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Advances in Brief

Immunoreactive Hepatocyte Growth Factor Is a Strong and Independent Predictor of Recurrence and Survival in Human Breast Cancer[1]

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Abstract

Hepatocyte growth factor (HGF) is a stromally derived modulator of epithelial cell proliferation and motility. In the present study, we have measured immunoreactive (ir)-HGF concentration in tumor extracts of 258 primary human breast cancers using an enzyme-linked immunosorbent assay and have evaluated its association with disease-free and overall survival. The median value of ir-HGF concentration was 11.0 ng/100 mg protein (range, 1.4–566.7 ng/100 mg protein). Correlation analyses between ir-HGF concentration and clinicopathological factors showed that the ir-HGF level was correlated only with tumor size ($P = 0.05$). No significant associations were found between ir-HGF content and age, menopausal status, nodal status, histological type, histological grade, vessel involvement, estrogen receptor, progesterone receptor, type of surgery, or postoperative adjuvant therapy. Breast cancer patients with high ir-HGF concentration had a significantly shorter relapse-free ($P = 0.001$) and overall survival ($P = 0.001$) rate when compared to those with low ir-HGF concentration at the cutoff point of 21.7 ng/100 mg protein, which was determined in another group of 82 patients. In multivariate analysis, ir-HGF level was found to be the most important independent factor in predicting relapse-free and overall survival, of greater import than lymph node involvement. The putative role of HGF in breast cancer growth and metastasis is hereby strengthened.

Introduction

HGF[3] was purified from human and rabbit plasma and rat platelets on the basis of its ability to stimulate mitogenesis of mature hepatocytes (1–3). HGF was initially considered to have a narrow target-cell specificity and merely act as a humoral mediator of liver regeneration. However, increasing evidence suggests that HGF is produced by mesenchymal cells and has more diverse biological activities on a variety of epithelial cells other than hepatocytes (4–6). Recently, the potential role of HGF in tumor progression has attracted attention because HGF affects both epithelial cell proliferation and motility (7).

Very recently, we have demonstrated that human breast cancer cells have no ability to produce ir-HGF, but a large amount of ir-HGF is present in tissue extracts from human breast cancer, which may presumably be derived from the stromal component of breast cancer tissues (8). Furthermore, Rubin *et al.* (9) reported that human mammary epithelial cells are particularly sensitive to the mitogenic effects of HGF, suggesting a possible role of this peptide as a paracrine mediator in the progression of human breast cancer. These observations prompted us to investigate the relationship between the tissue concentration of ir-HGF and the clinicopathological status in human breast cancer. We report here that patients with breast cancer containing high levels of ir-HGF have significantly shorter disease-free and overall survival times compared with those with low levels of ir-HGF.

Materials and Methods

Patients. Tumor specimens from 258 patients with primary breast cancer (132 node-negative and 126 node-positive) were evaluated in this study. These patients underwent curative operation (radical mastectomy, 95 patients; modified mastectomy, 163 patients) in the Department of Surgery II, Kumamoto University Hospital, during the 6-year period from 1981 to 1987. Routine follow-up of these patients after surgery consisted of clinical evaluation every 3 months for the first 2 years and every 6 months thereafter. Disease recurrence was documented by physical examination, radiological and laboratory tests, and/or other relevant diagnostic procedures. Median follow-up period was 8.2 years (range, 6.0–11.2 years) for patients with high ir-HGF level and 8.5 years (range, 6.0–11.8 years) for those with low ir-HGF level.

The clinicopathological parameters studied for prognostic value were tissue level of ir-HGF concentration, age, menopausal status, tumor size, lymph node involvement, histological type, histological grade, vessel involvement, ER, PgR, type of surgery and postoperative, adjuvant therapy. When histological typing was performed according to the WHO classification (10), all tumors in this study were classified into the same category, *i.e.*, invasive ductal carcinoma. Therefore, each tumor was further analyzed according to the classification of the Japanese Breast Cancer Society (11) and was graded in parallel according to the criteria described by Bloom and Richardson (12) except for 7 comedocarcinomas. ER and PgR were determined by the dextran-coated charcoal method as described previously (13).

Assay for ir-HGF. Frozen tissue (0.2 g) was homogenized and extracted with 50 mM Tris-HCl buffer (2 ml), pH 7.4, containing 0.25% Triton X-100 as described previously (14). The tissue extracts were stored at −80°C until ir-HGF assay. ir-HGF concentration was measured using an enzyme-linked immunosorbent assay kit (Otsuka Assay Laboratories, Tokushima, Japan), which is a sandwich method consisting of three steps as reported previously (15). The detection limit of this assay is 0.10 ng/ml.

Statistics. The correlation of various disease parameters was analyzed by the χ^2 test. The relapse-free survival and overall survival curves were generated by the Kaplan-Meier method (16). The significance of survival differences between the patient groups was calculated according to the log-rank test (17). Multivariate analysis using Cox's proportional hazard regression model (18) was carried out to assess the independent contribution of each variable to disease-free and overall survival.

Results

ir-HGF in Tumor Extracts. A serial dilution curve of tissue extracts exhibited parallelism with that of standard human HGF (data not shown). When standard HGF was added to two tissue samples at final concentrations of 0.313, 1.250, 2.500, and 5.000 ng/ml, the recoveries of HGF were 100.9 ± 1.4%, 99.1 ± 1.3%, 104.3 ± 3.7%, and 105.4 ± 6.1%, respectively. For estimation of the reproducibility of the assay, seven samples were chosen, and each sample was measured five times. The coefficient of variation of SD was within 2.2–5.4%.

Fig. 1 shows the distribution of ir-HGF concentration in 258 breast cancer tissues. The distribution was not normal, and there was the

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[3] The abbreviations used are: HGF, hepatocyte growth factor; ir-HGF, immunoreactive HGF; ER, estrogen receptor; PgR, progesterone receptor; LOH, loss of heterozygosity.

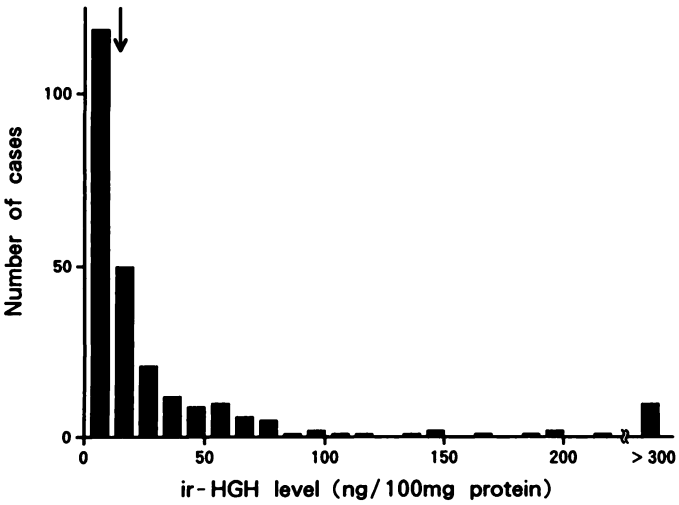

Fig. 1. Distribution of the 258 primary breast cancer tumors as a function of the tissue ir-HGF concentration. The class interval is 10 ng/100 mg protein. *Arrow*, the median value of 11.0 ng/100 mg protein.

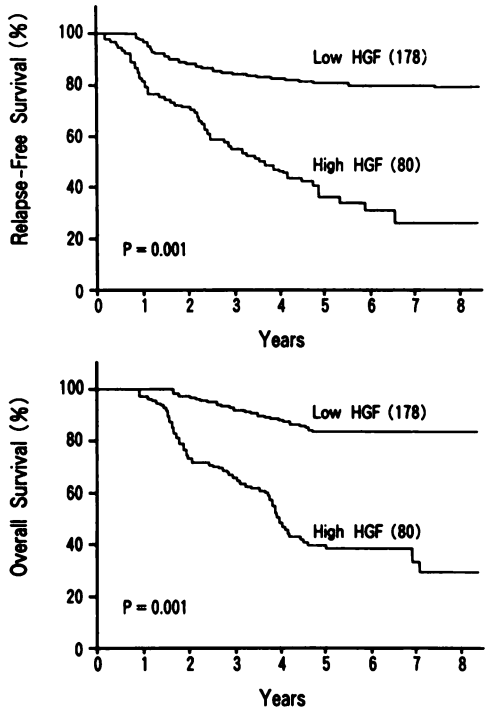

Fig. 2. Relapse-free and overall survival of 258 patients with breast cancer according to their ir-HGF levels. The cutoff value of ir-HGF was 21.7 ng/100 mg protein. Numbers in parentheses, the total number of patients per group.

median value of 11.0 ng/100 mg protein (range, 1.4–566.7 ng/100 mg protein). To evaluate ir-HGF as a prognostic factor in breast cancer, the cut-off point of 21.7 ng/100 mg protein was first determined in another group of 82 patients (8) to give the optimal separation between a low and high risk of relapse according to the method of Tandon *et al.* (19). This cutoff point identified 31% of the patients as having high ir-HGF levels in the present series.

Relations between dichotomized ir-HGF levels and other clinicopathological parameters were then determined. There was no significant association between levels of ir-HGF and age ($P = 0.61$), menopausal status ($P = 0.58$), nodal status ($P = 0.16$), histological type ($P = 0.40$), histological grade ($P = 0.53$), vessel involvement ($P = 0.72$), ER ($P = 0.66$), PgR ($P = 0.77$), type of surgery ($P = 0.64$), or type of adjuvant therapy ($P = 0.62$). Only tumor size (≥5.0 cm) was associated with high concentrations of ir-HGF ($P = 0.05$).

ir-HGF in Multivariate Prognostic Analysis. Fig. 2 shows disease-free and overall survival curves stratified by ir-HGF status. These curves visualize the increased hazard rates of our patients with ir-HGF-high primary tumors. In univariate analysis concerning relapse-free and overall survival rate, tumor size ($P = 0.036$; $P = 0.002$), lymph node involvement ($P = 0.002$; $P = 0.001$), histological grade ($P = 0.042$; $P = 0.063$), postoperative adjuvant therapy ($P = 0.042$; $P = 0.053$), and ir-HGF level ($P = 0.001$; $P = 0.001$) were significant prognostic factors in 258 breast cancer patients. Following the univariate study, multivariate analysis was conducted to test the independent prognostic role of these variables. When all variables were taken into account, through a stepwise analysis the model selected ir-HGF concentration as the single independent prognostic factor regarding relapse-free survival ($P = 0.001$; relative risk = 3.3) and overall survival (P = 0.001; relative risk = 3.5) (Table 1). Nodal status came next, short of significance ($P = 0.034$, relative risk = 2.5; and $P = 0.005$, relative risk = 2.1; respectively), whereas tumor size, histological grade, and postoperative adjuvant therapy were not significant. With respect to overall survival, the model added tumor size as a third variable for prediction ($P = 0.047$; relative risk = 1.4) (Table 1).

When the multivariate analysis was repeated separately within 132 node-negative and 126 node-positive groups, ir-HGF remained the most important variable for predicting disease-free ($P = 0.022$, relative risk = 3.5; and $P = 0.041$, relative risk = 2.8; respectively) and overall survival ($P = 0.008$, relative risk = 3.8; and $P = 0.036$, relative risk = 2.5; respectively). Then, we combined the two inde-

Table 1 *Multivariate Cox analysis of relapse-free survival and overall survival*

	Relapse-free survival		Overall survival	
	Relative risk	*P*	Relative risk	*P*
ir-HGF ≥21.7 *vs.* <21.7	3.3	0.001	3.5	0.001
Age ≥50 *vs.* <50		0.76		0.58
Menopausal status post *vs.* pre/peri		0.51		0.67
Tumor size ≥5.0 *vs.* <5.0		0.11	1.4	0.047
Node status n(+) *vs.* n(−)	2.5	0.034	2.1	0.005
Histological type[a] a_1 *vs.* a_2 *vs.* a_3		0.68		0.82
Histological grade[b] I *vs.* II *vs.* III		0.15		0.085
Vessel involvement present *vs.* absent		0.66		0.53
ER positive *vs.* negative		0.26		0.22
PgR positive *vs.* negative		0.17		0.19
Surgery radical *vs.* modified		0.54		0.61
Adjuvant therapy nonadjuvant *vs.* adjuvant		0.093		0.083

[a] Histological typing was performed according to the Japanese Breast Cancer Society (25). a_1, papillotubular carcinoma; a_2, solid-tubular carcinoma; a_3, scirrhous carcinoma.
[b] Seven comedocarcinomas were excluded from this analysis.

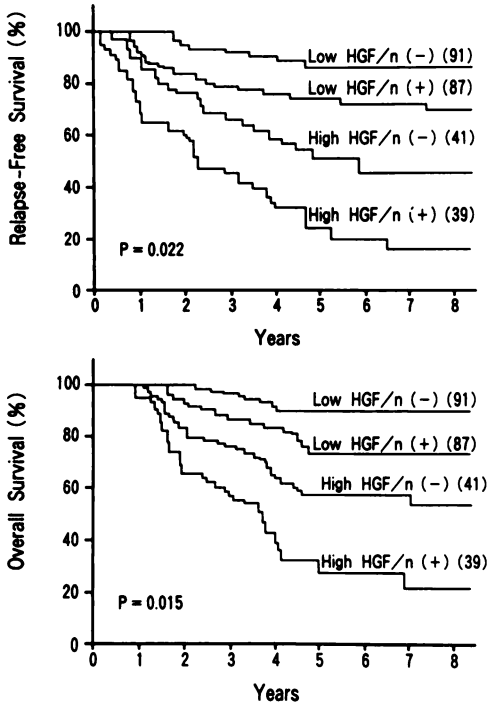

Fig. 3. Relapse-free and overall survival curves in patients with breast cancer. Four risk groups were separated according to lymph node status and ir-HGF concentration. The cut-off value of ir-HGF was 21.7 ng/100 mg protein. n(+) and n(−), the status of lymph node metastasis. Numbers in parentheses, the number of patients per group.

pendent prognostic factors, ir-HGF level and lymph node status, to define four risk groups. Patients with high ir-HGF concentrations had a poor relapse-free survival and overall survival; of these patients, the worst survival rate was seen in those with node-positive breast cancer and with high ir-HGF concentration. In contrast, patients with node-negative breast cancer and low ir-HGF concentration had better survival rates than did patients in any other groups (Fig. 3).

Discussion

Recent evidence suggests that HGF increases the invasiveness of cancer cells in an invasion assay system *in vitro* (20, 21) and also increases the motility and invasiveness of some cancer cells in cooperation with other factors (*e.g.*, fibroblast growth factors) *in vivo* (22). However, a few attempts have been made to examine the biological significance of HGF expressed in human breast cancer. Rubin *et al.* (9) reported that a broad-spectrum lung fibroblast-derived mitogen that is highly related to HGF elicits a significant stimulation of mitogenic-signaling tyrosine phosphorylation on B5/589 mammary epithelial cell lines. In the present study, we provide statistical evidence that HGF concentration in primary breast cancer tissue is a useful prognostic marker which identifies clearly high- and low-risk patients. Breast cancer patients with high ir-HGF concentration had a significantly shorter disease-free and overall survival rate when compared to those with low ir-HGF concentration. In multivariate analysis, HGF was found to be an independent and most powerful prognostic factor in breast cancer. Since the relative impact of prognostic factors on disease-free and overall survival reflects their respective role in tumor biology, we can speculate that HGF may play an important role in the progression of human breast cancer.

The mammary stroma is known to exercise a paracrine influence on the neighboring epithelial cell population (23). Recent experiments have suggested a potential importance of HGF in stromal epithelial communication in human breast cancer. Seslar *et al.* (24) reported that a delicate interaction may exist in fibroblast HGF expression between epithelial and mesenchymal compartments in human breast cancer depending upon the *in vivo* circumstances. HGF receptor is widely distributed in various epithelial cells including tumor cells but apparently not in mesenchymal cells (25). In contrast, HGF production was seen in the stromal component but not in the epithelial component of the breast (8, 24). Since it has been reported that HGF is a modulator of epithelial cell proliferation and motility for a broad spectrum of cell types (4–7), it seems conceivable that HGF produced by breast stromal cells plays a pathological role in facilitating breast cancer cell invasion and metastasis.

Several investigators reported that amplification of the c-*met* protooncogene that encodes the HGF receptor might participate in carcinogenesis and progression of human gastric cancer (26), colorectal cancer (27), and thyroid cancer (28). In contrast, Bieche *et al.* (29) demonstrated that patients with LOH on chromosome 7q31 (the c-*met* protooncogene locus) in primary breast cancer DNAs had significantly shorter disease-free survival and overall survival after surgery than patients without this alteration, indicating that the c-*met* protooncogene may act as a metastasis suppressor gene. This finding seems to be somewhat contradictory to our results. However, as it is not evident whether another allele of the c-*met* gene is also lost in the cases of LOH, normal or altered protein encoded by the remaining copy of this gene may be produced. In this context, c-*met* activity should be studied in such cases of LOH.

In conclusion, this is the first report showing that HGF concentration is an independent and most powerful prognostic factor in human breast cancer. This result supported the hypothesis that the overexpression of this peptide in human breast cancer tissue may play an active role in the tumor progression that leads to metastasis.

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References

1. Nakamura, T., Teramoto, H., and Ichihara, A. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. Proc. Natl. Acad. Sci. USA, *83:* 6489–6493, 1986.
2. Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S., and Daikuhara, Y. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J. Clin. Invest., *81:* 414–419, 1988.
3. Zarnegar, R., and Michalopoulos, G. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. Cancer Res., *49:* 3314–3320, 1989.
4. Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y., and Nakamura, T. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem. Biophys. Res. Commun., *174:* 831–838, 1991.
5. Matsumoto, K., Tajima, H., and Nakamura, T. Hepatocyte growth factor is a potent stimulator of human melanocyte DNA synthesis and growth. Biochem. Biophys. Res. Commun., *176:* 45–51, 1991.
6. Morimoto, A., Okamura, K., Hamanaka, R., Sato, Y., Shima, N., Higashio, K., and Kuwano, M. Hepatocyte growth factor modulates migration and proliferation of human microvascular endothelial cells in culture. Biochem. Biophys. Res. Commun., *179:* 1042–1049, 1991.
7. Gherardi, E., and Stoker, M. Hepatocytes and scatter factor. Nature (Lond.), *346:* 228, 1990.
8. Yamashita, J., Ogawa, M., and Beppu, T. Immunoreactive hepatocyte growth factor is present in tissue extracts from human breast cancer but not in conditioned medium of human breast cancer cell lines. Res. Commun. Chem. Pathol. Pharmacol., *82:* 249–252, 1993.
9. Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H., Taylor, W. G., Gech, A. C., Hirschfield, D. W., Wong, J., Miki, T., Finch, P. W., and Aaronson, S. A. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc. Natl. Acad. Sci. USA, *88:* 415–419, 1991.
10. World Health Organization. Histologic typing of breast tumours. *In:* International Histologic Classification of Tumours, No. 2. Geneva: World Health Organization, 1981.
11. Japan Mammary Cancer Society. Histologic classification of breast tumors. *In:* General Rule for Clinical and Pathological Record of Mammary Cancer, Ed. 9, pp. 21–57. Tokyo: Kanehara, 1988.
12. Bloom, H. J. G., and Richardson, W. W. Histologic grading and prognosis in breast

cancer. A study of 1049 cases of which 359 have been followed for 15 years. Br. J. Cancer, *11:* 359–377, 1957.
13. McGuire, W. L., De La Garza, M., and Chamness, G. C. Evaluation of estrogen receptor assays in human breast cancer tissue. Cancer Res., *37:* 637–639, 1977.
14. Yamashita, J., Horiuchi, S., Kimura, M., Nishimura, R., and Akagi, M. Plasminogen activator as a functional marker for estrogen dependence in human breast cancer cells. Jpn. J. Cancer Res., *77:* 177–181, 1986.
15. Tsubouchi, H., Niitani, Y., Hirono, S., Nakayama, H., Gohda, E., Arakaki, N., Sakiyama, O., Takahashi, K., Kimoto, M., Kawakami, S., Setoguchi, M., Tachikawa, T., Shin, S., Arima, T., and Daikuhara, Y. Levels of human hepatocyte growth factor in serum of patients with various liver diseases determined by an enzyme-linked immunosorbent assay. Hepatology (Baltimore), *13:* 1–5, 1991.
16. Kaplan, E. L., and Meier, P. Nonparametric estimation for incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.
17. Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Rep., *50:* 163–170, 1966.
18. Cox, D. R. Regression models and life tables (with discussion). J. R. Stat. Soc. Ser. B., *34:* 187–220, 1972.
19. Tandon, A. K., Clark, G. M., Chamness, G. C., Chirgwin, J. M., and McGuire, W. L. Cathepsin D and prognosis in breast cancer. N. Engl. J. Med., *322:* 297–302, 1990.
20. Weidner, K. M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J. Cell Biol., *111:* 2097–2108, 1990.
21. Rosen, E., Meromsky, L., Setter, E., Vinter, D. W., and Goldberg, I. D. Smooth muscle-derived factor stimulates mobility of human tumor cells. Invasion Metastasis, *10:* 49–64, 1990.
22. Yoshinaga, Y., Matsuno, Y., Fujita, S., Nakamura, T., Kikuchi, M., Shimosato, Y., and Hirohashi, S. Immunohistochemical detection of hepatocyte growth factor/scatter factor in human cancerous and inflammatory lesions of various organs. Jpn. J. Cancer Res., *84:* 1150–1158, 1993.
23. Oka, T., and Yoshimura, M. Paracrine regulation of mammary gland growth. J. Clin. Endocrinol. Metab., *15:* 79–97, 1986.
24. Seslar, S. P., Nakamura, T., and Stephen, W. B. Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. Cancer Res., *53:* 1233–1238, 1993.
25. Tajima, H., Matsumoto, K., and Nakamura, T. Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines. FEBS Lett., *291:* 229–232, 1991.
26. Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H., and Tahara, E. Frequent amplification of the c-*met* gene in scirrhous type stomach cancer. Biochem. Biophys. Res. Commun., *189:* 227–232, 1992.
27. Lui, C., Park, M., and Tsao, M-S. Overexpression of c-*met* proto-oncogene but not epidermal growth factor receptor or c-*erb*B-2 in primary human colorectal carcinomas. Oncogene, *7:* 181–185, 1992.
28. Di Renzo, M. F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Costantino, A., Vigneri, R., Pierotti, M. A., and Comoglio, P. M. Overexpression of the c-*met*/HGF receptor gene in human thyroid carcinomas. Oncogene, *7:* 2549–2553, 1992.
29. Bieche, I., Champeme, M. H., Matifas, F., Hacene, K., Callahan, R., and Lidereau, R. Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. Lancet, *339:* 139–143, 1992.

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