The role of neuregulin4 and HER4 in gastrointestinal malignant lymphoma

MASAHIDE EBI[1], HIROMI KATAOKA[1], TAKAYA SHIMURA[1], YOSHIKAZU HIRATA[1], TAKASHI MIZUSHIMA[1], TSUTOMU MIZOSHITA[1], MAMORU TANAKA[1], HIRONOBU TSUKAMOTO[1], KEIJI OZEKI[1], SATOSHI TANIDA[1], TAKESHI KAMIYA[1], HIROSHI INAGAKI[2] and TAKASHI JOH[1]

Departments of [1]Gastroenterology and Metabolism, and [2]Pathology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

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Abstract. The human epidermal growth factor (EGF) receptor (HER) family consists of four receptors that bind to ligands sharing an EGF-like motif. The HER family of receptor tyrosine kinases and their ligands (EGF family) are known to play a significant role in gastrointestinal cancer. In particular, the EGF receptor, HER1, is one of the main candidates for the molecular-targeted therapy of colon cancer, and HER2 is a candidate for the treatment of gastric cancer which overexpresses HER2. In contrast, the role of the HER and EGF families in malignant lymphoma has not been fully elucidated. In this study, we investigated the expression and function of the HER and EGF families in lymphoma cell lines and tumor samples. Reverse transcription polymerase chain reaction revealed that the ligands for HER1 were mainly expressed in gastric cancer and colon cancer cell lines, but not in lymphoma cell lines. On the other hand, the EGF family member, neuregulin (NRG) 4, was highly expressed in lymphoma cell lines. Immunohistochemical analyses of malignant lymphoma clinical samples revealed that NRG4 and HER4 were mainly expressed in mucosa-associated lymphoid tissue (MALT) and follicular lymphoma. Immunoprecipitation of Raji and Daudi cell lines revealed that recombinant NRG4 induced the tyrosine phosphorylation of HER4. Additionally, recombinant NRG4 activated the proliferation of lymphoma cell lines. These findings suggest that the NRG4-HER4 axis plays a major role in the proliferation of malignant lymphoma cells in the gastrointestinal tract.

Introduction

Neuregulins (NRGs) have recently been identified as new members of the epidermal growth factor (EGF) family (1,2). NRG-1-4 are ligands for the human EGF receptor (HER)3 and HER4. NRGs signal through HER3 and HER4 tyrosine kinases to activate downstream signaling pathways, such as phosphatidyl inositol 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) (3). In human cancers, NRG expression has been reported in papillary thyroid, ovarian, prostate and breast cancers (4-6). Out of these, the expression and function of NRGs have been intensively investigated in breast cancer (7-9), and NRG expression in breast cancer has been reported to correlate with the response to trastuzumab therapy targeted against the HER2 molecule (10).

Although the role of NRGs in several types of cancer has been reported, the expression and function of NRGs in lymphoma cells have not yet been fully elucidated. We previously investigated the expression and function of the EGF family in gastrointestinal cancer and cancer therapy (11-13). In the present study, we unexpectedly found that NRG4 was highly expressed in malignant lymphoma cells in the gastrointestinal tract. We reveal the expression and function of NRG4 and its receptor, HER4, in malignant lymphoma.

Materials and methods

Cell culture. KARPAS-422, NCU-L-4, Raji and Daudi lymphoma cell lines, as well as the MKN28 gastric cancer cell line were seeded in 10-cm dishes and cultured for 48 h with RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum and 1% ampicillin and streptomycin in 5% CO_2. The human colon cancer cell line, HT29, was cultured with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, and 1% ampicillin and streptomycin in 5% CO_2.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Eugene, OR, USA), according to the manufacturer's instructions. Reverse transcription was performed using a high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The synthesized cDNA from each sample was subjected to polymerase chain reaction (PCR) amplification using an Amplitaq Gold PCR Master Mix (Applied Biosystems)

Correspondence to: Dr Hiromi Kataoka, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
E-mail: hkataoka@med.nagoya-cu.ac.jp

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and primers. The primer sequences used for each product are shown in Table I. The PCR program was as follows: ten min of initial denaturation at 95˚C, 15 sec at 95˚C, 15 sec at 56-58˚C and 60 sec at 72˚C, repeated for 35 cycles. Amplified products were separated by 2% agarose gel electrophoresis, and bands were visualized by ethidium bromide staining. The gels were photographed under UV illumination.

Immunohistochemistry. We purchased the following antibodies: Rabbit anti-NRG4 polyclonal antibody (ab60090; Abcam, Cambridge, MA, USA), rabbit anti-HER4 polyclonal antibody (RB-9045-R7; Thermo Fisher Scientific, Worcester, MA, USA), mouse anti-HER4 monoclonal antibody (sc-8050; Santa Cruz Biotechnology, Delaware Avenue, CA, USA) and mouse anti-phospho-tyrosine, clone 4G10 (05-321; Millipore, Billerica, MA, USA). The immunohistochemical technique was as previously described (14). Briefly, 4-μm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, antigen retrieval was achieved by heating the samples in 10 mM citrate buffer (pH 6.0) in a microwave oven for 15 min at 98˚C. Sections were then incubated with primary antibodies. After thorough washing in PBS (-), the samples were incubated with biotinylated secondary antibodies and then with avidin-biotin horseradish peroxidase complexes (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, USA). Finally, immunocomplexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3-diaminobenzidine tetrachloride. Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Immunoprecipitation. Cells were treated with recombinant NRG4 protein (H00145957-P01; Abnova, Taipei, Taiwan, China) for 30 min. Cells were then lysed with 1 ml of lysis buffer. After centrifugation of the lysates at 15,000 rpm for 20 min, the supernatants were collected and incubated with 1 μg of anti HER4 antibody for 2 h at 4˚C with end-over-end rotation. Then 20 μl of protein G Sepharose beads (50% suspension; GE Healthcare, Uppsala, Sweden) were added to each lysate/antibody mixture, followed by incubation for 2 h at 4˚C with end-over-end rotation. The mixtures were then centrifuged and the protein G Sepharose beads were washed three times with lysis buffer, re-suspended in 15 ml of 2X SDS-PAGE sample buffer and boiled for 5 min. The bound proteins were analyzed by Western blotting using an anti-phosphorylation antibody.

Table I. Primer sequences.

Primers	Oligonucleotide sequences (5'-3')	Product size (bp)
EGF	F: caggtaatggagcgaagctttca R: gtgcatcgacatagttcattcttcttg	199
TGFα	F: ttaatgactgcccagattccca R: ggaggtccgcatgctcaca	133
AR	F: ccaaaacaagacggaaagtga R: tgttactgcttccaggtgctc	175
HB-EGF	F: cctcctctcggtgcggg R: agtcaccagtgccgagagaactg	86
EPR	F: atcatgtatcccaggagagtccag R: aagtgttcacatcggacaccagt	207
NRG1	F: ccattagaatatcagtatccacagaagg R: ccttctccgcacattttacaaga	99
NRG2	F: tccccagccttcctaccgtt R: tgtagtcgtgagttctttctgccg	102
NRG3	F: cgaggacagtgcaagcgaaa R: ttggtcaatgcagagtctctttgtatt	117
NRG4	F: aacagatcacgaagagccctgt R: tgggaatagtaggtatcacataacaaagc	86
HER1	F: ggagaactgccagaaactgacc R: gcctgcagcacactggttg	106
HER2	F: agccgcgagcacccaagt R: ttggtgggcaggtaggtgagtt	147
HER3	F: gtctgtgtgacccactgcaact R: gggtggcaggagaagcatt	80
HER4	F: ggctgctgagttttcaaggatg R: gcttcatacgatcatcaccctga	74
GAPDH	F: cggagtcaacggatttggtcgtat R: agccttctccatggtggtgaagac	307

Primer sequences for RT-PCR of the EGF family members, EGF, TGF-α, AR, HB-EGF, EPR, NRG1, NRG2, NRG3 and NRG4, and the HER family members, HER1, HER2, HER3 and HER4, and the internal control, GAPDH.

Results

NRG4 mRNA is more highly expressed in lymphoma cell lines than in gastric cancer and colon cell lines. We examined the mRNA expression of the EGF and HER family members in gastric and colon cancer, and malignant lymphoma cell lines using RT-PCR. As shown in Fig. 1, the EGF family members, EGF, amphiregulin (AR), epiregulin (EPR), transforming growth factor α (TGFα) and heparin binding-EGF (HB-EGF), were mainly detected in MKN28 and HT29 cell lines rather than in lymphoma cell lines. Furthermore, their cognate receptors, HER1 and HER2, were also detected in MKN28 and HT29 cell lines. In contrast, the EGF family member, NRG4, and its receptor, HER4, were mainly expressed in lymphoma cell lines.

Immunohistochemical analysis of the expression of NRG4 and HER4 proteins in gastrointestinal lymphoma tissue. As shown in Table II, immunohistochemical analyses of NRG4 and HER4 protein expression were performed using 26 clinical samples of gastrointestinal malignant lymphoma. There were 7 mucosa-associated lymphoid tissue (MALT) lymphomas, 6 follicular lymphomas (FLs), 2 mantle lymphomas, 7 diffuse large B cell lymphomas (DLBCLs), 1 T cell lymphoma and 3 Burkitt lymphomas. NRG4 was expressed in 13 out of 26 samples (positive rate, 48%) and HER4 was expressed in 14 samples (positive rate, 54%). Both NRG4 and HER4 tended to be expressed in MALT and FL. We also examined NRG4

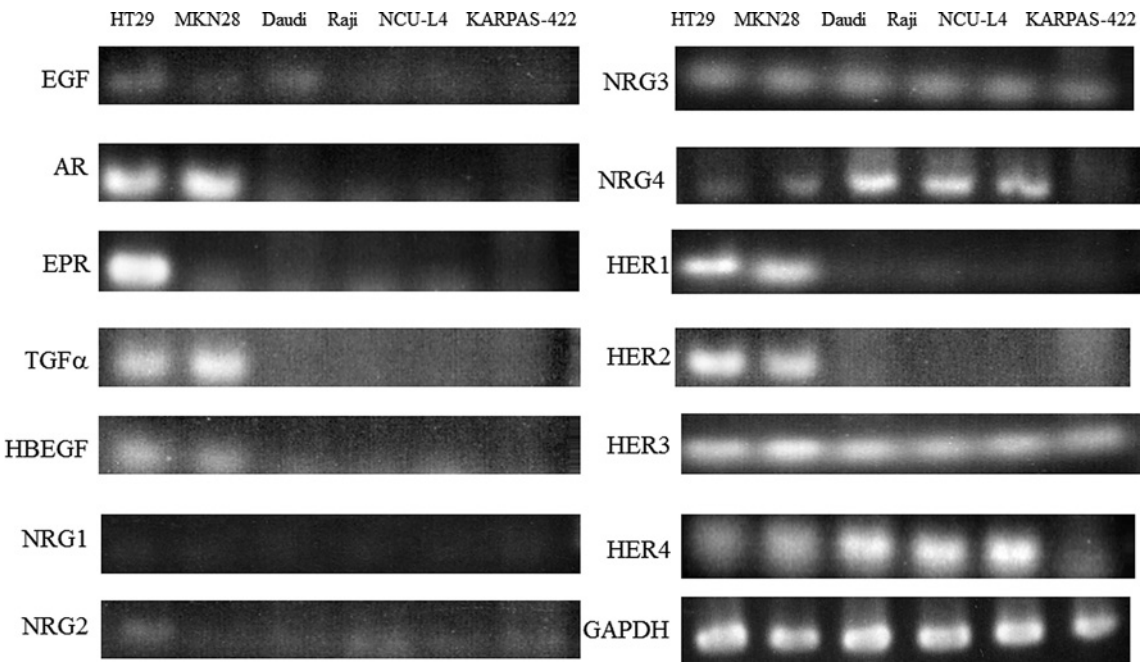

Figure 1. Analysis of EGF and HER family mRNA expression in gastric cancer, colon cancer and malignant lymphoma cell lines. A higher level of expression of HER4 and NRG4 mRNA was detected in lymphoma cell lines compared to gastric and colon cell lines. The expression of EGF and HER family genes in 4 lymphoma, 1 gastric cancer and 1 colon cancer cell line was analyzed using RT-PCR with specific oligonucleotide primer sets followed by agarose gel electrophoresis.

Table II. NRG4 and HER4 expression in ML.

		NRG4 expression		HER4 expression	
Histological type	No.	Positive	Negative	Positive	Negative
MALT	7	5	2	4	3
Follicular	6	4	2	3	3
Mantle	2	1	1	0	2
Diffuse	7	2	5	4	3
T cell	1	0	1	1	0
Burkitt	3	1	2	2	1

Immunohistochemical analyses of NRG4 and HER4 expression in human malignant lymphoma tissues.

expression in 2 normal lymph node clinical samples as the control, but they were negative. Representative immunohistochemical staining of NRG4 and HER4 is shown in Fig. 2.

NRG4 induces HER4 phosphorylation. NRG4 has been reported to induce HER4 tyrosine phosphorylation and MAPK activation (15). To determine whether NRG4 activates HER4 in lymphoma cells, we used Western blotting to examine the tyrosine phosphorylation of HER4 that was precipitated from lymphoma cells stimulated with recombinant NRG4 protein. As shown in Fig. 3, 1 μg/ml recombinant NRG4 induced HER4 phosphorylation.

NRG4 enhances lymphoma cell proliferation. Finally, we investigated cell growth regulation by NRG4. As shown in Fig. 4, the proliferation of two lymphoma cell lines was significantly increased by stimulation with 1 μg/ml recombinant NRG4 ($p<0.05$). These results indicate that NRG4 increases lymphoma cell growth via binding to HER4 and thus inducing HER4 phosphorylation.

Discussion

In this study we demonstrate a previously unknown role for the NRG family members and their receptors in gastrointestinal malignant lymphoma. The NRGs are EGF family members that function as ligands for the HER3 and HER4 receptors. Four members of the NRG family have been identified. NRG1α and NRG1β are ligands for HER3 and HER4. NRG2, NRG3 and NRG4 are ligands for HER4 (16). NRGs activate downstream signaling pathways, such as PI3K and MAPK (17,18).

EGF, TGFα, HB-EGF, AR, ligands of HER1 (EGF receptor), as well as HER1 and HER2 have been intensively studied in the area of cancer biology and cancer therapy (13,19-21). Recently,

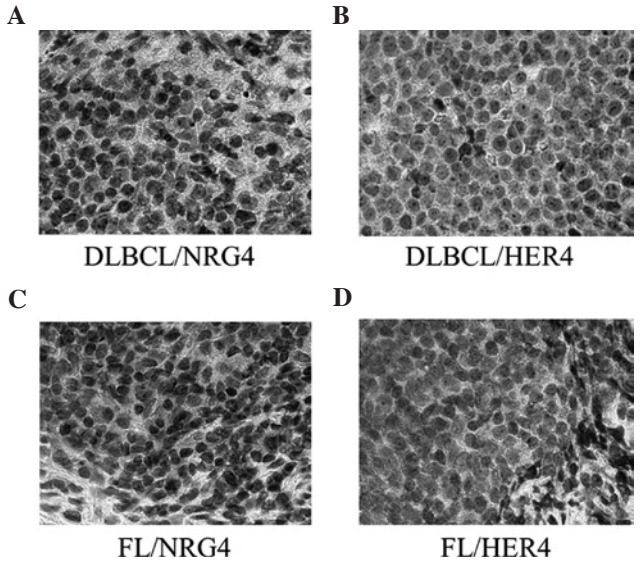

Figure 2. Immunohistochemical staining of DLBCL and FL with an antibody (A and C) against NRG4 or (B and D) against HER4.

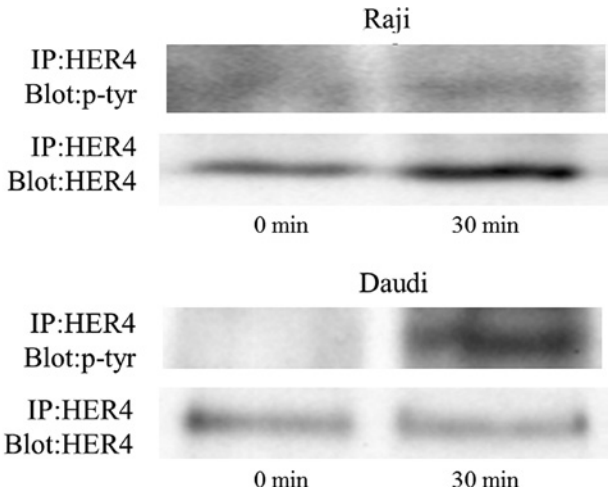

Figure 3. Analysis of NRG4 induction of HER4 tyrosine phosphorylation. Two kinds of lymphoma cell lines were treated with 1 μg/ml of recombinant NRG4 for 30 min. HER4 was immunoprecipitated from extracts of the lymphoma cells with an anti-HER4 antibody and was subsequently divided into two equal aliquots. One aliquot was subjected to Western blotting with an anti-P-Tyr antibody, and the second aliquot was subjected to Western blotting with an anti-HER4 loading control antibody.

a few monoclonal antibodies against HER1 and HER2, such as cetuximab, panitumumab and trastuzumab, have been clinically used for the treatment of cancers, such as breast, gastric and colorectal cancers (22-24). However, NRG family members and their receptors HER3 and HER4 are not considered to play a crucial role in cancer cells.

The contribution of several kinds of growth factors to the proliferation and survival of lymphoma cells has been reported. Recently, the vascular endothelial growth factor (VEGF) and its receptor, Flt-4, were reported to play a crucial role in the expansion of MALT lymphoma (25,26). A high level of expression of VEGF, as well as of its receptors, VEGF-receptor-1 and VEGF-receptor-2, was also reported in DLBCL (27). These studies revealed that the expression levels of VEGF and VEGF-receptor-1 could function as a prognostic marker of DLBCL with anthracycline-based chemotherapy. Paydas *et al* reported that the high expression of VEGF-C may be a risk factor for the prognosis of DLBCL (28). Single nuclear polymorphisms of the VEGF gene in mantle cell lymphoma (29) and a correlation between the VEGF concentration of platelets and tumor angiogenesis in canine non-Hodgkin lymphoma models (30) have also been reported. Apart from VEGF, high expression of the platelet-derived growth factor in Hodgkin's and non-Hodgkin's lymphoma has also been reported (31). However, there has only been one report regarding the expression of members of the EGF and HER family in lymphomas. In that study, published in 1985, the translocation of chromosome 2, on which TGFα is located, was reported in Burkitt's lymphoma (32).

In this study, we investigated the expression of the HER and EGF family members in lymphoma cell lines. Unexpectedly, we found high mRNA expression of NRG4 and its receptor, HER4, using RT-PCR in three out of four lymphoma cell lines. In contrast, NRG4 expression levels in gastric and colon cancer cells were much lower than those of lymphoma cells (Fig. 1).

Additionally, we detected NRG4 expression in 48% of lymphoma tissues assayed and MALT and FL in particular, frequently expressed NRG4 (71 and 67%, respectively). HER4 was also highly expressed in MALT and FL (57 and 50%, respectively) (Table II).

Furthermore, we confirm that NRG4 induces HER4 tyrosine phosphorylation and increases the proliferation of lymphoma cells (Figs. 3 and 4). These results indicate that the NRG4-HER4

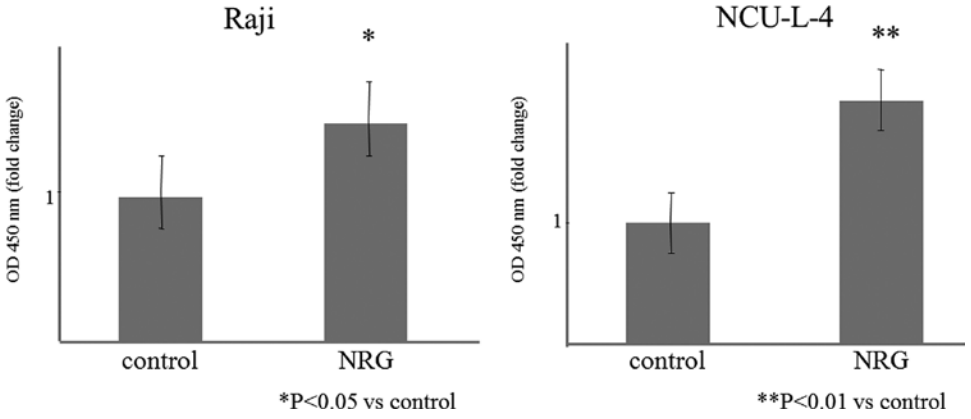

Figure 4. The effects of recombinant NRG4 stimulation on lymphoma cell proliferation. Two different lymphoma cell lines were incubated with 1 μg/ml recombinant NRG4 for 48 h. The Cell Counting Kit8 was used to analyze the cell proliferation of the two lymphoma cell lines.

axis might play a crucial role in the growth regulation in lymphoma cells, especially of MALT and FL cells.

In conclusion, NRG4 and HER4 are expressed in lymphoma cells, especially in MALT and FL cells of the gastrointestinal tract. The NRG4-HER4 axis may play an important role in lymphoma cell growth and could be a candidate for the molecular-targeted therapy of malignant lymphoma.

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