We recently showed that Th1 and Th17 cells contribute to chronic GVHD using a MHC-compatible, minor histocompatibility antigen-incompatible mouse model of chronic GVHD [21]. Th1 and Th2 responses were up-regulated early after HSCT, followed by up-regulation of Th17 cells [21]. Significantly greater numbers of Th17 cells infiltrated into the lung and liver from allogeneic recipients than from syngeneic recipients [21]. Infusion of IFN-$\gamma^{-/-}$ or IL-17$^{-/-}$ donor T cells attenuated chronic GVHD in the skin and salivary glands [21], confirming that Th1 and Th17 contribute to the development of chronic GVHD. We also identified a population of donor-derived IFN-γ/IL-17 double-positive cells following only allogeneic HSCT, not syngeneic HSCT, suggesting that this population is generated by allogeneic stimulation, but is not due to lymphopenia-induced proliferation [21].

Recently, the Th17 cell spectrum has been shown to range from "classical" to "alternative" Th17 cells. Classical Th17 cells depend on TGF-β, are more regulated, and less pathogenic. In contrast, "alternative" Th17 cells depend on IL-23, are less regulated, and more pathogenic [80]. The accumulated evidence suggests that T-bet and IFN-γ expression by Th17 cells is dependent on IL-23, but is inhibited by TGF-β and is thus a characteristic of alternative rather than classical Th17 cells [80-84]. Further investigations will be needed to clarify the difference(s) in the functions of IL-17 single-positive and IFN-γ/IL-17 double-positive cells, taking into consideration both classical and alternative Th17 cells, in chronic GVHD pathogenesis.

Retinoids for the Treatment of Chronic GVHD

Retinoic acid, the active metabolite of vitamin A, exerts multiple effects on cell differentiation and survival by binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [85]. All-*trans*-retinoic acid (ATRA) has been reported to suppress the differentiation of Th17 cells with reciprocal induction of Tregs [86]. Am80, a novel RARα/β-specific synthetic retinoid, has a biological activity approximately 10 times more potent than that of ATRA, and directly inhibits Th1 cytokine production [87]. Thus, we hypothesized that retinoids would down-regulate both Th1 and Th17 differentiation in donor T cells, resulting in attenuation of chronic GVHD. Recipient mice were orally administered Am80 from day 0 of HSCT. We found that Am80 significantly ameliorated the clinical and pathological chronic GVHD score, compared with controls [21]. Additionally, peripheral lymph nodes from Am80-treated recipients produced significantly less Th1 and Th17 cytokines, confirming that Am80 regulated both Th1 and Th17 responses, resulting in the attenuation of chronic GVHD [21]. We also demonstrated that Am80 was effective in the treatment setting; Am80 was orally administered to mice from day 21 of HSCT, when clinical signs of chronic GVHD had developed [21]. We are now planning a phase I/II clinical study of Am80 for the treatment of refractory chronic GVHD.

Conclusions

We reviewed many mediators that contribute to or regulate chronic GVHD. A better understanding of the biology of chronic GVHD will lead to the development of novel strategies for its prevention and treatment. Successful clinical studies of treatments for chronic GVHD would improve patient outcomes and result in the establishment of new standards of care.

References

1. Teshima T, Maeda Y and Ozaki K: Regulatory T cells and IL-17-producing cells in graft-versus-host disease. Immunotherapy (2011) 3: 833-852.
2. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, Shlomchik MJ and Emerson SG: Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science (1999) 285: 412-415.
3. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R and Ferrara JL: A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. Nat Med (2005) 11: 1244-1249.
4. Koyama M, Hashimoto D, Aoyama K, Matsuoka K, Karube K, Niiro H, Harada M, Tanimoto M, Akashi K and Teshima T: Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells. Blood (2009) 113: 2088-2095.
5. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, Ferrara JL and Teshima T: Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol (2004) 172: 7393-7398.
6. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR and Ferrara JL: Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med (2002) 8: 575-581.
7. Maeda Y, Levy RB, Reddy P, Liu C, Clouthier SG, Teshima T and Ferrara JL: Both perforin and Fas ligand are required for the regulation of alloreactive CD8$^+$ T cells during acute graft-versus-

host disease. Blood (2005) 105: 2023-2027.
8. Min CK, Maeda Y, Lowler K, Liu C, Clouthier S, Lofthus D, Weisiger E, Ferrara JL and Reddy P: Paradoxical effects of interleukin-18 on the severity of acute graft-versus-host disease mediated by $CD4^+$ and $CD8^+$ T-cell subsets after experimental allogeneic bone marrow transplantation. Blood (2004) 104: 3393-3399.
9. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF and Velardi A: Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science (2002) 295: 2097-2100.
10. Zeng D, Lewis D, Dejbakhsh-Jones S, Lan F, Garcia-Ojeda M, Sibley R and Strober S: Bone marrow NK1.1(−) and NK1.1(+) T cells reciprocally regulate acute graft versus host disease. J Exp Med (1999) 189: 1073-1081.
11. Maeda Y, Reddy P, Lowler KP, Liu C, Bishop DK and Ferrara JL: Critical role of host gammadelta T cells in experimental acute graft-versus-host disease. Blood (2005) 106: 749-755.
12. Teshima T, Wynn TA, Soiffer RJ, Matsuoka K and Martin PJ: Chronic graft-versus-host disease: how can we release Prometheus? Biol Blood Marrow Transplant (2008) 14: 142-150.
13. Socie G, Stone JV, Wingard JR, Weisdorf D, Henslee-Downey PJ, Bredeson C, Cahn JY, Passweg JR, Rowlings PA, Schouten HC, Kolb HJ and Klein JP: Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. N Engl J Med (1999) 341: 14-21.
14. Baker KS, Gurney JG, Ness KK, Bhatia R, Forman SJ, Francisco L, McGlave PB, Robison LL, Snyder DS, Weisdorf DJ and Bhatia S: Late effects in survivors of chronic myeloid leukemia treated with hematopoietic cell transplantation: results from the Bone Marrow Transplant Survivor Study. Blood (2004) 104: 1898-1906.
15. Wolff D, Schleuning M, von Harsdorf S, Bacher U, Gerbitz A, Stadler M, Ayuk F, Kiani A, Schwerdtfeger R, Vogelsang GB, Kobbe G, Gramatzki M, Lawitschka A, Mohty M, Pavletic SZ, Greinix H and Holler E: Consensus Conference on Clinical Practice in Chronic GVHD: Second-Line Treatment of Chronic Graft-versus-Host Disease. Biol Blood Marrow Transplant (2011) 17: 1-17.
16. Shustov A, Luzina I, Nguyen P, Papadimitriou JC, Handwerger B, Elkon KB and Via CS: Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. J Clin Invest (2000) 106: R39-47.
17. Serody JS and Hill GR: The IL-17 differentiation pathway and its role in transplant outcome. Biol Blood Marrow Transplant (2012) 18: S56-61.
18. Zhou L, Askew D, Wu C and Gilliam AC: Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. J Invest Dermatol (2007) 127: 281-292.
19. Lohr J, Knoechel B, Wang JJ, Villarino AV and Abbas AK: Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. J Exp Med (2006) 203: 2785-2791.
20. Zhang C, Todorov I, Zhang Z, Liu Y, Kandeel F, Forman S, Strober S and Zeng D: Donor $CD4^+$ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. Blood (2006) 107: 2993-3001.
21. Nishimori H, Maeda Y, Teshima T, Sugiyama H, Kobayashi K, Yamasuji Y, Kadohisa S, Uryu H, Takeuchi K, Tanaka T, Yoshino T, Iwakura Y and Tanimoto M: Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17. Blood (2012) 119: 285-295.
22. Flowers ME, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW, Pereira SE, Nash RA, Mielcarek M, Fero ML, Warren EH, Sanders JE, Storb RF, Appelbaum FR, Storer BE and Martin PJ: Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. Blood (2011) 117: 3214-3219.
23. Wolff D, Gerbitz A, Ayuk F, Kiani A, Hildebrandt GC, Vogelsang GB, Elad S, Lawitschka A, Socie G, Pavletic SZ, Holler E and Greinix H: Consensus conference on clinical practice in chronic graft-versus-host disease (GVHD): first-line and topical treatment of chronic GVHD. Biol Blood Marrow Transplant (2010) 16: 1611-1628.
24. Akpek G, Zahurak ML, Piantadosi S, Margolis J, Doherty J, Davidson R and Vogelsang GB: Development of a prognostic model for grading chronic graft-versus-host disease. Blood (2001) 97: 1219-1226.
25. Gilman AL, Chan KW, Mogul A, Morris C, Goldman FD, Boyer M, Cirenza E, Mazumder A, Gehan E, Cahill R, Frankel S and Schultz K: Hydroxychloroquine for the treatment of chronic graft-versus-host disease. Biol Blood Marrow Transplant (2000) 6: 327-334.
26. Browne PV, Weisdorf DJ, DeFor T, Miller WJ, Davies SM, Filipovich A, McGlave PB, Ramsay NK, Wagner J and Enright H: Response to thalidomide therapy in refractory chronic graft-versus-host disease. Bone Marrow Transplant (2000) 26: 865-869.
27. Akpek G, Lee SM, Anders V and Vogelsang GB: A high-dose pulse steroid regimen for controlling active chronic graft-versus-host disease. Biol Blood Marrow Transplant (2001) 7: 495-502.
28. Flowers ME, Apperley JF, van Besien K, Elmaagacli A, Grigg A, Reddy V, Bacigalupo A, Kolb HJ, Bouzas L, Michallet M, Prince HM, Knobler R, Parenti D, Gallo J and Greinix HT: A multicenter prospective phase 2 randomized study of extracorporeal photopheresis for treatment of chronic graft-versus-host disease. Blood (2008) 112: 2667-2674.
29. Olivieri A, Locatelli F, Zecca M, Sanna A, Cimminiello M, Raimondi R, Gini G, Mordini N, Balduzzi A, Leoni P, Gabrielli A and Bacigalupo A: Imatinib for refractory chronic graft-versus-host disease with fibrotic features. Blood (2009) 114: 709-718.
30. Furlong T, Martin P, Flowers ME, Carnevale-Schianca F, Yatscoff R, Chauncey T, Appelbaum FR, Deeg HJ, Doney K, Witherspoon R, Storer B, Sullivan KM, Storb R and Nash RA: Therapy with mycophenolate mofetil for refractory acute and chronic GVHD. Bone Marrow Transplant (2009) 44: 739-748.
31. Kim SJ, Lee JW, Jung CW, Min CK, Cho B, Shin HJ, Chung JS, Kim H, Lee WS, Joo YD, Yang DH, Kook H, Kang HJ, Ahn HS, Yoon SS, Sohn SK, Min YH, Min WS, Park HS and Won JH: Weekly rituximab followed by monthly rituximab treatment for steroid-refractory chronic graft-versus-host disease: results from a prospective, multicenter, phase II study. Haematologica (2010) 95: 1935-1942.
32. Jedlickova Z, Burlakova I, Bug G, Baurmann H, Schwerdtfeger R and Schleuning M: Therapy of sclerodermatous chronic graft-versus-host disease with mammalian target of rapamycin inhibitors. Biol Blood Marrow Transplant (2011) 17: 657-663.
33. Weng JY, Du X, Geng SX, Peng YW, Wang Z, Lu ZS, Wu SJ, Luo CW, Guo R, Ling W, Deng CX, Liao PJ and Xiang AP: Mesenchymal stem cell as salvage treatment for refractory chronic GVHD. Bone Marrow Transplant (2010) 45: 1732-1740.
34. Pidala J, Kim J, Roman-Diaz J, Shapiro J, Nishihori T, Bookout R,

Anasetti C and Kharfan-Dabaja MA: Pentostatin as rescue therapy for glucocorticoid-refractory acute and chronic graft-versus-host disease. Ann Transplant (2010) 15: 21-29.
35. Ho VT and Soiffer RJ: The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. Blood (2001) 98: 3192-3204.
36. Korngold R and Sprent J: Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J Exp Med (1978) 148: 1687-1698.
37. Sakoda Y, Hashimoto D, Asakura S, Takeuchi K, Harada M, Tanimoto M and Teshima T: Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. Blood (2007) 109: 1756-1764.
38. Zhang Y, Hexner E, Frank D and Emerson SG: $CD4^+$ T cells generated de novo from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease. J Immunol (2007) 179: 3305-3314.
39. Blazar BR, Weisdorf DJ, Defor T, Goldman A, Braun T, Silver S and Ferrara JL: Phase 1/2 randomized, placebo-control trial of palifermin to prevent graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). Blood (2006) 108: 3216-3222.
40. Anscher MS, Peters WP, Reisenbichler H, Petros WP and Jirtle RL: Transforming growth factor beta as a predictor of liver and lung fibrosis after autologous bone marrow transplantation for advanced breast cancer. N Engl J Med (1993) 328: 1592-1598.
41. Murase T, Anscher MS, Petros WP, Peters WP and Jirtle RL: Changes in plasma transforming growth factor beta in response to high-dose chemotherapy for stage II breast cancer: possible implications for the prevention of hepatic veno-occlusive disease and pulmonary drug toxicity. Bone Marrow Transplant (1995) 15: 173-178.
42. McCormick LL, Zhang Y, Tootell E and Gilliam AC: Anti-TGF-beta treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: a model for human scleroderma. J Immunol (1999) 163: 5693-5699.
43. Chu YW and Gress RE: Murine models of chronic graft-versus-host disease: insights and unresolved issues. Biol Blood Marrow Transplant (2008) 14: 365-378.
44. Liem LM, Fibbe WE, van Houwelingen HC and Goulmy E: Serum transforming growth factor-beta1 levels in bone marrow transplant recipients correlate with blood cell counts and chronic graft-versus-host disease. Transplantation (1999) 67: 59-65.
45. Baron C, Somogyi R, Greller LD, Rineau V, Wilkinson P, Cho CR, Cameron MJ, Kelvin DJ, Chagnon P, Roy DC, Busque L, Sekaly RP and Perreault C: Prediction of graft-versus-host disease in humans by donor gene-expression profiling. PLoS Med (2007) 4: e23.
46. Rubtsov YP and Rudensky AY: TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol (2007) 7: 443-453.
47. Trojanowska M: Role of PDGF in fibrotic diseases and systemic sclerosis. Rheumatology (Oxford) (2008) 47 Suppl 5: v2-4.
48. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A, Avvedimento EV and Gabrielli A: Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. N Engl J Med (2006) 354: 2667-2676.
49. Svegliati S, Olivieri A, Campelli N, Luchetti M, Poloni A, Trappolini S, Moroncini G, Bacigalupo A, Leoni P, Avvedimento EV and Gabrielli A: Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. Blood (2007) 110: 237-241.
50. Daniels CE, Wilkes MC, Edens M, Kottom TJ, Murphy SJ, Limper AH and Leof EB: Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis. J Clin Invest (2004) 114: 1308-1316.
51. Distler JH, Jungel A, Huber LC, Schulze-Horsel U, Zwerina J, Gay RE, Michel BA, Hauser T, Schett G, Gay S and Distler O: Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. Arthritis Rheum (2007) 56: 311-322.
52. Magro L, Mohty M, Catteau B, Coiteux V, Chevallier P, Terriou L, Jouet JP and Yakoub-Agha I: Imatinib mesylate as salvage therapy for refractory sclerotic chronic graft-versus-host disease. Blood (2009) 114: 719-722.
53. Nakasone H, Kanda Y, Takasaki H, Nakaseko C, Sakura T, Fujisawa S, Yokota A, Yano S, Usuki K, Maruta A, Abe D, Hoshino T, Takahashi S, Kanamori H and Okamoto S: Prophylactic impact of imatinib administration after allogeneic stem cell transplantation on the incidence and severity of chronic graft versus host disease in patients with Philadelphia chromosome-positive leukemia. Leukemia (2010) 24: 1236-1239.
54. Sakaguchi S, Miyara M, Costantino CM and Hafler DA: $FOXP3^+$ regulatory T cells in the human immune system. Nat Rev Immunol (2010) 10: 490-500.
55. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, Bellucci R, Alyea EP, Antin JH, Soiffer RJ and Ritz J: Reduced frequency of $FOXP3^+$ $CD4^+$ $CD25^+$ regulatory T cells in patients with chronic graft-versus-host disease. Blood (2005) 106: 2903-2911.
56. Buckner JH: Mechanisms of impaired regulation by CD4(+) CD25(+) FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol (2010) 10: 849-859.
57. Matsuoka K, Kim HT, McDonough S, Bascug G, Warshauer B, Koreth J, Cutler C, Ho VT, Alyea EP, Antin JH, Soiffer RJ and Ritz J: Altered regulatory T cell homeostasis in patients with $CD4^+$ lymphopenia following allogeneic hematopoietic stem cell transplantation. J Clin Invest (2010) 120: 1479-1493.
58. Setoguchi R, Hori S, Takahashi T and Sakaguchi S: Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med (2005) 201: 723-735.
59. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP 3rd, Armand P, Cutler C, Ho VT, Treister NS, Bienfang DC, Prasad S, Tzachanis D, Joyce RM, Avigan DE, Antin JH, Ritz J and Soiffer RJ: Interleukin-2 and regulatory T cells in graft-versus-host disease. N Engl J Med (2011) 365: 2055-2066.
60. Weiden PL, Sullivan KM, Flournoy N, Storb R and Thomas ED: Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med (1981) 304: 1529-1533.
61. Asakura S, Hashimoto D, Takashima S, Sugiyama H, Maeda Y, Akashi K, Tanimoto M and Teshima T: Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice. J Clin Invest (2010) 120: 2370-2378.
62. Reddy P, Maeda Y, Hotary K, Liu C, Reznikov LL, Dinarello CA and Ferrara JL: Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. Proc Natl Acad Sci U S A (2004) 101: 3921-3926.

63. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S and Negrin RS: $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med (2003) 9: 1144–1150.
64. Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, Hochberg EP, Wu CJ, Alyea EP, Cutler C, Ho V, Soiffer RJ, Antin JH and Ritz J: Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. Blood (2005) 105: 2973–2978.
65. Clark WB, Brown-Gentry KD, Crawford DC, Fan KH, Snavely J, Chen H, Savani BN, Kassim A, Greer JP, Schuening FG, Engelhardt BG and Jagasia MH: Genetic variation in recipient B-cell activating factor modulates phenotype of GVHD. Blood (2011) 118: 1140–1144.
66. Sarantopoulos S, Stevenson KE, Kim HT, Bhuiya NS, Cutler CS, Soiffer RJ, Antin JH and Ritz J: High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. Clin Cancer Res (2007) 13: 6107–6114.
67. She K, Gilman AL, Aslanian S, Shimizu H, Krailo M, Chen Z, Reid GS, Wall D, Goldman F and Schultz KR: Altered Toll-like receptor 9 responses in circulating B cells at the onset of extensive chronic graft-versus-host disease. Biol Blood Marrow Transplant (2007) 13: 386–397.
68. Ratanatharathorn V, Ayash L, Reynolds C, Silver S, Reddy P, Becker M, Ferrara JL and Uberti JP: Treatment of chronic graft-versus-host disease with anti-CD20 chimeric monoclonal antibody. Biol Blood Marrow Transplant (2003) 9: 505–511.
69. Cutler C, Miklos D, Kim HT, Treister N, Woo SB, Bienfang D, Klickstein LB, Levin J, Miller K, Reynolds C, Macdonell R, Pasek M, Lee SJ, Ho V, Soiffer R, Antin JH, Ritz J and Alyea E: Rituximab for steroid-refractory chronic graft-versus-host disease. Blood (2006) 108: 756–762.
70. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N and Anderson DR: Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood (1994) 83: 435–445.
71. Nishimori H, Matsuo K, Maeda Y, Nawa Y, Sunami K, Togitani K, Takimoto H, Hiramatsu Y, Kiguchi T, Yano T, Yamane H, Tabayashi T, Takeuchi M, Makita M, Sezaki N, Yamasuji Y, Sugiyama H, Tabuchi T, Kataoka I, Fujii N, Ishimaru F, Shinagawa K, Ikeda K, Hara M, Yoshino T, Tanimoto M and West-Japan Hematology Oncology Group: The effect of adding rituximab to CHOP-based therapy on clinical outcomes for Japanese patients with diffuse large B-cell lymphoma: a propensity score matching analysis. Int J Hematol (2009) 89: 326–331.
72. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA and Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol (1986) 136: 2348–2357.
73. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR and Weaver CT: Transforming growth factor-beta induces development of the T(H)17 lineage. Nature (2006) 441: 231–234.
74. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, Johnston H, Young J, Todorov I, Umetsu DT, Chen L, Iwakura Y, Kandeel F, Forman S and Zeng D: Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood (2009) 114: 3101–3112.
75. Yi T, Zhao D, Lin CL, Zhang C, Chen Y, Todorov I, LeBon T, Kandeel F, Forman S and Zeng D: Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. Blood (2008) 112: 2101–2110.
76. Kappel LW, Goldberg GL, King CG, Suh DY, Smith OM, Ligh C, Holland AM, Grubin J, Mark NM, Liu C, Iwakura Y, Heller G and van den Brink MR: IL-17 contributes to CD4-mediated graft-versus-host disease. Blood (2009) 113: 945–952.
77. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR and Serody JS: In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood (2009) 113: 1365–1374.
78. Tawara I, Maeda Y, Sun Y, Lowler KP, Liu C, Toubai T, McKenzie AN and Reddy P: Combined Th2 cytokine deficiency in donor T cells aggravates experimental acute graft-vs-host disease. Exp Hematol (2008) 36: 988–996.
79. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C and Yu XZ: Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. Blood (2011) 118: 5011–5020.
80. Peters A, Lee Y and Kuchroo VK: The many faces of Th17 cells. Curr Opin Immunol (2011) 23: 702–706.
81. Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, Ramos HL, Wei L, Davidson TS, Bouladoux N, Grainger JR, Chen Q, Kanno Y, Watford WT, Sun HW, Eberl G, Shevach EM, Belkaid Y, Cua DJ, Chen W and O'Shea JJ: Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. Nature (2010) 467:967–971.
82. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO and Weaver CT: Late developmental plasticity in the T helper 17 lineage. Immunity (2009) 30: 92–107.
83. Das J, Ren G, Zhang L, Roberts AI, Zhao X, Bothwell AL, Van Kaer L, Shi Y and Das G: Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. J Exp Med (2009) 206: 2407–2416.
84. Ahern PP, Schiering C, Buonocore S, McGeachy MJ, Cua DJ, Maloy KJ and Powrie F: Interleukin-23 drives intestinal inflammation through direct activity on T cells. Immunity (2010) 33: 279–288.
85. Mark M, Ghyselinck NB and Chambon P: Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol (2006) 46: 451–480.
86. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M and Cheroutre H: Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science (2007) 317: 256–260.
87. Nagai H, Matsuura S, Bouda K, Takaoka Y, Wang T, Niwa S and Shudo K: Effect of Am-80, a synthetic derivative of retinoid, on experimental arthritis in mice. Pharmacology (1999) 58: 101–112.

VOLUME 31 · NUMBER 35 · DECEMBER 10 2013

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Randomized, Open-Label, Phase III Study Comparing Irinotecan With Paclitaxel in Patients With Advanced Gastric Cancer Without Severe Peritoneal Metastasis After Failure of Prior Combination Chemotherapy Using Fluoropyrimidine Plus Platinum: WJOG 4007 Trial

Shuichi Hironaka, Shinya Ueda, Hirofumi Yasui, Tomohiro Nishina, Masahiro Tsuda, Takehiko Tsumura, Naotoshi Sugimoto, Hideki Shimodaira, Shinya Tokunaga, Toshikazu Moriwaki, Taito Esaki, Michitaka Nagase, Kazumasa Fujitani, Kensei Yamaguchi, Takashi Ura, Yasuo Hamamoto, Satoshi Morita, Isamu Okamoto, Narikazu Boku, and Ichinosuke Hyodo

Author affiliations appear at the end of this article.

Published online ahead of print at www.jco.org on November 4, 2013.

Written on behalf of the West Japan Oncology Group.

Supported by Yakult Honsha and Daiichisankyo.

Presented at the 48th Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, June 1-5, 2012, and European Society of Medical Oncology Congress, Vienna, Austria, September 28-October 2, 2012.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical trial information: University Hospital Medical Information Network–Clinical Trials Registry Japan No. 000001252.

Corresponding author: Shuichi Hironaka, MD, Clinical Trial Promotion Department, Chiba Cancer Center, 666-2 Nitona-cho Chuo-ku Chiba-shi, Chiba, 260-8717 Japan; e-mail: shironaka@ta2.so-net.ne.jp.

0732-183X/13/3135w-4438w/$20.00

DOI: 10.1200/JCO.2012.48.5805

ABSTRACT

Purpose
This phase III study compared treatment with weekly paclitaxel and biweekly irinotecan in patients with advanced gastric cancer refractory to treatment with fluoropyrimidine plus platinum.

Patients and Methods
Patients were randomly assigned to receive either paclitaxel (80 mg/m^2 on days 1, 8, and 15, every 4 weeks) or irinotecan (150 mg/m^2 on days 1 and 15, every 4 weeks). Primary end point was overall survival (OS), and secondary end points were progression-free survival (PFS), response rate, adverse events, and proportion of patients who received third-line chemotherapy.

Results
Of 223 patients, 219 were eligible for analysis. Median OS was 9.5 months in 108 patients allocated to the paclitaxel group and 8.4 months in 111 patients allocated to the irinotecan group (hazard ratio [HR], 1.13; 95% CI, 0.86 to 1.49; P = .38). Median PFS was 3.6 months in the paclitaxel group and 2.3 months in the irinotecan group (HR, 1.14; 95% CI, 0.88 to 1.49; P = .33). Response rate was 20.9% in the paclitaxel group and 13.6% in the irinotecan group (P = .24). Common grade 3 to 4 adverse events were neutropenia (paclitaxel group, 28.7%; irinotecan group, 39.1%), anemia (21.3%; 30.0%), and anorexia (7.4%; 17.3%). Treatment-related deaths occurred in two patients (1.8%) in the irinotecan group. Third-line chemotherapy was administered in 97 patients (89.8%) after paclitaxel treatment and in 80 patients (72.1%) after irinotecan treatment (P = .001).

Conclusion
No statistically significant difference was observed between paclitaxel and irinotecan for OS. Both are reasonable second-line treatment options for advanced gastric cancer.

J Clin Oncol 31:4438-4444. © 2013 by American Society of Clinical Oncology

INTRODUCTION

The outcomes in patients with unresectable gastric cancer are extremely poor; median survival times of 3 to 5 months have been reported with best supportive care (BSC) alone.[1-3] In randomized studies conducted in the 1990s, first-line chemotherapy for advanced gastric cancer provided survival benefit over BSC alone. After many clinical trials, at present, fluoropyrimidine plus platinum with or without epirubicin or docetaxel is regarded as standard first-line chemotherapy in the treatment of gastric cancer worldwide.[4-9]

Since S-1 was approved for treatment of advanced gastric cancer in Japan, several phase III studies have been conducted, such as the JCOG 9912 (Japan Clinical Oncology Group 9912; fluorouracil *v* S-1 *v* irinotecan plus cisplatin),[10] SPIRITS (S-1 Plus Cisplatin Versus S-1 in a Randomized Controlled Trial in the Treatment for Stomach Cancer; S-1 *v* S-1 plus cisplatin),[9] and GC0301/TOP-002 trials (Gastric Cancer 0301/Topotecin-002; S-1 *v* S-1 plus irinotecan).[11] On the basis of these study results, S-1 plus cisplatin is accepted as standard first-line chemotherapy for advanced gastric cancer

in Japan. Despite no robust evidence of survival benefit, > 70% of participants received second-line chemotherapy in these studies.[9-11]

Many phase II studies of second-line chemotherapy for advanced gastric cancer have been conducted.[12-20] In evaluations of taxanes, administration of both paclitaxel (210 mg/m^2) and docetaxel (60 mg/m^2) on a triweekly schedule resulted in high rates of grade 3 or 4 neutropenia (37% to 88%),[12-14] whereas lower rates of severe neutropenia (3% to 32%) were observed with weekly administration of paclitaxel (80 mg/m^2).[15-18] Regarding efficacy parameters, response rate (RR) and progression-free survival (PFS) were similar for patients on the triweekly and weekly schedules of paclitaxel. Two reports evaluated weekly paclitaxel as second-line chemotherapy, in which median overall survival (OS) was 5 and 6.9 months, respectively.[15,16] In other studies, combination chemotherapy including biweekly administration of irinotecan (150 mg/m^2) as second-line chemotherapy resulted in median OS of 8 to 10 months,[19,20] although toxicity seemed to be more severe than that seen with weekly paclitaxel. Thus, weekly paclitaxel has become the preferable second-line chemotherapy in Japan.

At present, taxanes and irinotecan are two main options for treatment of advanced gastric cancer refractory to fluoropyrimidine plus platinum. However, to our knowledge, no randomized study has directly compared the efficacy of these two treatments. The West Japan Oncology Group (WJOG) conducted a phase III trial (WJOG 4007) comparing paclitaxel with irinotecan in patients with advanced gastric cancer.

PATIENTS AND METHODS

Patients

Eligible patients were age 20 to 75 years with histologically confirmed metastatic or recurrent gastric adenocarcinoma. Other inclusion criteria were Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 to 2; disease progression confirmed by computed tomography (CT), endoscopy, or other imaging technique during or within 1 month after last dose of first-line chemotherapy with fluoropyrimidine plus platinum; no prior chemotherapy with taxanes or irinotecan; and no severe peritoneal metastasis. Severe peritoneal metastasis was defined as ileus or subileus suggested on barium enema examination and moderate to severe ascites exceeding the pelvic cavity on spine CT scan caused by peritoneal metastasis. In case of treatment with adjuvant or neoadjuvant chemotherapy consisting of fluoropyrimidine plus platinum, patients with disease progression during treatment or within 6 months after treatment completion were eligible. Adequate bone marrow, hepatic, and renal functions were also required.

Study Design

WJOG 4007 was a prospective, multicenter, randomized, open-label, parallel-group phase III clinical trial conducted at 37 centers in Japan. The protocol was approved by the independent ethics committee or institutional review board of each participating institution. This trial was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before study entry. The trial was registered with the University Hospital Medical Information Network.

After checking eligibility, patients were randomly assigned at a 1:1 ratio to receive either paclitaxel or irinotecan. Random assignment was carried out centrally at the data center using minimization method with the following adjustment factors: institution, ECOG PS (0 to 1 *v* 2), and measurable lesions (presence *v* absence). Neither investigators nor patients were blinded to the allocated treatment.

Treatment

Paclitaxel (80 mg/m^2) was administered intravenously on days 1, 8, and 15, every 4 weeks. Patients were premedicated with histamine receptor-1 and -2 blockers and dexamethasone for prophylaxis of allergic reactions 30 minutes before paclitaxel administration. Irinotecan (150 mg/m^2) was administered intravenously on days 1 and 15, every 4 weeks. Dose reduction and/or cycle delays were permitted according to predefined toxicity criteria. Treatment continued until disease progression, occurrence of unacceptable serious toxicity, or patient refusal of further treatment. Subsequent chemotherapy was not specified.

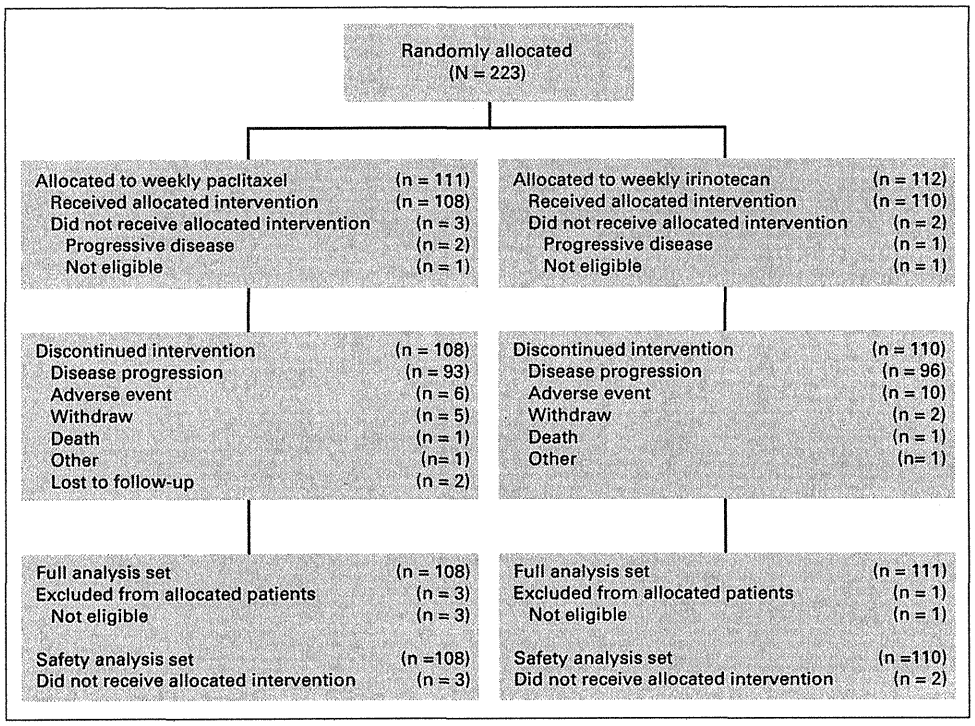


Fig 1. CONSORT diagram.

Assessments

Vital signs, ECOG PS, and laboratory tests were assessed within 7 days before study entry. Physical examinations and hematology and biochemistry tests were conducted during drug administration throughout the treatment course. Tumor assessments using CT scans of the chest, abdomen, and pelvis were performed within 28 days before study entry and repeated every 2 months after random assignment until discontinuation of protocol treatment. RECIST (version 1.0) was used to evaluate treatment responses.[21] Safety assessments were repeated every 2 weeks until initiation of subsequent chemotherapy or 6 weeks after the last protocol treatment. Severity of adverse events was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). The WJOG Data and Safety Monitoring Committee reviewed serious adverse events for trial safety during the protocol treatment. Investigators assessed response, progression, and toxicities in their patients; independent central assessments of response and disease progression were not performed.

Statistical Analysis

The primary end point was OS, defined as time from random assignment to death resulting from any cause. Secondary end points were PFS, defined as time from random assignment to disease progression or death resulting from any cause; RR; toxicity; and proportion of patients who received subsequent chemotherapy.

Previous single-arm studies showed median OS of 5 and 6.9 months in paclitaxel-[15,16] and 8 and 10 months in irinotecan-containing regimen.[19,20] Irinotecan was contraindicated for patients with severe peritoneal metastasis, because its biliary-excreted metabolites caused severe toxicities. In gastric cancer, peritoneal metastasis often developed along with disease progression, and we therefore speculated that subsequent irinotecan after paclitaxel would be more difficult to apply in patients compared with the reverse treatment sequence. On the basis of these previous results and our assumption, this study was designed to detect 50% improvement in median OS from 5 months in the paclitaxel group to 7.5 months in the irinotecan group (hazard ratio [HR], 0.67). Assuming accrual and follow-up periods of 36 and 12 months, respectively, and using a two-sided log-rank test with 5% α and 20% β errors, 220 patients were required for the study. No interim analyses were planned.

A full analysis set (FAS) included all randomly assigned patients who met the eligibility criteria (patients found to be ineligible after random assignment were excluded). The safety analysis set (SAS) included all randomly assigned patients who received ≥ one dose of study medication. OS and PFS were analyzed in the FAS and estimated using the Kaplan-Meier method. RR was assessed in patients with ≥ one measurable lesion at baseline. Toxicity was analyzed in the SAS.

The primary analysis was planned for 1 year after enrollment of the last patient or approximately 205 events, whichever came first. An independent statistician and data analysis center performed the primary analysis for OS with unstratified log-rank test in the FAS population. All investigators remained blinded to the data until the analysis was completed. Cox proportional hazards models were used to calculate HRs and CIs. Fisher's exact test was used to assess differences in RR, incidence of

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic	Weekly Paclitaxel (n = 108) No.	%	Irinotecan (n = 111) No.	%
Sex				
Male	84	77.7	87	78.4
Female	24	22.2	24	21.6
Age, years				
Median	64.5		65	
Range	37-75		38-75	
ECOG PS				
0 to 1	104	96.3	107	96.4
2	4	3.7	4	3.6
Prior gastrectomy				
Yes	37	34.3	39	35.1
No	71	65.7	72	64.9
Prior chemotherapy				
S-1 plus cisplatin	92	85.2	102	91.9
Capecitabine plus cisplatin	13	12.4	8	7.2
S-1 plus oxaliplatin	3	2.8	1	0.9
Target lesion				
Yes	91	84.3	88	79.3
No	17	15.7	23	20.7
Histology				
Intestinal	54	50.0	54	48.6
Diffuse	54	50.0	57	51.4
Peritoneal metastasis				
Yes	28	25.9	28	25.2
No	80	74.1	83	74.8
No. of metastatic sites				
One	57	52.8	64	57.7
Two or more	51	47.2	47	42.3

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status.

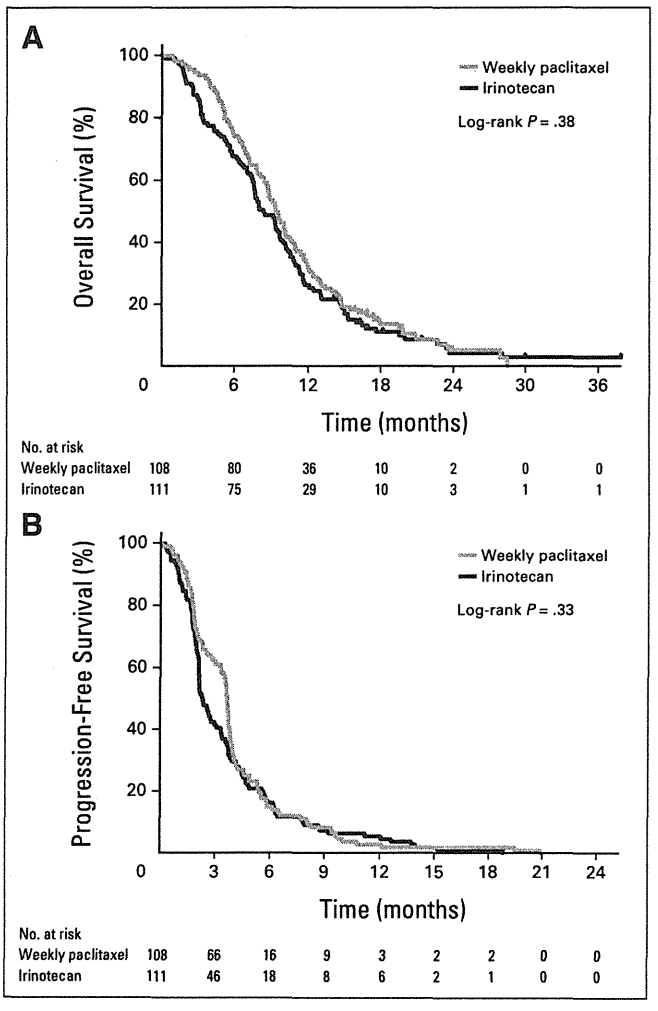


Fig 2. Kaplan-Meier curves of (A) overall and (B) progression-free survival.

adverse events, and proportion of patients who received third-line chemotherapy. Exploratory subgroup analyses of OS were performed using stratification and prognostic variables.

RESULTS

Patients

Between August 2007 and August 2010, 223 patients were enrolled from 37 centers in Japan. Of these patients, 111 were allocated to the paclitaxel group and 112 to the irinotecan group (Fig 1). Four patients, who either had received prior fluoropyrimidine monotherapy (paclitaxel group, n = 2; irinotecan group, n = 1) or had radiologically unconfirmed disease progression (paclitaxel group, n = 1), were ineligible for the study. Thus, the FAS consisted of 108 patients in the paclitaxel group and 111 patients in the irinotecan group. After random assignment, three patients in the paclitaxel group and two in the irinotecan group did not receive the protocol treatment. Thus, the SAS consisted of 108 patients in the paclitaxel group and 110 patients in the irinotecan group. Baseline characteristics of patients in the FAS were well balanced between the two treatment groups (Table 1). ECOG PS scores of 0 or 1 were found in a majority of patients. The most common first-line chemotherapy was S-1 plus cisplatin (88.6%), followed by capecitabine plus cisplatin with or without anti–epidermal growth factor receptor or anti–vascular endothelial growth factor antibodies (9.6%) and S-1 plus oxaliplatin (1.8%). One or more measurable lesions were present in approximately 80% of patients, and mild or moderate peritoneal metastasis was detected in approximately 25% of patients in both groups. Two or more metastatic sites were found in < 50% of patients.

Exposure to Chemotherapy

Median number of administrations was 11.5 (range, one to 46) in the paclitaxel group and 4.5 (range, one to 39) in the irinotecan group. Reasons for discontinuation of treatment included: disease progression (86.7%), adverse events (7.3%), withdrawal of consent (3.2%), and other reasons (2.8%). The proportion of patients in whom treatment was discontinued because of toxicity was 5.6% in the paclitaxel group and 9.1% in the irinotecan group.

Third-line chemotherapy was administered to 97 patients (89.8%) in the paclitaxel group and 80 patients (72.1%) in the irinotecan group ($P = .001$). In the paclitaxel group, third-line chemotherapy containing irinotecan was used in 81 patients (75.0%), and in the irinotecan group, a taxane-containing regimen was used in 67 patients (60.4%). Including later lines, 87 patients (80.6%) in the paclitaxel group received irinotecan, and 75 patients (67.6%) in the irinotecan group received paclitaxel.

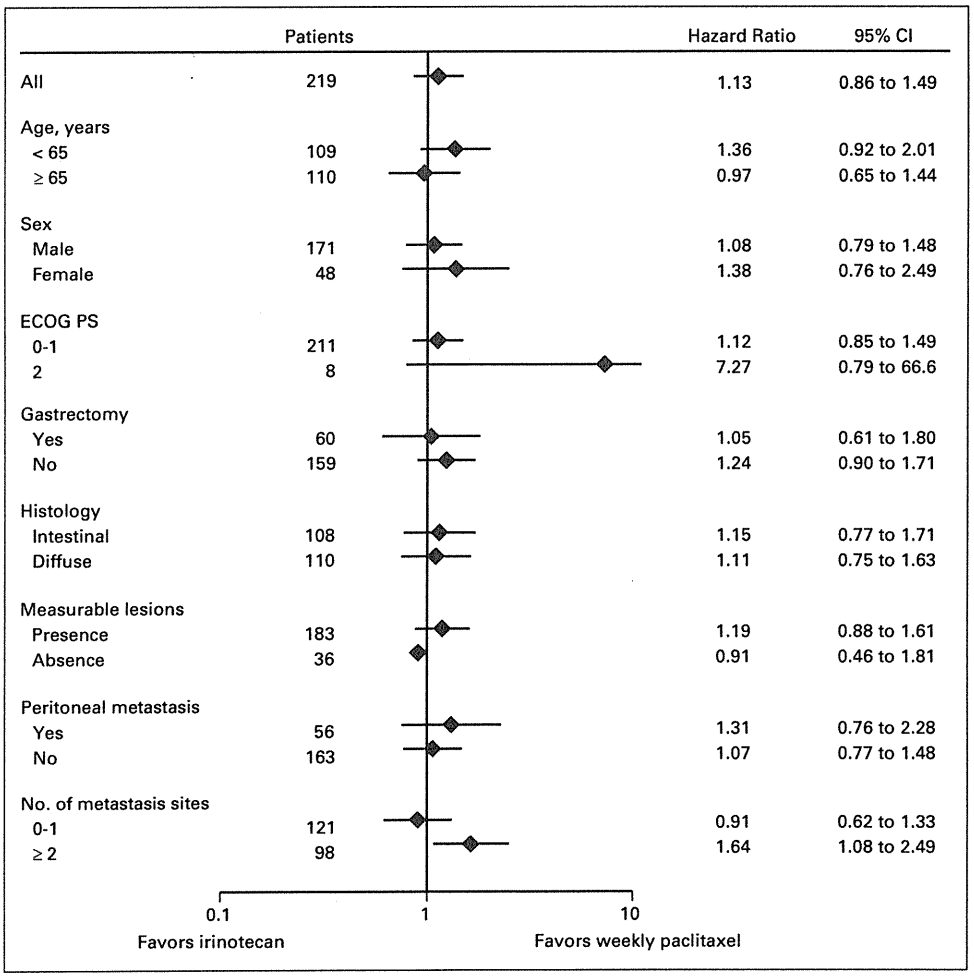


Fig 3. Forest plot of subgroup analyses. ECOG PS, Eastern Cooperative Oncology Group performance status.

Efficacy

In August 2011, after a median follow-up period of 17.6 months, 203 deaths (92.7%) were reported in the patient cohort. For the primary end point of OS, no statistically significant difference was observed between paclitaxel and irinotecan groups (HR, 1.13; 95% CI, 0.86 to 1.49; two-sided $P = .38$). Median OS was 9.5 months (95% CI, 8.4 to 10.7) in the paclitaxel group and 8.4 months (95% CI, 7.6 to 9.8) in the irinotecan group (Fig 2A). Median PFS was 3.6 months (95% CI, 3.3 to 3.8) in the paclitaxel group and 2.3 months (95% CI, 2.2 to 3.1) in the irinotecan group. This difference was not statistically significant (HR, 1.14; 95% CI, 0.88 to 1.49; two-sided $P = .33$; Fig 2B). RR was 20.9% (19 of 91 patients) in the paclitaxel group and 13.6% (12 of 88) in the irinotecan group (Fisher's exact $P = .24$).

Results of the subgroup analysis of OS are shown in Figure 3. Although treatment with weekly paclitaxel conferred a slight survival advantage in almost all subgroups, no significant interactions were observed in any subgroup. In an exploratory analysis, OS was analyzed in patients who received irinotecan and paclitaxel during second- and later-line chemotherapies. Median OS was 10.1 months in each group, and the survival curves of these two subgroups almost overlapped (HR, 0.96; 95% CI, 0.69 to 1.32; two-sided $P = .96$).

Safety

Table 2 lists adverse events and the proportion of patients experiencing adverse events during treatment in the SAS. The most common grade 3 or 4 adverse events were leukopenia (20.4%), neutropenia (28.7%), and anemia (21.3%) in the paclitaxel group. Leukopenia (19.1%), neutropenia (39.1%), anemia (30.0%), anorexia (17.3%), and hyponatremia (15.5%) were common in the irinotecan group. Grade 3 or 4 sensory neuropathy was observed in the paclitaxel group (7.4%) only. Grade 3 or 4 febrile neutropenia was more prevalent in the irinotecan group (9.1%) than in the paclitaxel group (2.8%). Three (2.7%) and four deaths (3.6%) resulting from any cause occurred within 30 days after the last administration in the paclitaxel and irinotecan groups, respectively. Treatment-related death confirmed by the independent data safety monitoring committee was observed in two patients (1.8%) in the irinotecan group. Causes of death included serious pneumonia in one patient and gastric perforation in the other.

Table 2. Adverse Events

	Weekly Paclitaxel (n = 108)				Irinotecan (n = 110)			
	All Grade		Grade 3 to 4		All Grade		Grade 3 to 4	
Adverse Event	No.	%	No.	%	No.	%	No.	%
Leukocytopenia	88	81.4	22	20.4	76	69.4	21	19.1
Neutropenia	85	78.7	31	28.7	77	70.0	43	39.1
Hemoglobin	69	63.9	23	21.3	84	76.4	33	30.0
Thrombocytopenia	6	5.6	1	0.9	15	13.6	2	1.8
Febrile neutropenia	3	2.8	3	2.8	10	9.1	10	9.1
Nausea	33	30.6	2	1.9	61	55.5	5	4.5
Vomiting	22	20.4	3	2.8	40	36.4	1	0.9
Anorexia	50	46.3	8	7.4	78	70.1	19	17.3
Diarrhea	21	19.4	1	0.9	49	44.5	5	4.5
Neuropathy (sensory)	62	57.4	8	7.4	2	1.8	0	0
Bilirubin	10	9.3	3	2.8	21	19.1	4	3.6
AST	32	29.6	4	3.7	42	38.2	9	8.2
ALT	24	22.2	3	2.8	41	37.3	3	2.7
Hyponatremia	21	19.4	4	3.7	35	31.8	17	15.5
Treatment-related death	0	0	0	0	2	1.8	2	1.8

DISCUSSION

To our knowledge, this was the first randomized phase III trial comparing paclitaxel and irinotecan in second-line chemotherapy for advanced gastric cancer. No statistically significant differences were observed between paclitaxel and irinotecan for the primary end point of OS or for other parameters evaluated in this study, including PFS and RR. Activity, feasibility, and tolerability of paclitaxel and irinotecan were comparable for second-line treatment of advanced gastric cancer.

When we planned this study, OS in patients who received second-line chemotherapy seemed to be longer than OS in patients who received BSC alone in previous trials.[12-16,19,20] Because > 70% of patients were receiving second-line chemotherapy as part of routine clinical practice at that time, conducting a trial of second-line chemotherapy compared with BSC alone was difficult in Japan. Since then, the survival benefit of second-line chemotherapy over BSC has been demonstrated in two randomized trials[22,23]: the AIO (Arbeitsgemeinschaft Internistische Onkologie) trial using irinotecan and Korean trial using irinotecan or docetaxel during the same time period as this WJOG 4007 study. On the basis of these results, second-line chemotherapy using irinotecan or docetaxel has been recognized as the standard of care for patients with gastric cancer. However, further comparison between irinotecan and taxane regimens would be valuable for strategic planning of treatment in patients with advanced gastric cancer.

In the Korean trial, choice of chemotherapy regimen—docetaxel or irinotecan—depended on investigator discretion. A subgroup analysis showed no significant difference in survival between regimens (median OS: docetaxel, 5.2 months *v* irinotecan, 6.6 months; $P = .116$).[23] In addition, Ji et al[24] conducted a retrospective analysis of 725 patients with gastric cancer treated with second-line chemotherapy; they found no relevant difference in OS between taxane and irinotecan treatment. In our exploratory subgroup analysis, no interaction was observed among several clinical factors; results favored neither paclitaxel nor irinotecan. Thus, either taxane or irinotecan can be recommended as a treatment option for second-line chemotherapy in patients with advanced gastric cancer.

Longer OS was achieved in this study than in previous phase III studies.[22,23] Many patients in good condition with small tumor burdens were enrolled onto our study. ECOG PS of 0 or 1 was recorded in almost all patients, and only one metastatic site was detected in > half of all patients. Additionally, excluding patients with severe peritoneal metastasis resulted in a lower proportion of patients (25.6%) with peritoneal metastasis, compared with those in the AIO (43%) and Korean (45%) trials.[22,23] These are well known as prognostic factors in advanced gastric cancer, and these patient-selection biases might have led to longer survival in our study.

In gastric cancer, peritoneal metastasis often develops along with disease progression, and irinotecan would be toxic for patients with

severe peritoneal metastasis. Indeed, the proportion of patients receiving subsequent irinotecan after second-line paclitaxel was only 24% in the previous report.[16] In this study, excluding patients with severe peritoneal metastasis seemed to result in a high proportion of patients (> 70%) receiving third-line chemotherapy, whereas 30% to 40% of patients did so in previous studies.[23,24] Although evidence is limited with regard to the efficacy of third-line chemotherapy in advanced gastric cancer, this therapy may have contributed to prolonged OS, and the unexpected higher proportion of those receiving third-line chemotherapy might have diluted a difference in OS between the paclitaxel and irinotecan groups.

Overall toxicity in both treatment arms was acceptable for second-line chemotherapy. In the paclitaxel group, common grade 3 or 4 toxicities (≥ 10%) included leukocytopenia, neutropenia, and anemia. Grade 3 sensory neuropathy, which was specific to paclitaxel, occurred at an incidence < 10% in this study. These toxicity profiles and severity levels are consistent with those in previous reports.[15,16] In the irinotecan group, leukocytopenia, neutropenia, anemia, anorexia, and hyponatremia were commonly observed. Frequency and severity of these toxicities were also consistent with those in previous reports.[22,23] Severe diarrhea, which is a well-known adverse reaction to irinotecan, generally occurs less frequently in Asian patients than in Western patients. In fact, grade 3 or 4 diarrhea was observed in 4.5% of patients in this trial, 8% of those in the Korean trial,[23] and 26% of those in the AIO trial.[22] Although ethnic diversity in metabolism of irinotecan has been suggested, the dosage of irinotecan is commonly higher in Western countries than in Asian countries. This may explain the different incidence of severe diarrhea between this and other studies.

Our study has several limitations. Participants were all Japanese; tumor biology may differ from that in Western patients.[25] In addition, a majority of patients received S-1 plus cisplatin as first-line chemotherapy, whereas S-1 is not popular in Western countries. However, a large, global phase III study (FLAGS [First-Line Therapy in Patients With Advanced Gastric Cancer Study] trial) demonstrated S-1 plus cisplatin to be similar in efficacy to fluorouracil plus cisplatin.[7] This difference in regimens used as first-line chemotherapy may have had little influence on interpretation of results of our study. Because patients with severe peritoneal metastasis were excluded from our study to avoid confounding effects of serious adverse events resulting from irinotecan, our results are not applicable to patients with severe peritoneal metastasis. Another trial is needed to determine the most appropriate treatment in such patients. As for statistical consideration, our hypothesis was 50% improvement in median OS in the irinotecan group over weekly paclitaxel group, and this resulted in a relatively small sample size. Therefore, if a small but true benefit existed in either group, this study may have been underpowered to detect it.

In conclusion, no difference in OS between paclitaxel and irinotecan groups was observed in this study. Both are considered reasonable second-line treatment options. The differences in toxicity profile and treatment schedule between both treatments will help in choosing either irinotecan or paclitaxel. Currently, several randomized trials investigating additional benefits of molecular targeting agents in second-line chemotherapy are planned or being conducted using weekly paclitaxel or irinotecan as a platform or reference regimen. The findings of our study are relevant to these future trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** Tomohiro Nishina, Yakult Pharmaceutical Industry, Bristol-Myers Squibb; Satoshi Morita, Bristol-Myers Squibb, Yakult Pharmaceutical Industry, Daiichi Sankyo; Narikazu Boku, Daiichi Sankyo, Yakult Pharmaceutical Industry; Ichinosuke Hyodo, Yakult Pharmaceutical Industry **Research Funding:** Naotoshi Sugimoto, Yakult Pharmaceutical Industry; Taito Esaki, Yakult Pharmaceutical Industry; Ichinosuke Hyodo, Yakult Pharmaceutical Industry, Daiichi Sankyo **Expert Testimony:** None **Patents:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Shuichi Hironaka, Satoshi Morita, Narikazu Boku, Ichinosuke Hyodo
Administrative support: Takashi Ura, Isamu Okamoto, Narikazu Boku
Provision of study materials or patients: Shuichi Hironaka, Tomohiro Nishina, Toshikazu Moriwaki, Kensei Yamaguchi, Yasuo Hamamoto
Collection and assembly of data: Shuichi Hironaka, Shinya Ueda, Hirofumi Yasui, Tomohiro Nishina, Masahiro Tsuda, Takehiko Tsumura, Naotoshi Sugimoto, Hideki Shimodaira, Shinya Tokunaga, Toshikazu Moriwaki, Taito Esaki, Michitaka Nagase, Kazumasa Fujitani, Kensei Yamaguchi, Takashi Ura, Yasuo Hamamoto
Data analysis and interpretation: Shuichi Hironaka, Satoshi Morita, Isamu Okamoto, Ichinosuke Hyodo
Manuscript writing: All authors
Final approval of manuscript: All authors

REFERENCES

1. Murad AM, Santiago FF, Petroianu A, et al: Modified therapy with 5-fluorouracil, doxorubicin, and methotrexate in advanced gastric cancer. Cancer 72:37-41, 1993
2. Glimelius B, Hoffman K, Haglund U, et al: Initial or delayed chemotherapy with best supportive care in advanced gastric cancer. Ann Oncol 5:189-190, 1994
3. Pyrhönen S, Kuitunen T, Nyandoto P, et al: Randomised comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus supportive care with supportive care alone in patients with non-resectable gastric cancer. Br J Cancer 71:587-591, 1995
4. Cunningham D, Starling N, Rao S, et al: Capecitabine and oxaliplatin for advanced esophagogastric cancer. N Engl J Med 358:36-46, 2008
5. Bang YJ, Van Cutsem E, Feyereislova A, et al: Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): A phase 3, open-label, randomised controlled trial. Lancet 376:687-697, 2010
6. Kang YK, Kang WK, Shin DB, et al: Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: A randomised phase III noninferiority trial. Ann Oncol 20:666-673, 2009
7. Ajani JA, Rodriguez W, Bodoky G, et al: Multicenter phase III comparison of cisplatin/S-1 with cisplatin/infusional fluorouracil in advanced gastric or gastroesophageal adenocarcinoma study: The FLAGS trial. J Clin Oncol 28:1547-1553, 2010

8. Ohtsu A, Shah MA, Van Cutsem E, et al: Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: A randomized, double-blind, placebo-controlled phase III study. J Clin Oncol 29:3968-3976, 2011

9. Koizumi W, Narahara H, Hara T, et al: S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): A phase III trial. Lancet Oncol 9:215-221, 2008

10. Boku N, Yamamoto S, Fukuda H, et al: Fluorouracil versus combination of irinotecan plus cisplatin versus S-1 in metastatic gastric cancer: A randomised phase 3 study. Lancet Oncol 10:1063-1069, 2009

11. Narahara H, Iishi H, Imamura H, et al: Randomized phase III study comparing the efficacy and safety of irinotecan plus S-1 with S-1 alone as first-line treatment for advanced gastric cancer (study GC0301/TOP-002). Gastric Cancer 14:72-80, 2011

12. Yamada Y, Shirao K, Ohtsu A, et al: Phase II trial of paclitaxel by three-hour infusion for advanced gastric cancer with short premedication for prophylaxis against paclitaxel-associated hypersensitivity reactions. Ann Oncol 12:1133-1137, 2001

13. Yamaguchi K, Tada M, Horikoshi N, et al: Phase II study of paclitaxel with 3-h infusion in patients with advanced gastric cancer. Gastric Cancer 5:90-95, 2002

14. Taguchi T, Sakata Y, Kanamaru R, et al: Late phase II clinical study of RP56976 (docetaxel) in patients with advanced/recurrent gastric cancer: A Japanese Cooperative Study Group trial (group A) [in Japanese]. Gan To Kagaku Ryoho 25:1915-1924, 1998

15. Arai T, Hamaguchi T, Shirao K: Weekly paclitaxel in patients with heavily treated advanced gastric cancer. Proc Am Soc Clin Oncol 22:321, 2003 (suppl; abstr 1291)

16. Hironaka S, Zenda S, Boku N, et al: Weekly paclitaxel as second-line chemotherapy for advanced or recurrent gastric cancer. Gastric Cancer 9:14-18, 2006

17. Kodera Y, Ito S, Mochizuki Y, et al: A phase II study of weekly paclitaxel as second-line chemotherapy for advanced gastric cancer (CCOG0302 study). Anticancer Res 27:2667-2671, 2007

18. Matsuda G, Kunisaki C, Makino H, et al: Phase II study of weekly paclitaxel as a second-line treatment for S-1-refractory advanced gastric cancer. Anticancer Res 29:2863-2867, 2009

19. Hamaguchi T, Shirao K, Ohtsu A, et al: A phase II study of biweekly mitomycin C and irinotecan combination therapy in patients with fluoropyrimidine-resistant advanced gastric cancer: A report from the Gastrointestinal Oncology Group of the Japan Clinical Oncology Group (JCOG0109-DI Trial). Gastric Cancer 14:226-233, 2011

20. Giuliani F, Molica S, Maiello E, et al: Irinotecan (CPT-11) and mitomycin-C (MMC) as second-line therapy in advanced gastric cancer: A phase II study of the Gruppo Oncologico dell' Italia Meridionale (prot. 2106). Am J Clin Oncol 28:581-585, 2005

21. Therasse P, Arbuck SG, Eisenhauer EA, et al: New guidelines to evaluate the response to treatment in solid tumors: European Organisation for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205-216, 2000

22. Thuss-Patience PC, Kretzschmar A, Bichev D, et al: Survival advantage for irinotecan versus best supportive care as second-line chemotherapy in gastric cancer: A randomised phase III study of the Arbeitsgemeinschaft Internistische Onkologie (AIO). Eur J Cancer 47:2306-2314, 2011

23. Kang JH, Lee SI, Lim DH, et al: Salvage chemotherapy for pretreated gastric cancer: A randomized phase III trial comparing chemotherapy plus best supportive care with best supportive care alone. J Clin Oncol 30:1513-1518, 2012

24. Ji SH, Lim do H, Yi SY, et al: A retrospective analysis of second-line chemotherapy in patients with advanced gastric cancer. BMC Cancer 9:110, 2009

25. Kim J, Sun CL, Mailey B, et al: Race and ethnicity correlate with survival in patients with gastric adenocarcinoma. Ann Oncol 21:152-160, 2010

Affiliations

Shuichi Hironaka, Chiba Cancer Center, Chiba; Shinya Ueda and Isamu Okamoto, Kinki University, Osakasayama; Hirofumi Yasui, Shizuoka Cancer Center, Shizuoka; Tomohiro Nishina, National Hospital Organization Shikoku Cancer Center, Matsuyama; Masahiro Tsuda, Hyogo Cancer Center, Akashi; Takehiko Tsumura, Osaka Red Cross Hospital; Naotoshi Sugimoto, Osaka Medical Center for Cancer and Cardiovascular Diseases; Shinya Tokunaga, Osaka City General Hospital; Kazumasa Fujitani, Osaka National Hospital, Osaka; Hideki Shimodaira, Tohoku University Hospital, Sendai; Toshikazu Moriwaki and Ichinosuke Hyodo, University of Tsukuba, Tsukuba; Taito Esaki, National Kyushu Organization Kyushu Cancer Center, Fukuoka; Michitaka Nagase, Jichi Medical University, Shimono; Kensei Yamaguchi, Saitama Cancer Center, Saitama; Takashi Ura, Aichi Cancer Center Hospital, Nagoya; Yasuo Hamamoto, Tochigi Cancer Center, Utsunomiya; Satoshi Morita, Yokohama City University Graduate School of Medicine, Yokohama; and Narikazu Boku, St Marianna University School of Medicine, Kawasaki, Japan.

Breast Cancer Res Treat (2013) 137:773–782
DOI 10.1007/s10549-012-2398-5

CLINICAL TRIAL

High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival

Akimitsu Yamada · Takashi Ishikawa · Ikuko Ota · Mariko Kimura · Daisuke Shimizu · Mikiko Tanabe · Takashi Chishima · Takeshi Sasaki · Yasushi Ichikawa · Satoshi Morita · Koh-ichiro Yoshiura · Kazuaki Takabe · Itaru Endo

Received: 26 November 2012 / Accepted: 18 December 2012 / Published online: 4 January 2013

Abstract ATP-binding cassette (ABC) transporters are membrane proteins that efflux various compounds from cells, including chemotherapeutic agents, and are known to affect multidrug resistance. Recent reports disagree on whether ABCC11 is a risk factor for breast tumorigenesis, but its expression in breast cancer is poorly investigated. We hypothesized that both frequency and expression levels of ABC transporters in breast tumors would vary by cancer subtype, and be associated with prognosis. Here, we constructed a tissue microarray breast tumor samples from 281 patients, and analyzed expressions of ABCB1, ABCC1, ABCC11, and ABCG2 immunohistochemically. Breast cancer subtypes were determined by immunohistochemistry of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). Protein expression was correlated to clinicopathological characteristics, clinical follow-up, and pathological complete response to neoadjuvant chemotherapy. The tissue microarray comprised 191 luminal A (68.0 %), 17 luminal B (6.0 %), 27 HER2 (9.6 %), and 46 triple-negative (16.4 %) samples. ABCC1 and ABCC11 expressions were associated with significantly shorter disease-free survival ($P = 0.027$ and $P = 0.003$, respectively). ABCC1, ABCC11, and ABCG2, but not ABCB1, were expressed significantly more, and more frequently, in aggressive subtypes. Patients with HER2+ and triple-negative tumor subtypes that expressed high levels of ABCC11 had significantly worse disease-free survival ($P = 0.017$ and $P < 0.001$, respectively). We have shown, for the first time, that ABCC1, ABCC11, and ABCG2 are highly expressed in aggressive breast cancer subtypes, and that tumor ABCC11 expression is associated with poor prognosis.

Keywords Breast cancer · ATP-binding cassette transporters · ABCC11 · Tissue microarray · Subtype

Introduction

Breast cancer is a heterogeneous disease [1]. DNA microarray profiling studies on breast cancer have identified distinct subtypes: luminal A, luminal B, human epidermal

A. Yamada · M. Kimura · T. Chishima · Y. Ichikawa · I. Endo
Department of Clinical Oncology and Breast Surgery, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa, Japan

T. Ishikawa (✉) · I. Ota · D. Shimizu
Department of Breast and Thyroid Surgery, Yokohama City University Medical Center, 4-57 Urafunecho, Minami-ku, Yokohama, Kanagawa, Japan
e-mail: tishik@urahp.yokohama-cu.ac.jp

M. Tanabe · T. Sasaki
Department of Pathology, Yokohama City University Medical Center, 4-57 Urafunecho, Minami-ku, Yokohama, Kanagawa, Japan

S. Morita
Department of Biostatics and Epidemiology, Yokohama City University Medical Center, 4-57 Urafunecho, Minami-ku, Yokohama, Kanagawa, Japan

K. Yoshiura
Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki, Japan

K. Takabe
Division of Surgical Oncology, Department of Surgery, Virginia Commonwealth University School of Medicine, 7-402 West Hospital, 1200 E Broad Street, Richmond, VA, USA

growth factor receptor 2 (HER2)-enriched, and triple-negative (which is sometimes further subdivided into the core-basal and five-negative subtypes) [2]. These subtypes are reportedly associated with differences in resistance to chemotherapy [3–5] and subsequent outcomes [6, 7]. Several mechanisms affect how cancer cells become resistant to cytotoxic drugs, which include efflux of the drug compound from cancer cells, and others such as mutation, overexpression of the drug's targets, and drug inactivation [8].

The ATP-binding cassette (ABC) transporters are transmembrane proteins that use ATP to transport various molecules across extra- and intra-cellular membranes. This function is thought to have evolved as a xenobiotic protective mechanism [9]. Of the 49 human ABC transporters so far identified (which have been classified into seven subfamilies), ABCA2, ABCB1, ABCC1–6, ABCC11, and ABCG2 have been associated with chemoresistance in breast cancer [8]. Unfortunately, all clinical trials that have targeted ABC transporters failed to improve outcomes [10]. One explanation for this is that they all targeted ABCB1 [also known as MDR1, permeability glycoprotein 1 (P-glycoprotein or Pgp), and cluster of differentiation 243 (CD243)]. This led us to hypothesize that other ABC transporters may be more important for drug resistance.

ABCC11 is a member of the ABCC1 (also known as MDR-associated protein) sub-family. A single nucleotide polymorphism (SNP) in the *ABCC11* gene was shown to be responsible for "wet earwax" in humans [11]. Reports as to whether *ABCC11* is a risk factor for breast tumorigenesis conflict; although this gene was originally shown to be a risk factor for development of breast cancer among Japanese women [12], it is reportedly not the case in Caucasian women [13, 14]. There has been no investigation of ABCC11 protein expression levels in breast tumors or their association with cancer subtype and prognosis. We hypothesized that both frequency and expression levels of ABC transporters (ABCB1, ABCC1, ABCC11, and ABCG2) in breast tumors would differ by cancer subtype and be associated with prognosis. Here, utilizing a tissue microarray newly constructed from 281 breast cancer samples, we analyzed the expression of these transporters in light of breast cancer subtype and prognosis, as well as investigating the effects of neoadjuvant chemotherapy.

Methods

Tissue sources and clinical characteristics

Tissues for this study were obtained from 281 patients treated in Yokohama City Medical Center, Japan, between 2006 and 2008, involving all stages of breast cancer. This study was approved by the Institutional Review Board of Yokohama City University, Kanagawa, Japan, and the patients gave their informed consent before their inclusion in the study. Core biopsy samples taken prior to treatment were obtained from 50 patients who received neoadjuvant chemotherapy (35 patients received anthracycline followed by taxane; 14 received anthracycline alone; and one received taxane alone). One hundred and eight patients received adjuvant chemotherapy after surgery (45 received anthracycline followed by taxane; 38 received anthracycline alone; 15 received taxane alone; and 10 received other regimens) and 208 patients received adjuvant hormonal therapy (tamoxifen and luteinizing hormone-releasing hormone-agonist for 61 premenopausal patients; tamoxifen or aromatase inhibitor for 147 postmenopausal patients). None of the tissues described here was obtained after any treatment. All the patients were followed up at least every 3 months after surgery. The mean observation period was 49 months (range: 28–60 months). The clinical characteristics are presented in Table 1.

Table 1 Patients' characteristics

	N	%
Age		
<65	197	70.1
65≦	80	28.5
	4	1.4
Menstruation states		
Pre menopause	87	31.0
Post menopause	154	54.8
NA	40	14.2
Estrogen receptor		
Positive	210	74.8
Negative	71	25.2
NA	0	0.0
Progesterone receptor		
Positive	162	42.7
Negative	119	57.3
NA	0	0.0
HER2 overexpression		
Present	44	15.7
Absent	237	84.3
NA	0	0.0
Basal markers		
Basal	34	12.1
Non basal	235	83.4
NA	12	4.5
Subtype		
Luminal A	191	68.0
Luminal B	17	6.0
HER2	27	9.6

Table 1 continued

	N	%
Triple negative	46	16.4
Core basal	26	9.3
Five-negative	20	7.1
Tumor stage		
T1	123	43.8
T2	122	43.4
T3	11	3.9
T4	19	6.8
NA	6	2.1
Node		
N0	150	53.4
N1	83	29.5
N2	23	8.2
N3	11	3.9
NA	14	5.0
Metastases		
M0	259	92.2
M1	6	2.1
NA	16	5.7
TNM stage		
1	106	37.8
2	122	43.4
3	31	11.0
4	6	2.1
NA	16	5.7
Observation time (days)	1458 ± 509[a]	

[a] Expressed as mean ± standard deviation

Tissue microarray

The tissue microarray was constructed by taking 3.0-mm cores from representative areas of surgical specimens from patients using a KIN-2 tissue arrayer (Azumaya, Tokyo, Japan), and re-embedding these cores into a gridded paraffin block. Tissue cores were excluded from the tissue microarray if they fail to adhere to the glass slide, did not include invasive carcinoma, or were a non-interpretable specimen.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were sliced into 5-μm sections. The sections were baked at 60 °C, deparaffinized in xylene, and gradually rehydrated in ethanol. Sections were boiled in antigen retrieval solution (Funakoshi, Japan) for 30 min. Activity of endogenous peroxidase was blocked by 20 min of quenching in 0.3 % H_2O_2 and methanol; the sections were then incubated in 5 % rabbit serum for ABCB1 and ABCC1, or goat serum for ABCC11 and ABCG2. Immunohistochemical reactions were performed overnight at 4 °C using monoclonal mouse antibodies against ABCB1 (C219; 1:100; Abcam, UK), monoclonal rat antibodies against ABCC1 (MRPr1; 1:40; Monosan, The Netherlands), polyclonal rabbit antibodies against ABCC11 (1:500) [15], or monoclonal mouse antibodies against ABCG2 (BXP-21; 1:100; Abcam). For the triple-negative subtype, cytokeratin 5/6 (D5/16 B4; Dako, Denmark) and epidermal growth factor receptor (EGFR; Roche Diagnostics K.K, Japan,) were used for subdivision into the core-basal or non-basal (five-negative) subtypes. After washing, the slides were incubated with biotinylated antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (5 min, room temperature). 3,3′-diaminobenzidine (Dako Japan, Tokyo, Japan) was used as the chromogen. All sections were counterstained with Meyer's hematoxylin.

Evaluation of staining

Staining results were assessed by two pathologists independently, using a 4-point scoring system as shown in Fig. 1: 0 = invasive tumor cells present in the tissue core with no staining; 1 = invasive tumor cells present with weak staining intensity; 2 = invasive tumor cells present with strong staining intensity and <30 % of tumor cells stained or intermediate staining intensity in ≥30 % of tumor cells; and 3 = invasive tumor cells present with strong staining in ≥30 % of tumor cells. To evaluate positivity, both membranous and/or cytoplasmic staining scoring 2 or above was considered positive (high expression). CK5/6 and EGFR were considered positive when cytoplasmic and/or membranous staining of invasive carcinoma cells was observed, regardless of intensity.

Genotyping

Genotyping of ABCC11 by the SmartAmp method was performed as previously reported [12].

Statistical analysis

Statistical analysis used SPSS 19.0 for Windows software (SPSS Inc., Chicago, IL). Correlations among the clinicopathologic parameters and each transporter were evaluated by the Pearson χ^2 test, the Fisher exact test, and the Mann–Whitney test. Tukey-type multiple comparison analyses with the χ^2 test and Mantel test were carried out to compare expression of each transporter among the subtypes. Patient outcomes were assessed by disease-free survival. Survival distributions were estimated by the Kaplan–Meier method; differences were compared using the log-rank test. The multivariate Cox proportional hazard regression method

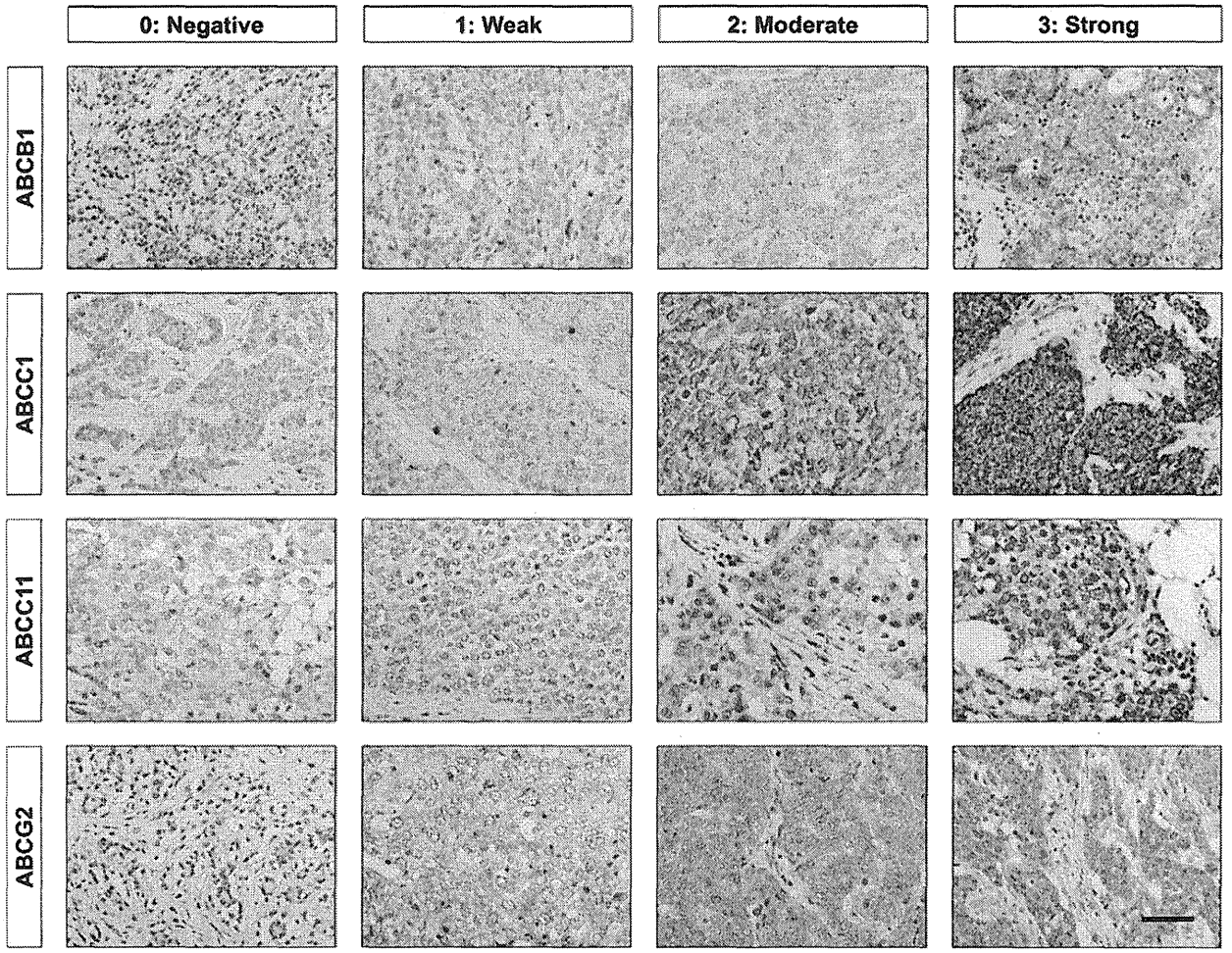


Fig. 1 4-Point scoring system for ABCB1, ABCC1, ABCC11, and ABCG2 protein expression. Our tissue microarray contained 281 breast tumor tissues, and was stained with antibodies against ABCB1 (1:100), ABCC1 (1:40), ABCC11 (1:500), and ABCG2 (1:100). Stain intensity was graded as negative (0), weak (1), moderate (2), or strong (3). Representative images are shown under high magnification. *Scale bar*: 50 μm

was used to determine the independent prognostic value. $P < 0.05$ was considered statistically significant.

Results

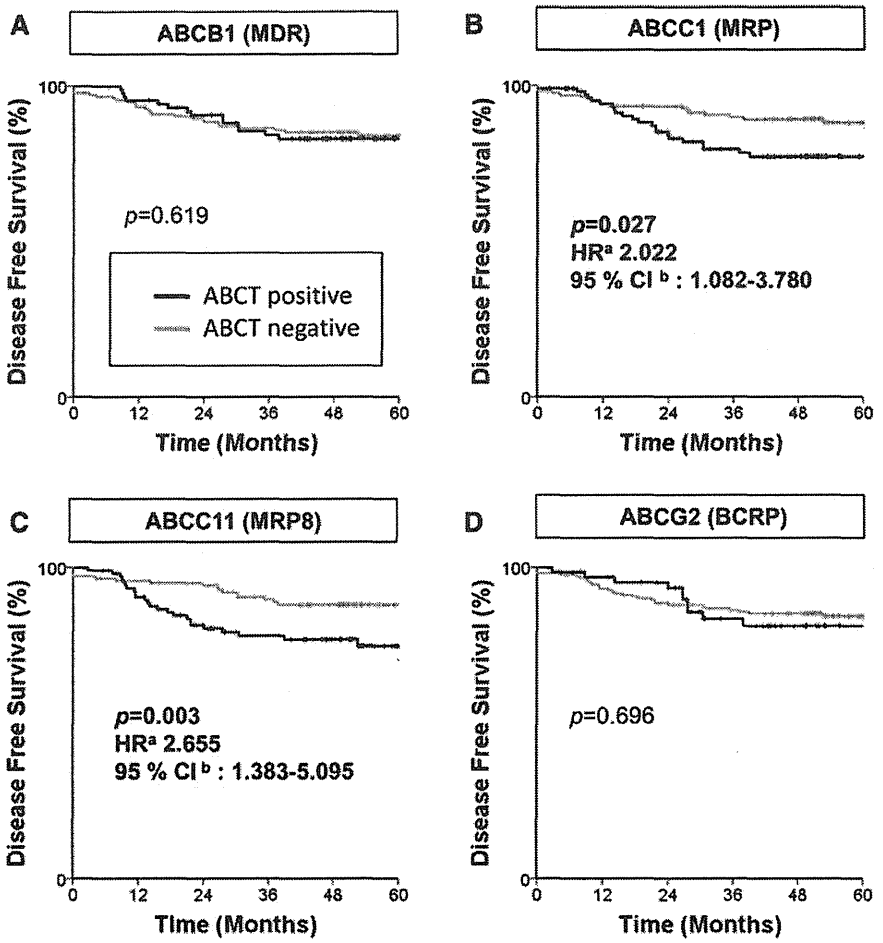
Characteristics of samples used for the tissue microarray

Subtypes of the 281 samples on the tissue microarray were determined using immunohistochemistry for the estrogen receptor (ER), progesterone receptor (PgR), and HER2, as previously reported [5, 16]. Patients' and tumor characteristics used for the tissue microarray are summarized in Table 1. The numbers of cases of the respective subtypes were: luminal A (ER+ and HER2−): 191 (68.0 %); luminal B (ER+ and HER2+): 17 (6.0 %); HER2 (ER− and HER2+): 27 (9.6 %); and triple-negative (ER− and HER2−): 46 (16.4 %). Triple-negative tumors were further sub-divided into two groups, core-basal (CK5/6+ and/or EGFR+) and five-negative (CK5/6− and EGFR−). The core-basal subtype constituted 56.5 % (26/46) of triple-negative tumors.

Associations between ABC transporter expression and clinical features of the tumors are shown in Table 2. ABCB1 was detected in 32.4 % (91/277) of the tumors, ABCC1 in 39.1 % (110/279), ABCC11 in 40.2 % (113/259), and ABCG2 in 24.2 % (68/278). There was no association between ABCB1 expression and any clinical features. ABCG2 was more frequently highly expressed in young premenopausal patients. High expressions of ABCC1 and ABCG2 were significantly more frequent in ER− tumors than in ER + ones ($P = 0.001$ and $P = 0.006$, respectively). There was no association between HER2 expression and ABC transporter expression.

Table 2 The expression of ABC transporters and clinical features

	ABCB1			*p* Value	ABCC1			*p* Value	ABCC11			*p* Value	ABCG2			*p* Value
	Negative	Positive	NA		Negative	Positive	NA		Negative	Positive	NA		Negative	Positive	NA	
N (%)	186 (66.2 %)	91 (32.4 %)	4 (1.4 %)		169 (60.2 %)	110 (39.1 %)	2 (0.7 %)		146 (52.0 %)	113 (40.2 %)	22 (7.8 %)		210 (74.7 %)	68 (24.2 %)	3 (1.1 %)	
Age																
<65	125 (63.5 %)	68 (34.5 %)	4 (2 %)	0.14	119 (60.4 %)	76 (38.6 %)	2 (1 %)	0.54	97 (49.2 %)	83 (42.1 %)	17 (8.7 %)	0.13	138 (70.0 %)	56 (28.4 %)	3 (1.6 %)	<0.01
65≦	58 (72.5 %)	22 (27.5 %)	0		49 (61.2 %)	31 (38.8 %)	0		47 (58.8 %)	28 (35.0 %)	5 (6.2 %)		69 (86.3 %)	11 (13.7 %)	0	
Menstruation status																
Pre menopause	55 (63.2 %)	29 (33.3 %)	3 (3.5 %)	0.40	51 (58.6 %)	36 (41.4 %)	0	0.32	41 (47.2 %)	37 (42.5 %)	9 (10.3 %)	0.35	54 (62.1 %)	31 (35.6 %)	2 (2.3 %)	<0.01
Post menopause	104 (67.5 %)	49 (31.8 %)	1 (0.7 %)		95 (61.7 %)	57 (37.0 %)			81 (52.6 %)	63 (40.9 %)	10 (6.5 %)		81 (52.6 %)	63 (40.9 %)	10 (6.5 %)	
Estrogen receptor																
Negative	50 (70.4 %)	21 (29.6 %)	0	0.61	30 (42.3 %)	39 (54.9 %)	2 (2.8 %)	<0.01	37 (52.1 %	27 (38.0 %)	7 (9.9 %)	0.65	43 (60.6 %)	27 (38.0 %)	1 (1.4 %)	<0.01
positive	135 (64.5 %)	70 (33.5 %)	4 (2.0 %)		139 (66.5 %)	70 (33.5 %)	0		108 (51.7 %)	86 (41.1 %)	15 (7.2 %)		166 (79.4 %)	41 (19.6 %)	2 (1.0 %)	
Progesterone receptor																
Negative	78 (65.5 %)	38 (31.8 %)	3 (2.7 %)	0.78	63 (52.9 %)	54 (45.4 %)	2 (1.7 %)	0.06	57 (47.9 %)	49 (41.2 %)	13 (10.9 %)	0.55	81 (68.0 %)	35 (29.4 %)	3 (2.6 %)	0.15
Positive	107 (66.5 %)	53 (32.9 %)	1 (0.6 %)		106 (65.8 %)	55 (34.2 %)	0		88 (54.7 %)	64 (40.0 %)	9 (5.3 %)		128 (79.5 %)	33 (20.5 %)	0	
HER2 expression																
Absent	155 (66.0 %)	77 (32.8 %)	3 (1.2 %)	1.00	146 (62.1 %)	88 (37.4 %)	1 (0.5 %)	0.18	123 (52.4 %)	97 (41.2 %)	15 (6.4 %)	1.00	179 (76.1 %)	55 (23.4 %)	1 (0.5 %)	0.33
Present	179 (76.1 %)	55 (23.4 %)	1 (0.5 %)		22 (50.0 %)	21 (47.7 %)	1 (2.3 %)		21 (47.7 %)	16 (36.4 %)	7 (15.9 %)		29 (65.9 %)	13 (29.5 %)	2 (4.6 %)	



[a] *HR* hazard ratio, [b] *CI* confidence interval

Fig. 2 Kaplan–Meier disease-free survival curves according to expression of ABCB1 (**a**), ABCC1 (**b**), ABCC11 (**c**), and ABCG2 (**d**). The *thick bold line* indicates positivity; and the *light gray line* indicates negativity, for the respective transporters. Only the ABCC1+ and ABCC11+ groups showed significantly improved survival ($P = 0.027$ and $P = 0.003$, respectively)

Expression of ABCC1 and ABCC11 is associated with poor patient survival

We compared expression of each transporter and patient disease-free survival (Fig. 2). In the entire study group, patients with ABCC1+ or ABCC11+ tumors had significantly shorter disease-free survival compared to patients with corresponding ABCC1− or ABCC11− tumors ($P = 0.027$ or $P = 0.003$, respectively).

ABC transporters are more frequently highly expressed in aggressive subtypes of breast cancer

Because breast cancer subtypes are associated with different clinical behaviors [2], we further analyzed clinical outcomes according to cancer subtype and ABC transporter expression. Expression of each transporter according to breast cancer subtype is shown in Fig. 3. The percentage of patients whose tumors expressed ABCB1 did not differ among the subtypes. ABCC1 and ABCG2 were more frequently highly expressed in triple-negative subtype, especially in the core-basal subtype, compared with the luminal A subtype, whereas highly expressed ABCC11 was more common in HER2-enriched, core-basal, and luminal A subtypes. Although core-basal tumors tended to express ABC transporters more often than five-negative tumors did, only ABCC11 showed significantly more frequent high expression in the core-basal subtype. Semi-quantification of ABC transporters expression is shown in Fig. 3b. ABCC1, ABCC11, and ABCG2 were more highly expressed in HER2-enriched and/or the core-basal subtypes, which is consistent with frequency data shown in Fig. 3a.

Patients whose tumors expressed high levels of ABCC11 tended towards decreased pathological complete responses to neoadjuvant chemotherapy

We next investigated whether there was any association between the "wet earwax" genotypes and ABCC11 expression. Figure 4a and b show the relationship between *ABCC11* genotypes and ABCC11 expression in breast

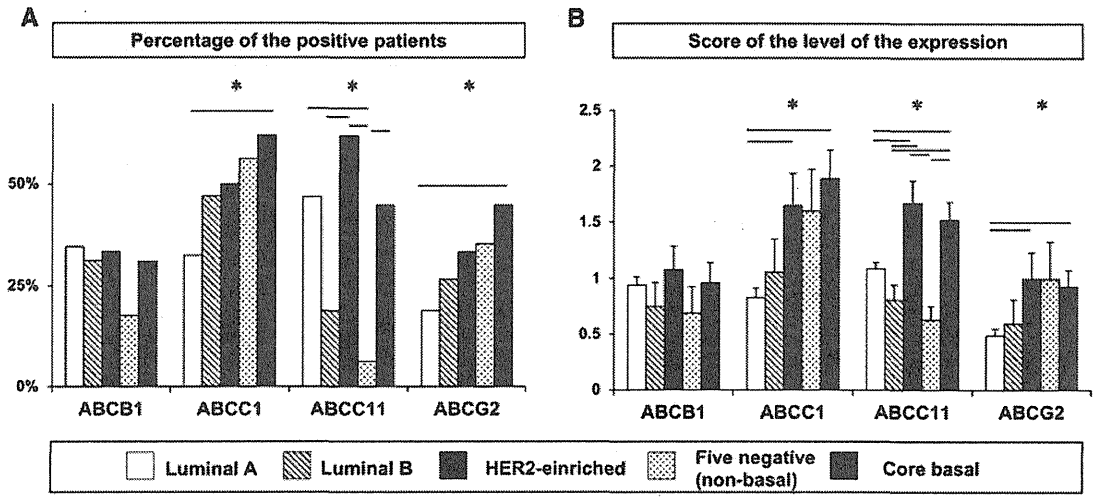


Fig. 3 Frequency (**a**) and intensity (**b**) of high ABC transporter expression classified by subtype, including luminal A (*open columns*), luminal B (*hatched columns*), HER2-enriched (*gray columns*), five-negative (*dotted columns*), and core-basal (*filled columns*). **a** Percentage of patients who showed high expression of each transporter. **b** Semi-quantification of expression level of each transporter, using a 4-point scoring system

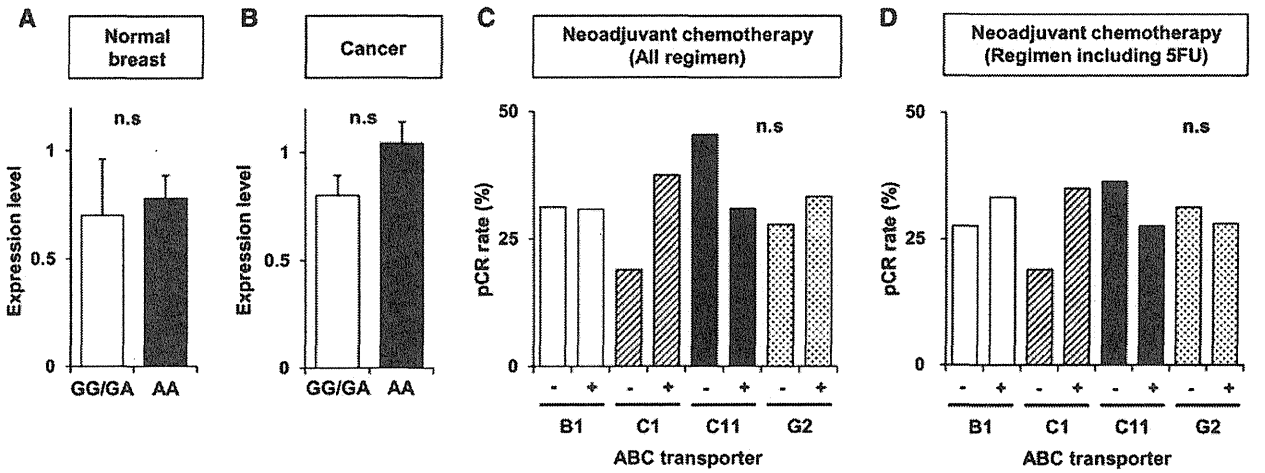


Fig. 4 Semi-quantification of ABCC11 expression levels in normal breast tissue (**a**) and cancer tissue (**b**) in patients carrying 538G/G, 538G/A (white open column, GG/GA, wet earwax phenotype), and 538A/A alleles (*black filled column* AA, dry earwax phenotype). **c**, **d** Pathological complete response ratios to neoadjuvant chemotherapy of all regimens (**c**) and regimens including 5-FU (**d**). *Bars* indicate ABCB1 (*white columns*), ABCC1 (*hatched columns*), ABCC11 (*black columns*), and ABCG2 (*dotted columns*)

cancer tissues. ABCC11 expression did not differ among the wet earwax genotype (538G/G + 538G/A) and the dry earwax genotypes (538A/A), in either normal breast tissues or breast cancer tissues.

As ABCC11 is known to efflux fluoropyrimidines (5-FU) in vitro [17], assessment of responses of ABCC11+ tumors to 5-FU-based regimens could be particularly valuable. Analysis of the association between ABC transporter expression and pathological complete response to neoadjuvant chemotherapy showed no statistically significant differences, regardless of regimen, but patients whose cancers expressed high levels of ABCC11 tended to have decreased pathological complete responses to neoadjuvant chemotherapy (Fig. 4c, d).

ABCC11+ tumors show worse prognoses among aggressive breast cancer subtypes

Because patients with ABCC1+ or ABCC11+ tumors tend to have poor prognoses, we investigated prognosis according to subtype. Patients with ABCC1+ tumors ended to have worse prognoses for luminal A tumors, but not significantly so ($P = 0.096$). Interestingly, patients with ABCC11+ tumors had significantly worse prognoses than did patients

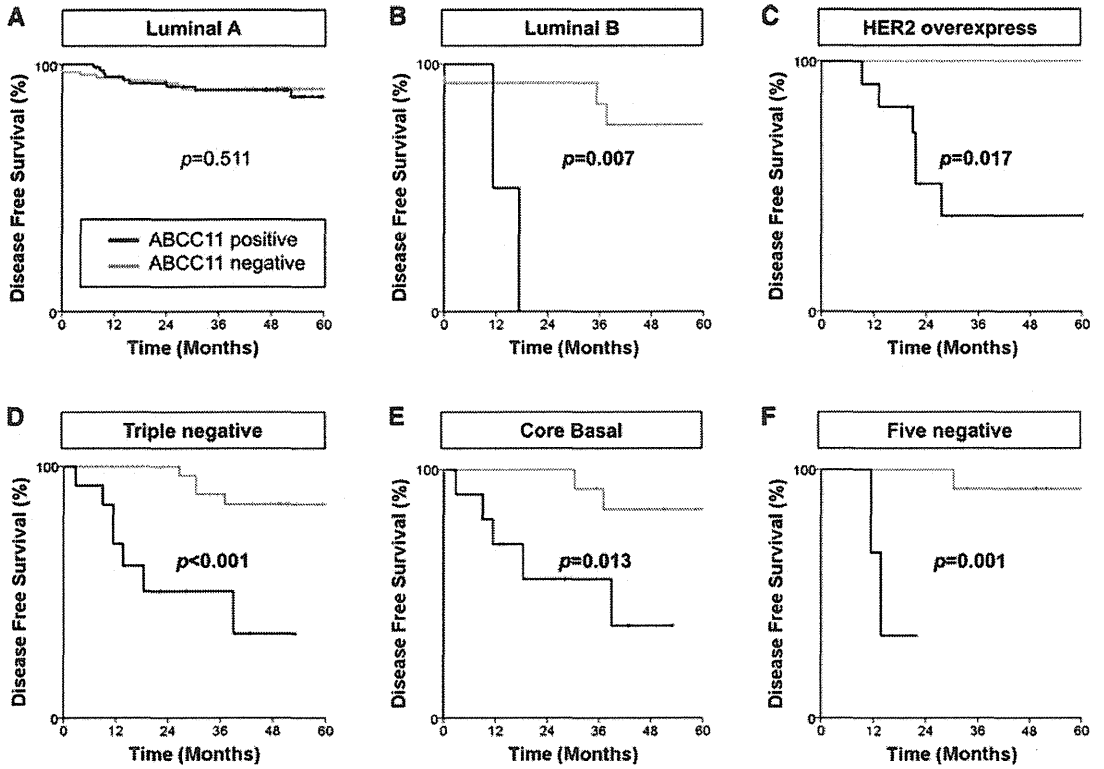


Fig. 5 Kaplan–Meier disease-free survival curve according to the subtype of breast cancer: luminal A (**a**), luminal B(B), HER2-enriched (**c**), triple-negative (**d**), core-basal (**e**), and five-negative (**f**). The *thick bold line* indicates ABCC11+, and the *light gray line* indicates ABCC11−

with ABCC11− tumors, except for the luminal A subtype, which is known to have a better prognosis than the other subtypes (Fig. 5a–f).

Discussion

Different subtypes of breast cancer have different biological behaviors, including responses to systemic and local therapies [3–5] and subsequent clinical outcomes [6, 7]. The two hormone receptor-negative subtypes, triple-negative and HER2-enriched, have poor outcomes compared with the luminal subtypes. Among the triple-negative subtypes, the core-basal subtype, which responds poorly to cytotoxic chemotherapy, has the worst prognosis. Thus, there is a particular need to elucidate drug resistance mechanisms for this subtype.

Expression of ABC transporters is reportedly related to chemoresistance [9]. Some ABC transporters, namely ABCB1, ABCC1, and ABCG2, have been identified as MDR proteins in breast cancer, which contribute to drug resistance via ATP-dependent drug efflux pumps [8]. Because ABCB1 effluxes drugs important for breast cancer—anthracyclines (doxorubicin, epirubicin, and daunomycin) and taxanes (paclitaxel, docetaxel)—ABC transporter inhibitors were the subjects of several widely anticipated clinical trials. Unfortunately, these agents proved disappointing [8, 18]. The vast majority of clinical trials targeting ABC transporters focused on ABCB1 (the most investigated ABC transporter) but data that associates patients' clinicopathological factors with ABCB1 expression tends to conflict [10]. This led us to investigate expression of multiple ABC transporters that are associated with MDR, in the context of different breast cancer subtypes. We felt that this information would be particularly relevant for the triple-negative subtype.

Patient characteristics and our tissue microarray staining data generally agree with previous reports [10, 19, 20]. The proportion of breast cancer subtypes may differ among different races or geographic populations; e.g., prevalence of the luminal A subtype may be higher, and the triple-negative subtype may be lower, in Asian women than in Western women [19]. The demographics of our tissue microarray are consistent with the prevalence among Japanese women. Leonessa et al. [10] reported that the detection rate of ABCB1 and ABCC1 in untreated tumors by immunohistochemistry was 40 % (range: 0–100 %) and 49 % (range: 20–100 %), respectively, with no clear association between ABCB1 and hormone receptors. In

agreement, our results also showed no association between ABCB1 expression and clinical features.

Among ABC transporters, ABCC11 is at relatively early stages of investigation. ABCC11 is lipophilic anion pump that can confer resistance to chemotherapeutic agents such as methotrexate and 5-FU [17]. We previously reported that a SNP in *ABCC11* is associated with the risk of developing breast cancer among Japanese women [12], although the association of *ABCC11* with breast cancer risk is unclear in Caucasian and European women [13, 14]. These reports mentioned host factors that might differ among races and thus modify the impact of this gene on breast cancer risk. *ABCC11* mRNA is reportedly over-expressed in breast tumors and breast cancer cell lines [9, 21, 22], but few studies discuss expression of the ABCC11 protein in human tumors [23]. Although the breast cancer risk conferred by the SNP in *ABCC11* is not within the scope of this study, we did not see significant differences in breast cancer prognosis by SNP genotype in our samples.

Core-basal and HER2-enriched subtypes are associated with poor clinical outcome [5]. In our series, high expressions of ABCC1 and ABCG2 were more common in aggressive subtypes such as core-basal. Strikingly, high expression of ABCC11 was more frequent and intense in both the HER2-enriched and core-basal subtypes, which implies that ABCC11 may promote the aggressive behavior of these subtypes. Indeed, ABCC11 has been shown to export not only drugs but also other factors that affect cancer biology. In agreement, our results show that patients with high tumor expression of ABCC11 have worse outcomes, particularly among the HER2-enriched and core-basal subtypes. This is the first study to show such an association.

Reportedly, ABCC11 expression is related to sensitivity and resistance to chemotherapy [17, 24–26]. In our data, only ABCC11, but not other transporters, tended to correlate with neoadjuvant chemotherapy response. Interestingly, this was true of chemotherapy regimens that both did and did not include 5-FU, which suggests that ABCC11 possesses unidentified supportive functions for drug resistance other than simple drug efflux. For example, we reported that ABCC1 and ABCG2 in breast cancer cells export sphingosine-1-phosphate [27], a bioactive lipid mediator known to affect drug resistance; we cannot exclude the possibility that ABCC11 possesses such a function. In that case, ABCC11 could become a new target in suppressing drug resistance.

Interestingly, it has been suggested that ABCB1 and ABCG2 may affect the role of cancer stem cells in drug resistance [8]. Although we do not currently have data on this relationship, it is intriguing to speculate that the worse prognosis of ABCC11-expressing tumors may be related to cancer stem cells.

Our study is limited in that it is a retrospective analysis of prospectively collected breast tumor samples, and that it shows only association of these transporters with breast cancer prognosis. To evaluate adequately the role of ABCC11 in breast cancer drug resistance, further studies of the mechanism of resistance are needed.

In conclusion, this is the first demonstration that ABCC11 expression in breast cancer is associated with aggressive subtypes and poor disease-free survival.

Acknowledgments The authors thank Dr. Harry D. Bear (Virginia Commonwealth University) for his critical review and valuable input to improve this manuscript. Kazuaki Takabe is supported by the US National Institutes of Health (R01CA160688, K12HD055881) and a Susan G. Komen for the Cure Investigator Initiated Research Grant and Career Catalyst Research Grant (KG090510).

Ethical standards This study was approved by the Institutional Review Board of Yokohama City University, Kanagawa, Japan.

Conflicts of interest The authors declare that they have no conflict of interest.

References

1. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ (2011) Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22(8):1736–1747. doi:10.1093/annonc/mdr304
2. Perou CM, Sørlie 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, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406(6797):747–752. doi:10.1038/35021093
3. 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
4. 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
5. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkila P, Heikkinen T, Nevanlinna H, Akslen LA, Begin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, Garcia-Closas M, Caldas C, Pharoah PD, Huntsman D (2010) Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 7(5):e1000279. doi:10.1371/journal.pmed.1000279