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Case Report

Brain Metastases after Achieving Local Pathological Complete Responses with Neoadjuvant Chemotherapy

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Background: We encountered two patients with inflammatory breast carcinoma who developed symptomatic brain metastases after achieving local pathological complete responses (pCR) with neoadjuvant chemotherapy (NAC).

Case presentations: The first patient is a 39-year-old woman (Case 1), who underwent NAC with AC (doxorubicin + cyclophosphamide) followed by weekly paclitaxel. After achieving a clinical CR (cCR), we conducted a modified radical mastectomy. Pathological evaluation confirmed no residual malignant cells within the breast tissue or lymph nodes. However, she developed neurological symptoms from brain metastases one month postoperatively. The second patient is a 44-year-old woman (Case 2). Again, no residual malignant cells were detected within the breast tissue or lymph nodes following NAC, but the patient developed symptomatic brain metastases eight months postoperatively. When primary breast tumors are locally advanced, it may be worthwhile to rule out brain metastases even if pCR is obtained after NAC.

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Key words: Brain metastasis, Pathological complete response, Breast cancer

Introduction

Neoadjuvant chemotherapy (NAC) is a standard treatment option for patients with locally advanced and/or inflammatory breast cancers. The outcomes of patients achieving pCR of their primary tumors are significantly better than those with residual disease[1-3]. Here, we introduce two patients who developed symptomatic brain metastases shortly after documented pCRs following NAC and surgery.

Case Report

Case 1

A 39-year-old premenopausal woman sought medical attention for erythematous induration of her left breast. With a working diagnosis of inflammatory breast cancer, fine needle aspiration cytology revealed adenocarcinoma. The patient was referred to the National Cancer Center Hospital for further treatment in February 2005. Physical examination revealed an indistinct 12 cm mass in the upper area of the left breast, and the surface of this lesion exhibited a peau d'orange appearance. Axillary and supraclavicular lymph nodes were palpable and measured 4 and 2 cm in diameter, respectively. The axillary lymph node was fixed to the surrounding tissue. Ultrasonography (US) revealed a 7 cm breast mass with dermal thickening, edematous subcutaneous tissue, and enlarged lymph nodes (Fig 1a). These findings were also observed on computed tomography (CT) and magnetic resonance imaging (MRI).

Core needle biopsy led to a pathological diagnosis of invasive ductal carcinoma (grade 3, nuclear grade 3, and HER-2 negative) (Fig 2a). The tumor was negative for both estrogen and progesterone receptors. Chest X-ray, bone scintigraphy, abdominal US, and chest and abdominal CT revealed no distant metastases. Due to the presumed low incidence of brain metastases at this clinical stage, brain imaging was not done at

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Abbreviations:
pCR, Pathological complete response; NAC, neoadjuvant chemotherapy; US, ultrasonography; CT, Computed tomography; MRI, Magnetic resonance imaging

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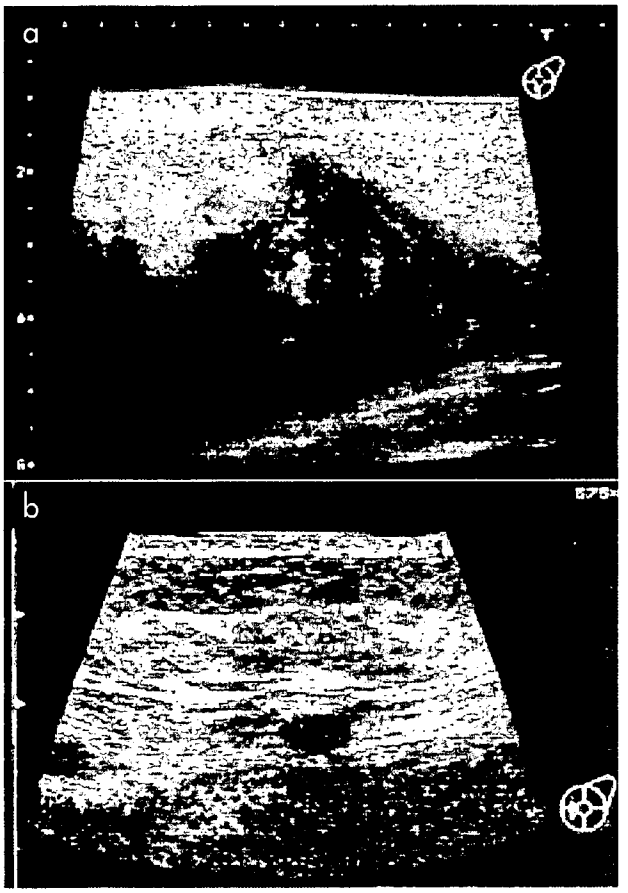

Fig 1. (a) US reveals a 7 cm breast mass with overlying skin thickening, edematous subcutaneous tissue. (b) US reveals no residual tumor following neoadjuvant chemotherapy.

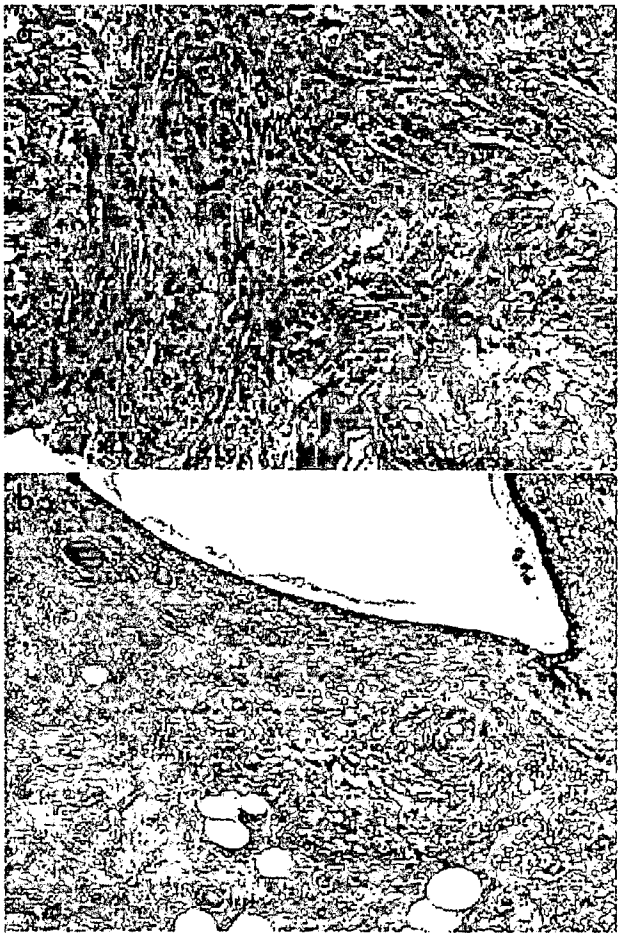

Fig 2. (a) Core needle biopsy reveals invasive ductal carcinoma, grade 3, nuclear grade 3. (b) No residual tumor is detected. The presence of inflammatory cells surrounding a duct with an increased number of enlarged capillary vessels, typical after tumor disappearance, is observed. (hematoxylin-eosin staining, × 100).

this point. Inflammatory breast cancer of the left breast was initially diagnosed, T4dN3M0, Stage IIIC, according to the general rules for clinical and pathological grading of breast cancers[4]. She received NAC from February to July consisting of doxorubicin and cyclophosphamide (60/600 mg/m^2) 4 times every 3 weeks, followed by paclitaxel (80 mg/m^2) weekly for 12 weeks. Following NAC, only induration of her left breast was apparent upon physical examination, and no breast masses or axillary lymph nodes were detected by US (Fig 1b) and CT. Additionally, serum levels of tumor markers (CEA, CA 15-3, ST 439) remained within normal limits before and after chemotherapy. We subsequently conducted a modified radical mastectomy in August, and no malignant cells were detected in the resected breast tissue and dissected axillary lymph nodes (Fig 2b). However, the patient presented with vertigo and severe headache prior to the initiation of radiotherapy to the left chest wall in September. Brain MRI revealed multiple metastatic lesions in her right frontal lobe, temporal lobe, and bilateral cerebellum (Fig 3). To control her symptoms, whole-brain radiotherapy with a total dose of 30 Gy/10 fractions was incorporated in October. However, her condition deteriorated, and she expired in December.

Case 2

A 44-year-old premenopausal woman was seen at a nearby hospital with a chief complaint of an erythematous enlarged right breast. Inflammatory breast cancer was suspected, so she was referred to our institution in December 2004.

On initial examination, the right breast was firm, erythematous, and edematous with a thickened dermis. Axillary and supraclavicular lymph nodes were palpable and measured 5 cm and 1 cm

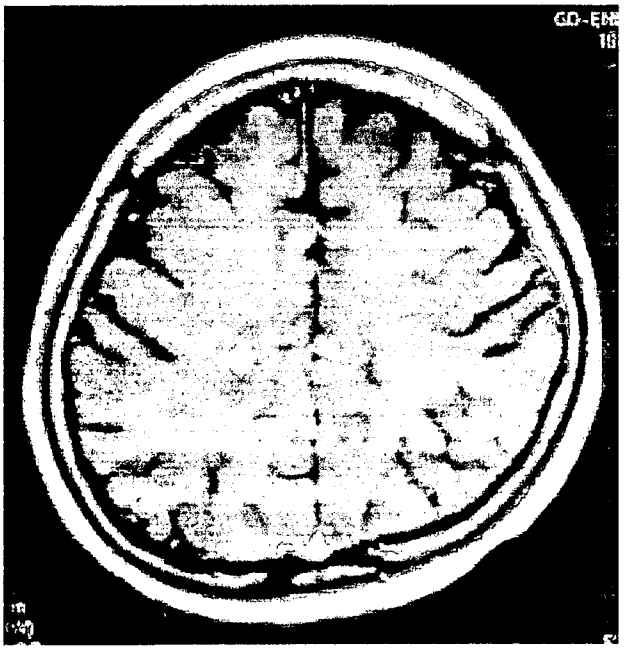

Fig 3. The metastatic lesions exhibited high signal intensity in the right temporal lobe by T1 weighted MRI.

in diameter, respectively. CT showed a large right breast mass with an edematous dermis and subcutaneous tissue.Additionally, the axillary and supraclavicular lymph nodes were enlarged (Fig 4a). The specimen obtained by the core needle biopsy was consistent with an invasive ductal carcinoma (solid tubular type, grade 3, nuclear grade 3, HER-2 negative, estrogen and progesterone receptor negative) (Fig 5a). No metastatic lesions were detected by bone scintigraphy, chest X-ray, chest CT, or abdominal US, though diagnostic brain imaging was not performed at that time. Serum tumor markers were elevated, with a CEA of 52.4 ng/ml, CA 15-3 of 279 U/ml, and NCC-ST 439 of 910 U/ml. Inflammatory breast cancer, T4dN3M0, Stage IIIC[4] was diagnosed. She underwent NAC from December to May 2005, using the same treatment regimen as Patient 1. Following NAC, physical examination revealed only induration of the right breast with slight thickening of the overlying skin. CT revealed a slightly enhanced, 3-cm lesion in the breast (Fig 4b) without enlarged lymph nodes. All tumor markers were within normal limits after chemotherapy. We performed a modified radical mastectomy in July, and no tumor cells were pathologically detected in the breast tissue and axillary lymph nodes (Fig 5b). Following surgery, we performed local radiotherapy with a total dose of 60 Gy/30 fractions from August through October. However, the patient developed

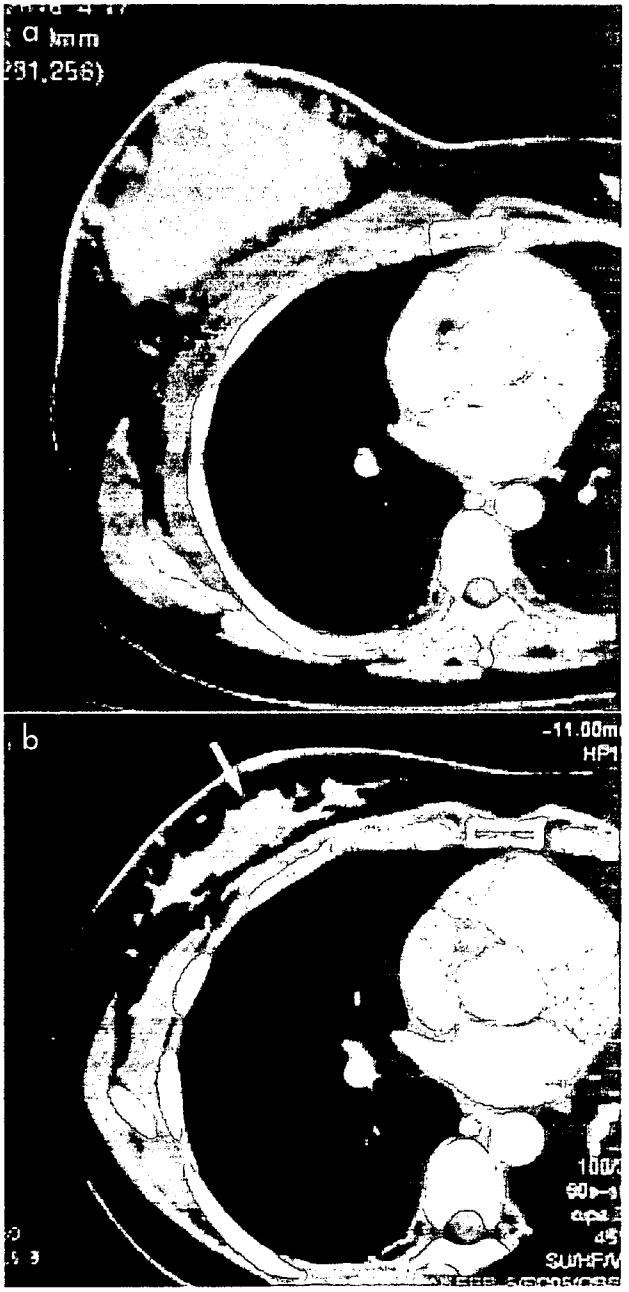

Fig 4. (a) CT shows a large right breast mass with overlying edematous subcutaneous tissue and thickened skin. This is not the early phase but late phase scan of breast CT, because only chest CT without an early phase scan was performed to detect distant metastasis instead of breast CT. (b) CT scan reveals a mass-like lesion measuring 3 cm, without enhancement, in the right breast.

headache and ambulatory disturbance in early December. Brain CT and MRI scans performed in March 2006 detected a tumor measuring 5 cm in diameter in her right temporal lobe with surrounding edema (Fig 6). A right frontotemporal craniotomy followed by whole-brain radiotherapy of 37.5 Gy/15 fractions was carried out from

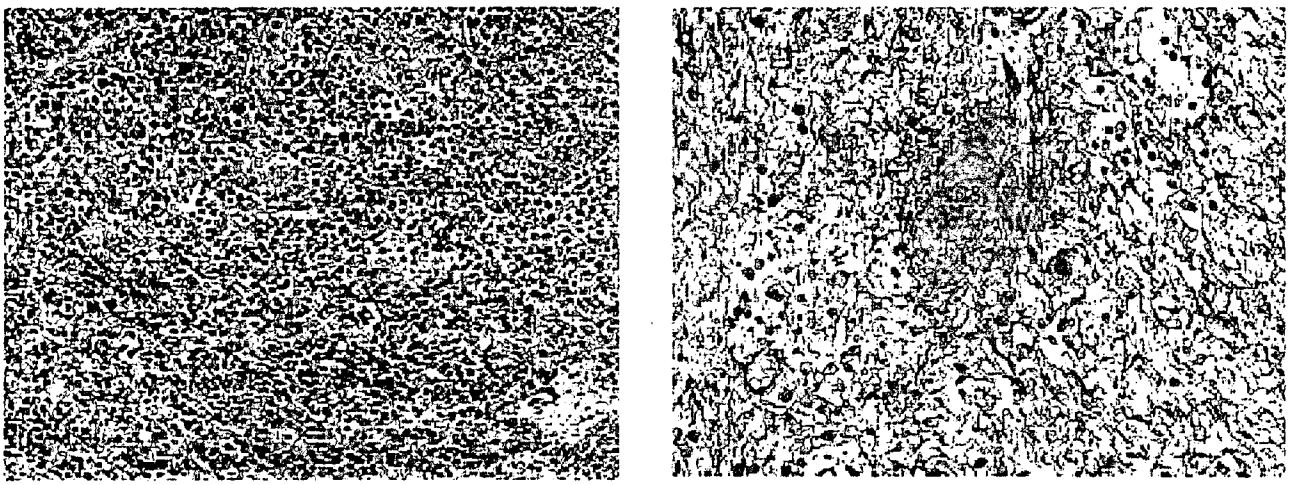

Fig 5. (a) Core needle biopsy reveals invasive ductal carcinoma, grade 3, nuclear grade 3. (b) No residual tumor is detected. Many foamy cells and a disturbance of the fiber rows after the disappearance of the tumor are observed (hematoxylin and eosin staining, ×100).

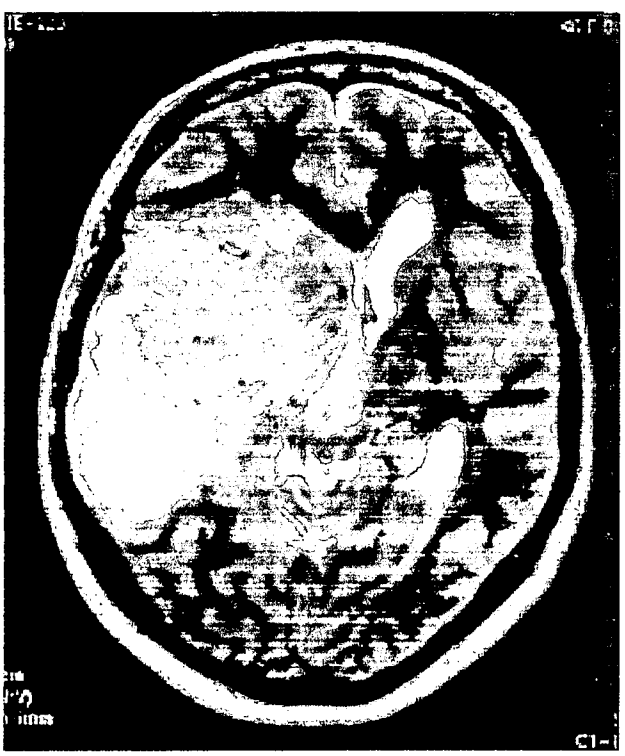

Fig 6. MRI demonstrates a tumor measuring 5 cm in diameter, with surrounding edema, in the right temporal lobe.

March through April. Intracranial recurrence is now controlled three months after radiotherapy.

Discussion

Several studies have indicated that breast cancer patients with pCR following NAC have better overall survival and disease-free survival rates[1-3]. Moreover, pCR of axillary lymph nodes is an excellent prognostic factor for locally advanced breast cancers[5-8]. The two cases presented were first diagnosed with inflammatory breast cancer with axillary and supraclavicular lymph node metastases. The patients achieved pCR for both the main tumors and the axillary lymph nodes following NAC, and favorable prognoses were expected from the published literature. However, both patients developed symptomatic brain metastases soon after mastectomy. The interval between surgery and the occurrence of neurological signs was only one month for Patient 1 and five months for Patient 2. This led us to the theory that the blood brain barrier restricted access of the chemotherapeutic agents to the central nervous system. Therefore despite locally effective NAC, occult brain metastases may continue to progress into clinical significance. This theory may help us understand the progression of brain metastases in these patients[9]. There have been no reports examining the rates of brain metastasis following NAC. Yet there are reports of patients receiving adjuvant chemotherapy having an increased incidence of brain metastases as the site of first recurrence compared to control[10, 11]. In the present cases, we suspect that subclinical metastases were present in the brain before initiating NAC. It is likely that, because of inadequate delivery of cytotoxic agents to the brain, these metastases continued to grow despite effective tumor control elsewhere the body.

Several studies have identified risk factors for brain metastases in patients with breast cancer. Young age[12, 13], unresponsiveness to the hormonal

therapies, and HER-2 over expression are reported risk factors[14-17]. Intracranial metastases are also related to the use of trastuzumab[18]. In the two patients presented here, relatively young age and the absences of both estrogen and progesterone receptor were concordant risk factors for developing brain metastases.

The combination of NAC and surgery can lead to favorable outcomes in many cases of breast cancer, but effective control over the primary lesions and the extracranial micrometastases by the cytotoxic agents may not predict future intracranial event. The blood brain barrier would likely prevent chemotherapeutic agents from reaching the central nervous system. As a consequence, brain metastases may continue to grow and become symptomatic despite pCR of primary sites and lymph node metastases. This can be a concerning factor, especially in patients at risk for developing brain metastases. Further investigations are warranted to identify the mechanisms leading to intracranial metastases, as well as pretherapeutic risk factors.

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COMMENTARY

The next step to approaching central nervous system metastasis in HER-2-positive metastatic breast cancer patients

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Trastuzumab is a recombinant humanized monoclonal antibody which inhibits tumor cell growth by targeting the HER-2/neu receptor. The efficacy of trastuzumab has been demonstrated by randomized controlled trials not only of metastatic breast cancer[1] but also in adjuvant treatment of primary breast cancer[2,3] This agent has dramatically changed the treatment strategy of HER-2-positive breast cancer.

Increased central nervous system (CNS) metastasis related to the use of trastuzumab has been of concern. Considering that breast cancer itself is a tumor which is often accompanied by CNS metastasis[4] the incidence of this may be still high.

In this issue of the *Asia–Pacific Journal of Clinical Oncology*, Dawson *et al.* reported the results of a retrospective study of 28 patients with HER-2-positive breast cancer treated with trastuzumab. In their study, the incidence of CNS metastasis was 39%, median survival time from diagnosis of CNS metastasis was 12.1 months. The uniqueness of their study is a long follow-up period and the quality of pathological information: 93% of HER-2-positive cases were confirmed by immunohistochemical staining (IHC) or Fluorolescence *in situ* hybridization (FISH). The limitation of their study was the small sample size and lack of control group.

We also investigated 70 patients with HER-2-positive breast cancer consecutively treated with trastuzumab from 1999 to 2002 in National Cancer Center Hospital, Japan.[5] All tumors were confirmed to be HER-2 positive by either IHC or FISH. The median follow-up time was 36 months. In our cohort, the incidence of CNS metastasis was 40% (28 patients out of 70) and median overall survival was 11 months, both observation being very similar to Dawson's study. This may be due to consistency in patient background such as the high proportion of patients to be confirmed as HER-2 positive and similar median follow up period.

The findings from previous and current studies are summarized in Table 1. There are some studies addressing the high incidence of CNS metastasis after treatment with trastuzumab, although others do not. The incidence varies from 10 to 48%.[6–10] This variation may be due to the nature or biases of retrospective studies, such as patient selection method or the method of defining of HER-2 positivity. The cohorts with high proportion of pathologically proven HER-2-positive cases seem to have higher incidence of CNS metastasis than those with low proportion of HER-2-positive cases. Only in one study was the incidence of brain metastasis very high, 48%. This is perhaps due to selection bias in that study, because patients were selected from an imaging database.

A short follow-up can bias survival data. Survival from diagnosis of CNS metastasis until death varies form 5 months to 25 months, as shown in the Table 1. However, if we confine to studies with adequate follow up period, the survival comes around one year, from 11 to 13 months.

Although patient background should be taken into account when we see the result of the studies, in overall, we may conclude that for breast cancer patients with CNS metastasis with properly examined HER-2 status and adequate follow-up period, the incidence and median survival of CNS metastasis is around 25–40% and about 12 months. The high incidence is more likely because of better systemic control by trastuzumab rather than the biology of HER2-posituve tumor.[11] With new active agents developed against systemic disease, e.g. bevacizumab, an antivascular endothelial growth factor monoclonal antibody, the increase in CNS

Table 1 Findings from previous and current studies of patients with HER-2-positive breast cancer

Author	N	BM%	HER2conf%	TTC	mf/u	OS	Source
Miller[8]	155	15	80	–	–	5 month	*Ann Oncol* 2003
Bendell[5]	122	34	–	6 month	22 month	13 month	*Cancer* 2003
Clayton[6]	93	25	100	10 month	11 month	6 month	*Br J Cancer* 2004
Lai[7]	79	48	47	–	–	25 month	*Cancer* 2004
Shmueli[9]	41	31	100	10 month	–	–	*Eur J Cancer* 2004
Dawson	28	39	93	12.1 month	31 month	12 month	This issue
Matsumoto	70	40	100	11.5 month	38 month	11 month	This issue

N, number of patients; BM%, percentage of patients with brain metastasis; Her2conf%, percentage of patients whose HER2 status was pathologically confirmed; TTC, time from start of trastuzumab to diagnosis of CNS metastasis; mf/u, median follow up period; OS, overall survival after CNS metastasis diagnosis.

metastasis has become of a further threat, because most available agents cannot cross the blood brain barrier (BBB).

Unfortunately we currently have limited treatment options for CNS metastasis, those are the local treatments such as irradiation, surgery or intrathecal chemotherapy. Dawson *et al.* mentioned two possible treatment approaches against CNS metastasis. The first one is to use more aggressive local therapy, such as prophylactic cranial irraditaion or repeating stereotacitic radio-surgery. Little is known about the safety and efficacy of such aggressive treatment. Considering the limitation of radiotherapy such as irreversible late toxicity or difficulties in re-irradiation, we need to investigate the way to select the best suitable patient population for aggressive local therapy. A second approach involves using new systemic agent which can pass through BBB. Temozolamide (TMZ) is a novel oral alkylating agent which can enter the BBB. The addition of TMZ to radiotherapy for newly diagnosed glioblastoma resulted in a clinically meaningful and statistically significant survival benefit, proven in recently reported phase III study.[12] Assessing this kind of new agents may make a breakthrough.

Furthermore, the current standard care does not recommend routine CNS screening, because there is no evidence to support the benefit of early diagnosis and treatment of CNS metastasis. Rather, some studies have recommended that prognoses of patients with symptomatic or asymptomatic CNS metastasis seem to be the same, but depending mainly on the control of extracranial metastasis.[9] This suggests that improved local treatment for CNS metastasis may improve the outcome of selected patients with better systemic control. Therefore, efforts for developing new surveillance strategy should be combined with new treatment development strategy. Rigorous challenges against CNS metastasis in both treatment and surveillance are now strongly warranted.

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ORIGINAL PAPER

Tumor-marker analysis and verification of prognostic models in patients with cancer of unknown primary, receiving platinum-based combination chemotherapy

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Abstract
Objectives: To evaluate the usefulness of tumor-marker measurements and to identify prognostic factors in patients with cancer of unknown primary (CUP), receiving platinum-based combination chemotherapy and to verify the adjustment of previously reported prognostic models in this population.
Methods: We conducted univariate and multivariate analyses in consecutive patients with CUP receiving platinum-based combination chemotherapy. Previously reported prognostic models were then validated in this population.
Results: A total of 93 patients were analyzed and the response rate to platinum-based chemotherapeutic regimens among the 93 patients was 39.8%. The median time to progression and overall survival period were 4.1 and 12.4 months, respectively. The ST-439 level was significantly higher in patients with histologically confirmed adenocarcinoma than in patients with poorly differentiated adenocarcinoma or poorly differentiated carcinoma. A multivariate analysis indicated that performance status, the number of involved organs, and the serum lactate dehydrogenase level were the prognostic factors of the outcome. Both the previously reported prognostic models for predicting the duration of survival in this population were shown to be valid.
Conclusion: Tumor-marker measurements are not helpful in the management of patients with CUP. Previously reported prognostic models may be useful for selecting indication for chemotherapy or for stratifying the patients in clinical trial.

Keywords Tumor marker · Chemotherapy · Cancer of unknown primary · Prognostic model · Stratification

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Introduction

Cancer of unknown primary (CUP) represents a group of heterogeneous malignancies and is defined by the presence of a metastatic disease without an identifiable primary tumor site on presentation. CUP accounts for approximately 2–3% of all newly diagnosed patients with solid malignancies. Approximately half of these patients will be diagnosed as having adenocarcinoma, 30% as having poorly differentiated adenocarcinoma or carcinoma, 15% as having squamous cell carcinoma, and the remaining 5% as having undifferentiated neoplasms (Greco and Hainsworth 2005).

Serum tumor markers for human chorionic gonadotropin β subunit (β-HCG), α-fetoprotein (AFP), and prostate-specific antigen (PSA) are useful for identifying treatable germ cell tumors or metastatic prostate cancer. In female patients, carbohydrate antigen 125 (CA125) can be of some help in diagnosing peritoneal carcinoma, which is usually treated as ovarian cancer (Greco and Hainsworth 2005; Varadhachary et al. 2004). A few studies have reported that common

serum tumor markers like carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3), and carbohydrate antigen 19-9 (CA19-9) were not generally useful in diagnostic or prognostic tests (Greco and Hainsworth 2005; Varadhachary et al. 2004; Pavlidis et al. 2003). However, routine measurements of various tumor markers in CUP and the role of tumor-marker measurements, besides indicating favorable subsets, have not been previously studied.

The prognosis of CUP is generally poor, with a median survival period of approximately 6–12 months. Some favorable subsets of patients with either clinical or pathologic features require specific treatment approaches and have the potential for prolonged survival (Greco and Hainsworth 2005; Pavlidis et al. 2003). However, most patients fit into an unfavorable subset that does not benefit from specific treatments and that potentially includes patients with broadly heterogeneous malignancies. In the 1980s, the use of platinum agents was shown to produce better responses and prolonged survival. With the introduction of new antineoplastic agents (i.e., paclitaxel, docetaxel, gemcitabine, and irinotecan), platinum-based combination chemotherapy provided treatment options for a large group of patients. However, since all of the studies were performed in non-randomized control settings, the benefits of the current therapy remains limited (Greco and Hainsworth 2005; Pavlidis et al. 2003). Many investigators have called for designed randomized trials in CUP patients belonging to the unfavorable subset, thereby generating the need for methods to select indication for chemotherapy or stratifying randomized trials, appropriately (van der Gaast et al. 1995; Culine et al. 2002).

Identifying prognostic models for patients with CUP is a challenge because of the vast heterogeneous nature of CUP. Previous studies used multivariate Cox regression analysis to identify prognostic factors for estimating the survival. Many prognostic factors were identified, including age, performance status, smoking history, number of metastatic sites, the presence of liver metastasis, and elevated serum alkaline phosphatase (ALP), or lactate dehydrogenase (LDH) levels (van der Gaast et al. 1995; Culine et al. 2002; Hainsworth et al. 1992; Abbruzzese et al. 1995; van de Wouw et al. 2004). Two studies described simple prognostic models for predicting survival. The previous study presented, but did not validate, a prognostic model based on the performance status and serum-ALP level (van der Gaast et al. 1995). Another study reported an externally validated prognostic model based on the performance status and serum-LDH level (Culine et al. 2002). However, these models may not be widely accepted in practical settings or clinical trials because both models were based on data from European populations and because CUP includes patients with heterogeneous cancers. Thus, these models may not be applicable to other populations or institutions. Consequently, these prognostic models should be verified in different populations; such an effort might contribute to advances in treatment strategies for CUP.

The aims of this study were as follows: (1) to evaluate the usefulness of various tumor-marker measurements for primary unknown cancer patients receiving platinum-based combination chemotherapy, (2) to identify predictive factors for response to chemotherapy and prognostic factors in this population, and (3) to verify the adjustment of previously reported simple prognostic models.

Patients and methods

This study retrospectively analyzed a total of 93 consecutive patients with CUP, who were treated with platinum-based combination chemotherapy between November 1997 and December 2005 at the National Cancer Center Hospital, Tokyo. All patients were diagnosed as having CUP if no primary tumor site could be identified after a thorough history and physical examination, complete blood cell counts and blood chemistry using routine tumor-marker measurements, chest radiography, a computed tomography scan between the neck and pelvis, upper gastrointestinal endoscopy, lower gastrointestinal endoscopy or barium enema imaging, urologist examination (male patients), mammography and gynecologist examination (female patients), and radiologic work-up for any symptomatic areas. All the pathological specimens were carefully evaluated by two or three pathologists to confirm the epithelial origin of the disease and to exclude other malignancies and specific tumor sites. All patients were examined for the presence of routine tumor markers, including AFP, β-HCG, protein induced by vitamin K absence-2, CEA, sialyl-specific embryonic antigen, cytokeratin 19 fragment (Cyfra), squamous-cell carcinoma antigen, CA19-9, CA15-3, sialyl Tn antigen, national cancer center-ST439 (ST-439), neuron-specific enolase (NSE), and progastrin-releasing peptide. In addition, the presence of PSA was examined in men and the presence of CA125 was examined in woman. Patients with squamous cell carcinoma or neuroendocrine carcinoma and patients with carcinomas belonging to any of the favorable subsets requiring well-defined treatments were excluded from the present study. All patients were required to provide written informed consent to review

medical chart and imaging, which approved by the institutional review board at the National Cancer Center.

We used the World Health Organization criteria to assess the response to treatment of patients with measurable lesions (Miller et al. 1981). We also used Response Evaluation Criteria in Solid Tumors to evaluate the response to treatment (Therasse et al. 2000).

Time to progression was measured from the first day of treatment with platinum-based combination chemotherapy until disease progression, and the overall survival time was measured from the first day of treatment until death. Event-free cases at the final day of the follow-up period were censored in time to event analyses. The median time to progression and the median overall survival period were estimated using the Kaplan–Meier method, and differences between survival curves were assessed using a log-rank test. Observed differences in proportion were tested using the Fisher exact test. A multivariate logistic regression analysis was performed to determine the predictive factors for response to chemotherapy. A Cox regression analysis using a stepwise procedure was used to evaluate prognostic factors that were significantly related to survival in the univariate analysis performed in this study as well as the previously reported factors, including age, performance status, smoking history, number of metastatic sites, the presence of liver metastasis, and elevated serum-ALP and -LDH levels (van der Gaast et al. 1995; Culine et al. 2002; Hainsworth et al. 1992; Abbruzzese et al. 1995; van de Wouw et al. 2004). Statistical analysis was performed using SPSS 12.0 J (SPSS Inc., Chicago, IL, USA), the significance level for the results was set at 0.05 (two-sided) and the multiplicity of the statistical test was not corrected.

Results

Patient characteristics

A total of 93 patients including 48 men, were included in the analysis. The median age was 60 years (range, 28–76 years), and the median performance status was 1 (range, 0–3). The histologic types consisted of 48 patients with adenocarcinoma, 21 patients with poorly differentiated adenocarcinoma, and 27 patients with poorly differentiated carcinoma. The median number of involved organs was 1 (range, 1–5). Most patients (78%) had lymph-node metastasis, but liver metastasis (n=15), lung metastasis (n=16), bone metastasis (n=18), and brain metastasis (n=4) were also seen. Almost all the patients (91 of 93 patients) exhibited an elevated serum tumor marker. The median number of elevated serum tumor markers was 5 (range, 0–11); the serum tumor marker characteristics are listed in Table 1. Elevations in Cyfra and ST-439 were significantly associated with histologically confirmed adenocarcinoma in a univariate analysis (P=0.04 and P=0.005, respectively), and an elevation in ST-439 was associated with histologically confirmed adenocarcinoma in a multivariate analysis (P=0.006).

A total of 340 courses of platinum-based combination chemotherapy were administered and the median number of administered courses was 4 (range, 1–6). Approximately two-thirds of the patients in this study received a taxanes plus platinum regimen (37 patients received paclitaxel plus carboplatin, 36 patients received docetaxel plus cisplatin) and the remaining 20

Table 1 Characteristics of serum tumor markers

Tumor markers	Percentage of patients with elevated levels (%)	Increased levels[a] (mean ± SD)
AFP (>10 ng/ml)	5.3	3,700.8±7,178.3
β-HCG (>0.5 mIU/ml)	54.8	14.1±53.0
PIVKA-II (≥40 mAU/ml)	10.7	1,650.0±4,268.2
CEA (>5.0 ng/ml)	44.1	658.3±1,957.5
SLX (>38 U/ml)	57.0	133.7±217.6
Cyfra (>2.2 ng/ml)	69.9	55.4±144.2
SCC (>1.5 ng/ml)	7.5	8.2±10.0
CA19-9 (>37 U/ml)	38.7	4,085.5±10,303.9
CA15-3 (>28 U/ml)	28.0	288.5±734.1
Erastase (>300 ng/dl)	9.7	866.2±801.5
STN (>45 U/ml)	46.2	694.5±1,660.2
ST-439 (>4.5 U/ml)	36.6	496.9±1,594.4
NSE (>15 ng/ml)	32.3	38.7±43.8
ProGRP (≥46 pg/ml)	12.9	77.5±36.1
PSA in male (>2.7 ng/ml)	8.3	5.54±2.18
CA125 in female (>35 U/ml)	64.4	299.5±356.0

AFP α-fetoprotein, *β-HCG* human chorionic gonadotropin β subunit, *PIVKA-II* protein induced by vitamin K absence-2, *CEA* carcinoembryonic antigen, *SLX* sialyl-specific embryonic antigen, *Cyfra* cytokeratin 19 fragment, *SCC* squamous-cell carcinoma antigen, *CA19-9* carbohydrate antigen 19-9, *CA15-3* carbohydrate antigen 15-3, *STN* sialyl Tn antigen, *ST-439* national cancer center-ST439, *NSE* neuron-specific enolase, *ProGRP* progastrin-releasing peptide, *PSA* prostate-specific antigen, *CA125* carbohydrate antigen 125

[a] The mean serum tumor marker level in patients with elevated levels

patients received irinotecan plus carboplatin. The response rate of the 93 patients was 39.8% (95% confidence interval, 29.9–49.7%). No treatment-related deaths occurred in this study. The median time to progression and overall survival period were 4.1 and 12.4 months, respectively (Fig. 1). At the time of the analysis, 64 of the 93 patients had died.

Prediction of response to treatment and prognostic models

Table 2 shows the relationship between patient characteristics, including the presence of elevated tumor markers, and response to platinum-based combination chemotherapy. No significant predictive factors of response to chemotherapy were seen in the univariate and multivariate analyses. The results of the univariate analysis for prognostic factors are listed in Table 3. Poor performance status (>1), number of involved organs (>2), and elevated serum-LDH and -NSE levels were significantly associated with survival in this univariate analysis. The multivariate analysis indicated that performance status, number of involved organs, and elevated serum-LDH levels were the prognostic factors (P=0.01, P=0.033, P=0.006, respectively).

All 93 patients with a complete data set were analyzed to verify the previously reported prognostic models. The prognostic model by Culine et al. significantly divided these patients into two groups with median survival times of 21.0 and 10.1 months, respectively (P=0.003, Fig. 2). The other prognostic model by Van der Gaast et al. significantly divided these patients into three groups with median survival times of 19.6, 12.2, and 6.7 months, respectively (Fig. 3).

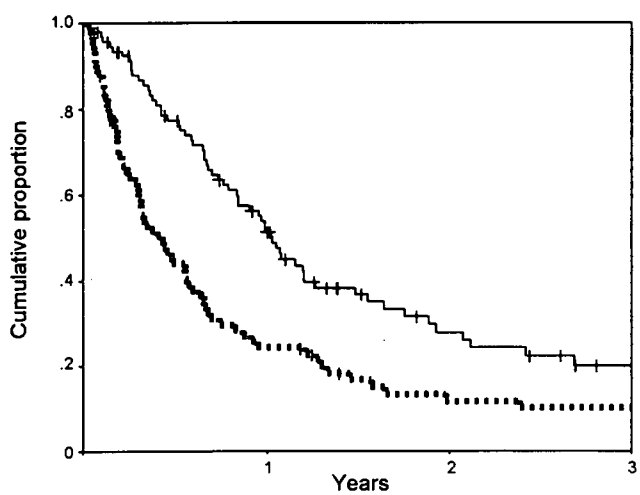

Fig. 1 Kaplan–Meier analysis of time to progression (*dotted line*) and overall survival (*solid line*). *Vertical bars* indicate censored cases

Table 2 Univariate analysis of response to chemotherapy

Variables	No. of patients	Response rate (%)	P value
Sex			
Male	48	37.5	0.68
Female	45	42.2	
Age (years)			
≥60	46	43.4	0.53
<60	47	36.2	
Performance status			
>1	18	22.2	0.11
0 or 1	75	44	
Smoking history			
Past or current smoking history	41	46.3	0.29
No smoking history	52	34.6	
Histologic type			
Adenocarcinoma	45	33.3	0.22
PDA or PDC	48	45.8	
No. of involved organs			
>2	26	38.5	0.99
1 or 2	67	40.3	
Presence of liver metastasis			
Yes	15	33.3	0.78
No	78	41.0	
ALP (U/l)			
Elevated (>359)	24	25.0	0.1
Normal (≤359)	69	44.9	
LDH (U/l)			
Elevated (>229)	43	39.5	0.99
Normal (≤229)	50	40.0	
CRP (mg/dl)			
>1.0	33	30.3	0.19
≤1.0	60	45.0	
No. of elevated tumor markers			
>5	41	36.6	0.67
≤5	52	42.3	
AFP (ng/ml)			
Elevated (>10)	5	80.0	0.08
Normal (≤10)	88	37.5	
β-HCG (mIU/ml)			
Elevated (>0.5)	51	37.3	0.67
Normal (≤0.5)	42	42.9	
PIVKA-II (mAU/ml)			
Elevated (≥40)	10	61.0	0.19
Normal (<40)	83	37.3	
CEA (ng/ml)			
Elevated (>5.0)	41	41.5	0.83
Normal (≤5.0)	52	38.5	
SLX (U/ml)			
Elevated (>38)	53	33.9	0.21
Normal (≤38)	40	47.5	
Cyfra (ng/ml)			
Elevated (>2.2)	65	40.0	0.99
Normal (≤2.2)	28	39.3	
SCC (ng/ml)			
Elevated (>1.5)	7	42.9	0.99

Table 2 Continued

Variables	No. of patients	Response rate (%)	*P* value
Normal (≤1.5)	86	38.6	
CA19-9 (U/ml)			
Elevated (>37)	36	33.3	0.39
Normal (≤37)	57	43.9	
CA15-3 (U/ml)			
Elevated (>28)	26	34.6	0.64
Normal (≤28)	67	41.8	
Erastase (ng/dl)			
Elevated (>300)	9	44.4	0.99
Normal (≤300)	84	39.3	
STN (U/ml)			
Elevated (>45)	43	39.5	0.99
Normal (≤45)	50	40.0	
ST-439 (U/ml)			
Elevated (>4.5)	34	41.2	0.99
Normal (≤4.5)	59	39.0	
NSE (ng/ml)			
Elevated (>15)	30	43.3	0.66
Normal (≤15)	63	38.1	
ProGRP (pg/ml)			
Elevated (≥46)	12	25.0	0.35
Normal (<46)	81	42.0	

PDA poorly differentiated adenocarcinoma, *PDC* poorly differentiated carcinoma, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *CRP* C-reactive protein, *AFP* α-fetoprotein, *β-HCG* human chorionic gonadotropin β subunit, *PIVKA-II* protein induced by vitamin K absence-2, *CEA* carcinoembryonic antigen, *SLX* sialyl-specific embryonic antigen, *Cyfra* cytokeratin 19 fragment, *SCC* squamous-cell carcinoma antigen, *CA19-9* carbohydrate antigen 19-9, *CA15-3* carbohydrate antigen 15-3, *STN* sialyl Tn antigen, *ST-439* national cancer center-ST439, *NSE* neuron-specific enolase, *ProGRP* progastrin-releasing peptide

Discussion

Based on the results of the present study, various routine tumor-marker measurements were not useful for predicting either the response to chemotherapy or survival in patients with CUP. Poor performance status, the number of involved organs, and an elevated serum-LDH level were the prognostic factors for survival in patients receiving platinum-based combination chemotherapy, including taxanes or irinotecan. In addition, the previously reported prognostic models were validated in this population. To our knowledge, this is the first report to verify prognostic models for patients with CUP.

Serum tumor markers are substances that can be measured quantitatively using laboratory methods and that can be used to detect cancer and possibly the organ where it resides, as well as being useful for monitoring responses to therapy. Several tumor markers like PSA,

Table 3 Univariate analysis of survival period

Variables	No. of patients	Median survival (months)	*P* value
Sex			
Male	48	12.2	0.24
Female	45	14.3	
Age (years)			
≥60	46	12.6	0.86
<60	47	12.2	
Performance status			
>1	18	7.1	<0.01
0 or 1	75	14.3	
Smoking history			
Past or current smoking history	41	12.4	0.85
No smoking history	52	12.6	
Histologic type			
Adenocarcinoma	45	12.4	0.69
PDA or PDC	48	12.9	
No. of involved organs			
>2	26	7.9	0.01
1 or 2	67	14.3	
Presence of liver metastasis			
Yes	15	10.1	0.11
No	78	12.9	
ALP (U/l)			
Elevated (>359)	24	11.8	0.66
Normal (≤359)	69	12.8	
LDH (U/l)			
Elevated (>229)	43	10.1	0.01
Normal (≤229)	50	17.7	
CRP (mg/dl)			
>1.0	33	11.8	0.49
≤1.0	60	12.8	
No. of elevated tumor markers			
>5	41	11.4	0.48
≤5	52	14.3	
AFP (ng/ml)			
Elevated (>10)	5	7.1	0.18
Normal (≤10)	88	12.4	
β-HCG (mIU/ml)			
Elevated (>0.5)	51	10.2	0.62
Normal (≤0.5)	42	12.9	
PIVKA-II (mAU/ml)			
Elevated (≥40)	10	10.1	0.31
Normal (<40)	83	12.4	
CEA (ng/ml)			
Elevated (>5.0)	41	12.4	0.83
Normal (≤5.0)	52	12.6	
SLX (U/ml)			
Elevated (>38)	53	11.4	0.15
Normal (≤38)	40	15.1	
Cyfra (ng/ml)			
Elevated (>2.2)	65	11.4	0.21
Normal (≤2.2)	28	17.6	

Table 3 Continued

Variables	No. of patients	Median survival (months)	*P* value
SCC (ng/ml)			
Elevated (>1.5)	7	7.2	0.12
Normal (≤1.5)	86	12.8	
CA19-9 (U/ml)			
Elevated (>37)	36	9.5	0.13
Normal (≤37)	57	15.1	
CA15-3 (U/ml)			
Elevated (>28)	26	12.4	0.93
Normal (≤28)	67	12.6	
Elastase (ng/dl)			
Elevated (>300)	9	10.9	0.56
Normal (≤300)	84	12.4	
STN (U/ml)			
Elevated (>45)	43	12.6	0.86
Normal (≤45)	50	11.8	
ST-439 (U/ml)			
Elevated (>4.5)	34	12.2	0.84
Normal (≤4.5)	59	12.8	
NSE (ng/ml)			
Elevated (>15)	30	8.8	0.01
Normal (≤15)	63	14.4	
ProGRP (pg/ml)			
Elevated (≥46)	12	6.4	0.09
Normal (<46)	81	12.9	

PDA poorly differentiated adenocarcinoma, *PDC* poorly differentiated carcinoma, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *CRP* C-reactive protein, *AFP* α-fetoprotein, *β-HCG* human chorionic gonadotropin β subunit, *PIVKA-II* protein induced by vitamin K absence-2, *CEA* carcinoembryonic antigen, *SLX* sialyl-specific embryonic antigen, *Cyfra* cytokeratin 19 fragment, *SCC* squamous-cell carcinoma antigen, *CA19-9* carbohydrate antigen 19-9, *CA15-3* carbohydrate antigen 15-3, *STN* sialyl Tn antigen, *ST-439* national cancer center-ST439, *NSE* neuron-specific enolase, *ProGRP* progastrin-releasing peptide

CA125, AFP, and β-HCG have been useful for screening for cancer, monitoring treatment, and detecting recurrence. Although a positive correlation exists between tumor mass and the marker level, most tumor markers are elevated in various types of cancer and sometimes even in benign conditions. At present, the role of serum tumor markers in the management of various cancers might be limited (Canil and Tannock 2002).

A few studies have examined tumor markers in patients with CUP, but their implications for the management of CUP are controversial. Koch and McPherson suggested that a CEA above 10 ng/ml indicated that the tumor site was more likely to be an endodermally derived organ, like a breast or ovary, containing a mucinous carcinoma (Koch and McPherson 1981). However, a previous study reported that CEA, CA19-9, CA15-3, and CA125 levels were not correlated with histologic type, the number of involved organs, or the disease site but that an elevated CEA level was a significant prognostic factor for survival (Milovic et al. 2002). Yet another study reported that elevated CA19-9 levels were related to histologic adenocarcinoma and the presence of liver metastasis (Pavlidis et al. 1994). However, CEA, CA19-9, CA15-3, CA125, β-HCG, and AFP were not reported as predictive factors for response to chemotherapy or survival in two other reports (Pavlidis et al. 1994; Currow et al. 1996). In our study, almost all of the patients exhibited several elevated serum tumor markers, suggesting that patients with CUP exhibit a non-specific over-expression of serum tumor marker. Based on an analysis of our study and a previous one (Pavlidis et al. 1994), we concluded that the routine measurement of tumor markers does not offer any diagnostic or therapeutic assistance to patients with CUP, except for identifying some specific cancers such as germ-cell tumors, prostate cancer, and peritoneal carcinoma.

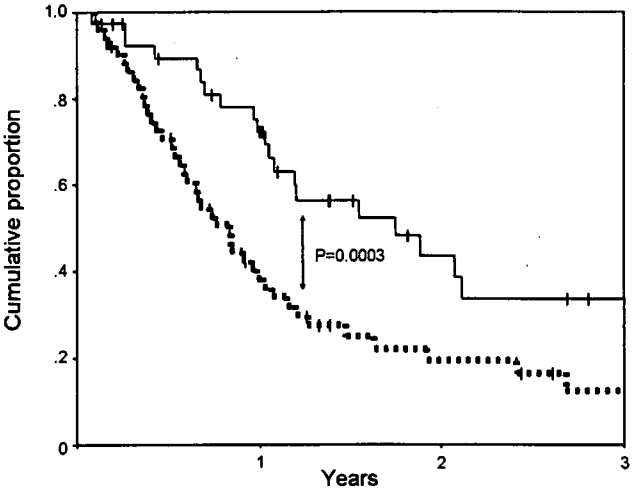

Fig. 2 Overall survival according to a previously reported prognostic model using performance status and serum LDH level (Culine et al. 2002). The *solid line* indicates good risk patients (performance status of 0 or 1 and a normal LDH level), and the *dotted line* represents poor-risk patients (performance status >1 or elevated LDH level). *Vertical bars* indicate censored cases

We retrospectively analyzed 93 consecutive patients with CUP, who had been treated with platinum-based combination chemotherapy. In this study, the response rate and the median survival period were 39.8% and 12.4 months, respectively; these results are similar to those of the previous reports on taxanes-plus-platinum-based combination chemotherapy (Pavlidis et al. 2003; Greco et al. 2000; Greco and Hainsworth 2005; Briasoulis et al. 2000). Since all the patients in this study received new-generation anticancer drugs plus platinum agents, the median survival time was longer than those of the previous studies reporting prognostic models (van der Gaast et al. 1995; Culine et al. 2002). In a previous prognostic report conducted in France, 10% of the patients only

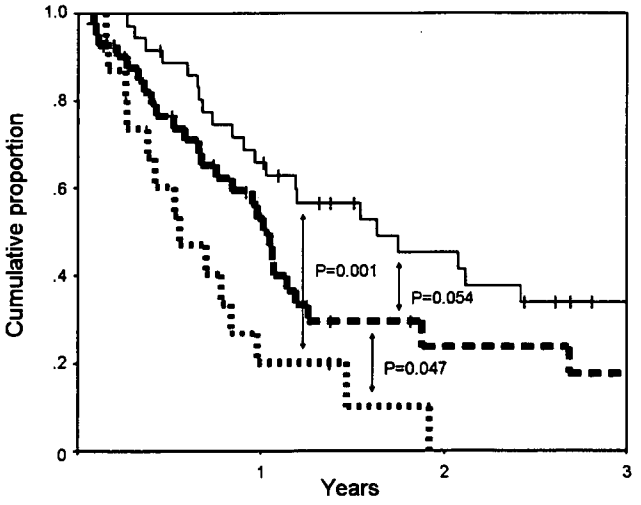

Fig. 3 Overall survival according to a previously reported prognostic model using performance status and serum-ALP level (van der Gaast et al. 1995). The *solid line* indicates patients with a performance status of 0 and an ALP level <1.25 N, the *broken line* indicates patients with a performance status ≥1, or an ALP level ≥1.25 N, and the *dotted line* represents patients with a performance status ≥1 and an ALP level ≥1.25 N. *Vertical bars* indicate censored cases

received the best supportive care and most of the patients (41%) received doxorubicin-plus-etoposide-plus-cyclophosphamide-plus-platinum agent therapy (van der Gaast et al. 1995). Patients in another study were treated with either bleomycin-plus-etoposide-plus-cisplatin therapy or etoposide-plus-cisplatin therapy (Culine et al. 2002). Although there are many differences in the patient characteristics, chemotherapy regimens, and treatment results among these previous reports and ours, it is noteworthy that the prognostic factors for survival are similar (van der Gaast et al. 1995; Culine et al. 2002; van de Wouw et al. 2004). In addition, the two previously reported prognostic models fitted our results for Japanese patients with CUP quite well. Though the investigators published a regression tree analysis in 1,000 consecutive patients in a US population (Hess et al. 1999), that kind of analysis requires a much larger data set and was not feasible in this study. Even using a simple prognostic assessment, however, the verification of prognostic models using an independent data set may be useful for establishing therapeutic strategies for patients with CUP.

Concerning the indications for chemotherapy, previous studies have suggested that patients in the poor-risk group should not be offered chemotherapy routinely, since the median survival time of poor-risk patients is less than 4 months (van der Gaast et al. 1995; Culine et al. 2002). However, in our study, the median survival times of the poor-risk patients according to the prognostic models were 10.1 and 6.7 months, respectively. Whether, poor-risk patients should be offered palliative therapy or chemotherapy may be difficult to determine without conducting a randomized trial. Since survival times have been prolonged by advances in new anticancer drugs, the utility of platinum-based combination chemotherapy or single-agent chemotherapy for poor-risk CUP patients should be carefully considered in a prospective trial.

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Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients

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Summary

Drug resistance is a major obstacle to the successful chemotherapy. Several ATP-binding cassette (ABC) transporters including ABCB1, ABCC1 and ABCG2 have been known to be important mediators of chemoresistance. Using oligonucleotide microarrays (HG-U133 Plus 2.0; Affymetrix), we analyzed the ABC transporter gene expression profiles in breast cancer patients who underwent sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy. We compared the ABC transporter expression profile between two classes of pretreatment tumor samples divided by the patients' pathological response to neoadjuvant chemotherapy (residual disease [RD] versus pathologic complete response [pCR]) ABCB3, ABCC7 and ABCF2 showed significantly high expression in the pCR. Several ABC transporters including ABCC5, ABCA12, ABCA1 ABCC13, ABCB6 and ABCC11 showed significantly increased expression in the RD ($p < 0.05$). We evaluated the feasibility of developing a multigene predictor model of pathologic response to neoadjuvant chemotherapy using gene expression profiles of ABC transporters. The prediction error was evaluated by leave-one-out cross-validation (LOOCV). A multigene predictor model with the ABC transporters differentially expressed between the two classes ($p \leq 0.003$) showed an average 92.8% of predictive accuracy (95% CI, 88.0–97.4%) with a 93.2% (95% CI, 85.2–100%) positive predictive value for pCR, a 93.6% (95% CI, 87.8–99.4%) negative predictive value, a sensitivity of 88.1%(95% CI, 76.8–99.4%), and a specificity of 95.9% (91.1% CI, 87.8–100%). Our results suggest that several ABC transporters in human breast cancer cells may affect the clinical response to neoadjuvant chemotherapy, and transcriptional profiling of these genes may be useful to predict the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

Introduction

Resistance to chemotherapy is a significant obstacle to appropriate treatment of cancer patients. Various cellular pathways may play a role in drug resistance and ATP-binding cassette (ABC) transporters are one of the most well known mediators leading to drug resistance and treatment failure. To date 49 ABC transporter genes have been identified and classified into seven groups, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG (database of ABC transporters available at http://nutrigene.4t.com/humanabc.htm).

Extensive studies have been conducted on the individual proteins or genes of ABC transporter members regarding their role in chemoresistance. ABCB1 (MDR1-P-gp) [1,2], ABCC1 (MRP1) [3], and ABCG2 (MXR) [4] are particularly well known as mediators leading to resistance to several chemotherapeutic agents including paclitaxel [5], topoisomerase inhibitors [6], anthracyclin [7] and tyrosine kinase inhibitors [8]. Although little has been known about most of ABC transporter members, other members of this family sharing sequence and structural homology may play roles in absorption, distribution, and excretion of chemotherapeutic agents and probably influence the response to chemotherapy.

Recently, using ABC transporter gene expression profiling, studies on the relationship of drug resistance and ABC transporter were performed in cancer cell lines [9,10].

The characterization of the comprehensive expression of these genes in relation to the clinical response to chemotherapy may be useful to determine on an individual basis the patient's underlying risk and choose the optimal therapeutic regimen to which the individual cancer patient is most likely to respond. We studied the relationship between ABC transporter gene expression and the responsiveness to chemotherapy in early breast cancer patients who underwent sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy and evaluated the feasibility of developing a multigene predictor model of pathologic response using differentially expressed ABC transporters on the basis of microarray data.

Materials and methods

Patient and sample preparation

This study was performed at the National Cancer Center Hospital, Tokyo, Japan. This study was approved by the institutional review boards of the National Cancer Center. Twenty-one pretreatment samples were obtained from breast cancer patients who underwent neoadjuvant chemotherapy from 2002 to 2004. All patients underwent pretreatment core needle biopsy (CNB) of the primary tumor tissue before starting neoadjuvant chemotherapy. The core needle biopsy was done using 14–16 gauge needles.

The patients received 4 cycles of FEC (5-Fluorouracil 500 mg/m^2, Epirubicin 100 mg/m^2 and Cyclophosphamide 500 mg/m^2) every three weeks followed by 12 cycles of weekly paclitaxel (80 mg/m^2). Additionally, in the case of HER2 positive determined by immunohistochemical staining (IHC), the specific inhibitory antibody of HER2 receptor, Trastuzumab (Herceptin®) was added in the course of the paclitaxel (Herceptin 4 mg/kg on day1 then 2 mg/kg weekly). Samples that showed 3+ IHC staining were considered as HER2 positive.

Every patient underwent surgery on the completion of the neoadjuvant chemotherapy, and histopathologic examination was performed. As described previously [11], pathologic complete response (pCR) was defined as no pathologic evidence of any residual invasive cancer cells in the breast and axillary lymph nodes, and residual disease (RD) was defined as any residual cancer cells on the histopathologic examination. Informed consent was obtained from all patients for voluntary participation in the study.

Tissue preparation and microarray

Samples for the microarray were collected into tubes containing Isogen (Nippon gene, Toyama) and stored at −80 °C. Total RNA was extracted by the single step method of Chomczynski et al. [12] with acid guanidinium thiocyanate phenol chloroform after homogenizing the tissue using a high speed homogenizer. The mean yield of RNA was 23.1 μg (ranged from 12.3 to 31.6 μg) from each collected samples. RNA that had distinct ribosomal RNA band by electrophoresis and had A_{260}/A_{280} absorbance ratio ranging from 1.8 to 2.1 was used for cDNA synthesis. Gene expression profiles were analyzed on a high-density oligonucleotide microarray (GeneChip® HG-U133 Plus 2.0; Affymetrix, Santa Clara, CA) containing 54,675 probe sets. The oligonucleotide microarray procedure for generation of the biotin-labeled cyclic RNA (cRNA) by *in vitro* transcription, hybridization to the array and scanning were performed according to the manufacturer's instructions. The amplification cycle of RNA to cDNA and cDNA to cRNA was performed using the GeneChip® 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit including SuperScript II reverse transcriptase and a T7-$(dT)_{24}$ primer (Affymetrix). The synthesized cRNA was biotinylated using GeneChip 3′-amplification reagents for IVT labeling. The labeled cRNA was then purified and chemically fragmented at 94 °C for 35 min using the GeneChip Sample Cleanup Module (Affymetrix). The labeled fragmented cRNA was next hybridized to the GeneChip® at 45 °C for 16 h according to the manufacturer's instructions. The hybridized probe array was washed and stained with streptavidin-phycoerythrin. The stained probe array was scanned with a GeneChip® Scanner3000 (Affymetrix) at 570 nm. The signal intensity of the gene expression level was calculated by GeneChip Operating Software, Ver.1 (Affymetrix).

Data analysis

Microarray data analyses were performed with BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam. (http://linus.nci.nih.gov/BRB-ArrayTools.html) which provides a variety of tools for the analysis of gene expression profile. Gene expression data were log transformed (base 2) and normalized to the median expression value of all genes on each array. Any genes in which the expression levels did not differ by at least by 1.5 fold from the median in at least 20% of the arrays were filtered out, for the exclusion of the genes showing minimal variation across the set of arrays. In addition, if an expression value was missing or filtered out in more than 50%, these data were excluded. The final data set included 50,508 clones, and contained all 49 ABC transporter genes. The list of transcripts on ABC transporters was obtained using GeneSprings software (http://www.silicongenetics.com/cgi/SiG.cgi/index.smf) from Agilent Technologies (Waldbronn, Germany). (Supplementary data).

Class comparison

To identify informative genes differentially expressed between the two classes of patients grouped by their pathologic response, we used supervised classification methods applying the random variance *t*-test to data using the BRB Array Tools and was accompanied by multivariate permutation tests in order to minimize false-positives with the maximum allowed number of

false positives set at 10, a false discovery rate of 0.1, and confidence 90%. Genes with a parametric *p*-value less than 0.05 were considered statistically significant.

Class prediction

To develop a prediction model of pathologic response using the ABC transporter gene expression profiles, we used the class prediction tools of BRB ArrayTools in which six multivariate classification methods were available including a compound covariate predictor [13], a *K*-nearest neighbor analysis ($K=1$, 3), a nearest centroid analysis, a support vector machine [14] and a diagonal linear discriminate analysis.

For the evaluation of the feasibility of developing a multigene predictor model of response to neoadjuvant chemotherapy using differentially expressed ABC transporters, six different multivariate classification models were examined. Firstly, we determined the number of genes that were included in the classifier model using a paired *t*-test applying multiple univariate parametric significance thresholds, and developed a classifier model based on these selected genes at the univariate parametric significance thresholds. With changes in the parametric significance thresholds, the multivariate classification algorithms were performed iteratively evaluating the classification error and the classifier *p*-value to identify the best classifier, and the processes were iteratively performed for each number of genes included in the classifier (determined by the significance threshold). The prediction error of each model was evaluated by leave-one-out cross-validation (LOO-CV) [15]. This validation procedure was performed in a manner that removed the left-out sample before selecting the discriminate genes [15,16]. The classifier *p*-value, the probability that similar low error rate happen by chance, was obtained by a random permutation test performed 2000 times.

Results

The patient characteristics

All the patients received 4 courses of FEC (5-fluorouracil, epirubicin and cyclophosphamide) combination chemotherapy followed by 12 courses of weekly paclitaxel. In those patients who were HER-2 positive by IHC, Trastuzumab (Herceptin®) was added in the course of the treatment. We divided the patients into two groups from the results of the histopathologic examination performed after the completion of the neoadjuvant chemotherapy. Pathologic data were available for nineteen patients. Patients with no pathologic evidence of any residual invasive cancer cells in breast were classified as 'pCR', and if any residual cancer cells were found in the histopathologic study, these patients were classified as 'RD' group. Thirty-six point eight percent (7) of the nineteen patients showed no pathologic evidence of any residual invasive cancer cells in the breast and were classified as pCR and 63.2% (12) of patients were classified as RD.

Gene expression profiling of differentially expressed ABC transporters

Using gene expression data of the pretreatment tumor sample, we compared the ABC transporter gene expression profile between the two groups (RD versus pCR). A probe set on all of the 49 human ABC transporters genes known so far was contained in the microarray chip we used (HG-U133 Plus 2.0; Affymetrix). To identify differentially expressed ABC transporter genes potentially associated with the clinical response to neoadjuvant chemotherapy, a supervised class comparison analysis was performed. The random variance model *t*-test was used to discover differentially expressed genes and was accompanied by a multivariate 1000 permutation tests in order to minimize false-positives with the maximum allowed number of false positives set at 10, a false discovery rate of 0.1 and 90% confidence.

By comparing the average expression level of each transcript on ABC transporters between the two classes of patients, the median expression level in the RD group was 107.8 (range 15.8–6009.1) and 104.4 in the pCR group (range 17.9–5690.6). The median of fold difference (RD: pCR) of transcripts on the ABC transporters was 1.0, ranging from 0.3 to 7.6. Several ABC transporters showed prominently high expression at over 50 fold of the median value although the tumor samples were all from the pretreatment chemotherapy-naïve patients. The highest average expression level in the RD group, 6009.1, was observed in ABCC5 (AF146074, RD: pCR = 6009.1:2427.5, fold ratio 2.48) and the highest expression level in the pCR group, 5690.6, was observed in TAP1 (ABCB2, NM_000593, RD: pCR = 4551.4:5690.6, fold ratio 0.8), the transporter associated with antigen processing (Table 1).

The ABC transporters, which were significantly differentially expressed with a parametric *p*-value of less than 0.05, are listed in Table 2. Several transcripts (ABCC5, TAP2/ABCB3) selected overlapped for the microarray chip (HG-U133 Plus 2.0) containing 54,675 probe sets, more than 30,000 human transcripts were detected, derived from more than 20,000 loci within the human genome and some transcripts represented the same human gene.

ABC transporters, the expression of which in the RD group was significantly increased, included ABCC5 (fold ratio 2.48, $p=0.000368$), ABCA12 (fold ratio 7.64, $p=0.000795$), ABCA1 (fold ratio 3.30, $p=0.000859$), ABCC13 (fold ratio 7.54, $p=0.0194$), ABCB6 (fold ratio 2.17, $p=0.0271$), and ABCC11 (fold ratio 2.71, $p=0.0486$) (Table 2). These genes all showed over 2 fold increases in RD compared with pCR tumors. ABCC5 was recently reported to confer resistance to

Table 1. Clinical characteristics of the patients

	No. of patients
Age, years	
Median	51
Range	30–61
Menstruation status	
Pre menopause	12
Post menopause	7
TNM stage	
IIA	8
IIB	7
IIIA	2
IIIB	2
Histology	
Invasive ductal	17
Mixed ductal/lobular	
Invasive lobular	1
Invasive mucinous	1
Nuclear grade	
1	1
2	9
3	9
HER2 status	
HER2-positive	4
HER2-negative	15
ER status	
ER-positive*	5
ER-negative	14
Pathologic response	
Pathologic complete response	7
Residual disease	12
Treatment arm	
A[a]	15
B[b]	4

*Cases in which more than 10% of tumor cells stained positive for ER by IHC classified as ER positive.
[a]Treatment arm A; 4 courses of FEC* followed by 12 courses of weekly paclitaxel.
[b]Treatment arm B; 4 courses of FEC* followed by 12 courses of weekly paclitaxel with Trastuzumab.
*FEC combination chemotherapy (5-fluorouracil, epirubicin and cyclophosphamide).

5-fluorouracil [17] selected with the lowest p-value and it showed the highest gene expression level in tumors with decreased response. (AF146074, expression level RD: pCR = 6009.1: 2427.5, fold ratio 2.48).

CFTR (NM_000492, ABCC7, fold ratio 0.27, p= 0.007030), ABCF2 (NM_005692, fold ratio 0.32, p= 0.015901) and ABCB3 (M74447, TAP2, fold ratio 0.54, p= 0.019345), the transporter associated with antigen processing, showed increased expression in the pCR group but the biological significance concerning responsiveness to chemotherapy remains to be elucidated. The differentially expressed ABC transporter genes are shown in Figure 1 in hierarchical clustering view.

Development of multigene predictor model using the ABC transporter gene expression profile

To evaluate the feasibility of developing a multigene predictor model of response to neoadjuvant chemotherapy using the ABC transporter expression profile, six different multivariate classification models were examined.

Firstly, we determined the number of discriminate genes that were included in the classifier model by applying multiple univariate parametric significance thresholds, and developed a classifier model based on these selected genes at the significance thresholds. With changes in the parametric significance thresholds, the classification error and classifier p-value for each multivariate classification algorithms were evaluated iteratively by LOOCV (leave one out cross validation) [15] and the random permutation test to identify the best classifier model. The classifier p-value, the probability that a similar low error rate could happen by chance, was calculated by 2000 random permutation tests. We calculated the average of the classification error and the classifier p-value of six classifier models at each significance threshold. Figure 2 shows the change in the average classifier p-value for six multivariate classification models from the permutation test and the average of the classification error rate relative to multiple univariate parametric significance thresholds.

During this iterative process, the average estimated misclassification error and classifier p-value also dropped as the significance threshold decreased to 0.003, but applying further stringent significance thresholds caused a steep increase in the classification error. When the ABC transporters differentially expressed between the two classes at a significance threshold level of 0.003 were used for class prediction, the average of the classification error was minimal, 0.072 (92.8% of predictive accuracy, 95% CI, 88.0–97.4%), with the classifier p = 0.012, 93.2% (95% CI, 85.2–100%) positive predictive value for the pCR group, 93.6% (95% CI, 87.8–99.4%) negative predictive value, sensitivity for the pCR group 88.1% (95% CI, 76.8–99.4%), and a specificity of 95.9% (91.1% CI, 87.8–100%). The respective values for each model are represented in Table 3. On applying the compound covariate predictor classifier model, the predictive accuracy reached 100% with a classifier p-value of 0.0005. The ABC transporters selected as the best classifiers are presented in Table 4. The list included ABCA1, ABCA12 and ABCC5, recently reported to confer resistance to cyclic nucleotides including 5-fluorouracil [17].

Our results suggest that the ABC transporter genes expression pattern may be useful in predicting the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

Discussion

To determine the optimal therapeutic regimen to which the individual cancer patient is most likely to respond on