the present study, there was no significant relationship between the presence of TIL and tumor cell apoptosis in TNBC. However, recent studies demonstrated that tumor cell death induced by chemotherapy can promote cytotoxic T-lymphocyte response that confers permanent antitumor immunity [30, 31]. We used histological examination only to identify apoptotic cancer cells. However, it would be more informative to add other techniques, such as the TUNEL method or immunohistochemistry, to identify apoptosis from multiple angles.

We revealed no correlation between the expression of basal-like markers and response to NAC in all of the breast subtypes examined. Although the significance of basal-like markers for clinical outcome is controversial [32–34], a lack of association between basal-like markers and chemosensitivity or prognosis has been demonstrated when breast cancers are divided into subtypes on the basis of ER and HER2 positivity [33, 34]. Nuclear p53 has been shown to be frequent in TNBC [35], but the significance of p53 as a predictive marker for pCR is also controversial [36]. In the present study we were unable to demonstrate any significant impact of p53 as such a marker.

It is unknown whether TILs cause susceptibility to chemotherapy, or they are simply a possible marker of chemosensitivity. There are reports that showed TILs are a predictor of response to neoadjuvant chemotherapy in breast cancer [37, 38]. Hornychova et al. reported that the infiltration of $CD3^+$ T-lymphocytes and $CD83^+$ dendritic cells were correlated with the effectiveness of primary chemotherapy, evaluated as pCR [38]. Denkert et al. showed that T-cell-related markers CD3D and CXCL9 expression were significantly associated with pCR [37]. Several studies suggested possible mechanisms of tumor-immune interaction in response to chemotherapy. pCR to neoadjuvant chemotherapy was shown to be associated with an immunologic profile combining the absence of immunosuppressive $Foxp3^+$ regulatory T cells and the presence of a high number of $CD8^+$ T cells and cytotoxic cells [28]. These reports suggest subsets of TILs caused susceptibility to chemotherapy.

In conclusion, we have demonstrated that the various breast cancer subtypes classified by ER, PgR, and HER2 status have different pathological characteristics and predictive factors for response to chemotherapy. TNBC with a high score for TIL and apoptosis is more likely to respond to chemotherapy. Therefore, in patients with TNBC, the immune response appears to influence on the response to chemotherapy. Further examination is warranted to elucidate the mechanism involved in the immune response component of chemosensitivity.

References

1. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406(6797):747–752. doi:10.1038/35021093
2. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98(19):10869–10874. doi:10.1073/pnas.191367098
3. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lonning PE, Brown PO, Borresen-Dale AL, Botstein D (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 100(14):8418–8423. doi:10.1073/pnas.0932692100
4. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC (2006) Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 295(21):2492–2502. doi:10.1001/jama.295.21.2492
5. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, Delorenzi M, Piccart M, Sotiriou C (2008) Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 14(16):5158–5165. doi:10.1158/1078-0432.CCR-07-4756
6. Hugh J, Hanson J, Cheang MC, Nielsen TO, Perou CM, Dumontet C, Reed J, Krajewska M, Treilleux I, Rupin M, Magherini E, Mackey J, Martin M, Vogel C (2009) Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 trial. J Clin Oncol 27(8):1168–1176. doi:10.1200/JCO.2008.18.1024
7. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10(16):5367–5374. doi:10.1158/1078-0432.CCR-04-0220
8. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L (2005) Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 11(16):5678–5685. doi:10.1158/1078-0432.CCR-04-2421
9. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13(15 Pt 1):4429–4434. doi:10.1158/1078-0432.CCR-06-3045
10. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML, Perou CM (2007) The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13(8):2329–2334. doi:10.1158/1078-0432.CCR-06-1109

11. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M, Cristofanilli M, Hortobagyi GN, Pusztai L (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26(8):1275–1281. doi: 10.1200/JCO.2007.14.4147

12. Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER, Wickerham DL, Begovic M, DeCillis A, Robidoux A, Margolese RG, Cruz AB Jr, Hoehn JL, Lees AW, Dimitrov NV, Bear HD (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16(8):2672–2685

13. Toi M, Nakamura S, Kuroi K, Iwata H, Ohno S, Masuda N, Kusama M, Yamazaki K, Hisamatsu K, Sato Y, Kashiwaba M, Kaise H, Kurosumi M, Tsuda H, Akiyama F, Ohashi Y, Takatsuka Y (2008) Phase II study of preoperative sequential FEC and docetaxel predicts of pathological response and disease free survival. Breast Cancer Res Treat 110(3):531–539. doi:10.1007/s10549-007-9744-z

14. Mazouni C, Peintinger F, Wan-Kau S, Andre F, Gonzalez-Angulo AM, Symmans WF, Meric-Bernstam F, Valero V, Hortobagyi GN, Pusztai L (2007) Residual ductal carcinoma in situ in patients with complete eradication of invasive breast cancer after neoadjuvant chemotherapy does not adversely affect patient outcome. J Clin Oncol 25(19):2650–2655. doi:10.1200/JCO.2006.08.2271

15. Rouzier R, Extra JM, Klijanienko J, Falcou MC, Asselain B, Vincent-Salomon A, Vielh P, Bourstyn E (2002) Incidence and prognostic significance of complete axillary downstaging after primary chemotherapy in breast cancer patients with T1 to T3 tumors and cytologically proven axillary metastatic lymph nodes. J Clin Oncol 20(5):1304–1310

16. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19(5):403–410

17. Turner NC, Reis-Filho JS (2006) Basal-like breast cancer and the BRCA1 phenotype. Oncogene 25(43):5846–5853. doi:10.1038/sj.onc.1209876

18. Allred DC, Harvey JM, Berardo M, Clark GM (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 11(2):155–168

19. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF (2007) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25(1):118–145. doi:10.1200/JCO.2006.09.2775

20. Carlomagno C, Perrone F, Lauria R, de Laurentiis M, Gallo C, Morabito A, Pettinato G, Panico L, Bellelli T, Apicella A et al (1995) Prognostic significance of necrosis, elastosis, fibrosis and inflammatory cell reaction in operable breast cancer. Oncology 52(4):272–277

21. Lipponen P, Aaltomaa S, Kosma VM, Syrjanen K (1994) Apoptosis in breast cancer as related to histopathological characteristics and prognosis. Eur J Cancer 30A(14):2068–2073

22. Lee AH, Gillett CE, Ryder K, Fentiman IS, Miles DW, Millis RR (2006) Different patterns of inflammation and prognosis in invasive carcinoma of the breast. Histopathology 48(6):692–701. doi:10.1111/j.1365-2559.2006.02410.x

23. Marques LA, Franco EL, Torloni H, Brentani MM, da Silva-Neto JB, Brentani RR (1990) Independent prognostic value of laminin receptor expression in breast cancer survival. Cancer Res 50(5):1479–1483

24. Nixon AJ, Neuberg D, Hayes DF, Gelman R, Connolly JL, Schnitt S, Abner A, Recht A, Vicini F, Harris JR (1994) Relationship of patient age to pathologic features of the tumor and prognosis for patients with stage I or II breast cancer. J Clin Oncol 12(5):888–894

25. Rilke F, Colnaghi MI, Cascinelli N, Andreola S, Baldini MT, Bufalino R, Della Porta G, Menard S, Pierotti MA, Testori A (1991) Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors. Int J Cancer 49(1):44–49

26. Kreike B, van Kouwenhove M, Horlings H, Weigelt B, Peterse H, Bartelink H, van de Vijver MJ (2007) Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Res 9(5):R65. doi:10.1186/bcr1771

27. Demaria S, Volm MD, Shapiro RL, Yee HT, Oratz R, Formenti SC, Muggia F, Symmans WF (2001) Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. Clin Cancer Res 7(10):3025–3030

28. Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, Fumoleau P, Ghiringhelli F (2008) Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res 14(8):2413–2420. doi:10.1158/1078-0432.CCR-07-4491

29. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C (2007) An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. Genome Biol 8(8):R157. doi:10.1186/gb-2007-8-8-r157

30. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13(9):1050–1059. doi:10.1038/nm1622

31. Lake RA, Robinson BW (2005) Immunotherapy and chemotherapy—a practical partnership. Nat Rev Cancer 5(5):397–405. doi:10.1038/nrc1613

32. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO (2008) Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 14(5):1368–1376. doi:10.1158/1078-0432.CCR-07-1658

33. Jumppanen M, Gruvberger-Saal S, Kauraniemi P, Tanner M, Bendahl PO, Lundin M, Krogh M, Kataja P, Borg A, Ferno M, Isola J (2007) Basal-like phenotype is not associated with patient survival in estrogen-receptor-negative breast cancers. Breast Cancer Res 9(1):R16. doi:10.1186/bcr1649

34. Tischkowitz M, Brunet JS, Begin LR, Huntsman DG, Cheang MC, Akslen LA, Nielsen TO, Foulkes WD (2007) Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC Cancer 7:134. doi:10.1186/1471-2407-7-134

35. Bidard FC, Matthieu MC, Chollet P, Raoefils I, Abrial C, Domont J, Spielmann M, Delaloge S, Andre F, Penault-Llorca F (2008) p53 status and efficacy of primary anthracyclines/alkylating agent-based regimen according to breast cancer molecular classes. Ann Oncol 19(7):1261–1265. doi:10.1093/annonc/mdn039

36. Harris LN, Broadwater G, Lin NU, Miron A, Schnitt SJ, Cowan D, Lara J, Bleiweiss I, Berry D, Ellis M, Hayes DF, Winer EP, Dressler L (2006) Molecular subtypes of breast cancer in relation to paclitaxel response, outcomes in women with metastatic disease: results from CALGB 9342. Breast Cancer Res 8(6):R66. doi:10.1186/bcr1622

37. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, von

Torne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28(1):105–113. doi:10.1200/JCO. 2009.23.7370

38. Hornychova H, Melichar B, Tomsova M, Mergancova J, Urminska H, Ryska A (2008) Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. Cancer Invest 26(10):1024–1031. doi:10.1080/07357900 802098165

Clinicopathological analyses of triple negative breast cancer using surveillance data from the Registration Committee of the Japanese Breast Cancer Society

Hirotaka Iwase · Junichi Kurebayashi · Hitoshi Tsuda ·
Tomohiko Ohta · Masafumi Kurosumi · Kazuaki Miyamoto ·
Yutaka Yamamoto · Takuji Iwase

Abstract

Background Triple negative (TN) breast cancer is defined as a subtype that is negative for estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). To clarify the characteristics of TN breast cancer, surveillance data of the Registration Committee of the Japanese Breast Cancer Society were analyzed. *Method* Of 14,748 cases registered in 2004, 11,705 (79.4%) were examined for ER, PgR, and HER2. Of these, the most prevalent (53.8%) was a hormone-responsive subtype with ER positive/PgR positive/HER2 negative, followed by TN subtype (15.5%).

Results The proportion of postmenopausal patients was relatively high in the TN subtype. This cancer was diagnosed at a slightly advanced stage and with more cases positive for lymph node metastases than other subtypes. Morphologically, the TN subtype was more frequently classified as solid-tubular carcinoma. Mucinous, tubular, or secretary carcinomas were frequently found in the hormone receptor positive/HER2 negative subtype, while squamous cell carcinoma, spindle cell carcinoma, and metaplastic

H. Iwase · J. Kurebayashi · H. Tsuda · T. Ohta · M. Kurosumi ·
K. Miyamoto
Member of the Research Group,
Japanese Breast Cancer Society, Tokyo, Japan

H. Iwase (✉) · Y. Yamamoto
Department of Breast and Endocrine Surgery,
Kumamoto University, 1-1-1 Honjo,
Kumamoto 860-8556, Japan
e-mail: hiwase@kumamoto-u.ac.jp

Y. Yamamoto
e-mail: ys-yama@triton.ocn.ne.jp

J. Kurebayashi
Department of Breast and Thyroid Surgery,
Kawasaki Medical University, Okayama, Japan
e-mail: kure@med.kawasaki-m.ac.jp

H. Tsuda
Pathology Section, Clinical Laboratory Division,
National Cancer Center Hospital, Tokyo, Japan
e-mail: hstsuda@ncc.go.jp

T. Ohta
Division of Breast and Endocrine Surgery,
Department of Surgery, St. Marianna University School
of Medicine, Kawasaki, Japan
e-mail: to@marianna-u.ac.jp

M. Kurosumi
Department of Pathology, Saitama Prefecture Cancer Center,
Saitama, Japan
e-mail: mkurosumi@cancer-c.pref.saitama.jp

K. Miyamoto
Department of Surgery, National Hospital Organization Kure
Medical Center/Chugoku Cancer Center, Kure, Japan
e-mail: miyamotok@kure-nh.go.jp

T. Iwase
Director of the Registration Committee of the Japanese Breast
Cancer Society, Tokyo, Japan

T. Iwase
Department of Breast Clinic, Cancer Institute Ariake Hospital,
Tokyo, Japan
e-mail: takiwase@nifty.com

carcinoma with bone/cartilage metaplasia were very frequently found in the TN group. Apocrine carcinoma was also found very frequently in the TN group. Selection of chemotherapy was not based on receptor subtypes, but was determined by the degree of tumor progression.

Conclusions Although TN types are similar to basal-like breast tumor, as determined by gene profiling, their diagnosis needs verification by determination of the level of epidermal growth factor receptor or cytokeratin 5/6 expression. TN type should be examined further for immunohistochemical features and analyzed for prognostic details in this cohort.

Keywords Triple negative tumor · Breast cancer · Surveillance data

Introduction

Triple negative (TN) breast cancer represents a subtype that is negative for the three main prognostic/predictive receptors for breast cancer, namely, estrogen receptor (ER), progesterone receptor (PgR), and HER2 (human epidermal growth factor receptor type 2) [1]. ER and/or PgR positive cancer, which means hormone receptor (HR)-positive cancer, usually responds to endocrine therapy. Cancers scored immunohistochemically as 3+ or 2+ and that are 'fluorescence in situ hybridization' (FISH)-positive are regarded as HER2-positive and are targets for treatment with trastuzumab and other agents aimed at HER2. However, currently no targeted therapeutic agents have been identified specifically for TN breast cancer, and the only option at present is conventional systemic chemotherapy. In this context, it is essential to be familiar with the biological features of TN breast cancer in order to develop the best therapeutic strategy [1–3].

An alternative approach to subtyping breast cancers has been developed by Sørlie et al. [4, 5], who classified breast cancer into four or more intrinsic subtypes on the basis of gene profiling acquired from microarray analyses of a large number of breast cancer tissue specimens. In their classification, the first was called a basal-like subtype; it shared some characteristics with basement membrane cells and had a high proliferative capability [6]. The second was the HER2 (ErbB2) subtype, in which HER2 and related genes were overexpressed and ER-related genes were under expressed. This subtype was also relatively highly proliferative and expected to respond to trastuzumab. The third subtype had normal epithelium (normal-like subtype), but its other significant features have yet to be established. The fourth was called the luminal subtype, which expressed various amounts of ER-related genes that could be further subclassified into luminal A or B. If a connection can be found between the intrinsic subtypes and 'classic' breast cancer subtypes based on receptor status, the correlation is best understood by contrasting the basal-like subtype to TN breast cancer, as the former is positive for cytokeratin 5/6 or epidermal growth factor receptor (EGFR) (HER1).

The basal-like subtype accounts for 15–20% of breast cancers, irrespective of the method of analysis or ethnic group [6]. However, premenopausal African–American patients have a significantly higher incidence of this subtype compared to other patients [7]. It is well known that the pathological and biological characteristics of breast cancer are significantly worse in young African–American patients and that they show a clinically poor prognosis. In contrast, the basal-like subtype is relatively uncommon in breast cancers diagnosed in Japanese women; in 793 breast cancer patients, only 8% were this genetic subtype [8]. A significant overlap has been repeatedly demonstrated between the biological and clinical characteristics of sporadic TN breast cancers and basal-like subtypes, and breast carcinomas arising in BRCA1 mutation carriers [5].

In the receptor subtype determination, there is an ongoing debate on how to determine what to take as the cutoff value for deciding the positive/negative expression levels of hormone receptors. For example, there is no agreement at present on whether 'negative' should be based on: (1) no expression, (2) a score of 0 or 2 on the Allred Score [9], which takes into account the number of positive cells and the intensity of staining for the receptor in question, or (3) the proportion of receptor-positive cells less than 10% [10].

The purpose of this study was to disclose clinicopathological features of TN breast cancer. With the support of the Registration Committee of the Japanese Breast Cancer Society, we analyzed about 11,000 cases registered in 2004 in order to classify them by receptor subtypes based on expression levels of ER/PgR/HER2 and to analyze the clinicopathological characteristics of TN tumors.

Materials and methods

Basic data of patients

Comprehensive data on breast cancer patients diagnosed in Japan in 2004 were registered by the Registration Committee of the Japanese Breast Cancer Society, who reported the final registry data in 2008, although patient outcome data have not been published yet. The registrations were made by 352 institutions and included 14,749 cases. The data collected were: age, clinicopathological features of the tumor including size, presence of lymph node metastases, and receptor status (ER, PgR, and HER2), surgical techniques, and regimens of chemotherapy.
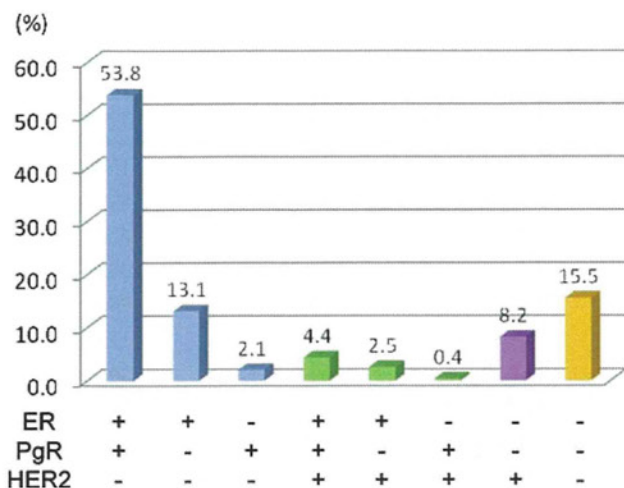
Fig. 1 Breast cancer surveillance data reported by the Japanese Breast Cancer Society

Individual participating institutions determined ER, PgR, and HER2 status by their own in-house method, as well as the other criteria for the registration. In 2004 the status of ER and PgR was being determined by the immunohistochemical (IHC) technique using monoclonal antibodies. Additionally, the cutoff level was mainly adopted to a score of between 2 and 3 on the Allred Score [9], or 10% as a staining proportion [10]. Tumors that were immunohistochemically scored as 3+, or scored 2+ with FISH-positive, were regarded as HER2-positive in a majority of individual participating institutions.

Subanalysis of receptors

Subanalysis was performed by permission of the Registration Committee and the Board of the Japanese Breast Cancer Society. Status of ER, PgR, and Her2 had been determined in 11,705 cases (79.4% of all registered cases), of which 1,819 cases (15.5%) were registered as negative for any one of ER/PgR/HER2. The most prevalent subtype was ER+/PgR+/HER2− (53.8%), followed by TN breast cancer (15.5%) (Fig. 1).

Receptor subtypes were divided according to their ER/PgR/HER2 profiles: the HR+/HER2− subtype was positive for ER and/or PgR and negative for HER2; the HR+/HER2+ subtype was positive for ER and/or PgR and positive for HER2; the HR−/HER2+ subtype was negative for both ER and PgR and positive for HER2; the triple negative (TN) subtype was negative for all three receptors, ER, PgR, and HER2 (Table 1)

Statistical processing

Fischer's exact test was used to compare various prevalence rates among the groups. Unpaired *t* test was

Table 1 Receptor subtype and status

Subtype	ER/PgR/HER2 status	
	Receptor profile	ER/PgR/HER2
HR+/HER2−	ER+ and/or PgR+, HER2−	+/+/−, +/−/−, −/+/−
HR+/HER2+	ER+ and/or PgR+, HER2+	+/+/+, +/−/+, −/+/+
HR−/HER2+	ER− and PgR−, HER2+	−/−/+
Triple negative	ER−, PgR− and HER2−	−/−/−

+, Positive; −, negative

employed to make inter-group comparisons in the number of cases and mean values. A significance level was set at less than 0.01 when multiple comparisons were required between four groups.

Results

Patient backgrounds

The relative proportions of the four cancer subtypes were: HR+/HER2− subtype, 68.7%; HR+/HER2+ subtype, 7.6%; HR−/HER2 subtype, 8.3%; and TN subtype, 15.4%. There was no difference in mean age between the groups. The incidence of bilateral breast cancer was significantly lower in HER2-positive subtypes than in HER2-negative subtypes ($P = 0.040$). The proportion of premenopausal patients was significantly greater in HR-positive groups (i.e., HR+/HER2− and HR+/HER2+ subtypes) than in the HR-negative groups (i.e., HR−/HER2+ or TN subtype) ($P < 0.001$). There were no significant differences between subtypes with respect of family history of breast cancer, height, body weight, or body mass index (BMI) (Table 2). Regarding disease stage, 37.2% of the HR+/HER2− subtype were diagnosed at stage I, indicating relatively early initiation of therapy, whereas the prevalence of stage I at diagnosis in HER2 was only 14.2%, which meant these patients received their first treatment at the slightly advanced stages of II–IV (Fig. 2).

Clinical findings

HR+/HER2− subtype was detected at an earlier stage than the other subtypes, that is, when the tumor was somewhat smaller in diameter, and compared advantageously in the incidence of node metastases especially with the ER−/HER2+ subtype. There was a tendency for the incidence of distant metastases to be higher in the HER2-positive groups (i.e., ER+/HER2+ and ER−/HER2+ subtypes) and for breast-conserving therapy to be less frequently performed in patients with the HR−/HER2+ subtype (Table 2).

**Table 2** Patient background and clinicopathological data

	Receptor subtype			
	HR+/HER2−	HR+/HER2+	HER2	TN
Number of patients (%)	8,039 (68.7)	892 (7.6)	977 (8.3)	1,797 (15.4)
Age median (range)	56 (NR–100)	54 (23–93)	56 (22–95)	57.5 (NR–94)
Ratio of bilateral breast cancer (%)	6.6[b]	5.9[a]	4.8[a]	6.2[b]
Incidence of breast cancer family history (%)	8.6	8.4	24.1	28.1
Ratio of premenopausal patients (%)	37.1[c]	38.8[c]	24.1[d]	28.1[d]
Height (cm) mean ± SD	154.3 ± 6.3	154.9 ± 6.1	154.0 ± 6.2	153.8 ± 6.3
Weight (kg) mean ± SD	54.7 ± 9.0	54.5 ± 8.8	53.9 ± 8.6	54.2 ± 9.0
BMI mean ± SD	23.0 ± 3.7	22.7 ± 3.5	22.7 ± 3.3	22.9 ± 3.5
Tumor size (cm) mean ± SD	2.6 ± 2.1[e]	3.2 ± 2.2	3.5 ± 2.6[f]	3.4 ± 2.7
Incidence of positive lymph node involvement (%)	20.6	34.9	38.5	32.2
Incidence of distant metastasis (%)	2.5	5.6	5.6	3.2
Incidence of breast-conserving surgery (%)	53.9	41.3	35.1	45.0

NR no record

[a] HER2 positive versus [b]HER2 negative according to ratio of bilateral breast cancer; $P = 0.040$, Fisher's exact probability test
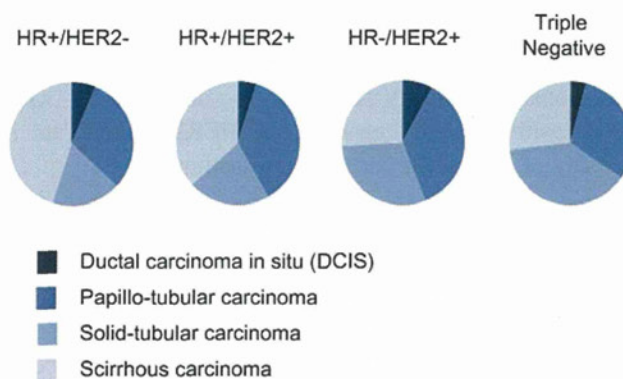
[c] Hormone receptor-positive group versus [d]hormone receptor-negative group according to the ratio of premenopausal patients; $P < 0.0001$, Fisher's exact probability test

[e] HR+/HER2− subtype versus [f]TN subtype according to tumor size; $P < 0.00001$, standard t test of mean and standard deviation (SD)
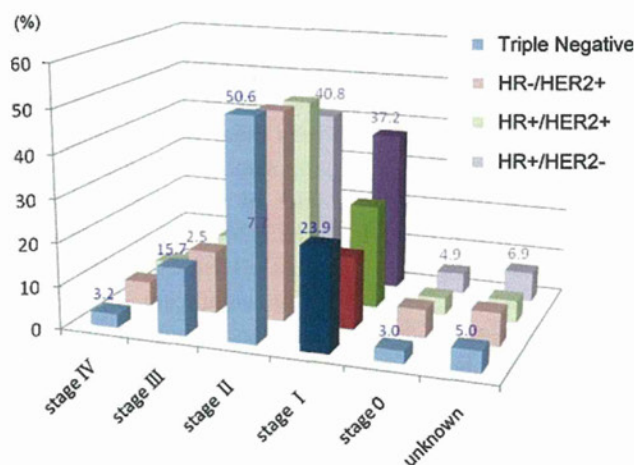
Fig. 2 Stage at diagnosis by receptor subtype

Fig. 3 DCIS and histological subtypes of invasive ductal carcinoma (IDC) by receptor subtype

Pathological findings

From a viewpoint of morphologic classification, whereas scirrhous carcinoma was most frequently found in ER+/HER2− subtype, solid-tubular carcinoma prevailed in TN breast cancer (Fig. 3). As to breast cancers of special types, mucinous carcinoma occurred rarely in the TN group, but was quite frequent among ER+/HER2− subtype patients (Fig. 4a). Invasive lobular carcinoma was found reasonably frequently in the HR+/HER2− type (Fig. 4b). Tubular and secretary carcinomas were mostly found in patients with a HR+/HER2− subtype (Fig. 4c).

Medullary carcinomas were observed frequently in the TN group (Fig. 4d). Squamous cell carcinoma, spindle cell carcinoma, or metaplastic carcinoma with bone/cartilage metaplasia was likewise very common in patients in the TN subtype (Fig. 4e). This group also included the highest percentages of apocrine carcinomas (Fig. 4f).

Selection of chemotherapeutic regimens

Two main chemotherapeutic regimens were administered: (1) anthracycline-containing regimens (ACR), which included: doxorubicin plus cyclophosphamide (AC), epirubicin plus C (EC), C plus A plus 5-fluorouracil (CAF) and CEF, and (2) taxane (paclitaxel or docetaxel)-containing
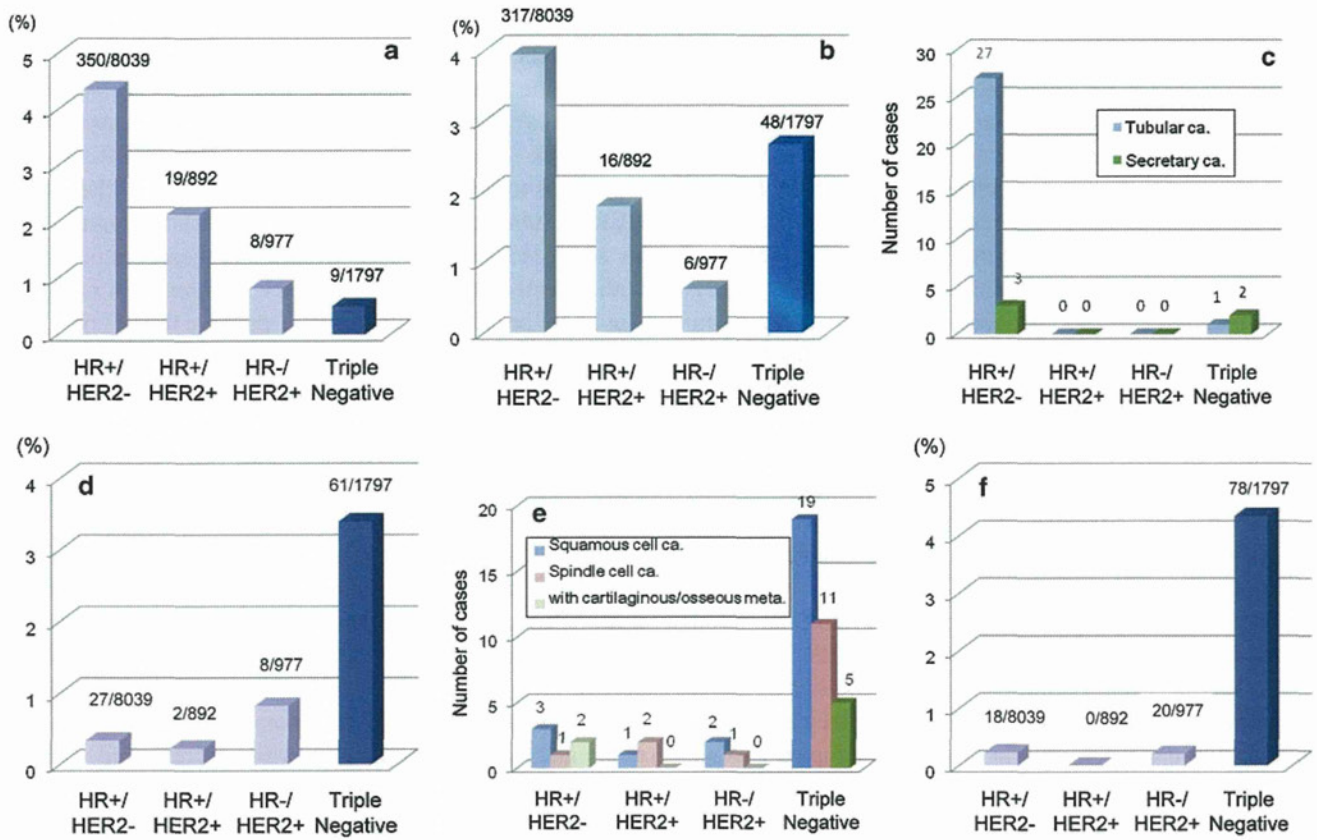
Fig. 4 a Incidence of mucinous carcinoma by receptor subtype. **b** Incidence of invasive lobular carcinoma by receptor subtype. **c** Incidence of tubular or secretary carcinomas by receptor subtype. **d** Incidence of medullary carcinoma by receptor subtype. **e** Incidence of metastatic carcinoma by receptor subtype. **f** Incidence of apocrine carcinoma by receptor subtype

Table 3 Chemotherapy according to receptor subtype

	Subtype			
	HR+/HER2−	HR+/HER2+	HR−/HER2+	TN
Number of cases	8,039	892	977	1,797
Node positive cases (%)	20.6	34.9	38.5	32.2
Number of patients treated by chemotherapy	3,913	696	940	1,563
Incidence of patients treated by ACR (%)	28.3	45.0	53.7	49.5
Incidence of patients treated by taxanes (%)	16.9	29.9	36.7	29.2
Incidence of neoadjuvant chemotherapy (%)	25.0	33.9	27.2	25.0

ACR anthracycline-containing regimen

regimens. In one patient there was a possibility that these two main regimens might have been administered concomitantly. ACR was administered to 28.3% of HR+/HER2− subtype tumors and taxane-based regimens to 16.9%. However, the incidence of axillary lymph nodes metastases was 20.6% in this subtype, which was smaller compared to other subtypes. In the other three subtype groups, as shown in Table 3, patients invariably received ACR or taxane regimens. Although the incidence of neoadjuvant chemotherapy was almost identical in each subtype group, the HR+/HER2+ group tended to be treated

with this type of chemotherapy a little more frequently (Table 3).

Discussion

Of 14,749 breast cancer cases in Japan in 2004, 11,705 (79.4%) were examined for their ER, PgR, and HER2 status. TN tumors, defined as negative for all three receptors, accounted for 15.5% (1,819 cases). This was the largest collection of data on the prevalence rate of TN

tumors in Japan and was gathered from a large patient sample. Although there were no restrictions placed on the various centers for the methods they used for determining the presence of the receptors, or the criteria employed for their definition, we assumed that most cases had been examined using immunohistochemistry, since this technique for the detection of the receptors was in widespread use in 2004. It is possible that the hormone receptor status of some cases in this current study were incorrectly determined, because the definition criteria had not been established at that time in 2004. Most Japanese institutions regarded 0 or 2 on the Allred score as negative; others used a cutoff value of 10% for the determination of ER/PgR. Currently, the criteria for HER2 positivity are: 3+ with the IHC method, or 2+ with the IHC method and positive with the FISH method. However, in 2004, a tumor was defined as positive for HER2 if the IHC method resulted in 3+ alone, or in 2+ 3+.

Hence, in the present analyses, there were no strict criteria in place for the determination of ER, PgR, or HER2, leaving each institution to apply its own criteria. Now, however, it is considered that standardized analytical methods and definition criteria have been adopted nationwide, so that future analyses will be more reliable. Nevertheless, despite this limitation, we consider that the results of this population study are clinically very significant because of the large number of cases (over 11,000) and participating institutions (over 350).

The basal-like subtype accounts for 15–20% of breast cancers, irrespective of the method of analysis or ethnic group [6]. However, premenopausal African–American patients have a significantly higher incidence of this subtype compared to other patients [7, 11]. It is well known that the pathological and biological characteristics of breast cancer are significantly worse in young African–American patients and that they show a clinically poor prognosis. Therefore, the high incidence of basal-like subtype in young African–American patients correlates with the high histological grade of the tumors and the poor prognosis of the disease in this specific patient subgroup. On the other hand, it has been reported that this basal-like subtype is comparatively rare in Japanese women, with an incidence of only 8% documented in a recent study of 793 breast cancer cases in Japan [8]. We were interested in a possible familial nature of TN tumors, because of suggestions of a link to BRCA1 mutation. In the present study, however, we could not find any evidence of a family history of breast cancer in this subtype or that it affected younger women than other subtypes. This may have been due to the fact that various subtypes of breast cancer are included in the definition of 'TN,' although the basal-like subtype is thought to comprise 40–80% of cases [7, 12].

On the other hand, HR+/HER2− subtype tumors tended to be smaller and the patients freer of lymph node metastases at the time of diagnosis, while the HER2 subtypes were often positive for regional or distant lymph node metastases. This finding indicates the HR+/HER2− subtype tends to be detected at an earlier stage than the HR−/HER2+ subtype, which was characteristically diagnosed at an advanced stage. However, even if these tumors were both detected at an early stage, the difference in outcome would not be affected since HER2-positive tumors progress more rapidly.

Regarding histological subtypes, scirrhous carcinoma and solid-tubular carcinoma tended to be found more frequently in the HR+/HER2− and the TN subtypes, respectively. Although invasive lobular carcinoma was also found in the TN type, its true incidence is unclear as it is rarely difficult to distinguish from scirrhous carcinoma. This TN subgroup also included many cases of medullary and metaplastic carcinomas. Spindle cell and squamous cell carcinomas of the TN tumors showed metaplasia derived from invasive ductal carcinoma and exhibited characteristics of basal-like tumors [12]. However, medullary and apocrine carcinomas, which were included in the metaplastic carcinomas, have a better prognosis than the common type and, among TN breast cancers, should be regarded as different from the more common basal-like breast cancer.

There was no apparent correlation between the choice of chemotherapeutic regimen and tumor receptor subtype, both an anthracycline-containing regimen (ACR) and taxanes were used depending on the degree of progression. Neoadjuvant therapy was used in 27.2 and 25% of HR−/HER2+ and TN tumors, respectively, indicating that in 2004 this therapy was being used in large resectable tumors.

In conclusion, we analyzed data from a large number of breast cancer cases registered by the Japanese Breast Cancer Society in order to characterize and advance our understanding of the TN subtype of breast cancer. The present study demonstrated that it was important to establish standard analytical methods and criteria for detection of ER, PgR, and HER2 and that in particular it was necessary to define TN breast cancer more carefully. In the future, we need to follow up the prognosis and response to chemotherapy in these TN breast cancer cases in an attempt to characterize the subtype in more detail [2]. TN breast cancer simulates basal-like tumor, which has been classified from gene profiles. The basal-like type of TN breast cancer is diagnosed by IHC methods based on the expression of EGFR and cytokeratin 5/6. We are looking into further analyzing the cases from the 2004 registry from the perspectives of immunohistochemistry, prognosis, and use of adjuvant chemotherapy.

References

1. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. Cancer. 2007;109:1721–8.

2. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res. 2007;13:2329–34.

3. Nishimura R, Arima N. Is triple negative a prognostic factor in breast cancer? Breast Cancer. 2008;15:303–8.

4. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA. 2001;98:10869–74.

5. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA. 2003;100:8418–23.

6. Kobayashi S. Basal-like subtype of breast cancer: a review of its unique characteristics and their clinical significance. Breast Cancer. 2008;15:153–8.

7. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006;295:2492–502.

8. Kurebayashi J, Moriya T, Ishida T, Hirakawa H, Kurosumi M, Akiyama F, et al. The prevalence of intrinsic subtypes and prognosis in breast cancer patients of different races. Breast. 2007;16(Suppl 2):S72–7.

9. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol. 1999;17:1474–81.

10. Umemura S, Kurosumi M, Moriya T, Oyama T, Arihiro K, Yamashita H, et al. Immunohistochemical Evaluation for hormone receptors in breast cancer: a practically useful evaluation system and handling protocol. Breast cancer. 2006;13:232–5.

11. Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Dressler LG, et al. Epidemiology of basal-like breast cancer. Breast Cancer Res Treat. 2008;109:123–39.

12. Reis-Filho JS, Milanezi F, Steele D, Savage K, Simpson PT, Nesland JM, et al. Metaplastic breast carcinomas are basal-like tumours. Histopathology. 2006;49:10–21.

PRECLINICAL STUDY

Expression pattern of stromal cell-derived factor-1 chemokine in invasive breast cancer is correlated with estrogen receptor status and patient prognosis

Takayuki Kobayashi · Hitoshi Tsuda · Tomoyuki Moriya ·
Tamio Yamasaki · Ryoko Kikuchi · Shigeto Ueda ·
Jiro Omata · Junji Yamamoto · Osamu Matsubara

Abstract Chemokine receptor CXCR4 is known to be crucially involved in tumor progression, but the role of its ligand, stromal cell-derived factor-1 (SDF-1), remains unclear. The present study was conducted to clarify the clinicopathological and prognostic impact of SDF-1 expression in invasive breast cancers. Expression of SDF-1 mRNA and protein was examined in five breast cancer cell lines with or without estradiol treatment. In 52 surgically resected breast cancers, the level of SDF-1 mRNA in frozen samples and the pattern of SDF-1 protein immunoreactivity in formalin-fixed paraffin-embedded tissue sections were compared. In another cohort of 223 breast cancers, the correlation between SDF-1 immunoreactivity and clinicopathological parameters was examined using a tissue microarray. Estradiol treatment markedly increased the expression of SDF-1 mRNA and protein in the estrogen receptor (ER)-positive cell lines, MCF-7 and T47D. Among the 52 resected breast cancers, those with a cytoplasmic-dominant pattern of SDF-1 expression showed higher SDF-1 mRNA levels (median 27.4) than those with a membrane-dominant or negative pattern (median 13.6, $P = 0.0017$). Accordingly, the cytoplasmic-dominant pattern was defined as "high SDF-1 expression," and other patterns were defined as "low SDF-1 expression." Among the cohort of 223 tumors, "high SDF-1 expression" was detected in 158 (70.9%) and was significantly correlated with ER positivity ($P < 0.0001$), HER2 negativity ($P = 0.021$), and lower grade ($P < 0.0001$). Univariate analysis demonstrated that "high SDF-1 expression" was a significant indicator of better clinical outcome in both the entire patient cohort ($P = 0.017$) and the 133 patients with ER-positive tumors ($P = 0.036$), but not in the 90 patients with ER-negative tumors. Multivariate analysis showed that SDF-1 status was an independent factor related to overall survival in patients with ER-positive tumors ($P = 0.046$). SDF-1 status is a significant prognostic factor and may be clinically useful for assigning adjuvant therapy to patients with ER-positive invasive breast cancers.

T. Kobayashi · H. Tsuda (✉) · R. Kikuchi · O. Matsubara
Department of Basic Pathology, National Defense Medical
College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan
e-mail: htsuda@ndmc.ac.jp

T. Moriya · S. Ueda · J. Omata · J. Yamamoto
Department of Surgery, National Defense Medical College,
3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

T. Yamasaki
Department of General Medicine, National Defense Medical
College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

H. Tsuda
Pathology Section, Clinical Laboratory Division, National
Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku,
Tokyo 104-0045, Japan

Abbreviations

SDF-1	Stromal cell-derived factor-1
ERG	Estrogen-regulated gene
TMA	Tissue microarray
ER	Estrogen receptor alpha
PR	Progesterone receptor

Introduction

Breast cancer is one of the most serious and prevalent diseases affecting women worldwide. Although age-adjusted

🖄 Springer

rates of breast cancer incidence are decreasing in the United States and the United Kingdom, they are still increasing in Asian countries including Japan [1]. The clinical outcome of patients with breast cancer in Japan has improved over the last 50 years [2], and this has been partly attributable to advances in adjuvant endocrine therapy and chemotherapy [3].

Approximately 70% of breast cancers are known to express estrogen receptor alpha (ER) and are considered to be hormone-dependent. Estrogens regulate the expression of various genes, and these estrogen-regulated genes (ERGs), including those encoding progesterone receptor (PR), transforming growth factor (TGF)-alpha, cyclin D1, bcl-2, and estrogen-responsive finger protein (Efp), have been shown to play various roles in estrogen signaling. Furthermore, ERGs are considered to be potential predictive biomarkers of response to hormonal therapy [4]. Currently, however, PR is the only ERG whose expression is used routinely to determine whether endocrine therapy is needed [5]. Recently, Hall and Korach [6] have revealed that the transcriptional activation and protein expression of stromal cell-derived factor-1 (SDF-1), also known as CXCL12, are regulated by estradiol in ER-positive breast cancer and ovarian cancer cell lines.

SDF-1, which was initially cloned from murine bone marrow stromal cells and characterized as a pre-B-cell growth-stimulating factor [7, 8], is a small chemotactic cytokine belonging to the CXC chemokine family. In cooperation with its cognate receptor, CXCR4 [chemokine (C-X-C motif) receptor 4], the CXCR4/SDF-1 axis plays various roles in many normal and pathological processes including embryogenesis, hematopoiesis, immunological homeostasis, human immunodeficiency virus infection, and the progression of rheumatoid arthritis. Furthermore, in various types of cancer, including breast cancer, CXCR4 on tumor cells has been shown to be critically involved in tumor progression [9]. In contrast, only a limited number of studies have investigated tumor-derived SDF-1, and the significance of this molecule in tumor biology is not fully understood [10].

In order to elucidate the clinicopathological role of tumor-derived SDF-1 in breast cancer, we examined the expression of SDF-1 protein using immunohistochemistry and that of SDF-1 mRNA by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis in surgically resected specimens of invasive breast cancer. In parallel, we also examined SDF-1 expression in five human breast cancer cell lines with and without estradiol treatment to acquire supportive evidence for SDF-1 expression in breast cancer tissues.

Materials and methods

Cell lines

The human breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-435, and MDA-MB-436 were purchased from the American Type Culture Collection (Rockville, MD, USA). Experiments using these cell lines were performed within 6 months. The cells were cultured in Dulbecco's MEM (DMEM) containing 10% fetal bovine serum and penicillin–streptomycin (100 U/ml). Before all of the experiments, the cells were cultured for 48 h in phenol red-free DMEM with 10% charcoal/dextran-treated fetal bovine serum.

Quantitative RT-PCR

Total RNA was extracted by using RNeasy Mini (Qiagen, Hilden, Germany) from the five cell lines, 52 samples of frozen tumor tissue, and 13 samples of frozen mammary gland tissue without any apparent histological abnormality. Each RNA sample was subjected to complementary DNA synthesis, and quantitative RT-PCR was performed with the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Scoresby VIC, Australia). The TaqMan® Gene Expression Assay used was **Hs00171022_m1** for SDF-1. The expression of 18s-rRNA was assessed as an internal control in order to verify mRNA integrity. In quantitative RT-PCR analysis using frozen tissue samples, the expression level of SDF-1 mRNA was calculated by the delta-delta Ct method for each sample.

Enzyme-linked immunosorbent assay (ELISA)

For quantification of SDF-1 secreted into the supernatant of the cell lines, a DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions. Each experiment was performed three times, and mean values were calculated.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by a wash with 0.5% Tween 20 in phosphate-buffered saline (PBS). The cells were then incubated with mouse monoclonal anti-human SDF-1 antibody (MAB350, R&D Systems) or murine IgG isotype control monoclonal antibody (MAB002, R&D systems), and SDF-1 protein and murine IgG background staining were visualized using fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibody as a secondary antibody (AP192F, Chemicon, Temecula, CA, USA).

Nuclei were visualized by 4,6-diamidino-2-phenylindole counterstaining.

Patients and tumor tissues

This study was carried out after obtaining approval from the internal review board. Frozen samples of invasive breast carcinoma tissue were obtained from a case series of 52 patients who had undergone surgery at the National Defense Medical College Hospital, Tokorozawa, Japan, between 2005 and 2007. These frozen tumor samples were used to evaluate levels of SDF-1 mRNA by quantitative RT-PCR. From formalin-fixed, paraffin-embedded samples of the corresponding 52 tumors, 4-μm-thick sections were prepared and subjected to immunohistochemistry for SDF-1 protein expression.

From among 247 consecutive patients who had undergone mastectomy or breast-conserving surgery for unilateral invasive breast carcinoma at the National Defense Medical College Hospital between 1995 and 1999, complete medical records and appropriate tissue samples for constructing tissue microarrays were available for 223 patients. The tumors resected from these 223 patients were included in another retrospective immunohistochemical study of SDF-1 expression.

The 223 patients had been followed up for a median period of 74 months (range, 1–151 months), during which there were 58 relapses and 30 deaths. After surgical therapy, the patients with ER- and/or PR-positive breast cancer had received endocrine therapy for 2 years or more, and the patients with large tumors and/or nodal metastasis had received adjuvant chemotherapy. Fifteen patients with locally advanced breast cancer had received preoperative chemotherapy, for example, two or more courses of the cyclophosphamide-epirubicin-5-fluorouracil (CEF) regimen. Two-hundred and twenty-one patients were females and two were males. Additional patient characteristics are summarized in Table 1. For these 223 tumors, histological types and nuclear grade were re-examined for the present study by two observers (T. K. and H. T.). Other clinicopathological data were collected from the medical records and pathology reports.

Tissue microarray construction

We constructed tissue microarray blocks as described previously [11]. Briefly, double tissue cores 2 mm in diameter were taken from each donor block, and these core specimens were transferred to a recipient block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA). One Tissue microarray (TMA) block contained a maximum of 26 tumor samples, and 13 TMA sets were prepared for the present study.

Immunohistochemistry

Immunohistochemistry was performed on whole tissue sections from the 52 tumors, which were also subjected to quantitative RT-PCR, and on the TMA sections from the 223 tumors. Antibodies used were mouse monoclonal anti-human SDF-1 (R&D Systems), mouse monoclonal anti-human CXCR4 (MAB173, R&D systems), mouse monoclonal anti-human ER (clone 1D5, Dako, Glostrup, Denmark), rabbit polyclonal anti-HER2 antibody included in a HercepTest kit (Dako), and murine IgG isotype control antibody (R&D Systems). After deparaffinization of 4-μm-thick tissue sections, antigens were retrieved by microwaving in 10 mM sodium citrate (pH 6.0) for SDF-1, CXCR4, and murine isotype antibody, or by autoclaving in 10 mM Tris–HCl (pH 9.0) for ER. Non-specific binding was blocked by incubation in 1% normal swine serum (Dako) in PBS. The slides were incubated with the primary antibodies at 4°C overnight and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus; Dako) for 1 h at room temperature. Specific antigen–antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Counterstaining was performed using Mayer's hematoxylin.

The expression of SDF-1 was assessed according to the proportion of the stained area (<10% as negative, ≥10% as positive). Positive staining was further classified into two patterns according to the localization of SDF-1 immunoreactivity: cytoplasmic-dominant staining (C-pattern), and membrane-dominant staining (M-pattern). For CXCR4, nuclear staining in ≥10% and <10% of carcinoma cells was defined as positive and negative expression, respectively. A HER2 score was assigned according to the standard procedure, and a score of 3+ was classified as positive. ER was defined as positive if nuclear staining was seen in ≥10% of carcinoma cells. The results of immunohistochemistry were evaluated by two observers (T.K. and H.T.) independently, and cases with discrepant judgments were re-evaluated by discussion until consensus was obtained.

Statistical analysis

Comparisons between groups were evaluated using chi-squared test, Fisher's exact test, or Mann–Whitney U-test. Multiple comparisons (post hoc test) were carried out by the Bonferroni method. Survival curves of patients were drawn using the Kaplan–Meier method. Cox's univariate and multivariate proportional hazards models were used to explore the association of variables with disease-free and overall survival. For all the tests, differences at $P < 0.05$ were considered to be statistically significant. All analyzes were performed using the JMP 6.0 software package for Windows (SAS Institute Inc., Cary, NC, USA).

Table 1 Clinicopathological implication of SDF-1 expression in surgically resected breast cancers

	Total ($n = 223$)	SDF-1		
		"High expression" ($n = 158$) [n (%)]	"Low expression" ($n = 65$) [n (%)]	P value
Age				
Median y (range)		52 (30 ~ 82y)		
≤52	111	87 (79)	24 (37)	
>52	112	71 (45)	41 (63)	0.018
ER status				
Negative	90	49 (31)	41 (63)	
Positive	133	109 (69)	24 (37)	<0.0001
HER2 status				
Score 0–2+	202	148 (94)	54 (83)	
Score 3+	21	10 (6)	11 (17)	0.021
Nuclear grade				
1	45	38 (24)	7 (11)	
2	95	78 (49)	17 (26)	
3	83	42 (27)	41 (63)	<0.0001
Tumor size				
<5.0 cm	176	128 (81)	48 (74)	
≥5.0 cm	42	25 (16)	17 (26)	0.13
Unknown	5	5 (3)	0 (0)	
Lymph node metastasis				
(−)	117	86 (55)	31 (48)	
(+)	101	67 (42)	34 (52)	0.29
Unknown	4	4 (3)	0 (0)	
Distant metastasis				
(−)	211	151 (96)	60 (92)	
(+)	8	3 (2)	5 (8)	0.052
Unknown	4	4 (2)	0 (0)	
Stage				
1 or 2	181	131 (83)	50 (77)	
3 or 4	37	22 (14)	15 (23)	0.16
Unknown	5	5 (3)	0 (0)	
Tumor histology				
IDC	194	136 (86)	58 (89)	
ILC	10	10 (6)	0 (0)	
Special[a]	19	12 (8)	7 (11)	0.095
CXCR4				
Negative	62	35 (22)	27 (42)	
Positive	161	123 (78)	38 (58)	0.0033

Abbreviation: *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

[a] Mucinous carcinoma (6), Medullary carcinoma (3), Spindle cell carcinoma (1), Apocrine carcinoma (2), Tubular carcinoma (5), Micropapillary (1), Endocrine carcinoma (1)

Results

SDF-1 expression is induced by estradiol in ER-positive breast cancer cell lines

To determine whether breast cancer cells produce SDF-1, we subjected breast cancer cell lines to quantitative RT-PCR, ELISA, and immunofluorescence analysis. Quantitative RT-PCR detected a low level of SDF-1 mRNA in the ER-positive cell lines MCF-7 and T47D (clear columns in Fig. 1a), but the mRNA level increased about 7-fold and 21-fold, respectively, in response to estradiol treatment (filled columns in Fig. 1a). In contrast, the level of SDF-1 mRNA was very low in the ER-negative cell lines MDA-MB-231, MDA-MB435, and MDA-MB-436, and did not change after estradiol treatment (Fig. 1a).

The results of ELISA were similar to those of quantitative RT-PCR. Mean levels of secreted SDF-1 protein in
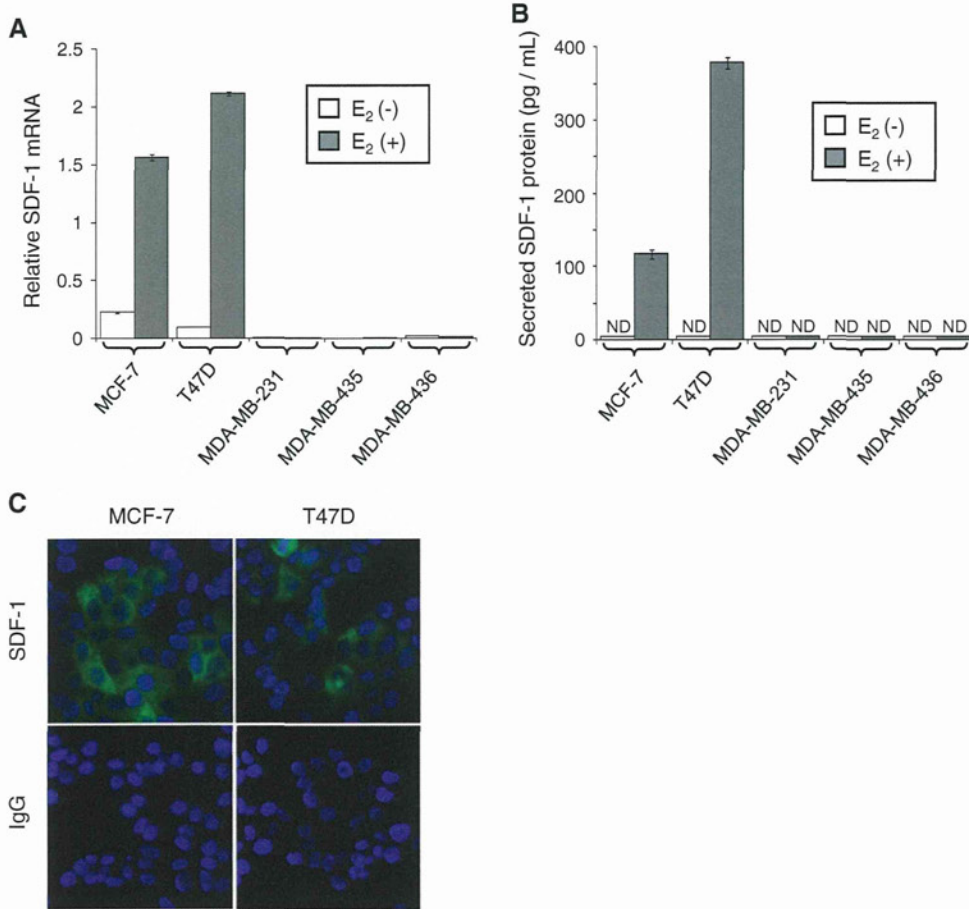
Fig. 1 SDF-1 expression in breast cancer cell lines. **a** Effect of estradiol on SDF-1 transcriptional activity in the five cell lines evaluated by quantitative RT-PCR. After treatment with 10 nM estradiol (*filled columns*) or ethanol vehicle (*clear columns*) for 48 h, total RNA was extracted. The relative SDF-1 mRNA values were calculated by estimating the ratio of SDF-1 copies to 18s-rRNA copies. *Columns*, mean values of three samples; *bars*, standard deviation; E2, estradiol. **b** Effect of estradiol on SDF-1 protein secretion by the five cell lines evaluated by ELISA. After treatment with 10 nM estradiol (*filled columns*) or ethanol vehicle (*clear columns*) for 72 h, the level of SDF-1 protein in the supernatant of each cell line was measured. *Columns*, mean values of three samples; *bars*, standard deviation; E2, estradiol; ND, not detectable. **c** Expression of SDF-1 protein detected by immunofluorescence in the ER-positive cell lines, MCF-7 and T47D. *Upper*: Cells were used after treatment with estradiol for 72 h. SDF-1 immunoreactivity was visualized in both cell lines. *Lower*: Immunofluorescence using murine IgG isotype control antibody showed no immunoreaction

the supernatant of MCF-7 and T47D cells after estradiol treatment were 117 and 378 pg/ml, respectively (filled columns in Fig. 1b). In contrast, SDF-1 protein was undetectable in the supernatant of these two ER-positive cell lines without estradiol treatment, or in the supernatant of the three ER-negative cell lines, irrespective of estradiol treatment (Fig. 1b).

Immunofluorescence analysis revealed diffuse and strong cytoplasmic SDF-1 immunoreactivity in almost all cells of the MCF-7 and T47D cell lines after estradiol treatment (Fig. 1c), whereas no, or only faint SDF-1 immunoreactivity was observed in the three ER-negative cell lines, regardless of estradiol treatment (data not shown). Taken together, these findings indicated that ER-positive breast cancer cell lines expressed SDF-1 in response to estradiol, whereas ER-negative breast cancer cell lines did not.

Correlation between protein expression pattern and mRNA level of SDF-1 in surgically resected breast cancers

Next, we conducted an immunohistochemical study to compare the expression levels of SDF-1 mRNA and protein in 52 samples of surgically resected invasive breast carcinoma and 13 samples of normal mammary gland tissue located adjacent to the tumors. Unremarkable mammary duct epithelia were found to have strong cytoplasmic immunoreactivity for SDF-1 (Fig. 2a). Among the 52 samples of breast cancer, 31 (60%), 18 (34%), and 3 (6%) showed C-, M-, and negative SDF-1 immunoreaction patterns, respectively (Fig. 2b–d). Cancer-associated fibroblasts (CAFs) sometimes showed weak SDF-1 staining (arrows in Fig. 2b–d), but the intensity and area of the
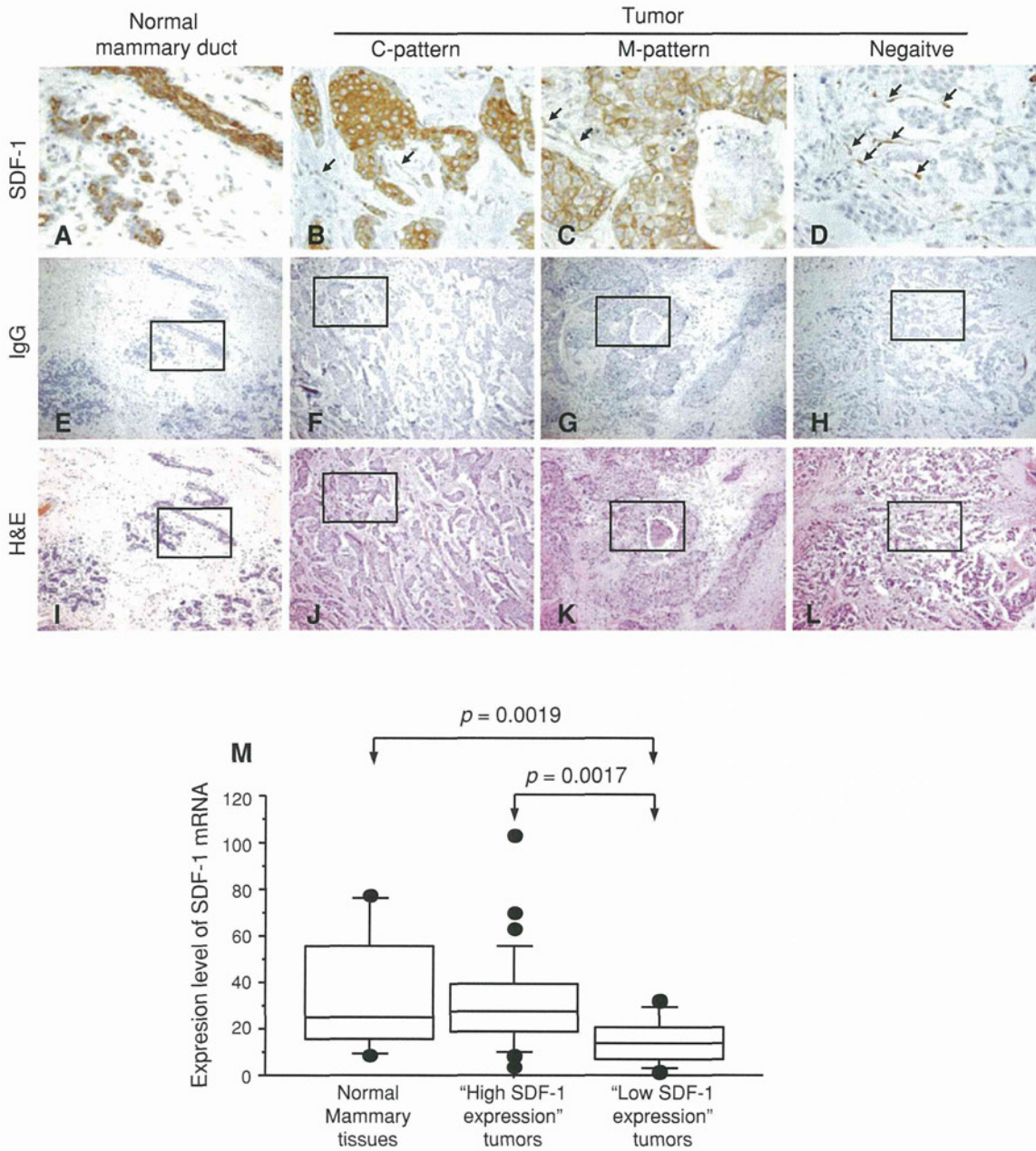
Fig. 2 SDF-1 expression in samples of human breast cancer. a–d SDF-1 immunoreaction pattern in surgically resected breast cancer tissues and normal mammary tissues. a Unremarkable mammary duct epithelia show strong cytoplasmic SDF-1 immunoreactivity. b C-pattern. Strong cytoplasmic-dominant immunoreactivity is evident in all constituent cells. c M-pattern. Moderate membrane-dominant immunoreactivity is evident in almost all constituent cells. d Negative immunoreaction pattern. e–h Immunohistochemistry using murine IgG control antibody for a–d. Murine IgG isotype control antibody showed no immunoreactivity. i–l, Hematoxylin and eosin (H&E) sections for a–d. H&E and IgG control images are shown at ×100 magnification with *boxed areas* indicating the ×400 magnification images shown for SDF-1 staining. Cancer-associated fibroblasts showed a weak immunoreaction for SDF-1 (*arrows* in Fig. b–d). m Correlation between results of immunohistochemistry and

quantitative RT-PCR. The median expression levels of SDF-1 mRNA in normal mammary gland tissues ($n = 13$), in the "high SDF-1 expression" group comprising tumors with the C-pattern ($n = 31$), and in the "low SDF-1 expression" group comprising tumors with the M- and negative patterns ($n = 21$), were 25.0, 27.4 and 13.6, respectively. Bonferroni-adjusted comparisons between groups showed that mRNA levels differed significantly between the tumors with "high" and "low SDF-1 expression" ($P = 0.0017$), and between the normal mammary tissues and the tumors with "low SDF-1 expression" ($P = 0.0019$). The *Y*-axis represents the relative level of SDF-1 mRNA expression normalized against 18s-rRNA, calculated relative to the sample with the lowest expression, which was assigned a value of 1.0. *Boxes and whiskers*, 25th to 75th and 10th to 90th percentiles, respectively; the median is the central line in each box; circles, outliers

SDF-1 immunoreactivity on tumor cells were much stronger and wider than on CAFs. Immunohistochemistry using mouse IgG control antibody showed no immunoreactivity (Fig. 2e–h). RT-PCR showed that the median levels of SDF-1 mRNA expression in the groups of tumors with C-, M-, and negative patterns were 27.4 (interquartile range, 18.2–39.8), 14.2 (interquartile range, 8.5–21.7), and 6.0 (interquartile range, 4.5–20.4), respectively. The median levels of SDF-1 mRNA differed significantly between the C- and M-pattern groups and between the C- and negative pattern groups ($P = 0.0011$ and 0.031, respectively, Mann–Whitney U-test).

When the 52 tumor samples were classified into two groups, i.e., "high SDF-1 expression" tumors comprising the C-pattern group, and "low SDF-1 expression" tumors comprising the negative plus M-pattern groups, the median levels of SDF-1 mRNA were 27.4 (interquartile range, 18.2–39.8) and 13.6 (interquartile range, 6.5–20.5) in the tumors showing "high" and "low SDF-1 expression", respectively. The median level of SDF-1 mRNA in samples of normal mammary gland tissue was 25.0 (interquartile range, 15.2–58.4). The levels of SDF-1 mRNA differed significantly between the tumors showing "high" and "low SDF-1 expression" ($P = 0.0017$), and between normal mammary gland tissue and the tumors showing "low SDF-1 expression" ($P = 0.0019$, post hoc Bonferroni test; Fig. 2m). Taken together, the data indicated that the pattern of immunoreactivity for SDF-1 protein was significantly correlated with the level of its mRNA.

Clinicopathological and prognostic implications of SDF-1 immunoreactivity for the entire patient cohort

We next evaluated the patterns of SDF-1 protein expression using another large cohort of patients with invasive breast cancer for whom long-term follow-up data were available, in order to clarify the clinicopathological and prognostic significance of tumor-derived SDF-1. One-hundred and fifty-eight patients (71%) had tumors showing "high SDF-1 expression" and 65 (29%) had tumors showing "low SDF-1 expression", the latter comprising 55 (24%) tumors with an M-pattern and 10 (5%) with a negative pattern. "High SDF-1 expression" showed a more significant association with younger patient age (≤52 years, $P = 0.018$), HER2 negativity ($P = 0.021$), and lower nuclear grade ($P < 0.0001$) than "low SDF-1 expression". In particular, positive ER staining was detected more frequently in tumors with "high SDF-1 expression" (109 of 158, 69%) than in those with "low SDF-1 expression" (24 of 65, 37%) ($P < 0.0001$, Table 1). Tumor size, lymph node status, or clinical stage was not significantly correlated with SDF-1 status. Tumor histology also showed no significant correlation with SDF-1 status, but all 10 invasive lobular carcinomas showed "high

SDF-1 expression". Nuclear CXCR4 staining was positive in 78% (123 of 158) of tumors with "high SDF-1 expression," and this proportion was significantly higher than that among tumors with "low SDF-1 expression" (58%, 38 of 65, $P = 0.0033$, Table 1).

Among the 223 patients examined, there was a significant difference in the disease-free and overall survival curves between the "high" and "low SDF-1 expression" groups ($P = 0.0058$ and 0.017, respectively; Fig. 3a, b). Univariate analysis also showed that clinical stage, nuclear grade, ER status, and HER2 status were significant or almost significant indicators of clinical outcome (Table 2). Multivariate analysis using the Cox proportional hazard model including these indicators showed that SDF-1 status was a factor significantly predictive of disease-free survival ($P = 0.036$), along with clinical stage ($P < 0.0001$) and nuclear grade ($P = 0.0012$). However, only clinical stage was selected as a significant prognostic factor for overall survival ($P < 0.0001$), and the impact of SDF-1 status was marginal ($P = 0.099$, Table 2). Univariate analysis showed that CXCR4 was of no prognostic significance (data not shown).

Clinicopathological and prognostic implications of SDF-1 immunoreactivity among ER-positive patients

As our data clearly indicated a marked correlation between the expressions of ER and SDF-1 in vitro and in vivo, we carried out further analysis to clarify the significance of SDF-1 expression in the subgroup of patients with ER-positive tumors. Among the 133 cases of ER-positive breast cancer, 109 (82%) showed "high SDF-1 expression." SDF-1 status was not significantly correlated with nuclear grade, tumor size, lymph node status, stage, histological type, or nuclear CXCR4 expression, but "high SDF-1 expression" was correlated with younger patient age ($P = 0.0025$; Table 3). A HER2 score of 3+ was detected in only two cases, both of which showed "low SDF-1 expression". Additionally, among only five cases that had distant metastasis at the time of diagnosis, two showed "high SDF-1 expression" and three showed "low SDF-1 expression".

In the 133 ER-positive patients, there was a significant difference in the disease-free and overall survival curves between the groups showing "high" and "low SDF-1 expression" ($P = 0.0047$ and 0.036, respectively; Fig. 3c, d). Furthermore, in these cases, multivariate analysis showed that SDF-1 status was an independent prognostic factor not only for disease-free survival, but also for overall survival ($P = 0.015$ and 0.046, respectively; Table 4). Nuclear grade and clinical stage also had a significant impact on overall survival (Table 4). Among ER-negative cases, there was no significant difference in the survival curves between the two groups (Fig. 3e, f).
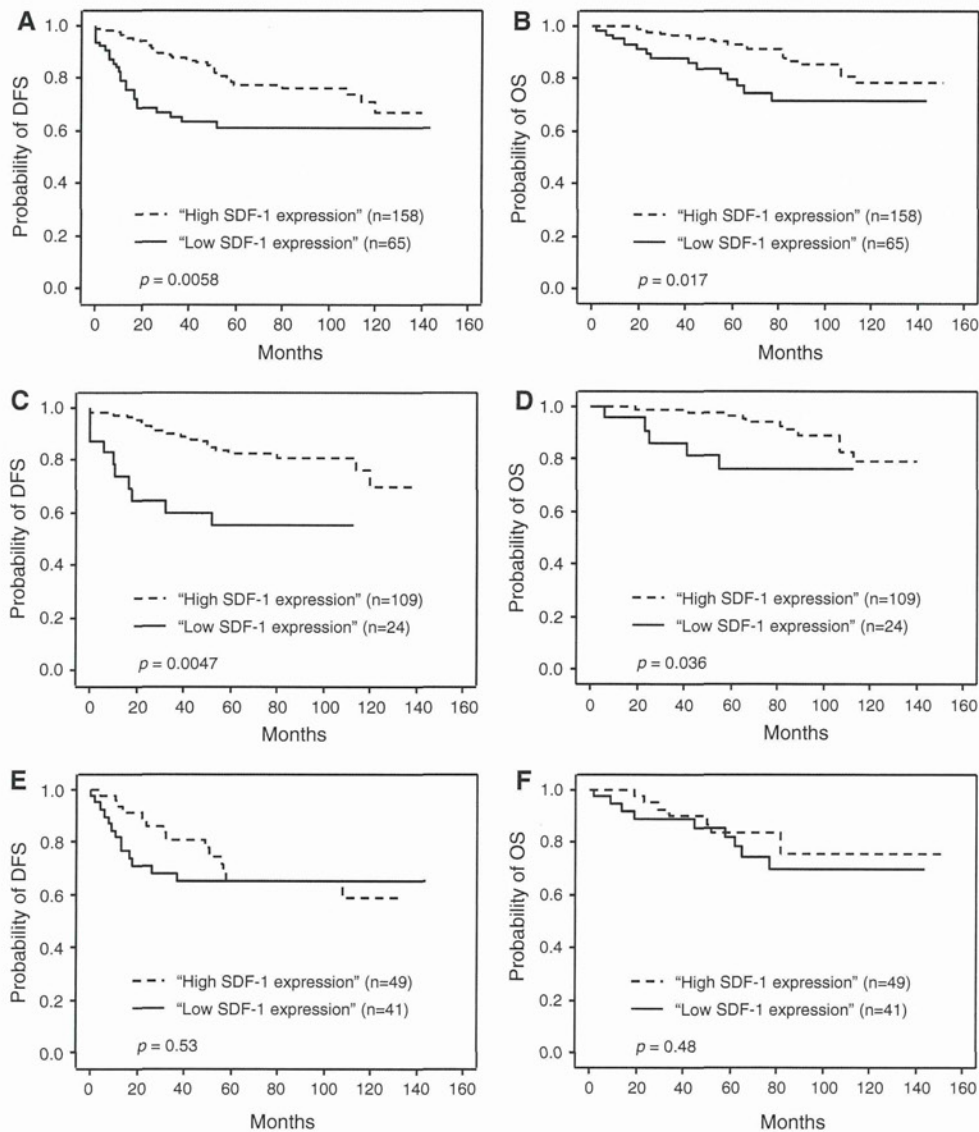
Fig. 3 Prognostic impact of SDF-1 status detected by immunohisto-chemistry in the patients with primary breast cancer. **a–b** all 223 patients; **c–d** 133 patients with ER-positive tumors; **e–f** 90 patients with ER-negative tumors. **a** Disease-free survival curves for the 158 patients whose tumors showed "high SDF-1 expression" and the 65 patients whose tumors showed "low SDF-1 expression." The two curves differ significantly ($P = 0.0058$). **b** Overall survival curves for the 158 patients whose tumors showed "high SDF-1 expression" and the 65 patients whose tumors showed "low SDF-1 expression." The two curves differ significantly ($P = 0.017$). **c** Disease-free survival curves for the 109 patients whose tumors showed "high SDF-1 expression" and the 24 patients whose tumors showed "low SDF-1 expression". The two curves differ significantly ($P = 0.0047$). **d** Overall survival curves for the 109 patients whose tumors showed "high SDF-1 expression" and the 24 patients whose tumors showed "low SDF-1 expression". The two curves differ significantly ($P = 0.036$). **e** Disease-free survival curves for the 49 patients whose tumors showed "high SDF-1 expression" and the 41 patients whose tumors showed "low SDF-1 expression". The two curves do not differ significantly ($P = 0.53$) **f** Overall survival curves for the 49 patients whose tumors showed "high SDF-1 expression" and the 41 patients whose tumors showed "low SDF-1 expression." The two curves do not differ significantly ($P = 0.48$). All P values were calculated using the Cox proportional hazards model

Discussion

In the present study, we showed that the expression of SDF-1 was regulated by estradiol in the ER-positive cell lines, MCF-7, and T47D, and found that SDF-1 status was significantly correlated with several clinically important factors, especially ER status, in samples of human breast cancer. In addition, we revealed that SDF-1 status was a statistically significant prognostic factor among cases of ER-positive breast cancer. To our knowledge, this is the first study to have demonstrated the importance of tumor-derived SDF-1 in ER-positive breast cancers.

Table 2 Prognostic indicators detected by Cox's univariate and multivariate analyses in patients with primary breast cancer

Variable	Univariate			Multivariate		
	HR	(95%CI)	P value	HR	(95%CI)	P value
Disease-free survival						
SDF-1						
"High expression"	1		0.0058	1		0.036
"Low expression"	1.45	(1.11–1.89)		1.38	(1.02–1.86)	
Clinical stage						
1 or 2	1		<0.0001	1		<0.0001
3 or 4	2.83	(2.16–3.72)		2.67	(2.01–3.54)	
Nuclear grade						
1	1		<0.0001	1		0.0012
2	1.30	(0.78–2.52)		1.51	(0.90–2.93)	
3	3.10	(1.93–5.90)		2.34	(1.40–4.55)	
ER						
Positive	1		0.10	1		0.39
Negative	1.24	(0.96–1.61)		0.88	(0.65–1.19)	
HER2						
Negative	1		0.025	1		0.21
Positive	1.57	(1.06–2.19)		1.29	(0.86–1.86)	
Overall survival						
SDF-1						
"High expression"	1		0.017	1		0.099
"Low expression"	1.56	(1.09–2.21)		1.39	(0.94–2.07)	
Clinical stage						
1 or 2	1		<0.0001	1		<0.0001
3 or 4	3.34	(2.34–4.88)		3.23	(2.23–4.78)	
Nuclear grade						
1	1		0.0002	1		0.16
2	1.22	(0.60–3.38)		1.32	(0.65–3.68)	
3	3.36	(1.79–9.13)		1.96	(0.96–5.49)	
ER						
Positive	1		0.047	1		0.58
Negative	1.42	(1.00–2.03)		1.23	(0.74–1.73)	
HER2						
Negative	1		0.0068	1		0.11
Positive	1.94	(1.22–2.87)		1.49	(0.90–2.35)	

Abbreviation: *95%CI* 95% confidence interval

Many researchers have investigated the role of the CXCR4/SDF-1 axis in tumor biology, and demonstrated the importance of CXCR4 expression for tumor progression in vitro [12–20] and in vivo [21–26]. In addition, some types of cancer cell lines have been shown to produce both SDF-1 and its receptor, CXCR4, and to use this signaling system in an autocrine manner [6, 27–29]. A number of clinical studies of glioma [30], colorectal cancer [31], gastric cancer [32], and oral squamous cell carcinoma [33] have concluded that higher SDF-1 expression is correlated with lymph node and/or distant metastasis and poorer clinical outcome.

In contrast, using a mouse model, Wendt et al. [34, 35] showed that breast and colon cancer cell lines with forced expression of SDF-1 established fewer secondary tumors than those with null SDF-1 expression. In patients with testicular germ cell tumors, high SDF-1 expression was correlated with longer relapse-free survival [36]. These differences in the clinical impact of tumor-derived SDF-1 might depend on the type of cancer.

🖄 Springer

Table 3 Clinicopathological implication of SDF-1 expression in surgically resected ER-positive breast cancers

	Total ($n = 133$)	SDF-1		
		"High expression" ($n = 109$) [n (%)]	"Low expression" ($n = 24$) [n (%)]	P value
Age				
≤52	67	60 (55)	7 (29)	
>52	66	49 (45)	17 (71)	0.025
Nuclear grade				
1	38	32 (29)	6 (24)	
2	65	56 (51)	9 (38)	
3	30	21 (20)	9 (38)	0.15
Tumor size				
<5.0 cm	109	90 (82)	19 (79)	
≥5.0 cm	20	15 (14)	5 (21)	0.53
Unknown	4	4 (4)	0 (0)	
Lymph node metastasis				
(−)	72	60 (55)	12 (50)	
(+)	57	45 (41)	12 (50)	0.65
Unknown	4	4 (4)	0 (0)	
Stage				
1 or 2	109	91 (83)	18 (75)	
3 or 4	20	14 (13)	6 (25)	0.21
Unknown	4	4 (4)	0 (0)	
Tumor histology				
IDC	116	94 (86)	22 (92)	
ILC	6	6 (6)	0 (0)	
Special[a]	11	9 (8)	2 (8)	0.49
CXCR4				
Negative	32	24 (22)	8 (33)	
Positive	101	85 (78)	16 (67)	0.29

Abbreviation: *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

[a] Mucinous carcinoma (5), Tubular carcinoma (5), Micropapillary (1)

Several previous reports and our results have indicated that SDF-1 is one of the ERGs [6, 37–39]. Hall and Korach [6] showed that SDF-1 induced proliferation of ER-positive breast and ovarian cancer cell lines, and proposed that SDF-1 might be a strong mediator of estrogen-induced cell proliferation. However, our clinical data demonstrated that tumors with high SDF-1 expression showed more indolent characteristics, i.e., ER positivity, a lower tumor grade, and lack of HER2 overexpression. At present, the function of SDF-1 as an ERG in ER-positive tumors in vivo remains unclear.

We conducted subset analysis of the prognostic significance of SDF-1 immunoreactivity in groups of patients with ER-positive and ER-negative tumors. In a subset of 133 ER-positive tumors, multivariate analysis showed that "high SDF-1 expression," as well as lower nuclear grade and earlier clinical stage, were independent indicators of better prognosis. On the other hand, in a subset of 90 ER-negative tumors, the "high" and "low SDF-1 expression" groups had similar clinical outcomes. In general, ERG

expression has been thought to reflect the activity of ER signaling pathways in breast cancers, and it has been proved that several ERGs, e.g., PR, are indicators of responsiveness to endocrine therapy [4, 40]. Therefore, it is possible that SDF-1 immunoreactivity might be a new and powerful predictor of endocrine responsiveness in breast cancer cells.

In our present study, 54% (49 of 90) of ER-negative breast cancers had "high SDF-1 expression." It has been shown that SDF-1 expression can be induced by hypoxia-induced factor-1 (HIF-1) in endothelial cells [41], by fibroblast growth factor-2 (FGF-2) in stromal cells [42], and by bone morphogenetic protein (BMP) in cancer-associated fibroblasts (CAFs) [43]. Thus, multiple alternative pathways mediated by these molecules may be involved in the upregulation of SDF-1 in ER-negative breast cancers.

Interestingly, the intracellular distribution pattern of SDF-1 immunoreactivity in surgically resected breast cancers was a distinctive characteristic, and significantly