standard deviation of LN SUVmax was larger than that of LN size, and LN size did not differ between responders and non-responders. Various basic studies have revealed different gene expression patterns between PT and LN,[18,38] and the latter is often observed to be biologically more malignant than the former.[39,40] Taken together, these findings suggest that the SUVmax of LN is more closely associated with biological malignancy than size. Thus, it would be advantageous for NACT to be more effective against LN tumors with high SUVmax. Another topic of interest is whether the clinical significance of SUVmax reduction differed between PT and LN, although both are independent prognostic factors. The response of LN might be associated with distant recurrence, while the response of PT might be associated with local recurrence. Further studies with larger sample sizes are needed to resolve this question.

The primary objective of PET evaluation in NACT is to predict patient survival and determine treatment strategies. We have discussed here that 3 independent prognostic factors, namely the number of LN and the NACT response for PT and LN, may indicate the presence of micrometastases, control of PT, and eradication of micrometastases, respectively. Theoretically, a novel and detailed NACT strategy for advanced esophageal cancer could be planned based on these PET-based prognostic factors. We could employ a more aggressive NACT protocol[41] when many PET-positive LN are detected, use additional irradiation when SUVmax reduction in PT is poor, and introduce taxan-based second-line chemotherapy when the SUVmax reduction in LN is poor.[41-43] We caution against rash decisions to conduct surgical resection following NACT. If the possibility of recurrence is high, surgery may not be indicated for esophageal cancer, because the physical invasiveness of this surgery is extremely high and the growth of esophageal cancer is usually very rapid. This study involved analysis of squamous cell carcinoma, which is frequently observed in East Asian countries. Therefore, our results are not applicable to adenocarcinoma of the esophagus, which is the main type in Western countries. For example, the relationship between the pathological response and postoperative prognosis was reported to be different between squamous cell carcinoma and adenocarcinoma.[44-46] However, the two cancer types share common problems. For example, lymph node metastasis is one of the most significant prognostic factors in both tumors. Another issue is that, unfortunately, the sample number was small in the present study due to the new and limited use of PET in the cancer field; thus, accordingly, our findings should be confirmed in a large cohort prospective study. However, we hope the findings of this study will offer useful information for surgeons dealing with this virulent disease.

REFERENCES

1. Girvin GW, Matsumoto GH, Bates DM, et al. Treating esophageal cancer with a combination of chemotherapy, radiation, and excision. Am J Surg 1995;169:557-9.
2. Urschel JD, Vasan H. A meta-analysis of randomized controlled trials that compared neoadjuvant chemoradiation and surgery to surgery alone for resectable esophageal cancer. Am J Surg 2003;185:538-43.
3. Walsh TN, Noonan N, Hollywood D, et al. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. N Engl J Med 1996;335:462-7.
4. Doki Y, Ishikawa O. Takachi k, et al. Association of the primary tumor location with the site of tumor recurrence after curative resection of thoracic esophageal carcinoma. World J Surg 2005;29:700-7.
5. Matsuyama J, Doki Y, Yasuda T, et al. The effect of neoadjuvant chemotherapy on lymph node micrometastases in squamous cell carcinomas of the thoracic esophagus. Surgery 2007;141:570-80.
6. Kijima F, Natsugoe S, Takao S, et al. Detection and clinical significance of lymph node micrometastasis determined by reverse transcription-polymerase chain reaction in patients with esophageal carcinoma. Oncology 2000;58:38-44.
7. Kato H, Kuwano H, Nakajima M, et al. Comparison between positron emission tomography and computed tomography in the use of the assessment of esophageal carcinoma. Cancer 2002;94:921-8.
8. Kobori O, Kirihara Y, Kosaka N, Hara T. Positron emission tomography of esophageal carcinoma using (11)C-choline and (18)F-fluorodeoxyglucose: a novel method of preoperative lymph node staging. Cancer 1999;86:1638-48.
9. Yoon YC, Lee KS, Shim YM, et al. Metastasis to regional lymph nodes in patients with esophageal squamous cell carcinoma: CT versus FDG PET for presurgical detection prospective study. Radiology 2003;227:764-70.
10. van Westreenen HL, Westerterp M, Bossuyt PM, et al. Systematic review of the staging performance of 18F-fluorodeoxyglucose positron emission tomography in esophageal cancer. J Clin Oncol 2004;22:3805-12.
11. Choi JY, Lee KH, Shim YM, et al. Improved detection of individual nodal involvement in squamous cell carcinoma of the esophagus by FDG PET. J Nucl Med 2000;41:808-15.
12. Brucher BL, Weber W, Bauer M, et al. Neoadjuvant therapy of esophageal squamous cell carcinoma: response evaluation by positron emission tomography. Ann Surg 2001; 233:300-9.
13. Flamen P, Van Cutsem E, Lerut A, et al. Positron emission tomography for assessment of the response to induction radiochemotherapy in locally advanced oesophageal cancer. Ann Oncol 2002;13:361-8.
14. Downey RJ, Akhurst T, Ilson D, et al. Whole body 18FDG-PET and the response of esophageal cancer to induction therapy: results of a prospective trial. J Clin Oncol 2003; 21:428-32.
15. Swisher SG, Erasmus J, Maish M, et al. 2-Fluoro-2-deoxy-D-glucose positron emission tomography imaging is predictive of pathologic response and survival after preoperative

chemoradiation in patients with esophageal carcinoma. Cancer 2004;101:1776-85.
16. Takayama N, Arima S, Haraoka S, et al. Relationship between the expression of adhesion molecules in primary esophageal squamous cell carcinoma and metastatic lymph nodes. Anticancer Res 2003;23:4435-42.
17. Aiko S, Yoshizumi Y, Ishizuka T, et al. Reduction rate of lymph node metastasis as a significant prognostic factor in esophageal cancer patients treated with neoadjuvant chemoradiation therapy. Dis Esophagus 2007;20:94-101.
18. Varambally S, Yu J, Laxman B, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell 2005;8:393-406.
19. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518-24.
20. Sobin LH, Wittekind C. TNM classification of malignant tumors. 6th ed. New York: John Wiley and Sons, Inc; 2002.
21. Yano M, Takachi K, Doki Y, et al. Preoperative chemotherapy for clinically node-positive patients with squamous cell carcinoma of the esophagus. Dis Esophagus 2006;19:158-63.
22. Akita H, Doki Y, Miyata H, et al. Clinical significance of the second cycle response to cisplatin-based chemotherapy as preoperative treatment for esophageal squamous cell carcinoma. J Surg Oncol 2006;93:401-9.
23. The National Cancer Center Institute Common Toxicity Criteria version 2.0
24. Miyata H, Yano M, Doki Y, et al. A prospective trial for avoiding cervical lymph node dissection for thoracic esophageal cancers, based on intra-operative genetic diagnosis of micrometastasis in recurrent laryngeal nerve chain nodes. J Surg Oncol 2006;93:477-84.
25. Paul AK, Tatsumi M, Higuchi I, et al. Gamma camera coincidence imaging with [18F]fluorodeoxyglucose in the pretreatment evaluation of patients with oesophageal cancer. Nucl Med Commun 2003;24:963-70.
26. Tatsumi M, Yutani K, Watanabe Y, et al. Feasibility of fluorodeoxyglucose dual-head gamma camera coincidence imaging in the evaluation of lung cancer: comparison with FDG PET. J Nucl Med 1999;40:566-73.
27. Fukunaga H, Sekimoto M, Tatsumi M, et al. Clinical relevance of fusion images using (18)F-2-fluoro-2-deoxy-D-glucose positron emission tomography in local recurrence of rectal cancer. Int J Oncol 2002;20:691-5.
28. Dittler HJ, Fink U, Siewert GR. Response to chemotherapy in esophageal cancer. Endoscopy 1994;26:769-71.
29. Jones DR, Parker LA Jr, Detterbeck FC, Egan TM. Inadequacy of computed tomography in assessing patients with esophageal carcinoma after induction chemoradiotherapy. Cancer 1999;85:1026-32.
30. Jager PL, Que TH, Vaalburg W, et al. Carbon-11 choline or FDG-PET for staging of oesophageal cancer? Eur J Nucl Med 2001;28:1845-9.
31. Flamen P, Lerut A, Van Cutsem E, et al. Utility of positron emission tomography for the staging of patients with potentially operable esophageal carcinoma. J Clin Oncol 2000;18:3202-10.
32. Ando N, Iizuka T, Ide H, et al. Surgery plus chemotherapy compared with surgery alone for localized squamous cell carcinoma of the thoracic esophagus: a Japan Clinical Oncology Group Study–JCOG9204. J Clin Oncol 2003;21:4592-6.
33. Group MRCOCW. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 2002;359:1727-33.
34. Cunningham D, Allum WH, Stenning SP, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med 2006;355:11-20.
35. Gebski V, Burmeister B, Smithers BM, et al. Survival benefits from neoadjuvant chemoradiotherapy or chemotherapy in oesophageal carcinoma: a meta-analysis. Lancet Oncol 2007;8:226-34.
36. Eisenhauer EA, Vermorken JB, van Glabbeke M. Predictors of response to subsequent chemotherapy in platinum pretreated ovarian cancer: a multivariate analysis of 704 patients. Ann Oncol 1997;8:963-8.
37. Heinmoller E, Renke B, Beyser K, et al. Piffalls in diagnostic molecular pathology–significance of sampling error. Virchows Arch 2001;439:504-11.
38. Lahdesmaki H, Hao X, Sun B, et al. Distinguishing key biological pathways between primary breast cancers and their lymph node metastases by gene function-based clustering analysis. Int J Oncol 2004;24:1589-96.
39. Matheus RS, Bernardi Fdel C, Gallo CP, et al. Nuclear markers (star volume, mitotic index, AgNOR and Ki-67) of the primary tumor and its metastasis in non-small cell lung carcinomas. Pathol Res Pract 2004;200:13-23.
40. Ferrari S, Bertoni F, Zanella L, et al. Evaluation of P-glycoprotein, HER-2/ErbB-2, p53, and Bcl-2 in primary tumor and metachronous lung metastases in patients with high-grade osteosarcoma. Cancer 2004;100:1936-42.
41. Ajani JA, Fodor MB, Tjulandin SA, et al. Phase II multi-institutional randomized trial of docetaxel plus cisplatin with or without fluorouracil in patients with untreated, advanced gastric, or gastroesophageal adenocarcinoma. J Clin Oncol 2005;23:5660-7.
42. Yoshioka T, Sakayori M, Kato S, et al. Dose escalation study of docetaxel and nedaplatin in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated using cisplatin, 5-fluorouracil, and radiation. Int J Clin Oncol 2006;11:454-60.
43. Armanios M, Xu R, Forastiere AA, et al. Adjuvant chemotherapy for resected adenocarcinoma of the esophagus, gastro-esophageal junction, and cardia: phase II trial (E8296) of the Eastern Cooperative Oncology Group. J Clin Oncol 2004;22:4495-9.
44. Rohatgi PR, Swisher SG, Correa AM, et al. Comparison of clinical stage, therapy response, and patient outcome between squamous cell carcinoma and adenocarcinoma of the esophagus. Int J Gastrointest Cancer 2005;36:69-76.
45. Rohatgi PR, Swisher SG, Correa AM, et al. Histologic subtypes as determinants of outcome in esophageal carcinoma patients with pathologic complete response after preoperative chemoradiotherapy. Cancer 2006;106:552-8.
46. Siewert JR, Ott K. Are squamous and adenocarcinomas of the esophagus the same disease? Semin Radiat Oncol 2007;17:38-44.

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Integrative approach for differentially overexpressed genes in gastric cancer by combining large-scale gene expression profiling and network analysis

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Gene expression profiling is a valuable tool for identifying differentially expressed genes in studies of disease subtype and patient outcome for various cancers. However, it remains difficult to assign biological significance to the vast number of genes. There is an increasing awareness of gene expression profile as an important part of the contextual molecular network at play in complex biological processes such as cancer initiation and progression. This study analysed the transcriptional profiles commonly activated at different stages of gastric cancers using an integrated approach combining gene expression profiling of 222 human tissues and gene regulatory dynamic mapping. We focused on an inferred core network with *CDKN1A* (*p21$^{WAF1/CIP1}$*) as the hub, and extracted seven candidates for gastric carcinogenesis (*MMP7, SPARC, SOD2, INHBA, IGFBP7, NEK6, LUM*). They were classified into two groups based on the correlation between expression level and stage. The seven genes were commonly activated and their expression levels tended to increase as disease progressed. NEK6 and INHBA are particularly promising candidate genes overexpressed at the protein level, as confirmed by immunohistochemistry and western blotting. This integrated approach could help to identify candidate players in gastric carcinogenesis and progression. These genes are potential markers of gastric cancer regardless of stage.
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Gastric cancer remains a major cause of cancer deaths worldwide despite early detection and curative surgery. Prognosis is favourable in early-stage disease with 5-year survival rates of 90% reported following gastrectomy and lymph node dissection. In contrast, patients diagnosed with advanced-stage cancer have 5-year survival rates of 20–30%, and the overall poor survival outcome for gastric cancer is attributed to these patient populations (Dicken *et al*, 2005). An efficient system for detecting disease status in gastric cancer regardless of its clinical stage is clearly needed to improve overall survival.

Gastric cancer is routinely classified according to the tumour-node-metastasis parameters of the primary tumour, lymph nodes, and metastasis. This classification helps the clinician to stage the tumour and develop a management strategy, as well as to provide an indication of prognosis. However, this conventional classification is not strong enough to predict individual prognosis, rendering uniform adjuvant therapy of limited value because of unnecessary adverse events. The use of molecular markers or gene profiling coupled with multivariate predictive models is designed to attain more accurate prognostic models. Recent molecular analyses revealed that gastric cancers closely associate with alterations in several interesting genes, such as p53 (Tamura *et al*, 1991; Uchino *et al*, 1993), p21 (Czerniak *et al*, 1989), c-met (Kaji *et al*, 1996), TGF-β (Park *et al*, 1994; Nakamura *et al*, 1998), and β-catenin (Park *et al*, 1999). However, these single candidate molecules yield different results among studies and the available data are unconvincing. Thus, the potential use of combinations of multiple markers instead of a single marker has been previously commented upon for the understanding of cancer biology or the prediction of patient prognosis (Lee *et al*, 2007).

The past decade has seen a revolution in high-throughput technologies for molecular profiling in cancer research. Particularly, gene expression profiling has enabled researchers to quantify biological states and consequently uncover subtle phenotypes important in cancer. Such analyses of tumour tissues have provided unique opportunities to develop profiles that can distinguish, identify, and classify discrete subsets of disease, predict the disease outcome, and even predict the response to therapy (Golub *et al*, 1999; Perou *et al*, 2000; van 't Veer *et al*, 2002; van de Vijver *et al*, 2002; Pittman *et al*, 2004). For example, expression profiling in gastric cancer identified novel target molecules involved in gastric carcinogenesis by comparing cancerous and healthy tissues (Boussioutas *et al*, 2003; Kim *et al*, 2003, 2005).

Despite their potential power, gene expression profiling has major limitations. Interpreting the significance of identified genes

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without any unifying biological theme can be difficult, makeshift, and dependent on the biologist's area of expertise. It is frequently challenging to understand a specific regulatory network involving enormous numbers of proteins. Furthermore, an approach that ignores biological cues may generate poor reproducibility among different studies of the same biological system. To overcome these analytical challenges, several recent studies have focused on phenotypic analysis of primary tumours using gene expression profiling, with a view to further understanding the roles of signalling pathways deregulated by the oncogenic process (Rhodes and Chinnaiyan, 2005; Rhodes *et al*, 2005).

This study sought to identify transcriptional profiles commonly activated across a wide range of stages in gastric cancer, as well as core networks in gastric carcinogenesis. It used an integrated approach combining gene expression profiling of over 200 human tissues with dynamic gene mapping. We identified seven candidates among the network that reflected essential transcriptional features of neoplastic transformation and progression, and validated these quantitatively by real-time reverse transcription (RT)-PCR. We also evaluated the expression of the encoded proteins in gastric cancer tissues by immunohistochemistry and western blotting, and identified novel potential markers for detecting gastric cancers.

MATERIALS AND METHODS

Tissue samples

Samples were obtained from 222 patients with gastric cancer who underwent curative resection at the following institutions: Osaka University Hospital, National Osaka Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, Sakai Municipal Hospital, Toyonaka Municipal Hospital, Mino Municipal Hospital, NTT West Osaka Hospital, Kinki Central Hospital, Suita Municipal Hospital, and Kansai Rosai Hospital. None of the patients received chemotherapy or radiotherapy before surgery. Tissues were evaluated macroscopically and microscopically according to the general rules for gastric cancer study in surgery and pathology in Japan. All cancers showed a depth of invasion beyond the subserosa. The clinical and pathological features are listed in Table 1. All aspects of our study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government, and each subject provided informed consent.

Extraction of RNA and quality assessment

The tumour specimens were cut into pieces (approximately 8 mm^3) within 2 h after surgical resection and stored in RNAlater™ (Ambion, Austin, TX) at −80°C until use. Total RNA was purified from clinical samples using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the protocol supplied by the manufacturer. RNA integrity was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNAs with intact 18S and 28S sequences were used for the subsequent analysis. Fifteen RNA samples extracted from normal gastric epithelium were mixed as a reference control.

Table 1 Clinical and pathological features of 222 patients

Age (years) median (range)	68 (23–92)
Sex (male/female)	156:66
Location	
Upper	62
Middle	70
Lower	90
Histopathological type	
Differentiated	102
Undifferentiated	120
Pathological stage	
I	30
II	58
III	81
VI	53

Preparation of fluorescently labelled aRNA targets and hybridisation

Extracted RNA samples were amplified with T7 RNA polymerase using the Amino Allyl MessageAmp™ aRNA kit (Ambion) according to the protocol provided by the manufacturer. The quality of each Amino Allyl-aRNA sample was checked on the Agilent 2100 Bioanalyzer. Five μg of control and experimental aRNA samples were labelled with Cy3 and Cy5, respectively, mixed, and then hybridised on an oligonucleotide microarray covering 30 000 human probes (AceGene Human 30K; DNA Chip Research and Hitachi Software Engineering Co, Yokohama, Japan). The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. The microarrays were scanned using a ScanArray 4000 (GSI Lumonics, Billerica, MA, USA).

Analysis of microarray data

Signal values were calculated by DNASISArray software (Hitachi, Tokyo). Following background subtraction, data with low signal intensities were excluded from additional investigation. In each sample, the Cy5/Cy3 ratio values were log-transformed and globally equalised to remove deviation of the signal intensity between whole Cy3- and Cy5-fluorescence by subtracting the median of all log (Cy5/Cy3) values from each log (Cy5/Cy3) value. Supplementary information is available on our website (http://www.dna-chip.co.jp/).

Network analysis

The Ingenuity Pathway (INGP) analysis was used to depict several networks in gastric cancer. The INGP software is a web-delivered application that enables biologists to discover, visualise, and explore therapeutically relevant networks significant to gene expression data sets. A detailed description of INGP analysis is available at Ingenuity Systems website (http//www.ingenuity.com). The average $\log_2$ expression values were used to calculate the fold change between gastric cancer and normal epithelium. The data set containing gene identifiers and their corresponding expression values were then uploaded into the INGP as a tab-delimited text file for analysis. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base.

To understand how the genes identified by inferential statistics are related as focus genes, we uploaded the target genes into the Ingenuity Knowledge Base and generated several networks. On the basis of focus genes, new and expanded pathway maps, connections, and specific gene-gene interactions were inferred, functionally analysed, and used to build on the existing pathway knowledge base. To generate networks, the knowledge base was queried for interactions between focus genes and all other gene objects stored therein. The output, displayed graphically as nodes (genes) and edges (the biological relationship between the nodes), represented a significantly consistent number of biological pathways and functions implicated by the empirical data sets.

RT reaction

Complementary DNAs (cDNAs) were generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) using the protocol recommended by the manufacturer. Briefly, 1 μg of RNA was mixed with RT reagents including oligo-$(dT)_{15}$ primer and incubated at 42°C for 15 min, followed by heating at 95°C for 5 min for enzyme inactivation.

Quantitative RT-PCR with the LightCycler™

To validate the microarray data, quantitative PCR was performed using real-time PCR with a LightCycler (Idaho Tech, ID, USA). PCR reagents contained 1x LightCycler DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.2 μM of each primer, 3 mM $MgCl_2$, and 2 μl of cDNA template. PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62°C for 5 s, and 72°C for 10 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified quantitatively at the same time to verify the integrity of RNA and to improve the diagnostic quality of the technique. The intensity of fluorescence was calculated at each cycle and a standard curve was constructed with 10-fold serial dilutions of cDNA obtained from the mixture of normal gastric epitheliums. The primer sequences for PCR amplification are listed below: *MMP7* forward primer, 5′→GTCTCGGAGGAGATGC TCAC→3′ and reverse, 3′→GAGGAATGTCCCATACCC→5′; *SPARC* forward primer, 5′→CATTGACGGGTACCTCTCCC→3′ and reverse, 3′→CGATATCCTTCTGCTTGATGC→5′; *INHBA* forward primer, 5′→ATCATTGCTCCCTCTGGCTA →3′ and reverse, 3′→ ACGATTTGAGGTTGGCAAAG→5′; *IGFBP7* forward primer, 5′→AAGTAACTGGCTGGGTGCTG→3′ and reverse, 3′→TATAGCTCGGCACCTTCACC →5′; *NEK6* forward primer, 5′→TGTCTGCTGTACGAGATGGC→3′ and reverse, 3′→GATGC ACATGCTGACCAGTT→5′; *LUM* forward primer, 5′→GACATAA AGAGCTTCTGCAA→3′ and reverse, 3′→TTGTTCCAGGATA CAGATATT→5′; *SOD2* forward primer, 5′→GCAAGGAACA ACAGGCCTTA→3′ and reverse, 3′→CAGCATAACGATCGTG GTTT→5′; *GAPDH* forward primer, 5′→CAACTACATGGTTTAC ATGTTC →3′ and reverse, 3′→GCCAGTGGACTCC ACGAC →5′.

Immunohistochemistry

Sections (3.5-μm thick) were deparaffinised in xylene and rehydrated. They were subjected to immunohistochemical analysis using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA, USA). The tissue sections were incubated overnight with the primary antibodies; anti-human INHBA (Serotec, Oxford, UK; 1:300 dilution) and anti-human NEK6 (GeneTex, San Antonio, TX; 1:200 dilution), at 4°C. Negative control staining was performed with the use of normal mouse or goat IgG instead of the primary antibody, yielding negative results in all patients.

Western blotting

Frozen tumour and noncancerous tissues were homogenised in 0.5 ml radioimmunoprecipitation assay buffer (25 mmol l^{-1} Tris (pH 7.4), 50 mmol l^{-1} NaCl, 0.5% sodium deoxycholate, 2% NP40, and 0.2% SDS) containing protease inhibitors (1 mmol l^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} leupeptin). The homogenate was centrifuged at 12 000 g for 20 min at 4°C. The resulting supernatant was collected and total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Then, 100 μg of the total protein was premixed with loading buffer (0.05 mol l^{-1} Tris-HCl (pH 6.8), 2% SDS, 0.2 mol l^{-1} n-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue), boiled for 5 min, and subjected to SDS-PAGE on 10% gels. Proteins were then transferred onto polyvinylidene difluoride membrane (Boehringer Mannheim) using a transblot apparatus in a buffer containing 0.02 mol l^{-1} Tris-HCl (pH 8.3), 0.2 mol l^{-1} glycine, and 20% methanol. After blocking in 10% skim milk, the membrane was incubated overnight with anti-human INHBA (1:200 dilution), anti-human NEK6 (Abgent, San Diego, CA, USA; 1:500 dilution) at 4°C, or anti-actin (Sigma-Aldrich, St Louis, MO, USA; 1:1000 dilution) for 1 h at room temperature. After three washes each for 10 min with TBS (0.02 mol l^{-1} Tris-HCl (pH 7.5) and 0.1 mol l^{-1} NaCl) containing 0.2% Tween 20, the filter was incubated with secondary antibody at 1:1000 dilution. The protein bands were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA) according to the instructions supplied by the manufacturer.

RESULTS

Analysis of microarray data

The gene expression profiles of 222 primary gastric cancers were analysed on a 30K oligonucleotide DNA microarray. Of the full gene sequences (29 638 expressed genes excluding control spots), 271 (0.9%) genes showed >1.5-fold change in differential expression in at least 100 samples. Among these 271 genes, 50 had been described previously in gastric cancers, whereas 187 genes were previously not described in gastric cancer and 34 genes were categorised into ESTs (expressed sequence tags).

Network analysis

Analysis of the commonly overexpressed 271 genes using the Ingenuity Knowledge Base generated several networks that identified 203 genes as focus genes. The knowledge base generated 17 networks composed of focus genes and all other gene objects stored in the base (Table 2). On the basis of overlapping networks, network-5 was found to be central (Supplementary Figure 1). The centred network-5 (network-5 and close relevant networks) included a substantial number of genes already implicated in gastric carcinogenesis (Figure 1), with numerous focus genes connected by several neighbourhood genes. Furthermore, the network analysis mapped *CDKN1A* ($p21^{WAF1/CIP1}$) to the core of the centred network-5, acting as a hub by interacting with surrounding focus genes. *CDKN1A* is associated with disease progression and prognosis in gastric cancer (Czerniak *et al*, 1989; Kasper *et al*, 1998).

We selected seven focus genes showing >2-fold change in differential expression for further analysis. Three of these are known to be involved in gastric cancer: *MMP7* (Yamashita *et al*, 1998), *SPARC* (Wang *et al*, 2004), and *SOD2* (Janssen *et al*, 2000), and the other four have no such reported associations (*INHBA, IGFBP7, NEK6,* and *LUM*).

Correlation between activation of candidate gene and pathological stage

To assess the clinical significance of each and common activation of the seven genes, we correlated microarray expression level and pathological stage. By comparing the expression level of each gene in early stage (stages I and II) and late stage (III and IV), we found that they could be classified into two groups: group 1 consisted of *MMP7, IGFBP7,* and *NEK6*; their expression levels correlated significantly with pathological stage ($P = 0.0087$, 0.01, and 0.0085, respectively, Student's *t*-test) (Figure 2A–C), whereas the expression levels of genes of group 2 (*SOD2, SPARC, LUM, INHBA*) showed no such correlation ($P = 0.25$, 0.6, 0.86 and 0.32, respectively, Student's *t*-test) (Figure 2E–H). Interestingly, the mean

Table 2 Seventeen networks identified in the data set

ID	Molecules in network	Score	Focus molecules
1	AEBP1, Ap1, APOC1, APOC2, APOE, BCL2A1, BID, CCL20, COX2, COX3, CTSL1, GDF15, GLA, HSPE1, IL1, IL32, INDO, LDL, LTA, LY96, MEOX2, MGP, MMP1, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFκB, NR4A2, PDGF, Rar, RIPK2, Rxr, SERPINF1, SOD2, STK10, TNF receptor, TNFSF13B	45	26
2	Akt, COL1A1, CSE1 L, CXCL10, CYR61, FAP, FBN1, Fibrin, FN1, IFN-γ, Igfbp, IGFBP7, INHBA, Integrin, ITGB2, LTBP2, MIF, MMP, MMP7, MMP9, MMP12, PCOLCE, PI3K, PLAUR, PRKAA1, SLC3A2, SPARC, SULF1, TGFβ, TGFBI, THBS1, THY1, TIMP1, VCAN, VEGF	45	26
3	ACP5, ACTN1, ADORA3, AGXT, AIF1, CEBPB, CKS1B, CLEC4E, COL10A1, COL1A2, COL3A1, CREB, CREM, Cyclin A, Cyclin E, DNAJA1, E2f, ERK1/2, Histone h3, HLA-DPA1, HLA-DPB1, IFITM3, MAPK, MHC2α, PCNA, PKA, PTTG1, RFC4, SKP2, SPP1, STMN1, TGFBR1, UBE2C, Vitamin D3-VDR-RXR, ZNF160	43	25
4	C13ORF15, CACYBP, CDC2, CDKN3, COL4A1, FCER1G, FCGR2A, FCGR2B, FCGR3A, FOXM1, FPR1, GZMB, HOMER1, IGE, JNK, LAMA4, LAMB1, LAMC1, LGALS1, MAD2L1, MEK, MEK1/2, NFAT, P38 MAPK, PKC(s), PLA2G7, Rac, RAN, RANBP1, Ras, RGS1, Rsk, SRGN, TCR, UBD	41	24
5	Ap1, BUB3, CDKN1A, CKS1B, CKS2 (includes EG:1164), CLEC2B, E2F4, epinephrine, F9, fructose-2,6-diphosphate, GCNT1, HMCN1 (includes EG:83872), HMGB3, HSPE1, IGF1R, IL15, INS1, KIAA0101, LGALS3BP, LUM, MAD2L1, NEK6, NPHS2, PBK, PDCD5, RPS21, S100A11 (includes EG:6282), SOD2, SPAG5, SPARC, ST8SIA1, STMN1, UBE2T, VKORC1, ZNF84	31	20
6	Actin, ASB2, ATP6, ATP2B1, ATP5E, ATP6V1F, Caspase, CD163, Ck2, CLNS1A, F Actin, GEMIN5, H+-transporting two-sector ATPase, Insulin, JUB, LMNA, NEXN, PDGF BB, PFDN1, PFDN2, PFDN4, PFDN6, PLC, POLR2K, RNA polymerase II, RNU1B, S100A11 (includes EG:6282), SNRPD1, SNRPE, SNRPF, SNRPG, TCEB1, Ubiquitin, UCHL1, VBP1	27	18
7	ACP5, ARF4, BUB1 (includes EG:699), C1ORF164, C20ORF24, CCR6, CCT3, CCT4, CCT5, CCT7, CCT8, CCT6A, CPNE3, CTSB, CTSK, DAPK1, DEFB103A, EBNA1BP2, FCGR3A, FGFR, HTRA1, IFI30, IL4, IL10RA, ITGB7, keratan sulphate, MBP, MRPS10, MYL6, NAB2, NNMT, PRSS3 (includes EG:5646), TFF3, TGFB1, TUBA1A	27	18
8	CDK10, GBP4 (includes EG:115361), GPNMB, GPR109B, HLA-DPB1, HLA-DRA, IFI30, IFITM1, IFNα, IFNβ, IFNAR1, IFNB1, IFNG, IFNK, ILF3, KIR2DL3, KIR2DS2, POMP, PTEN, PTP4A3, RARRES1, retinoic acid, RFX1, RFX5, RFXANK, RFXAP, RPS19, RPS20, SERPINA5, STX5, TBCB, TMSB10, TREM2, TREM3, TRIM22, TYROBP	25	17
9	CKLF, CLDN16, F2, GABRD, GGH, LAMP1, LAMP2, LEPRE1, MYC, MYCN, PAICS, PRDM5, Proteasome PA700/20s, PSMA, PSMA1, PSMA2, PSMA4, PSMA5, PSMA6, PSMA7, PSMB1, PSMB2, PSMB3, PSMB4, PSMB5, PSMB6, PSMB7, PSMD6, PSMD14, RPL31, RPL37, RPS19, RPS20, RPS27, TUBA1B	19	14
10	β-estradiol, BTK, dihydrotestosterone, EXOC1, EXOC2, EXOC3, EXOC5, EXOC7, EXOC8, FGF7, FGFR2, FSHB, GTP, GUCY1A3, INPP5F, ITGBL1, MLLT1, MME, NFE2L3, NME1, NME2, NUDT1, phosphatidylinositol-3,4,5-trisphosphate, PIB5PA, PRUNE, RAP1B, RND3, RRAD, SEC61G, SFRP2, SLC7A8, SLCO3A1, SPARC, TMEPAI, VHL	17	13
11	ABCB4, ACO1, ASXL1, ASXL2, ASXL3, C7ORF24, CBX2, CDKN2A, COL1A1, EZH2, FOS, FST, FTH1, FTL, FTMT, GAL, GNRHR, HIF1A, HIG2, iron, KNG1 (includes EG:3827), LOX, MELK, MYH4, NTF3, PCGF1, PCGF6, PCNX, PDGF Ab, PHF1, PHF19, progesterone, RPS6, SFRP4, TGFBI	12	10
12	ATP9B, Mg^{2+}-ATPase	2	1
13	*N*-acetylglucosaminylphosphatidylinositol deacetylase, PIGL	2	1
14	Mrlc, MYLIP	2	1
15	EXOSC4, LRRC8D	2	1
16	GDP-Gnat2-Gngt2-Transducin β (cone), GNGT2, Gngt2-transducin beta (cone)	2	1
17	ADP-D-mannose, ADP-D-ribose, ADP-sugar diphosphatase, ADPribose diphosphatase, AMP, D-ribose-5-phosphate, nucleoside-diphosphatase, NUDT5	1	1

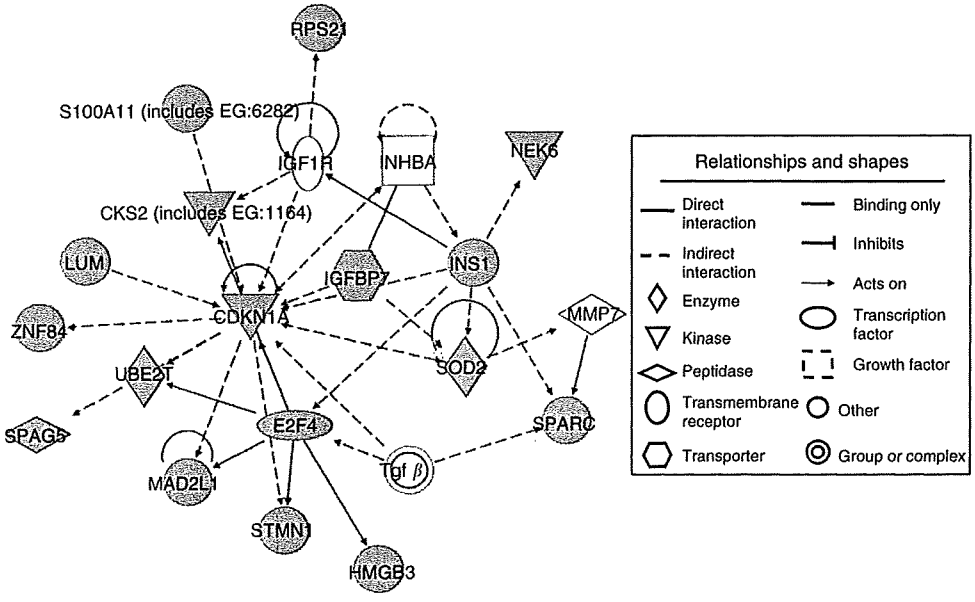

Figure 1 Inferential core network (network-5 and its close relevant networks) comprising many focus genes and several neighbourhood genes that connect the focus genes. Greyed nodes are part of network-5.

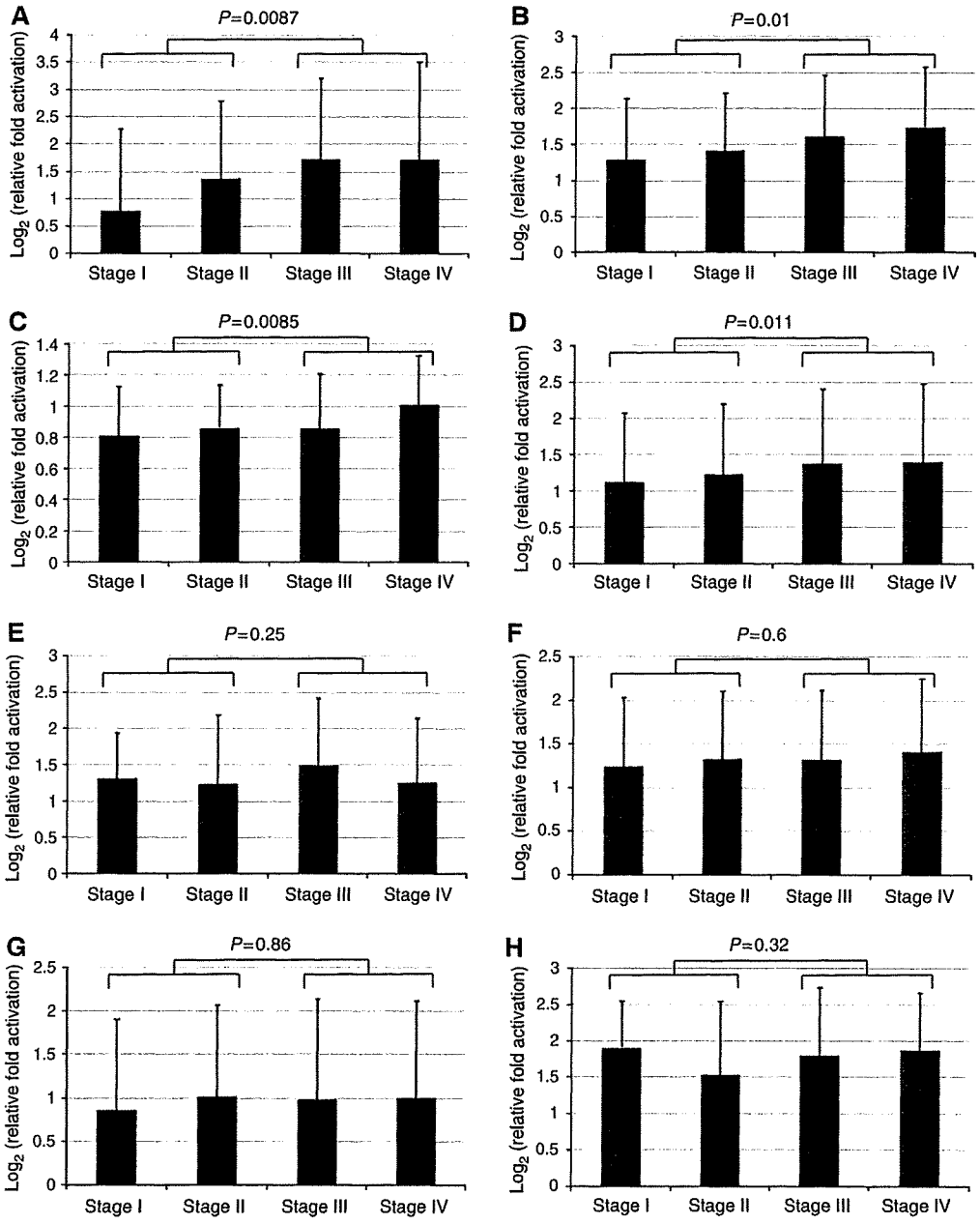

Figure 2 Correlation between activation of each candidate gene and pathological stage ($n = 222$). The expression levels of genes of group 1 ((**A**) *MMP7*, (**B**) *IGFBP7*, (**C**) *NEK6*) correlated significantly with pathological stage ($P = 0.0087$, 0.01, and 0.0085, respectively). The mean expression of the seven genes also correlated with pathological stage ($P = 0.011$) (**D**). The expression levels of genes of group 2 ((**E**) *SOD2*, (**F**) *SPARC*, (**G**) *LUM*, (**H**) *INHBA*) did not correlate significantly with pathological stage ($P = 0.25$, 0.6, 0.86, and 0.32, respectively).

expression of the seven genes correlated with the pathological stage ($P = 0.011$) (Figure 2D).

Validation of mRNA levels for selected genes using quantitative RT-PCR

To provide further quantitative validation of our microarray data for the 7 genes, we analysed 13 test tumour samples by quantitative RT-PCR and compared the results with the quantified mRNA expression levels on the microarray (Figure 3). All 7 genes were highly expressed across the 13 cancers and the microarray data agreed with those obtained by quantitative RT-PCR. Similar agreement was found in a subsequent comparative analysis of 14 validation tumour samples (Figure 3). We also compared the expression of the candidate genes with the mean expression level of the corresponding genes in 8 normal tissues that were used for microarray reference control. The results showed upregulation of each candidate gene compared with that in the normal tissues (Figure 3).

Protein expression of selected genes by immunohistochemistry and western blotting

Finally, we tested the encoded protein expression for each identified focus gene using immunohistochemistry and western blotting. Immunohistochemistry showed high expression of INHBA and NEK6 proteins in 14 of 20 and 24 of 27 tumour tissues, respectively (Figure 4A–D), whereas IGFBP7 and LUM

proteins showed little immunoreactivity in tumour tissue relative to adjacent healthy tissue (data not shown). Each of these proteins was expressed in >50% cells in each tissue examined and all were localised into the cytoplasm.

Western blotting showed strong bands for both NEK6 and INHBA in gastric cancer tissues compared to normal tissue in all three pairs (Figure 4E).

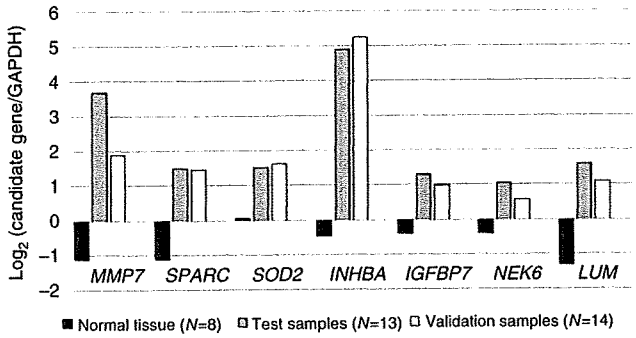

Figure 3 Bar chart shows mRNA levels of candidate genes using quantitative reverse transcription–PCR in normal gastric tissue ($n=8$, microarray reference control), test samples ($n=13$), and validation samples ($n=14$). Data are mean expression level of candidate gene relative to that of GAPDH in the examined tissues.

DISCUSSION

Comprehensive gene expression profiling is a useful tool for analysing several thousands of genes in multiple samples simultaneously. In gastric cancer, this approach successfully discriminated cancerous and noncancerous tissues (Hippo *et al*, 2002). Since then, several studies have searched for novel genes related to carcinogenesis of gastric cancer and novel clinical subtypes related to biological malignancy using comprehensive gene expression profiling (Hasegawa *et al*, 2002; Ji *et al*, 2002; Boussioutas *et al*, 2003; Kim *et al*, 2003, 2005; Oien *et al*, 2003; Jinawath *et al*, 2004; Motoori *et al*, 2005). However, these data were generally obtained from human cell lines or small-scale tissue samples. Here, we analysed the gene expression profiles of more than 200 tissue samples covering every pathological stage, and verified the findings at both the mRNA and protein levels to increase the universality of our microarray data. Such a study is more likely to identify specific expression profiles that are commonly activated and thus more reflective of crucial transcriptional features of neoplastic transformation and progression in gastric cancers. In fact, increasing recognition that this large-scale, systematic approach is necessary to view the overall molecular events responsible for carcinogenesis has spawned several recent studies combining large-scale analysis of gene expression with knowledge-based and relevance network analysis (Bredel *et al*, 2005; Abdel-Aziz *et al*, 2007). Using such an approach also

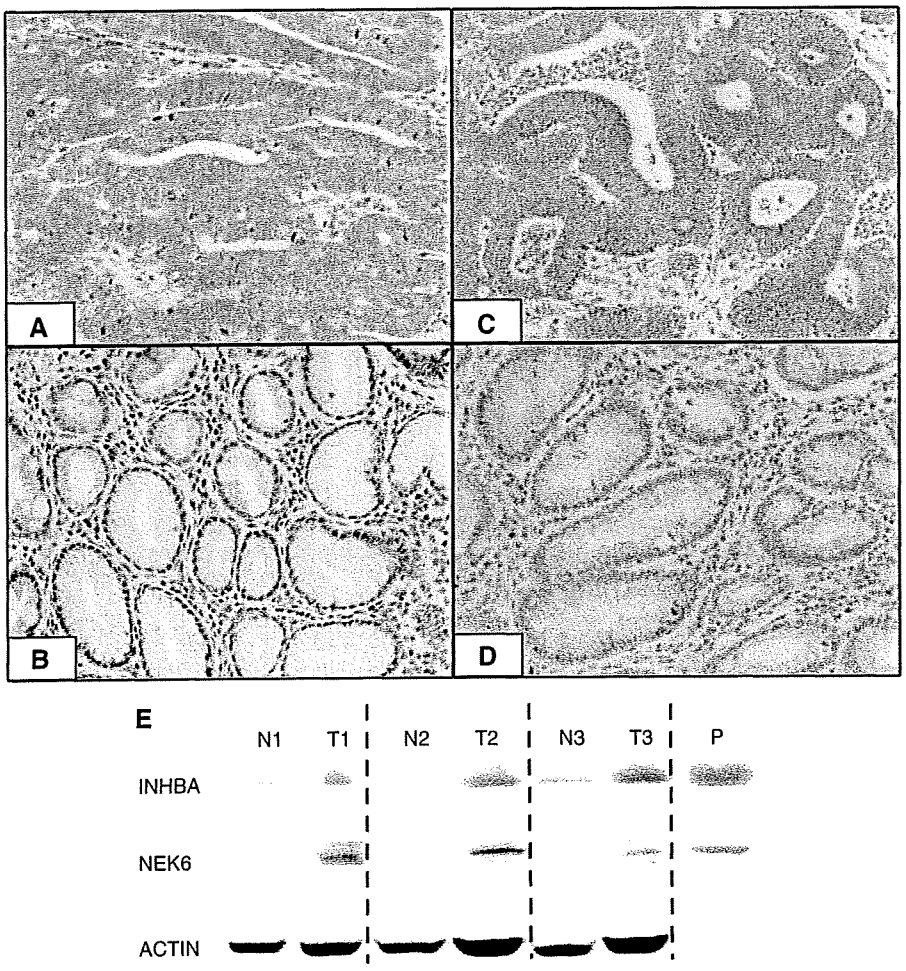

Figure 4 (**A**–**D**) Representative images of immunostaining for INHBA and NEK6. (**A**) Tumour tissue expressing INHBA; (**B**) healthy tissue for INHBA; (**C**) tumour tissue expressing NEK6; (**D**) healthy tissue for NEK6. Magnification, × 200. (**E**) Western blotting analysis of INHBA and NEK6 in three pairs of tumour (T) and normal (N) tissues. Anti-β-actin was used as control for protein level. P, positive control tissue.

identified significantly upregulated genes linked to activated pathways as potential key molecules in hepatocellular carcinoma (Kittaka *et al*, 2008).

Dynamic mapping of 271 genes differentially expressed in gastric cancer tissues in this study revealed links among the majority of genes (203 genes, 84%) based on the Ingenuity Pathway Knowledge Base. This finding indicates that such gene populations do not act as individual units, but rather collaborate closely in overlapping networks during gastric carcinogenesis. Among the 17 networks identified here, network-5 was mapped to the centre of the overlapping network and contained the largest number of focus genes, implicating it as a key network. Furthermore, the identified networks assumed a cluster of robust genes implicated in gastric cancer-related genes. Our network analysis also revealed *CDKN1A* ($p21^{WAF1/CIP1}$) as a hub gene that links to a large number of nodes and possibly determines the fundamental behaviour of the network.

The clinical significance of activation of our seven selected genes was further investigated by correlating the microarray expression data with the pathological stage. As indicated in Figure 2, we found these genes could be classified into two groups: the expression levels of genes of group 1 (*MMP7*, *IGFBP7*, and *NEK6*), but not those of group 2 (*SOD2*, *SPARC*, *LUM* and *INHBA*), correlated significantly with pathological stage. This finding indicates that although genes of group 2 may be involved in tumour formation and survival, those of group 1 may be involved in tumour progression. Their common activation seems to serve gastric carcinogenesis and tumour survival regardless of the pathological stage, based on the finding of overexpression of all seven genes in all samples. Furthermore, the gradual increase in the mean expression with cancer stage suggests that these genes cooperate in tumour progression. These results strengthen our proposal that such candidate genes are commonly activated during gastric carcinogenesis.

We also analysed the expression of the seven candidate genes based on age, sex, location, and histopathological type. Although the expression levels of *MMP7*, *NEK6*, *SOD2*, *SPARC*, and *INHBA* did not correlate with any of the above factors, *IGFBP7* and *LUM* were significantly upregulated in undifferentiated tumours compared to differentiated tumours (data not shown). These results suggest the involvement of these genes in tumour differentiation.

We also postulated that these genes are regulated by complex linkage between specific signalling pathways such as cell cycle signalling and TGF-β signalling, and that targeting several genes around *CDKN1A* ($p21^{WAF1/CIP1}$), which functions as a hub, can compensate each other. The differential expressions were also corroborated by quantitative RT–PCR data in some of the previously tested tissue samples and in 14 validation samples. Together, these findings implicate all seven genes in gastric carcinogenesis, including the four that were not previously related to human gastric cancer.

Transcript profiling studies require complementary protein analysis to fully understand the associated regulatory process in living organisms. By itself, profiling does not adequately reflect the fluctuating signalling events occurring at the proteomic level, based on the evidence that only a subset of proteins correlate significantly with mRNA abundance (Chen *et al*, 2002; Nishizuka *et al*, 2003; Tian *et al*, 2004). These seemingly anomalous results are explained partly by translational processes whereby microRNAs repress the translation of mRNA into proteins, and partly by post-translational modifications such as phosphorylation, methylation, acetylation, and ubiquitination. For that reason, the expression levels of proteins encoded by highly overexpressed genes related to gastric carcinogenesis require further investigation. This study detected protein expression for two gene products among the four previously noncancer-related genes. Furthermore, NEK6 protein was strongly stained in most of the cancer tissues, but showed less mRNA signal compared to the remaining six genes. This finding suggests that NEK6 might be significantly modified post-translationally.

Matrix metalloproteinases including MMP7 play important roles in determining tumour invasion and metastasis and *MMP7* gene expression correlates with vessel invasion and both lymphatic and hematogenous metastases (Yamashita *et al*, 1998). Increased SPARC expression is linked to advanced gastric cancer (Wang *et al*, 2004), although the expression of SOD2 (Mn-SOD; manganese superoxide dismutase) was significantly enhanced in cancer tissues compared with normal mucosa, and the Mn-SOD ratio was proposed as an independent prognostic parameter (Janssen *et al*, 2000). The *IGFBP7* gene was upregulated in diffuse-type gastric cancer (Boussioutas *et al*, 2003) and in 22 gastric cancer/nontumour mucosa paired tissues samples (Kim *et al*, 2003). Interestingly, recent study revealed that TGF-β signalling including *INHBA* accounted for some of the main differences between normal tissue and gastric cancer at the transcript level (Yang *et al*, 2007).

As stated, this study identified several genes, such as LUM and NEK6, which were not previously associated with human gastric cancer. LUM is a member of the small leucine-rich proteoglycan family that induces apoptosis and suppresses cell proliferation. Its reduced expression has been associated with poor outcome in invasive carcinoma (Vuillermoz *et al*, 2004; Schuetz *et al*, 2006). NIMA (never in mitosis, gene A) was originally identified in *Aspergillus nidulans* as a serine/threonine kinase critical for cell cycle progression (Osmani *et al*, 1988). Human NIMA-related kinases (Neks) have high homology to NIMA in the N-terminal catalytic domain sequences. *NEK6* is a Neks-family gene required for mitotic progression in human cells (Roig *et al*, 2002). Inhibition of *NEK6* by either overexpression of an inactive *NEK6* mutant or elimination of endogenous *NEK6* using siRNA-arrested cells in M phase and triggered apoptosis (Belham *et al*, 2003; Yin *et al*, 2003). A recent study demonstrated overexpression of *NEK6* transcripts in hepatocellular carcinoma (Chen *et al*, 2006), although it was found to be frequently expressed among 125 serine/threonine kinase genes implicated in breast cancer, colorectal cancer, lung cancer, and laryngeal cancer by *in situ* hybridisation (Capra *et al*, 2006). However, no previous studies have shown *NEK6* expression in gastric cancers or NEK6 protein expression in any cancerous tissues. In data not shown here, we also found higher levels of NEK6 protein in advanced cancer compared to early-stage samples by immunohistochemistry.

In conclusion, this study used an integrated approach combining gene expression profiling and dynamic mapping of gene expression data on large sample numbers to identify novel candidate genes that may contribute to gastric carcinogenesis. The identified genes were universally validated in additional samples. In particular, NEK6 and INHBA are promising potential markers of gastric cancer regardless of disease stage.

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REFERENCES

Abdel-Aziz HO, Takasaki I, Tabuchi Y, Nomoto K, Murai Y, Tsuneyama K, Takano Y (2007) High-density oligonucleotide microarrays and functional network analysis reveal extended lung carcinogenesis pathway maps and multiple interacting genes in NNK [4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone] induced CD1 mouse lung tumor. *J Cancer Res Clin Oncol* **133:** 107–115

Belham C, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J (2003) A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *J Biol Chem* **278:** 34897–34909

Boussioutas A, Li H, Liu J, Waring P, Lade S, Holloway AJ, Taupin D, Gorringe K, Haviv I, Desmond PV, Bowtell DD (2003) Distinctive patterns of gene expression in premalignant gastric mucosa and gastric cancer. *Cancer Res* **63:** 2569–2577

Bredel M, Bredel C, Juric D, Harsh GR, Vogel H, Recht LD, Sikic BI (2005) Functional network analysis reveals extended gliomagenesis pathway maps and three novel MYC-interacting genes in human gliomas. *Cancer Res* **65:** 8679–8689

Capra M, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M, Boldorini R, Pallotti F, Viale G, Gishizky ML, Draetta GF, Di Fiore PP (2006) Frequent alterations in the expression of serine/threonine kinases in human cancers. *Cancer Res* **66:** 8147–8154

Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG (2002) Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* **1:** 304–313

Chen J, Li L, Zhang Y, Yang H, Wei Y, Zhang L, Liu X, Yu L (2006) Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients. *Biochem Biophys Res Commun* **341:** 1059–1065

Czerniak B, Herz F, Gorczyca W, Koss LG (1989) Expression of ras oncogene p21 protein in early gastric carcinoma and adjacent gastric epithelia. *Cancer* **64:** 1467–1473

Dicken BJ, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM (2005) Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg* **241:** 27–39

Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286:** 531–537

Hasegawa S, Furukawa Y, Li M, Satoh S, Kato T, Watanabe T, Katagiri T, Tsunoda T, Yamaoka Y, Nakamura Y (2002) Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23 040 genes. *Cancer Res* **62:** 7012–7017

Hippo Y, Taniguchi H, Tsutsumi S, Machida N, Chong JM, Fukayama M, Kodama T, Aburatani H (2002) Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Res* **62:** 233–240

Janssen AM, Bosman CB, van Duijn W, Oostendorp-van de Ruit MM, Kubben FJ, Griffioen G, Lamers CB, van Krieken JH, van de Velde CJ, Verspaget HW (2000) Superoxide dismutases in gastric and esophageal cancer and the prognostic impact in gastric cancer. *Clin Cancer Res* **6:** 3183–3192

Ji J, Chen X, Leung SY, Chi JT, Chu KM, Yuen ST, Li R, Chan AS, Li J, Dunphy N, So S (2002) Comprehensive analysis of the gene expression profiles in human gastric cancer cell lines. *Oncogene* **21:** 6549–6556

Jinawath N, Furukawa Y, Hasegawa S, Li M, Tsunoda T, Satoh S, Yamaguchi T, Imamura H, Inoue M, Shiozaki H, Nakamura Y (2004) Comparison of gene-expression profiles between diffuse- and intestinal-type gastric cancers using a genome-wide cDNA microarray. *Oncogene* **23:** 6830–6844

Kaji M, Yonemura Y, Harada S, Liu X, Terada I, Yamamoto H (1996) Participation of c-met in the progression of human gastric cancers: anti-c-met oligonucleotides inhibit proliferation or invasiveness of gastric cancer cells. *Cancer Gene Ther* **3:** 393–404

Kasper HU, Schneider-Stock R, Mellin W, Roessner A (1998) P21 protein expression and ras-oncogene mutations in gastric carcinoma: correlation with clinical data. *Int J Oncol* **12:** 69–74

Kim B, Bang S, Lee S, Kim S, Jung Y, Lee C, Choi K, Lee SG, Lee K, Lee Y, Kim SS, Yeom YI, Kim YS, Yoo HS, Song K, Lee I (2003) Expression profiling and subtype-specific expression of stomach cancer. *Cancer Res* **63:** 8248–8255

Kim JM, Sohn HY, Yoon SY, Oh JH, Yang JO, Kim JH, Song KS, Rho SM, Yoo HS, Kim YS, Kim JG, Kim NS (2005) Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. *Clin Cancer Res* **11:** 473–482

Kittaka N, Takemasa I, Takeda Y, Marubashi S, Nagano H, Umeshita K, Dono K, Matsubara K, Matsuura N, Monden M (2008) Molecular mapping of human hepatocellular carcinoma provides deeper biological insight from genomic data. *Eur J Cancer* **44:** 885–897

Lee HS, Cho SB, Lee HE, Kim MA, Kim JH, Park do J, Kim JH, Yang HK, Lee BL, Kim WH (2007) Protein expression profiling and molecular classification of gastric cancer by the tissue array method. *Clin Cancer Res* **13:** 4154–4163

Motoori M, Takemasa I, Yano M, Saito S, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Doki Y, Kurokawa Y, Ueno N, Oba S, Ishii S, Monden M, Kato K (2005) Prediction of recurrence in advanced gastric cancer patients after curative resection by gene expression profiling. *Int J Cancer* **114:** 963–968

Nakamura M, Katano M, Kuwahara A, Fujimoto K, Miyazaki K, Morisaki T, Mori M (1998) Transforming growth factor beta1 (TGF-beta1) is a preoperative prognostic indicator in advanced gastric carcinoma. *Br J Cancer* **78:** 1373–1378

Nishizuka S, Charboneau L, Young L, Major S, Reinhold WC, Waltham M, Kouros-Mehr H, Bussey KJ, Lee JK, Espina V, Munson PJ, Petricoin III E, Liotta LA, Weinstein JN (2003) Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. *Proc Natl Acad Sci USA* **100:** 14229–14234

Oien KA, Vass JK, Downie I, Fullarton G, Keith WN (2003) Profiling, comparison and validation of gene expression in gastric carcinoma and normal stomach. *Oncogene* **22:** 4287–4300

Osmani SA, Pu RT, Morris NR (1988) Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell* **53:** 237–244

Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Roberts AB, Sporn MB (1994) Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. *Proc Natl Acad Sci USA* **91:** 8772–8776

Park WS, Oh RR, Park JY, Lee SH, Shin MS, Kim YS, Kim SY, Lee HK, Kim PJ, Oh ST, Yoo NJ, Lee JY (1999) Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. *Cancer Res* **59:** 4257–4260

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* **406:** 747–752

Pittman J, Huang E, Dressman H, Horng CF, Cheng SH, Tsou MH, Chen CM, Bild A, Iversen ES, Huang AT, Nevins JR, West M (2004) Integrated modeling of clinical and gene expression information for personalized prediction of disease outcomes. *Proc Natl Acad Sci USA* **101:** 8431–8436

Rhodes DR, Chinnaiyan AM (2005) Integrative analysis of the cancer transcriptome. *Nat Genet* **37**(Suppl): S31–S37

Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Barrette TR, Ghosh D, Chinnaiyan AM (2005) Mining for regulatory programs in the cancer transcriptome. *Nat Genet* **37:** 579–583

Roig J, Mikhailov A, Belham C, Avruch J (2002) Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes Dev* **16:** 1640–1658

Schuetz CS, Bonin M, Clare SE, Nieselt K, Sotlar K, Walter M, Fehm T, Solomayer E, Riess O, Wallwiener D, Kurek R, Neubauer HJ (2006) Progression-specific genes identified by expression profiling of matched ductal carcinomas *in situ* and invasive breast tumors, combining laser capture microdissection and oligonucleotide microarray analysis. *Cancer Res* **66:** 5278–5286

Tamura G, Kihana T, Nomura K, Terada M, Sugimura T, Hirohashi S (1991) Detection of frequent p53 gene mutations in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. *Cancer Res* **51:** 3056–3058

Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, Yi EC, Dai H, Thorsson V, Eng J, Goodlett D, Berger JP, Gunter B, Linseley PS, Stoughton RB, Aebersold R, Collins SJ, Hanlon WA, Hood LE (2004) Integrated genomic and proteomic analyses of gene expression in mammalian cells. *Mol Cell Proteomics* **3:** 960–969

Uchino S, Noguchi M, Ochiai A, Saito T, Kobayashi M, Hirohashi S (1993) p53 mutation in gastric cancer: a genetic model for carcinogenesis is common to gastric and colorectal cancer. *Int J Cancer* **54:** 759–764

van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven

RM, Roberts C, Linsley PS, Bernards R, Friend SH (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415:** 530–536

van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R (2002) A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* **347:** 1999–2009

Vuillermoz B, Khoruzhenko A, D'Onofrio MF, Ramont L, Venteo L, Perreau C, Antonicelli F, Maquart FX, Wegrowski Y (2004) The small leucine-rich proteoglycan lumican inhibits melanoma progression. *Exp Cell Res* **296:** 294–306

Wang CS, Lin KH, Chen SL, Chan YF, Hsueh S (2004) Overexpression of SPARC gene in human gastric carcinoma and its clinic-pathologic significance. *Br J Cancer* **91:** 1924–1930

Yamashita K, Azumano I, Mai M, Okada Y (1998) Expression and tissue localization of matrix metalloproteinase 7 (matrilysin) in human gastric carcinomas. Implications for vessel invasion and metastasis. *Int J Cancer* **79:** 187–194

Yang S, Shin J, Park KH, Jeung HC, Rha SY, Noh SH, Yang WI, Chung HC (2007) Molecular basis of the differences between normal and tumor tissues of gastric cancer. *Biochim Biophys Acta* **1772:** 1033–1040

Yin MJ, Shao L, Voehringer D, Smeal T, Jallal B (2003) The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. *J Biol Chem* **278:** 52454–52460

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Overexpression of Tyrosine Kinase B Protein as a Predictor for Distant Metastases and Prognosis in Gastric Carcinoma

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Distant metastases · Gastric carcinoma · Micrometastases · TrkB · Tyrosine kinases

Abstract
Objective: Tyrosine kinase B (TrkB) is associated with aggressive behavior and poor prognosis in various cancers. Here we examined the association between TrkB expression and distant metastases/prognosis in gastric carcinoma (GC). ***Patients and Methods:*** We analyzed TrkB expression in 161 GC patients by immunohistochemistry and Western blot analysis. The correlation of TrkB mRNA and protein expression levels was examined in 10 patients by RT-PCR assay. ***Results:*** TrkB expression was of level 1 in 97 (60.2%) and level 2 in 64 (39.8%) patients. Patients with level 2 expression had a significantly higher incidence of distant metastases ($p<0.0001$), well-differentiated tumors ($p<0.005$), deeper depth of invasion ($p<0.005$) and poorer disease-free and overall survival ($p<0.0001$ each) compared to patients with level 1. Multivariate analysis identified the level of TrkB expression as an independent prognostic factor for both disease-free and overall survival ($p<0.01$ and $p<0.0001$, respectively). Both lymph node metastasis (odds ratio = 10.7) and TrkB expression (odds ratio = 9.3) were independent predictors of distant metastases. ***Conclusion:*** A high level of TrkB expression was observed in well-differentiated GC subtypes and is a predictor for distant metastases and prognosis in GC.

Introduction

Although there has been a decline in both the incidence and mortality of gastric carcinoma (GC), it still represents a worldwide health concern and remains one of the most aggressive tumors [1]. The 5-year survival rate is as low as 7% in patients with metastases to distant organs [2]. Even patients with the most favorable form of the condition who undergo curative surgical resection often die from recurrent disease and distant metastases [3]. In such patients, there might be occult micrometastases at the time of surgery, from which cancer cells begin to proliferate and metastasize to distant organs [4, 5]. At present, the detection of micrometastases in distant organs is challenging by conventional methods, and advanced and reliable diagnostic methods to assess the metastatic potential of GC are important for the selection of appropriate therapeutic modalities.

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Trk is a receptor tyrosine kinase, a group of receptors of neurotrophin that includes TrkA, TrkB, and TrkC, which primarily regulates growth, differentiation and programmed cell death of neurons in both the peripheral and central nervous systems [6]. However, the Trk gene was originally cloned as an oncogene fused with the tropomyosin gene in the extracellular domain, conferring constitutive activation of its tyrosine kinase activity to induce continuous cell proliferation [7, 8]. Together with their ligands, they are involved in tumor proliferation, invasion and metastasis, and cause changes in cell morphology in a variety of cancers including medulloblastomas, neuroblastomas, lung, thyroid, breast and pancreatic cancers, lymphomas and soft tissue tumors [9–14]. In particular, high expression levels of TrkB and its ligand brain-derived neurotrophic factor (BDNF) are thought to be associated with more aggressive malignant behavior and a poor prognosis in human cancer, including lung, pancreatic and prostate carcinomas, Wilms' tumor and neuroblastomas [14–19]. Moreover, a recent study of functional screening for suppressors of anoikis in rat intestinal epithelial cells showed that TrkB generates a specific and potent pro-survival signal that renders epithelial cells resistant to anoikis, which is accompanied by the acquirement of potent tumorigenic, invasive and metastatic capacities, indicating that TrkB may directly contribute to human malignancies [20].

Although TrkB plays a plethora of roles in malignant cells, to our knowledge its role in GC has not been studied. The present study was designed to determine the expression of TrkB in GC and assess the clinical significance of protein expression to clinicopathological features and patient prognosis.

Patients and Methods

Patients

With institutional approval and written informed consent from all patients, we analyzed specimens from 169 patients who had undergone surgical resection for GC at the Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University Hospital (Osaka, Japan), during the period from December 1999 to December 2002. Surgical specimens were fixed in neutral buffered formaldehyde and processed for histopathological and immunohistochemical evaluation; fresh specimens were frozen at –80°C for Western blot and RT-PCR analysis.

There were 110 males and 51 females, with age ranging from 34 to 100 years (median, 63 years). The type of surgical resection used was total gastrectomy in 68 patients, proximal gastrectomy in 20 patients, distal gastrectomy in 67 patients and partial gastrectomy in 6 patients. Resected specimens were macroscopically examined to determine tumor location and size; tumors were located in the following areas: upper third of the stomach in 45 patients, middle third in 60 patients and lower third of the stomach in 56 patients. Main tumors ranged in size from 3 to 165 mm (mean, 36.7 mm). The histopathological subtype of GC was determined based on the criteria of the Japanese Research Society for Gastric Cancer [21]. Tumor stages were classified according to the tumor node metastasis (TNM) classification [22].

Laboratory tests were performed at follow-up postoperatively and included routine peripheral blood cell counts and serum carcinoembryonic antigen level at 1- to 6-month intervals, chest roentgenogram, liver ultrasonography, abdominal computerized tomographic scan and endoscopic examination of the rest of the stomach at 6- to 12-month intervals. Cisplatinum and 5-fluorouracil-based adjuvant chemotherapy was administered to 24 patients (preoperatively in 5 patients, postoperatively in 19 patients, both pre- and postoperatively in 5 patients) with a high risk for tumor recurrence (i.e. presence of lymph node metastasis, large tumor size – diameter >100 mm – and tumor invasion to the serosa).

Immunohistochemistry

Immunohistochemical staining was performed using 4-μm-thick formalin-fixed, paraffin-embedded sections. Sections were deparaffinized in xylene and rehydrated with a series of ethanol solutions. Antigen retrieval was performed by heating the sections in 10 mmol/l citrate buffer for 5 min. Rabbit polyclonal anti-TrkB (p145) antibody (sc-12, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as the primary antibody at a dilution of 1:500, and sections were incubated at 4°C for 12 h and then lightly counterstained using hematoxylin. Positive staining in endothelial cells was used as an internal positive control. For negative controls, phosphate-buffered saline was used as the primary antibody, and uniformly gave negative results. Stained sections were evaluated in a blinded manner without prior knowledge of the clinicopathologic parameters. Staining intensity in the cytoplasm of the tumor cells was compared to that of endothelial cells and categorized as follows: weaker than that of endothelial cells (level 1 TrkB expression) or equal to or stronger than that of endothelial cells (level 2). Samples showing combined level 1 and 2 intensity staining in different areas of the tumors were classified as level 2 expression.

Quantitative RT-PCR Analysis of TrkB Expression

Total RNA was extracted from frozen samples in 10 cases of GC with TRIzoL reagent (Invitrogen, Carlsbad, Calif., USA). DNaseI-treated total RNA (10 μg) was used for reverse transcription with Superscript II (Invitrogen). Resulting cDNA products were amplified using a LightCycler instrument. The total 10 μl of reaction mixture consisted of a master mixture (LightCycler DNA master hybridization probes; Roche, Mannheim, Germany), 4.0 mM $MgCl_2$, 0.25 μl of primers of TrkB or β-actin, 0.4 μM of each probe and 1 μl of template cDNA in a LightCycler capillary. Primers designed using published sequences are listed below. The TrkB set for the kinase domain amplified a 571-bp fragment: 5′ AGGGCAACCCGCCCACGGAA3′ and 3′GGATCGGTCTGGGGAAAAGG5′, and β-actin: 5′TCACCCACACTGTGCCCATCTACGA3′ and 3′GGTAACCGCTTACTCGCCAAGGC-

GAC5′. For TrkB amplification, 95°C (90 s) for hot start was followed by 40 rounds of amplification at 95°C (0 s) for denaturation, 50°C (10 s) for annealing and 72°C (10 s) for extension, with a temperature slope of 20°C/s, performed in the LightCycler. The same temperature profile was used, except for an annealing temperature of 55°C for β-actin. External standards for both TrkB mRNA and β-actin were included using known concentrations of ASPC-1 cell line (human pancreatic cancer cell line) RNA prepared by 10-fold serial dilutions. Each run consisted of five external standards, a negative control without a template and patient samples. Relative mRNA in each sample was then automatically quantitated by reference to the standard curve constructed each time according to the LightCycler software.

Western Blot Analysis

Freshly frozen tissue samples were thawed on ice and homogenized in the presence of radioimmunoprecipitation assay lysis buffer [150 mmol/l NaCl, 1% Triton X-100, 0.5% NaDOD, 0.1% sodium dodecyl sulfate and 50 mmol/l Tris (pH 8.0), containing proteinase inhibitors (5 μg/ml aprotinin and 1 μg/ml leupeptin)] at a temperature of 4°C throughout the procedure]. All lysates were centrifuged at 11,000 *g* for 5 min. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratory, Richmond, Calif., USA) with bovine serum albumin as a standard protein. Proteins (50 μg) were separated by electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide gel. In addition, as a positive control for TrkB, rat brain samples were prepared as described previously [23]. After electrophoresis, proteins were transferred to a nitrocellulose membrane. After 1-hour incubation in a blocking solution (5% non-fat dry milk in PBS-0.5% Tween 20), the membrane was blotted with the anti-TrkB polyclonal antibody (1:200, sc-12, Santa Cruz Biotechnology) and an anti-β-actin monoclonal antibody (1:1,000; AC15, Sigma, St. Louis, Mo., USA). The blots were developed with peroxidase-labeled secondary antibodies. After extensive washing and incubation with the enhanced chemiluminescence plus detection reagent for 5 min, using a developer, specific bands were detected by exposing it to an autoradiography film (Hyperfilm Enhanced Chemiluminescence; Amersham Biosciences, Arlington Heights, Ill., USA) in the dark.

Statistical Analysis

Statistical analyses were performed using the SPSS-PC package (version 13.0; SPSS, Chicago, Ill., USA). The χ^2 test and Fisher's exact test were used to analyze the association between TrkB expression measured by immunohistochemistry and clinicopathologic features of GC. The following endpoints were examined for survival analyses: disease-free and overall survival. Survival time was calculated from the date of diagnosis until death (for overall survival) or, if the patient was still alive, until the last follow-up visit (disease-free survival and overall survival). Local, regional or distant tumor progression was taken into account as adverse events for disease-free survival. Death from any cause was considered for overall survival. The Kaplan-Meier method with the log-rank test was used to calculate survival rates and differences in survival curves, and life tables with Wilcoxon test were used for 5-year survival rates [24]. The Cox proportional hazards regression model with a stepwise procedure was used to analyze the simultaneous influence of prognostic factors. Using multivariate analysis, a logistic regression model was built to identify the variables independently associated with onset of distant metastases, of which the presence of distant metastasis was considered as a dependent variable, and TrkB expression levels together with clinicopathologic factors were considered the covariates [25, 26]. Data are expressed as means ± SD or medians. $p < 0.05$ was considered statistically significant.

Results

TrkB Expression in GC

Eight (4.7%) of 169 sections that did not show endothelial immunohistochemistry staining were regarded as having poor antigen preservation and were excluded from further analyses. The remaining 161 sections that showed endothelial staining were evaluated for TrkB expression. In 97 (60.2%) and 52 (32.3%) patients, cancer cells showed constant level 1 or 2 TrkB expression, respectively, in all areas of the specimens, whereas in 12 patients tumor cells showed combined level 1 and 2 staining. In total, 64 patients (39.7%) were judged as having level 2 TrkB expression (fig. 1). Quantitative RT-PCR analysis was performed in 10 GC samples, of which 6 were well differentiated and four poorly differentiated. The relative ratio of TrkB/β-actin expression in 6 well-differentiated cases and 4 poorly differentiated cases was 10.1 ± 3.6 and 2.1 ± 1.2, respectively ($p < 0.001$; fig. 2) and was strongly consistent with the immunohistochemical results. In these cases, TrkB protein expression was also confirmed by Western blotting (fig. 3).

Uni- and Multivariate Analyses of Prognostic Factors in GC Patients

Table 1 summarizes the results of χ^2 and Fisher's exact tests of TrkB expression and various clinicopathological factors. In comparison to GC samples with level 1 TrkB expression, level 2 GC samples showed higher rates in the proximal location (57.7% in level 1 and 76.6% in level 2), in the well-differentiated histological subtype (47.4% in level 1 and 68.8% in level 2), in distant metastases (3.1% in level 1 and 21.9% in level 2; distant metastasis sites were the liver in 14, the lung in 2 and the bone in 1 patient) and a deeper depth of invasion (pT2–pT4; 42.3% in level 1 and 68.8% in level 2).

Patients with level 1 GC had better 5-year survival rates than those with level 2 GC (disease-free survival, 62.0 vs. 19.0%, $p < 0.0001$; overall survival, 53.0 vs. 22.0%, $p < 0.0001$). Univariate analysis revealed that TrkB expression, depth of tumor invasion and lymph node metastasis significantly correlated with disease-free and overall survival rates (table 2). Vascular inva-

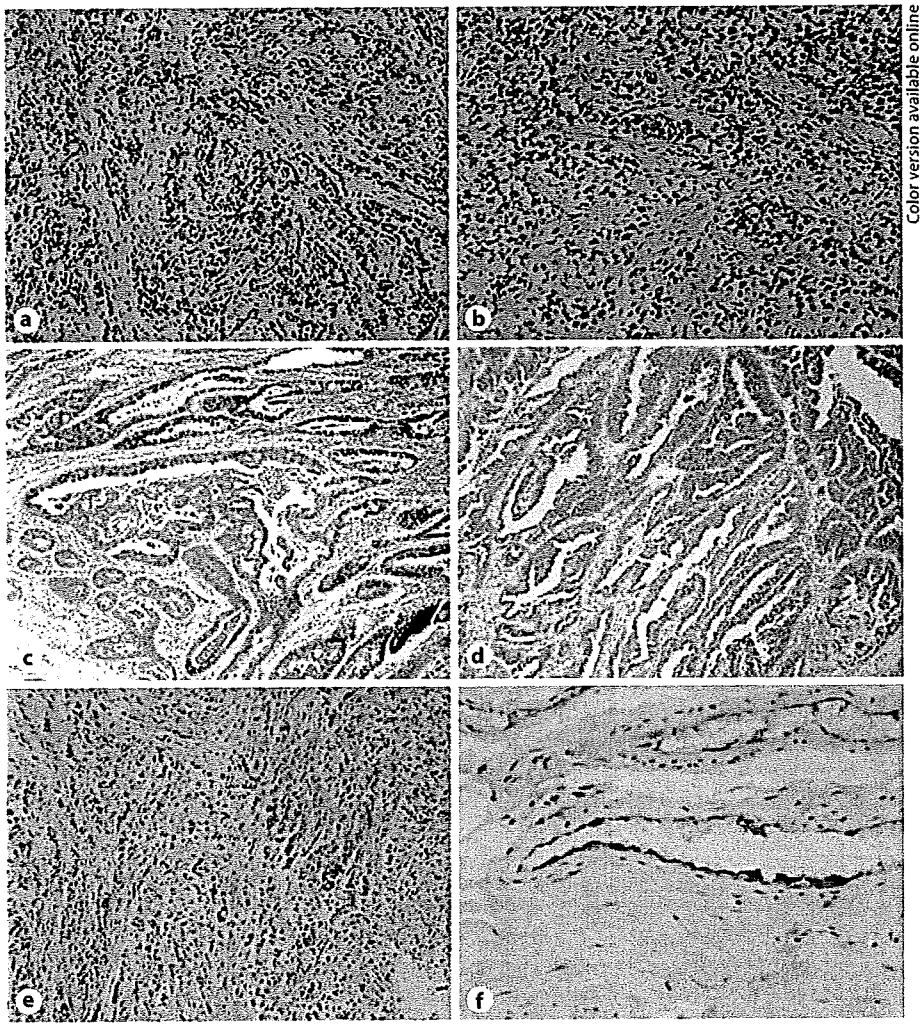

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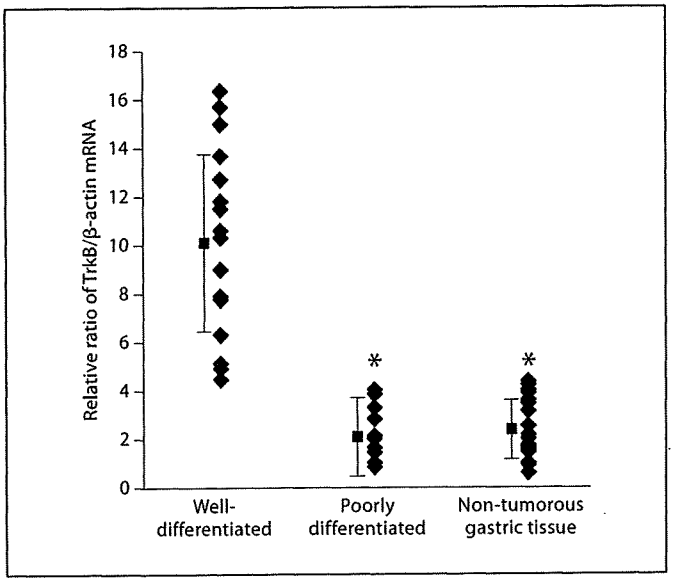
Fig. 1. a, b TrkB level in poorly differentiated GC. Tumor cells exhibited weak or no TrkB staining in the cytoplasm. **c, d** TrkB level 2 in well-differentiated GC. **e** TrkB level 2 in poorly differentiated GC from a patient with distant metastasis. Tumor cells exhibited strong cytoplasmic TrkB staining (magnification ×100). **f** Strong cytoplasmic TrkB staining in endothelial cells (magnification ×200).



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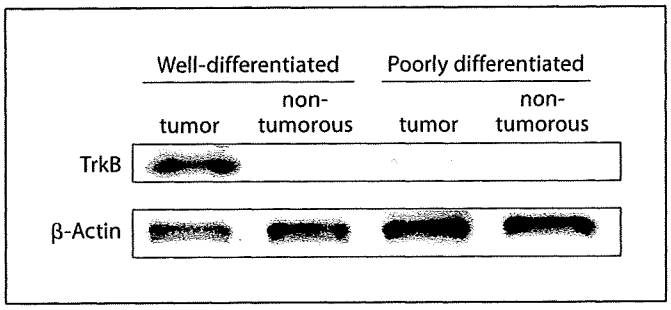

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Fig. 2. Relative ratio of TrkB/β-actin mRNA expression in well- and poorly differentiated GC and non-tumorous gastric tissue. Values are means (± SD) of three independent determinations. * $p < 0.001$, vs. poorly differentiated GC and non-tumorous gastric tissue.

Fig. 3. TrkB expression in GC. TrkB protein was detected in well-differentiated tumors by Western blotting using anti-TrkB antibody.

Table 1. Association between TrkB expression and clinicopathologic factors of 161 GC patients

Factors	Category	Total patients	Patients with TrkB				p value
			level 1 (n = 97)		level 2 (n = 64)		
			n	%	n	%	
Age, years			62.4 ± 12.2		63.1 ± 9.9		NS
Sex	male	110	67	60.9	43	39.1	NS
	female	51	30	58.8	21	41.2	
Tumor location	upper third	45	24	53.3	21	46.7	<0.05
	middle third	60	32	53.3	28	46.7	
	lower third	56	41	73.2	15	26.8	
Tumor size	<50 mm	106	64	60.4	42	39.6	NS
	≥50 mm	55	33	60.0	22	40.0	
Histologic differentiation	undifferentiated	71	51	71.8	20	28.2	<0.005
	differentiated	90	46	51.1	44	48.9	
Vascular invasion	absent	94	55	58.5	39	41.5	NS
	present	67	42	62.7	25	37.3	
Lymphatic invasion	absent	82	53	64.6	29	35.4	NS
	present	79	44	55.7	35	44.3	
Lymph node metastasis	absent	100	63	63.0	37	37.0	NS
	present	61	34	55.7	27	44.3	
Depth of tumor invasion	pT1	76	56	73.7	20	26.3	<0.005
	pT2	36	19	52.8	17	47.2	
	pT3	33	17	51.5	16	48.5	
	pT4	16	5	31.3	11	68.8	
Hepatic branch vagus nerve	not preserved	72	40	55.6	32	44.4	NS
	preserved	89	57	60.4	32	36.0	
Type of gastrectomy	proximal	20	8	40.0	12	60.0	<0.05
	distal	67	43	64.2	24	35.8	
	partial	6	1	16.7	5	83.3	
	total	68	45	66.2	23	33.8	
Distant metastasis	absent	144	94	65.3	50	34.7	<0.0001
	present	17	3	17.6	14	82.4	
Chemotherapy	not performed	137	89	65.0	48	35.0	<0.005
	performed	24	8	33.3	16	66.7	

sion and lymphatic invasion were also significant factors for both disease-free and overall survival, and tumor location was a significant factor for disease-free survival (table 2). Kaplan-Meier curves for disease-free and overall survival and for TrkB expression are shown in figure 4.

Multivariate analysis with factors proven to be significant in the univariate analysis revealed that the level of TrkB expression was an independent prognostic factor for both disease-free and overall survival. Depth of tumor invasion and lymph node metastasis were also independent and significant factors for both disease-free and overall survival (table 3).

Risk Factors for Distant Metastasis Analysis by a Logistic Regression Model

Multivariate analysis using a logistic regression model confirmed lymph node metastasis (odds ratio = 10.7) and TrkB expression (odds ratio = 9.3) as independent predictors of distant metastasis (table 4).

Table 2. Univariate analysis of clinicopathologic factors for disease-free and overall survival (means ± SD) of 161 GC patients

Factors	Category	Disease-free survival	p value	Overall survival	p value
TrkB expression	level 1	51.3 ± 2.6	<0.0001	51.3 ± 2.3	<0.0001
	level 2	27.4 ± 3.6		30.8 ± 3.1	
Age	<60 years	45.9 ± 3.4	NS	44.1 ± 3.1	NS
	≥60 years	40.5 ± 3.2		42.2 ± 2.6	
Sex	male	43.3 ± 2.8	NS	42.9 ± 2.4	NS
	female	44.3 ± 4.1		45.0 ± 3.6	
Tumor location	upper third	33.4 ± 4.2	<0.05	40.8 ± 3.8	NS
	middle third	44.8 ± 3.8		40.2 ± 3.3	
	lower third	49.5 ± 3.6		48.5 ± 3.2	
Tumor size	<50 mm	46.9 ± 2.8	NS	45.1 ± 2.5	NS
	≥50 mm	35.9 ± 3.8		40.0 ± 3.3	
Histologic differentiation	undifferentiated	43.7 ± 3.5	NS	45.1 ± 2.7	NS
	differentiated	43.7 ± 3.1		41.7 ± 3.0	
Vascular invasion	absent	47.1 ± 2.9	<0.05	50.0 ± 2.6	<0.001
	present	38.3 ± 3.8		35.6 ± 2.9	
Lymphatic invasion	absent	51.3 ± 2.9	<0.001	51.1 ± 2.7	<0.0001
	present	33.4 ± 3.4		34.9 ± 2.7	
Lymph node metastasis	absent	50.8 ± 2.6	<0.0001	52.3 ± 2.3	<0.0001
	present	25.6 ± 2.7		30.3 ± 2.8	
Depth of tumor invasion	pT1	58.1 ± 2.3	<0.0001	56.8 ± 2.3	<0.0001
	pT2	33.3 ± 4.1		42.1 ± 3.9	
	pT3	22.9 ± 3.6		30.3 ± 3.3	
	pT4	14.3 ± 3.0		11.8 ± 2.1	
Hepatic branch vagus nerve	not preserved	39.1 ± 3.5	NS	42.8 ± 3.0	NS
	preserved	47.0 ± 3.1		44.1 ± 2.7	
Type of gastrectomy	proximal	31.6 ± 4.3	<0.01	36.7 ± 5.7	<0.05
	distal	47.3 ± 3.4		46.4 ± 2.9	
	partial	16.2 ± 7.6		23.5 ± 7.3	
	total	43.2 ± 3.6		44.5 ± 3.1	
Chemotherapy	not performed	45.1 ± 2.5	NS	44.9 ± 2.1	NS
	performed	34.3 ± 5.1		36.7 ± 5.1	

Discussion

Although the rate of long-term survival after curative resection has increased in GC patients, increasing the survival of patients with distant metastases remains a challenge for clinicians. Since most patients with distant metastases die due to disease progression, advanced and reliable diagnostic methods to identify metastasis sites at an early stage for effective therapy are worthy of further research. However, at present, there are no specific markers to predict distant metastasis at an early stage after curative resection [27].

Cancer cells metastasize to distant organs through various mechanisms including basement membrane invasion, migration, adhesion to the vessel walls and angiogenesis [28]. Recently, TrkB has been reported to increase the invasive and metastatic ability of many cancers by inducing neoangiogenesis, suppressing anoikis and mediating endothelial adhesion [9–20, 29–34].

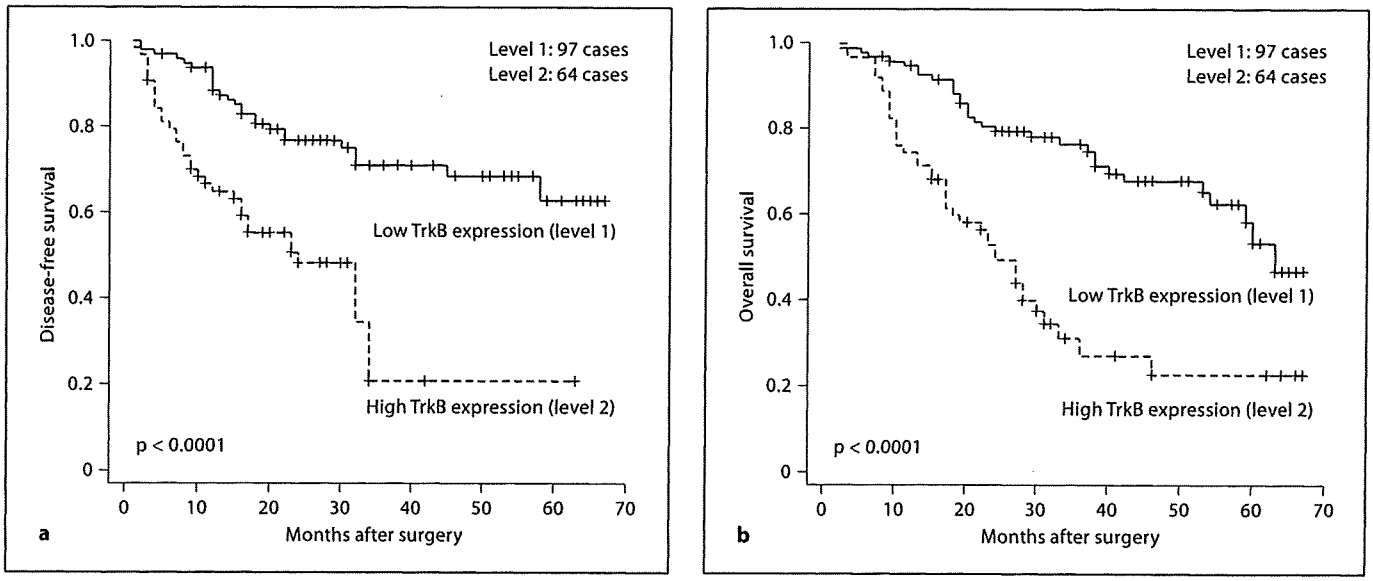

Fig. 4. Disease-free (**a**) and overall survival (**b**) of patients with TrkB expression levels 1 and 2 in GC. A significant difference was observed between these groups.

Table 3. Multivariate analysis of clinicopathologic factors for disease-free and overall survival of 161 GC patients

Factors	Disease-free survival		Overall survival	
	p value	RR (95% CI)	p value	RR (95% CI)
TrkB expression				
Level 2 vs. 1	<0.001	2.78 (1.51–5.12)	<0.0001	3.07 (1.73–5.44)
Lymph node metastasis				
Present vs. absent	<0.05	1.97 (1.03–3.76)	<0.05	2.04 (1.14–3.66)
Depth of tumor invasion				
pT3/pT4 vs. pT1/pT2	<0.001	1.80 (1.29–2.52)	<0.0001	2.03 (1.50–2.75)

CI = Confidence interval; RR = relative risk.

Table 4. Identification of features contributing to distant metastases based on a logistic regression analysis

Variable	β	χ^2 value	Odds ratio (95% CI)	p value
Lymph node metastasis	2.37	7.21	10.7 (1.89–60.3)	<0.01
TrkB expression	2.23	6.46	9.3 (1.67–51.89)	<0.05

CI = Confidence interval.

In fact, overexpression of TrkB has been demonstrated to be involved in the process of tumor invasion and metastasis in medulloblastomas, neuroblastomas, lung, thyroid, breast, pancreatic and prostate cancers, lymphoma, Wilms' tumor and soft tissue tumors [9–20, 28–33]. Moreover, recent study of the mutational analysis of Trk in colorectal cancer showed the gain-of-function mutations within the kinase domain of TrkB [19]. Using a cDNA microarray, TrkB overexpression was demonstrated in highly metastatic pancreatic cancer cell lines and pancreatic adenocarcinomas, which correlated with perineural invasion and shorter latency to development of liver metastasis by activation of mitogen-activated pro-

tein kinase and the activator protein-1 (AP-1), which can induce the expression of vascular endothelial growth factor and interleukin-8 [35]. TrkB was shown to suppress caspase-associated anoikis in epithelial cells by activating the phosphatidylinositol-3-OH kinase/protein kinase B pathway, thus enabling cells to survive and proliferate in suspension, and promoting these cells to form rapidly growing tumors that infiltrate lymphatic and blood vessels to colonize distant organs [20]. These changes were accompanied by potent tumorigenicity, and invasive and metastatic capacities [20]. Interestingly, resistance to anoikis also appears to be involved in the highly expressed TrkB-associated non-epithelial cancers such as melanoma [36] and neuroblastoma [37, 38]. These observations demonstrate the potent oncogenic effects of TrkB and a specific pro-survival function that contributes to its metastatic capacity.

In the present study, TrkB expression levels were examined by immunohistochemical, Western blot and RT-PCR analyses. A clear correlation in TrkB expression was demonstrated between mRNA and protein level indicating the reliability of immunohistochemistry for the evaluation of TrkB expression.

Among the clinicopathologic factors examined, results from our study revealed a significant association between TrkB expression and tumor location, depth of invasion, histological differentiation and distant metastases, indicating the close association between TrkB expression and GC growth and invasiveness. In the present study, patient characteristics such as sex, age and 5-year survival rates were similar to those in our previous report [39]. In addition, the incidence of level 2 staining for TrkB was significantly higher in well-differentiated than undifferentiated tumors. Both at the mRNA and protein level, the increased level 2 TrkB expression in well-differentiated tumors may explain previous reports describing a relationship between hematogenous recurrence and clinicopathologic features. These studies showed that hematogenous recurrence, in particular liver recurrence, develops more frequently from well-differentiated than poorly differentiated tumor types [40–47]. Furthermore, it has been suggested that GC seldom produces distant metastasis until it develops into stage T_3 [48–51]. Distant metastases were not only closely related to well-differentiated histological types but also to deeper depth of tumor invasion. Interestingly, of the 19 cases of distant metastasis, 3 were poorly differentiated and all showed strong TrkB staining.

BDNF, the ligand of TrkB, is a member of the neurotrophin family and mainly promotes the survival and differentiation of central and peripheral neurons [6]. BDNF involvement in the differentiation and proliferation of cancer and endothelial cells has recently been demonstrated [10–17, 30, 33, 52, 53]. TrkB itself, without BDNF, can effectively suppress cell death and enables cells to proliferate as large spheroid aggregates in suspension, promoting invasion and metastasis [20]. In addition, coexpression of TrkB with BDNF leads to a further increase in cell numbers [13–15]. This has also been observed in some other tumors [9–19]; however, it is interesting that BDNF was not expressed in GC or normal gastric tissues by immunohistochemistry (data not shown). In a cDNA microarray study, it has recently been demonstrated that BDNF mRNA may be downregulated in GC [54]. This interesting phenomenon may be interpreted by a late discovery, regarding the unique property of TrkB, that the transcriptional program of cells is modulated by Trk receptor expression and basal activation rather than by ligand-induced activation [55]. In general, a given cell does not express a growth factor receptor until it is ready for interaction with its ligand [56, 57]. BDNF may act as a diffusible attractant generated by some organ systems other than the central or peripheral neural system and is implicated as molecule guidance cue for attracting TrkB-expressing cells as it does in the neuronal development, growth and migration [58]. In comparison to other organs, BDNF is more pronounced in the liver, lung, bone marrow and brain [23, 59–62], and BDNF-rich microenvironments of these organs are likely targets for BDNF-responsive metastatic cells. This may represent a mechanism for the progression of TrkB-expressing cancers to highly aggressive metastasis to these organs. These mechanisms may explain our results showing a significant association between TrkB expression and distant metastases in GC patients.

Prognosis was poor in neoplasms located in the upper third of the stomach. In these cases, the incidence of distant metastases was almost doubled with respect to the distal ones, and a higher incidence of locoregional and distant recurrences was also observed, being probably related to the more advanced stage of upper-third neoplasms, because tumor location did not result in a significant prognostic factor in multivariate analysis. Our uni- and multivariate analyses also revealed that the level of TrkB expression was an independent prognosticator of both disease-free and overall survival. In addition, distant metastasis occurred significantly earlier in patients with level 2 TrkB expression tumors than level 1 tumors. Multivariate analysis using a logistic regression model also confirmed TrkB expression and lymph node metas-

tasis as independent predictors of distant metastasis. The direct association between lymph node metastasis and hematogenous spread from GC has already been thoroughly described in previous studies [63–66].

In conclusion, our results demonstrated the possible involvement of TrkB in the induction of distant metastases in GC and that TrkB expression, determined by immunohistochemistry, could be used as a new prognostic marker for GC. The latter may be a useful tool helping to define subgroups at high risk of developing distant metastases. Moreover, since pan-Trk-inactivating drugs are already being developed [67], specific drug-mediated inactivation of TrkB might provide a novel way to explore the effective therapeutic impact on distant metastatic capacities of GC with overexpressed or mutated TrkB.

References

1 World Health Organization. The World Health Report 1997. http://www.who.org/whr/1997/factse.htm.
2 Maruyama K, Kaminishi N, Katai H, et al: Committee of Nationwide Registry, Japanese Gastric Cancer Association Nationwide Registry of Gastric Cancer Patients in Japan. Jpn J Cancer Clin 1999;45:719–724.
3 Roder JD, Stein HJ, Bottcher K, et al: Surgical therapy for gastric cancer. J Infus Chemother 1995;5:97–103.
4 Maehara Y, Oshiro T, Endo K, et al: Clinical significance of occult micrometastasis lymph nodes from patients with early gastric cancer who died of recurrence. Surgery 1996; 119:397–402.
5 Okada Y, Fujiwara Y, Yamamoto H, et al: Genetic detection of lymph node micrometastases in patients with gastric carcinoma by multiple marker reverse transcriptase-polymerase chain reaction assay. Cancer 2001;92: 2056–2064.
6 Nakagawara A: Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett 2001;169:107–114.
7 Levi-Montalcini R: The nerve growth factor 35 years later. Science 1987;237:1154–1162.
8 Klein R, Jing SQ, Nanduri V, et al: The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 1991;65:189–197.
9 Hisaoka M, Sheng WQ, Tanaka A, et al: Gene expression of TrkC (NTRK3) in human soft tissue tumours. J Pathol 2002;197:661–667.
10 Ricci A, Greco S, Mariotta S, et al: Neurotrophins and neurotrophin receptors in human lung cancer. Am J Respir Cell Mol Biol 2001;25:439–446.
11 Descamps S, Lebourhis X, Delehedde M, et al: Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. J Biol Chem 1998;273:16659–16662.
12 D'Onofrio M, de Grazia U, Morrone S, et al: Expression of neurotrophin receptors in normal and malignant B lymphocytes. Eur Cytokine Netw 2000;11:283–291.
13 Zhu Z, Kleeff J, Kayed H, et al: Nerve growth factor and enhancement of proliferation, invasion, and tumorigenicity of pancreatic cancer cells. Mol Carcinog 2002;35:138–147.
14 Sclabas GM, Fujioka S, Schmidt C, et al: Overexpression of tropomysin-related kinase B in metastatic human pancreatic cancer cells. Clin Cancer Res 2005;11:440–449.
15 Nakagawara A, Azar CG, Scavarda NJ, et al: Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 1994;14:759–767.
16 Aoyama M, Asai K, Shishikura T, et al: Human neuroblastomas with unfavorable biologies express high levels of brain-derived neurotrophic factor mRNA and a variety of its variants. Cancer Lett 2001;164:51–60.
17 Eggert A, Grotzer MA, Ikegaki N, et al: Expression of the neurotrophin receptor TrkB is associated with unfavorable outcome in Wilms' tumor. J Clin Oncol 2001;19:689–696.
18 Brodeur GM: Neuroblastoma: biological insights into a clinical enigma. Nature Rev Cancer 2003;3:203–216.
19 Bardelli A, Parsons DW, Silliman N, et al: Mutational analysis of the tyrosine kinome in colorectal cancers. Science 2003;300:949.
20 Douma S, Laar T, Zevenhoven J, et al: Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. Nature 2004;430:1034–1039.
21 Japanese Gastric Cancer Association: Japanese Classification of Gastric Carcinoma (in English). Gastric Cancer 1998;1:10–24.
22 Sobin LH, Wittekind CH: TNM Classification of Malignant Tumours, ed 5. New York, Wiley, 1997, pp 59–62.
23 Labouyrie E, Dubus P, Groppi A, et al: Expression of neurotrophins and their receptors in human bone marrow. Am J Pathol 1999;154:405–415.
24 Kaplan EL, Meier P: Non-parametric estimation for incomplete observations. J Am Stat Assoc 1958;53:457–481.
25 Cox DR: Regression models and life tables. J R Stat Soc 1972;34:187–220.
26 Agresti A, Finlay B: Logistic Regression – Modeling Categorical Responses. Statistical Methods for Social Sciences, ed 3. Upper Saddle River, Prentice Hall, 1997, pp 575–619.
27 Sakamoto Y, Ohyama S, Yamamoto J, et al: Surgical resection of liver metastases of gastric cancer: an analysis of a 17-year experience with 22 patients. Surgery 2003;133:507–511.
28 Tomimatsu S, Ichikura T, Mochizuki H: Significant correlation between expression of interleukin-1α and liver metastasis in gastric carcinoma. Cancer 2001;91:1272–1276.
29 Liu ZZ, Zhu LQ, Eide FF: Critical role of TrkB and brain-derived neurotrophic factor in the differentiation and survival of retinal pigment epithelium. J Neurosci 1997;17: 8749–8755.
30 Duda DG, Jain RK: Pleiotropy of tissue-specific growth factors: from neurons to vessels via the bone marrow. J Clin Invest 2005;115: 596–598.
31 Liu Y, Encinas M, Comella JX, et al: Basic helix-loop-helix proteins bind to TrkB and p21(Cip1) promoters linking differentiation and cell cycle arrest in neuroblastoma cells. Mol Cell Biol 2004;24:2662–2672.
32 Donovan MJ, Lin MI, Wiegn P, et al: Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. Development 2000;127:4531–4540.
33 Duda DG, Jain RK: Neurotrophins promote revascularization by local recruitment of TrkB$^+$ endothelial cells and systemic mobilization of hematopoietic progenitors. J Clin Invest 2005;115:653–663.
34 Montano X, Djamgoz MB: Epidermal growth factor, neurotrophins and the metastatic cascade in prostate cancer. FEBS Lett 2004;571: 1–8.
35 Sclabas GM, Fujioka S, Schmidt C, et al: Overexpression of tropomysin-related kinase B in metastatic human pancreatic cancer cells. Clin Cancer Res 2005;11:440–449.