et al. (1997) Predictive value of c-erbB-2, p53, cathepsin-D and histology of the primary tumour in metastatic breast cancer. Br J Cancer 76:917–922
35. Rozan S, Vincent-Salomon A, Zafrani B, et al. (1998) No significant predictive value of c-erbB-2 or p53 expression regarding sensitivity to primary chemotherapy or radiotherapy in breast cancer. Int J Cancer 79:27–33
36. Vincent-Salomon A, Carton M, Freneaux P, et al. (2000) ERBB2 overexpression in breast carcinomas: no positive correlation with complete pathological response to preoperative high-dose anthracycline-based chemotherapy. Eur J Cancer 36:586–591
37. Geisler S, Lonning PE, Aas T, et al. (2001) Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res 61:2505–2512
38. Zhang F, Yang Y, Smith T, et al. (2003) Correlation between HER-2 expression and response to neoadjuvant chemotherapy with 5-fluorouracil, doxorubicin, and cyclophosphamide in patients with breast carcinoma. Cancer 97:1758–1765
39. Burcombe RJ, Makris A, Richman PI, et al. (2005) Evaluation of ER, PgR, HER-2 and Ki-67 as predictors of response to neoadjuvant anthracycline chemotherapy for operable breast cancer. Br J Cancer 92:147–155
40. Prisack HB, Karreman C, Modlich O, et al. (2005) Predictive biological markers for response of invasive breast cancer to anthracycline/cyclophosphamide-based primary (radio-) chemotherapy. Anticancer Res 25:4615–4621
41. Manna Edel F, Teixeira LC, Alvarenga M (2006) Association between immunohistochemical expression of topoisomerase II alpha, HER2 and hormone receptors and response to primary chemotherapy in breast cancer. Tumori 92:222–229
42. Konecny GE, Thomssen C, Luck HJ, et al. (2004) Her-2/neu gene amplification and response to paclitaxel in patients with metastatic breast cancer. J Natl Cancer Inst 96:1141–1151
43. Bozzetti C, Musolino A, Camisa R, et al. (2006) Evaluation of HER-2/neu amplification and other biological markers as predictors of response to neoadjuvant anthracycline-based chemotherapy in primary breast cancer: the role of anthracycline dose intensity. Am J Clin Oncol 29:171–177
44. Baselga J, Seidman AD, Rosen PP, et al. (1997) HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications. Oncology (Williston Park) 11:43–48
45. Sjostrom J, Collan J, von Boguslawski K, et al. (2002) C-erbB-2 expression does not predict response to docetaxel or sequential methotrexate and 5-fluorouracil in advanced breast cancer. Eur J Cancer 38:535–542
46. Di Leo A, Chan S, Paesmans M, et al. (2004) HER-2/neu as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. Breast Cancer Res Treat 86:197–206
47. Frassoldati A, Adami F, Banzi C, et al. (1997) Changes of biological features in breast cancer cells determined by primary chemotherapy. Breast Cancer Res Treat 44:185–192
48. Bonetti A, Zaninelli M, Leone R, et al. (1998) bcl-2 but not p53 expression is associated with resistance to chemotherapy in advanced breast cancer. Clin Cancer Res 4:2331–2336
49. Colleoni M, Orvieto E, Nole F, et al. (1999) Prediction of response to primary chemotherapy for operable breast cancer. Eur J Cancer 35:574–579
50. Kandioler-Eckersberger D, Ludwig C, Rudas M, et al. (2000) TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. Clin Cancer Res 6:50–56
51. Rahko E, Blanco G, Soini Y, et al. (2003) A mutant TP53 gene status is associated with a poor prognosis and anthracycline-resistance in breast cancer patients. Eur J Cancer 39:447–453
52. Ogston KN, Miller ID, Schofield AC, et al. (2004) Can patients' likelihood of benefiting from primary chemotherapy for breast cancer be predicted before commencement of treatment? Breast Cancer Res Treat 86:181–189
53. Berns EM, Foekens JA, Vossen R, et al. (2000) Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. Cancer Res 60:2155–2162
54. Geisler S, Borresen-Dale AL, Johnsen H, et al. (2003) TP53 gene mutations predict the response to neoadjuvant treatment with 5-fluorouracil and mitomycin in locally advanced breast cancer. Clin Cancer Res 9:5582–5588
55. Buchholz TA, Garg AK, Chakravarti N, et al. (2005) The nuclear transcription factor kappaB/bcl-2 pathway correlates with pathologic complete response to doxorubicin-based neoadjuvant chemotherapy in human breast cancer. Clin Cancer Res 11: 8398–8402
56. Sjostrom J, Blomqvist C, von Boguslawski K, et al. (2002) The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res 8:811–816
57. Krajewski S, Blomqvist C, Franssila K, et al. (1995) Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res 55:4471–4478

original article

Annals of Oncology 21: 255–262, 2010
doi:10.1093/annonc/mdp304
Published online 24 July 2009

Association between gain-of-function mutations in *PIK3CA* and resistance to HER2-targeted agents in *HER2*-amplified breast cancer cell lines

Y. Kataoka[1], T. Mukohara[2,3]*, H. Shimada[4], N. Saijo[4], M. Hirai[1] & H. Minami[2,3]

[1]*Hospital Pharmacy;* [2]*Cancer Center, Kobe University Hospital;* [3]*Medical Oncology, Department of Medicine, Kobe University Graduate School of Medicine, Chuo-ku, Kobe and* [4]*Research Center for Innovative Oncology, National Cancer Hospital East, Kashiwa, Japan*

Received 24 April 2009; accepted 4 May 2009

Background: The mechanism of resistance to human epidermal growth factor receptor 2 (HER2)-targeted agents has not been fully understood. We investigated the influence of *PIK3CA* mutations on sensitivity to HER2-targeted agents in naturally derived breast cancer cells.

Materials and methods: We examined the effects of Calbiochem (CL)-387,785, HER2 tyrosine kinase inhibitor, and trastuzumab on cell growth and *HER2* signaling in eight breast cancer cell lines showing *HER2* amplification and trastuzumab-conditioned BT474 (BT474-TR).

Results: Four cell lines with *PIK3CA* mutations (E545K and H1047R) were more resistant to trastuzumab than the remaining four without mutations (mean percentage of control with 10 μg/ml trastuzumab: 58% versus 92%; $P = 0.010$). While *PIK3CA*-mutant cells were more resistant to CL-387,785 than *PIK3CA*-wild-type cells (mean percentage of control with 1 μM CL-387,785: 21% versus 77%; $P = 0.001$), CL-387,785 retained activity against BT474-TR. Growth inhibition by trastuzumab and CL-387,785 was more closely correlated with changes in phosphorylation of S6K (correlation coefficient, 0.811) than those of HER2, Akt, or ERK1/2. Growth of most *HER2*-amplified cells was inhibited by LY294002, regardless of *PIK3CA* genotype.

Conclusions: *PIK3CA* mutations are associated with resistance to HER2-targeted agents. PI3K inhibitors are potentially effective in overcoming trastuzumab resistance caused by *PIK3CA* mutations. S6K phosphorylation is a possibly useful pharmacodynamic marker in HER2-targeted therapy.

Key words: breast cancer, HER2, *PIK3CA*, trastuzumab

introduction

Breast cancer is the leading cause of cancer death among women worldwide, with ~1 million new cases reported each year [1, 2]. Approximately 20% of breast cancer tumors show overexpression of the HER2 protein, which is mainly caused by gene amplification. HER2 overexpression has been repeatedly identified as a poor prognostic factor [3, 4]. Trastuzumab is a humanized mAb targeting the extracellular domain of the HER2 protein. From the late 1990s, clinical studies have intensively evaluated the therapeutic roles of trastuzumab. For the treatment of HER2-overexpressing metastatic breast cancers, studies report that a combination of trastuzumab and conventional chemotherapy shows significantly higher efficacy than chemotherapy alone [5]. The use of trastuzumab has extended to the treatment of operable HER2-overexpressing breast cancer as an adjuvant or neoadjuvant [6–8]. Despite promising usefulness in clinics, a modest percentage of patients are reported to benefit from trastuzumab therapy, with response rates to trastuzumab as a single agent of ~20% [9]. In addition, even when trastuzumab therapy leads to temporary tumor shrinkage, clinical relapse is observed for the vast majority of metastatic patients. To develop adequate therapies capable of overcoming primary and secondary resistance to trastuzumab, a better understanding of the resistance mechanism is crucial.

To date, several mechanisms of primary resistance to trastuzumab have been proposed. A series of studies indicated that trastuzumab resistance is due to the truncated form of HER2, which lacks an extracellular domain to which trastuzumab is indicated to attach [10, 11]. Nagata et al. [12] demonstrated that loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K, correlates with poor response to trastuzumab. More recently, the roles of *PIK3CA* in trastuzumab resistance have been under particular investigation. Somatic mutations of *PIK3CA* were first identified in 2004 in various malignant tumors including breast cancer [13]. Subsequent studies have reported that the E545K and H1047R hotspot mutations, found

Correspondence to: Dr T. Mukohara, Department of Medical Oncology, Kobe University Hospital, 7-5-2, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel: +81-78-382-5825; Fax: +81-78-382-5821; E-mail: mukohara@med.kobe-u.ac.jp

on exons 9 and 20, respectively, are the most frequent types of mutation, found in 8%–40% of breast cancer tumors [13–16]. Both hotspot mutations are gain-of-function mutations which transform normal mammary epithelial cells [17, 18]. Berns et al. [19] investigated the roles of gain-of-function mutations of the *PIK3CA* gene in trastuzumab resistance by transfecting wild-type and mutant (H1047R) forms of *PIK3CA* in SKBR-3 HER2-overexpressing breast cancer cells. Results showed that compared with green fluorescent protein (GFP) control, both wild-type and mutant *PIK3CA* transfections resulted in trastuzumab resistance. Further, analysis of *PIK3CA* genotypes in tumor samples obtained from breast cancer patients having undergone trastuzumab-based therapy showed an association between the presence of *PIK3CA* hotspot mutations and shorter time to progression after therapy [19].

Tyrosine kinase inhibitors (TKIs) have also been investigated as potential agents against trastuzumab resistance [20]. A clinical study in metastatic breast cancer patients having previously experienced tumor progression under trastuzumab-based therapies showed that compared with capecitabine alone, treatment using a combination of capecitabine with lapatinib, a dual inhibitor of epidermal growth factor receptor (EGFR) and HER2 tyrosine kinase, lead to significantly longer time to progression [21]. Eichhorn et al. [22], however, demonstrated that transfection of mutant *PIK3CA* (H1047R) in BT474 HER2-overexpressing breast cancer cells resulted in resistance to lapatinib compared with parental cells. Further, results showed that resistance was overcome using NVP-BEZ235, a PI3K and mammalian target of rapamycin dual inhibitor [22].

These findings based on gene manipulations indicate that gain-of-function mutations in the *PIK3CA* gene lead to resistance to trastuzumab, as well as HER2-TKI. To our knowledge, however, these findings have not been confirmed in naturally derived breast cancer cells. Here, trastuzumab resistance due to *PIK3CA* mutations was evaluated in eight naturally derived breast cancer cell lines harboring *HER2* gene amplification. Further, possible therapeutic means to overcome primary and secondary resistance to trastuzumab were investigated, as well as potential pharmacodynamic markers correlated with the growth-inhibitory effect of HER2-targeted drugs.

materials and methods

cell culture

MCF-7, MDA-MB-361, HCC1954, MDA-MB-453, UACC893, CAMA-1, MDA-MB-435S, MDA-MB-415, ZR75-30, HCC70, MDA-MB-468, and HCC1419 cell lines were purchased from the American Type Culture Collection (Manassas, VA). BT474, SKBR-3, BT549, T47D, ZR75-1, and MDA-MB-231 cells were kindly provided by Ian Krop of the Dana-Farber Cancer Institute. Of the 18 breast cancer cell lines, eight (ZR75-30, BT474, SKBR-3, HCC1419, MDA-MB-453, MDA-MB-361, HCC1954, and UACC 893) were reported to have *HER2* gene amplification [23], with levels of PTEN protein expression equivalent to those reported in our previous study [24]. Among the *HER2*-amplified cell lines, ZR75-30, SKBR-3, and HCC1419 were reported to contain the wild-type *PIK3CA* gene and MDA-MB-453, MDA-MB-361, HCC1954, and UACC893 hotspot *PIK3CA* mutations (Table 1) [14]. BT474 was reported to contain a relatively rare type of *PIK3CA* mutation at exon 2, K111N (Table 1) [14]. MDA-MB-435S, MDA-MB-468, and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (Cellgro; Mediatech, Inc., Herndon, CA) with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Inc., Woodland, CA), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine. The remaining cell lines were maintained in RPMI-1640 medium (Cellgro; Mediatech, Inc.) supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine. All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 and were in logarithmic growth phase at initiation of the experiments.

Table 1. Genotype of *PIK3CA* in *HER2*-amplified breast cancer cell lines

Cell line	Genotype of *PIK3CA*
BT474	K111N
ZR75-30	wt
SKBR-3	wt
HCC1419	wt
MDA-MB-361	E545K
MDA-MB-453	H1047R
HCC1954	H1047R
UACC893	H1047R

wt, wild-type.

drugs

Trastuzumab was obtained from the Kobe University Hospital pharmacy. CL-387,785, a dual inhibitor of EGFR and HER2 [25], and LY294002, a PI3K inhibitor, were purchased from Calbiochem (San Diego, CA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Before each experiment, drugs were diluted in fresh media. The final DMSO concentration was <0.1% for all experiments.

antibodies and western blotting

Cells were washed with ice-cold phosphate-buffered saline and scraped immediately after adding lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA] containing protease and phosphatase inhibitors (100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 2 μg/ml aprotinin, and 5 μg/ml leupeptin). Lysates were centrifuged at 14 000 relative centrifugal force for 10 min. Supernatants were collected as protein extract and then separated by electrophoresis on 7.6% polyacrylamide–sodium dodecyl sulfate gels, followed by transfer to nitrocellulose membranes (Millipore Corporate Headquarters, Billerica, MA) and detection by immunoblotting using an enhanced chemiluminescence system (New England Nuclear Life Science Products, Inc., Boston, MA). The resulting signals were digitally quantified using the ImageJ software (www.nih.gov). Phospho-HER2/ErbB2 (Thr1221/1222), phospho-p70 S6 Kinase (Thr389), phospho-Akt (Ser473)(D9E), and PathScan(R) Multiplex Western Cocktail I were purchased from Cell Signaling Technology (Beverly, MA). The phospho-1/2 (pT185/pY187) antibody was purchased from Biosource International Inc. (Camarillo, CA), the c-erbb-2 antibody from Chemicon (Billerica, MA), and β-actin antibody from Sigma-Aldrich (St Louis, MO).

cell growth assay

Growth inhibition was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI), a colorimetric method for determining the number of viable cells based on the bioreduction of MTS to a soluble formazan product, which is detectable by spectrophotometry at a wavelength of 490 nm. Cells were diluted in 160 μl/well of maintenance cell culture media and plated in 96-well flat-bottom plates (Corning, Inc., Corning, NY). After a 96-h growth period, the number of cells required to obtain an absorbance of 1.3–2.2, the linear range of the assay, was

determined for each cell line beforehand. The number of cells per well used in the subsequent experiments were as follows: MCF-7, 2000; MDA-MB-361, 8000; HCC1954, 2500; MDA-MB-453, 7000; UACC893, 7500; CAMA-1, 6000; MDA-MB-435S, 2000; ZR75-30, 7500; HCC70, 4000; HCC1419, 8000; BT474, 3000; SKBR-3, 2500; BT549, 2000; T47D, 2500; ZR75-1, 7500; MDA-MB-415, 5000; and MDA-MB-231, 2500. At 24 h after plating, cell culture media were replaced with 10% FBS-containing media with and without trastuzumab or CL-387,785, followed by incubation for an additional 120 h. Trastuzumab and CL-387,785 concentrations ranged from 33 ng/ml to 100 μg/ml and from 3.3 nM to 10 μM, respectively. A total of 6–12 plate wells were set for each experimental point, and all experiments were carried out at least in triplicate. Data are expressed as percentage of growth relative to that of untreated control cells.

generation of *in vitro* BT474-TR

To generate a cell line resistant to trastuzumab, BT474 cells were continuously exposed to 100 μg/ml trastuzumab. To confirm the emergence of resistant clones, MTS assays were carried out every five passages after allowing cells to grow in drug-free conditions for at least 4 days. After 11 months of drug exposure, cells showed sufficient resistance (Figure 1) and were designated as BT474-TR. For controls, BT474 parental cells were concomitantly maintained without trastuzumab, and drug sensitivity was compared with trastuzumab-conditioned cells. No significant change in the sensitivity to trastuzumab was observed in parental cells during the drug-exposure period (data not shown).

results

inhibitory effect of trastuzumab on growth in breast cancer cell lines

We first screened 17 breast cancer cell lines for *in vitro* growth inhibition using trastuzumab. We confirmed that all relatively sensitive cell lines were *HER2*-amplified (Figure 2A). Among eight *HER2*-amplified cell lines, those with hotspot mutations in *PIK3CA* appeared resistant compared with the remaining cell lines (Figure 2B and C). We categorized BT474 as a *PIK3CA* wild-type cell line in this study, based on reports showing that the K111N mutation lack ability of transformation and its influence on downstream signaling is negligible [18, 26]. A significant difference in sensitivity at 10 μg/ml trastuzumab was observed between *PIK3CA*-wild-type and -mutant cells (Figure 2C; $P = 0.010$). Protein expression levels of p110-α, the product of *PIK3CA*, were not correlated with sensitivity to trastuzumab (Figure 2C).

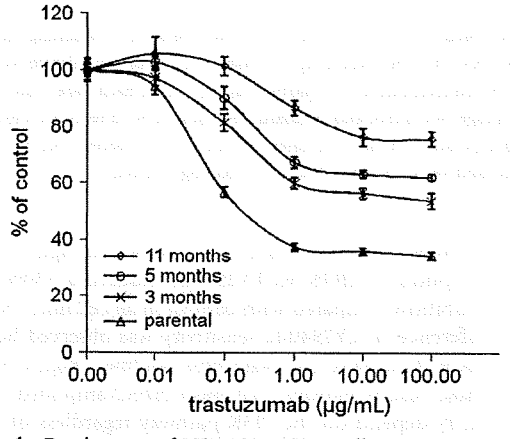

Figure 1. Development of BT474-TR. BT474 cells were continuously exposed to 100 μg/ml trastuzumab. BT-474 and trastuzumab-conditioned BT474 cells were grown in 10% serum-containing media for 5 days in the presence of various concentrations of trastuzumab. The percentage of viable cells is shown relative to that of the untreated control and plotted on the *y*-axis, whereas trastuzumab concentrations are plotted on the *x*-axis. Each data point represents the mean value and standard deviation of 6–12 replicate wells. Trastuzumab resistance increased in cells in a time-dependent manner. After 11 months, cells were designated as BT474-TR.

association between *PIK3CA* mutations and HER2-TKI resistance

Lapatinib, a HER2-TKI which may potentially overcome trastuzumab resistance, has been used in clinical settings [21]. We therefore tested a commercially available HER2-TKI, CL-387,785 [25], on *HER2*-amplified breast cancer cells. As shown in Figure 2D, cell lines with hotspot *PIK3CA* mutations showed resistance to CL-387,785. A statistically significant difference in sensitivity to 1 μM CL-387,785 was observed between *PIK3CA*-wild-type and -mutant cells (Figure 2C; $P = 0.001$) [24].

We then established a trastuzumab-resistant BT474 cell line (BT474-TR), a model of secondary resistant cells, by continuous exposure to trastuzumab (see 'Materials and methods' section). In contrast to *PIK3CA*-mutant cells, which showed primary resistance to trastuzumab, BT474-TR cells remained sensitive to CL-387,785 (Figure 3), which indicates that secondary resistant cells maintain dependency on HER2 signaling for growth.

association between phosphorylation change in S6K and growth inhibition by HER2-targeted agents

To identify potential pharmacodynamic markers of sensitivity to HER2-targeted therapy, we examined changes in phosphorylation of HER2 and representative downstream signaling molecules in 10% FBS-containing media with or without 10 μg/ml trastuzumab or 1 μM CL-387,785 (Figure 4A). The trastuzumab concentration was selected based on maintained growth inhibition (Figure 2B) and wide use in previous studies [11, 19]. The 1-μM CL-387,785 concentration was selected based on the approximate maximum plasma concentration of most TKIs available in clinics to date, including lapatinib [27], and use in previous studies [28, 29].

Trastuzumab treatment resulted in moderate phosphorylation inhibition of Akt and/or S6K in cell lines with wild-type *PIK3CA*. In contrast, no significant changes in Akt and S6K phosphorylation were observed in cell lines with hotspot mutant *PIK3CA*, as well as in BT474-TR cells. Although in ZR75-30, trastuzumab treatment appeared to inhibit phospho-ERK1/2, no significant changes were observed in other sensitive cells, namely BT474 and SKBR-3 (Figure 4A). In addition, phospho-ERK1/2 levels increased in MDA-MB-361 and UACC893, which indicates the presence of compensational cell signaling. Further, with the exception of HCC1419, treatment with CL-387,785 resulted in significant inhibition of Akt and S6K phosphorylation in BT474-TR and *PIK3CA*-wild-type cells, whereas residual phosphorylation signals were observed in all *PIK3CA* hotspot mutant cells.

doi:10.1093/annonc/mdp304