Oncogenic Fusion Gene CD74-NRG1 Confers Cancer Stem Cell–like Properties in Lung Cancer through a IGF2 Autocrine/Paracrine Circuit

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Abstract

The CD74-Neuregulin1 (NRG1) fusion gene was recently identified as novel driver of invasive mucinous adenocarcinoma, a malignant form of lung cancer. However, the function of the CD74-NRG1 fusion gene in adenocarcinoma pathogenesis and the mechanisms by which it may impart protumorigenic characteristics to cancer stem cells (CSC) is still unclear. In this study, we found that the expression of the CD74-NRG1 fusion gene increased the population of lung cancer cells with CSC-like properties. CD74-NRG1 expression facilitated sphere formation not only of cancer cells, but also of nonmalignant lung epithelial cells. Using a limiting dilution assay in a xenograft model, we further show that the CD74-NRG1 fusion gene enhanced tumor initiation. Mechanistically, we found that CD74-NRG1 expression promoted the phosphorylation of ErbB2/3 and activated the PI3K/Akt/NF-κB signaling pathway. Furthermore, the expression of the secreted insulin-like growth factor 2 (IGF2) and phosphorylation of its receptor, IGF1R, were enhanced in an NF-κB–dependent manner in cells expressing CD74-NRG1. These findings suggest that CD74-NRG1–induced NF-κB activity promotes the IGF2 autocrine/paracrine circuit. Moreover, inhibition of ErbB2, PI3K, NF-κB, or IGF2 suppressed CD74-NRG1–induced tumor sphere formation. Therefore, our study provides a preclinical rationale for developing treatment approaches based on these identified pathways to suppress CSC properties that promote tumor progression and recurrence.

Introduction

Cancer stem cells (CSC) are thought to be responsible for tumor recurrence and drug resistance (1). It is also believed that many cancer cells are actually differentiated cells generated from CSCs, similar to how normal tissues are derived from tissue-specific stem cells (1). By definition, CSCs represent a distinct cell population with self-renewal capacity that can be prospectively isolated. This population of cancer cells was initially identified in acute myeloid leukemia in 1997 (2). Since then, several properties of CSCs have been described, and cancer cells that exhibit some CSC properties have been detected in many solid tumors, including lung cancer and breast cancer (3–6). Because CSCs are thought to be resistant to various stressful conditions such as treatment with chemotherapy and molecular targeted drugs, they may survive regardless of tumor shrinkage. After some time, the small number of therapy-resistant CSCs may start to grow, leading to recurrence associated with drug resistance. Therefore, targeting molecules that play a critical role in maintenance of CSCs is an important therapeutic strategy to eradicate tumors and prevent recurrence.

Recently, oncogenic fusion genes have been discovered in solid tumors, especially in lung cancer. In lung adenocarcinomas, a major type of lung cancer, oncogene fusions frequently occur and it may act as driver gene aberrations as well as EGFR or KRAS oncogene mutations. The ALK, RET, and ROS1 fusion genes have already been reported to be involved in cancer development (7–10). Crizotinib, an inhibitor of ALK kinase, is clinically available and has been shown to be effective in lung adenocarcinomas with EML4-ALK fusion. Much effort has been devoted to developing targeted drugs against the tyrosine kinases in the fusion protein for improving therapeutic strategies. However, major concerns regarding recurrence and resistance to the targeted drugs against the tyrosine kinases have emerged, resulting in poor prognosis in cancer patients (11). In fact, it is largely unknown whether the fusion genes are functional in terms of initiation and maintenance of CSCs. If such mechanisms exist, novel therapeutic strategies based on these could be developed.

We and other researchers recently identified the CD74-Neuregulin1 (NRG1) fusion gene in a portion of invasive mucinous adenocarcinomas (IMA) of the lung (12, 13). IMA is a highly malignant type of lung adenocarcinoma that is mainly caused by KRAS mutations. However, this fusion gene is found in cancers...
that lack other targetable oncogene mutations such as KRAS, EGFR, BRAF, and ERBB2. Therefore, the fusion gene may play important roles as a driver gene aberration in the development of IMA and has the potential to be a novel therapeutic target. CD74 is a transmembrane protein that consists of extracellular, transmembrane, and intracellular domains. NRG1, also called heregulin, is a ligand for the ErbB3/HER3 tyrosine kinase (14). The CD74-NRG1 protein retains the transmembrane domain of CD74 and the EGF-like domain of NRG1 (NRG III-β3 form). The NRG III-β3 form protein has a cytosolic N-terminus and a membrane-tethered EGF-like domain, and mediates juxtacrine signaling through ErbB2/ErbB3, because the retained EGF-like domain has biologic activity. Following cleavage at the border of the EGF-like domain, NRG1 is also secreted and stimulates ErbB2/ErbB3 in an autocrine/paracrine manner. Recently, other types of fusion genes that include a part of NRG1 have been reported not only in IMA of the lung but also in ovarian cancer (15). The breakpoints in NRG1 occur in breast cancer tissues (16, 17), and the resulting fusion gene products are secreted by a breast cancer cell line (18). Typically, this part of NRG1 is thought to be able to stimulate ErbB2/ErbB3.

We previously reported that the NRG1 protein enhances tumor sphere formation by breast cancer cells by activating signaling pathways through ErbB2/ErbB3 heterodimers (19). The process of sphere formation partly recapitulates the tumorigenic process because only cells with CSC properties are thought to be resistant to anoikis, which is apoptosis caused by the loss of adhesive survival signals in suspension culture, and form clonal spheroids in sphere culture medium (SCM), which is serum-free but contains several growth factors and hormones (20, 21). Similar to breast CSCs, lung CSCs can be grown and isolated using the sphere culture protocol (6, 22, 23).

In this study, we showed that expression of the CD74-NRG1 fusion protein enhances the efficiency of tumor sphere formation in vitro and tumor-initiating ability in vivo, which are two important criteria for cells with CSC properties (24). The hypothesis that CD74-NRG1 initiates CSCs in lung tissues was further supported by the observation that expression of the fusion gene induced sphere formation by normal lung epithelial cells. We showed that the CD74-NRG1 protein activates the PI3K/Akt/IF-gamma signaling pathway. Moreover, the production and secretion of IGF2 protein, which is essential for this tumor sphere formation, was dependent on NF-kappaB activity. The resulting autocrine/paracrine circuit that involves IGF2 appears to maintain CSCs. To the best of our knowledge, this is the first study reporting that oncogenic fusion gene products indeed function at the level of CSCs. Thus, inhibition of this signaling pathway by targeting a single molecule or several molecules in combination is an efficient way to treat CD74-NRG1 fusion-positive cancers for eradication of cancer cells.

**Materials and Methods**

**Cell lines and cell culture**

Lung cancer cell line H322 and breast cancer cell line BT20 were purchased from the ATCC. Cells were cultured in RPMI1640 with 10% FBS (Gibco) and 1% penicillin-streptomycin (P/S; Nacalai). HEK293T cells (ATCC) for lentivirus production were cultured in DMEM: nutrient mixture with 10% FBS and 1% P/S. The cells were maintained in a humidified incubator at 5% CO2 at 37°C.

**Western blot analysis**

Immunoblotting was performed using standard procedures as described (19). Anti-ErbB2, p-ErbB2, p-ErbB3, Akt, p-Akt, Nanog, Oct-4, Sox2, IKBα, IκBβ, p-IκBα/β, IκBε, p-IGF1R, receptor for IGF2, and p-IGF1R antibodies were purchased from Cell Signaling Technology. Anti-ErbB3 and actin antibodies were purchased from Millipore. Anti-NRG1 and CD74 antibodies were purchased from Thermo Scientific and Abcam, respectively. Proteins were detected with horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare Life Sciences).

**Sphere formation assay**

Sphere formation assay was performed as described (19). Briefly, cells were plated as single cells on ultralow attachment 24-well plates (2,000–5,000 cells/well). They were grown in SCM, which consisted of serum-free DMEM/F-12 medium (Gibco) supplemented with 20 ng/mL EG2 (Millipore), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech), B27 (Gibco), and heparin (Stem Cell Technologies) or in DMEM/F-12 medium with or without inhibitors or antibodies. LY294002 and anti-IGF2 antibody were purchased from Cell Signaling Technology. Laptatinib and dasatinib were purchased from Selleck Chemicals. DHMEQ was a kind gift from K. Umezawa (Aichi Medical School, Aichi, Japan). Spheres with a diameter >75 μm were counted after 4 to 7 days.

**Proliferation assay**

Cells were seeded in a 12-well plate at low density (5,000 cells/well), and cultured in RPMI1640 with 10% FBS and 1% P/S. After 4 to 6 days, cells were harvested and counted.

**Construction of lentiviral vectors for expression of CD74-NRG1**

Expression vectors were constructed as described previously (12). Briefly, full-length cDNAs were amplified from tumor cDNA by PCR and then inserted into pLenti-6/V5-DEST plasmids (Invitrogen). By using Sanger sequencing, the integrity of inserted cDNA was verified.

**Viral infection**

H322 cells, BT20 cells, and small airway epithelial cells (SAEC) at 60% to 70% confluence were infected with empty lentiviruses or CD74-NRG1–expressing lentiviruses, and then treated with blastcidin (Invitrogen; 10, 20, and 5 μg/mL, respectively) for stable expression as described previously (12).

**Flow cytometry analysis**

To identify the breast CSC population, cells were stained with Alex Fluor 647–labeled anti-human CD24 and APC-H7–labeled anti-human CD44 antibodies (BD Pharmingen) at 4°C for 20 minutes. Then cells were analyzed with FACSVerse II flow cytometer (BD Biosciences). Dead cells were excluded by propidium iodide (PI) staining. Data were analyzed with FlowJo software (Treestar).

**Quantification of NF-κB activity by ELISA**

Nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif), and NF-κB subunit p65-DNA binding activity was measured with a TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif). All procedures were performed according to the manufacturer's protocol.
Measurement of IGF2 concentration in culture medium

Cells were seeded in 60-mm dishes and cultured in RPMI medium with 10% FBS. At 60% to 70% confluence, the medium was changed to 1.5 mL of RPMI without FBS. After 24-hour incubation, the medium was collected for IGF2 measurement assay. We concentrated 500 μL culture medium to 50 μL by using a microcon (Millipore) and then measured the IGF2 concentration with the IGF2 Human ELISA Kit (Mediagnost). All procedures were performed according to the manufacturer’s protocol.

Xenografts

Cells were admixed with 50 μL Matrigel (BD Biosciences) and the cell mixture was injected into the right flank of 8-week-old nude mice. Tumors larger than 200 mm³ were counted. Tumor volume was measured two times a week using the following formula: \( V = \frac{1}{2}(L \times W^2) \), where \( L \) equals length, and \( W \) equals width.

Statistical analysis

All data are presented as the mean ± SE. The unpaired Student t test was used to compare differences between two samples and values of \( P < 0.01 \) (\(*\)), \( P < 0.001 \)–\( 0.01 \) (**), or \( P < 0.001 \) (***), or \( P < 0.001 \) (***) were considered significant. Tumor-initiating frequency was calculated using the ELDA Software (25).

Study approval

Mice were handled according to the guidelines of National Cancer Center Research Institute, Institute of Medical Science, the University of Tokyo and Kanazawa University. The experiments were approved by the Committees for Animal Research.
at National Cancer Center Research Institute, Institute of Medical Science, the University of Tokyo and Kanazawa University.

Results
CD74-NRG1 protein induces sphere formation of cancer cells
We first examined whether the CD74-NRG1 fusion protein induces sphere formation by lung cancer cells. To evaluate the sphere-forming ability of CD74-NRG1–expressing cells, we infected lentivirus encoding cDNA for the CD74-NRG1 fusion gene, C6;N6 and C8;N6 variants, as reported by Nakaoku and colleagues (12), into H322 lung cancer cells (Fig. 1A and B). These two variants are different in the breakpoints of CD74. We chose H322 cells for this study because they have no KRAS mutations. When we cultured these cells in conventional SCM containing EGF, bFGF, and B27 supplement, they generated spheres with similar efficiency as cells infected with lentivirus carrying a control vector (Fig. 1C and D). Intriguingly, CD74-NRG1–expressing cells also generated spheres when cultured in medium without EGF, bFGF, or B27 supplement, whereas control cells did not (Fig. 1C and D). Because CSC-related function of NRG1 protein was originally identified in breast cancer cells (19), we constructed CD74-NRG1–expressing BT20 breast cancer cells to investigate the mammosphere-forming ability (Fig. 1B). CD74-NRG1–expressing breast cancer cells formed mammospheres even when cultured in a medium without EGF, bFGF, or B27 supplement (Fig. 1E and F). These findings indicate that the CD74-NRG1 fusion protein induces tumor sphere-forming ability in lung and breast cancer cells.

The CSC population increased in CD74-NRG1–expressing cells
Next, we examined expression levels of the stem cell marker proteins, Nanog, Oct-3/4, and Sox2 (26). These stem cell markers were expressed at higher levels in CD74-NRG1–expressing H322 cells than in control cells (Fig. 2A). Also, in CD74-NRG1–expressing BT20 cells, expression levels of the stem cell markers were higher than in control cells (Fig. 2B). In breast cancer, the CD44high/CD24–/low cell population is enriched with cancer cells with stem-like properties (3, 27). When we investigated the proportion of CD44high/CD24–/low CSC-enriched cells by flow cytometry, the percentages of the CD44high/CD24–/low population increased from 1.94% to 9.47% (C6;N6 variant) or 8.21%...
(C8;N6 variant; Fig. 2C). These data further support the idea that the CD74-NRG1 fusion gene can enhance CSC properties. On the other hand, the proliferation assay revealed that the CD74-NRG1 protein did not significantly induce cell growth in adherent cultures (Fig. 2D and E) in the medium containing 10% FBS or the starvation medium with 0.5% FBS (Fig. 2F).

The CD74-NRG1 protein activates the PI3K/Akt pathway and controls sphere formation ability

Because the CD74-NRG1 fusion protein contains the functional domain of NRG1, we hypothesized that activation of the ErbB2/ErbB3–regulated pathway contributes to sphere formation by CD74-NRG1–expressing cells. To test this hypothesis, we examined the phosphorylation levels of ErbB2, ErbB3, and Akt. Expression of the CD74-NRG1 fusion protein increased the phosphorylation levels of ErbB2, ErbB3, and Akt compared with vector control cells (Fig. 3A). Similar results were observed in CD74-NRG1–expressing BT20 cells (Fig. 3B). These data show that the CD74-NRG1 fusion protein activates ErbB2 and ErbB3 heterodimer receptors, leading to PI3K/Akt pathway activation. Then, to investigate whether activation of the ErbB signaling pathway is important for tumor sphere formation, we checked the effect of lapatinib, an inhibitory protein, in the cytoplasm (28). IKKα/β are the upstream kinases involved in the phosphorylation of IkBα, which results in its ubiquitination, proteasome-mediated degradation, and the subsequent release of NF-κB. The released NF-κB translocates to the nucleus and binds to the κB sequence, where it promotes the transcription of various genes. We compared phosphorylation levels of these proteins in CD74-NRG1–expressing cells and control cells. In CD74-NRG1–expressing cells, phosphorylation levels of IKKα/β and IkBα were increased (Fig. 4A). To examine the DNA-binding activity of NF-κB subunit p65 in CD74-NRG1–expressing cells, we quantified the intensity of the p65/DNA complex formation by ELISA. Expression of CD74-NRG1 fusion protein led to a marked increase in the DNA-binding activity of p65 (Fig. 4B and C). Thus, the CD74-NRG1 protein-stimulated ErbB2/ErbB3 signaling pathway appears to activate PI3K/Akt, leading to NF-κB activation. To test whether activation of PI3K or NF-κB is involved in the sphere-forming ability of CD74-NRG1–expressing cells, we treated these cells with LY294002 and DHMEQ, specific inhibitors of PI3K and NF-κB. 

NF-κB activation contributes to sphere formation by CD74-NRG1–expressing cells

The NF-κB transcription factor complex, a downstream target of Akt, is activated by the NRG1-stimulated ErbB2/ErbB3 signaling pathway (19). We then investigated whether NF-κB signaling is activated in CD74-NRG1–expressing cells. The NF-κB transcription factor complex is usually inactive and bound to IκBα, an inhibitory protein, in the cytoplasm (28). IKKα/β are the upstream kinases involved in the phosphorylation of IκBα, which results in its ubiquitination, proteasome-mediated degradation, and the subsequent release of NF-κB. The released NF-κB translocates to the nucleus and binds to the κB sequence, where it promotes the transcription of various genes. We compared phosphorylation levels of these proteins in CD74-NRG1–expressing cells and control cells. In CD74-NRG1–expressing cells, phosphorylation levels of IKKα/β and IkBα were increased (Fig. 4A). To examine the DNA-binding activity of NF-κB subunit p65 in CD74-NRG1–expressing cells, we quantified the intensity of the p65/DNA complex formation by ELISA. Expression of CD74-NRG1 fusion protein led to a marked increase in the DNA-binding activity of p65 (Fig. 4B and C). Thus, the CD74-NRG1 protein-stimulated ErbB2/ErbB3 signaling pathway appears to activate PI3K/Akt, leading to NF-κB activation. To test whether activation of PI3K or NF-κB is involved in the sphere-forming ability of CD74-NRG1–expressing cells, we treated these cells with LY294002 and DHMEQ, specific inhibitors of PI3K and NF-κB.

**Figure 3.** Activation of the PI3K/Akt pathway induces sphere formation. A and B, phosphorylation levels of ErbB2, ErbB3, and Akt in vector- or CD74-NRG1-transduced H322 cells (A) and BT20 cells (B) were determined by immunoblotting. C and D, sphere formation assay with vector- or CD74-NRG1-transduced H322 cells (C) or BT20 cells (D). Cells were incubated with or without 100 nmol/L lapatinib or 100 nmol/L dasatinib (N.T., not treated; n = 4; ***, P < 0.001).
respectively (29). LY294002 or DHMEQ suppressed sphere formation at the similar levels in both H322 and BT20 cells (Fig. 4D and E). These data indicate that PI3K/Akt/NF-κB pathway induces tumor sphere-forming ability.

IGF2 plays important roles in sphere formation induced by the CD74-NRG1 fusion protein

We have recently found that IGF2 is a downstream target of NF-κB upon stimulation with NRG (Tominaga K, Murayama T, and colleagues; unpublished data). We next measured secreted IGF2 protein in culture medium. The amount of IGF2 protein was increased by CD74-NRG1 expression: 0.91 ng/mL and 0.98 ng/mL in cells expressing C6;N6 and C8;N6 variants of CD74-NRG1, respectively, compared with 0.71 ng/mL in control cells (n = 2). To investigate whether secreted IGF2 is involved in sphere formation, we added IGF2-neutralizing antibody to the medium and measured sphere-forming efficiency in H322 cells and BT20 cells. The IGF2-neutralizing antibody greatly decreased sphere-forming efficiency in cells expressing either variant of CD74-NRG1 but not in control cells (Fig. 5A and B). To check whether IGF1R, a receptor for IGF2, is activated by secreted IGF2 that is induced by CD74–NRG1–NF-κB pathway, we treated H322 cells expressing C8;N6 variants of CD74-NRG1 with or without DHMEQ and examined phosphorylation of IGF1R. We found that the phosphorylation levels of IGF1R were increased by expression of CD74-NRG1 protein (Fig. 5C). The increased phosphorylation levels of IGF1R

Figure 4.
NF-κB is activated by CD74-NRG1 expression. A, phosphorylation levels of IKKα/β and IκBα in vector- or CD74-NRG1-transduced H322 cells were determined by immunoblotting. B and C, the DNA-binding activities of p65 in H322 (B) and BT20 (C) cells were quantified by ELISA (n = 3; *, P < 0.05; **, P < 0.01). D and E, sphere formation assay with vector- or CD74-NRG1-transduced H322 (D) and BT20 (E) cells. Cells were incubated with or without 1 μg/mL DHMEQ or 1 μmol/L LY294002 (N.T., not treated; n = 4; ***, P < 0.001).
The CD74-NRG1 protein induces sphere formation of normal lung epithelial cells

Finally, we extended our analysis to noncancerous cells. Using the lentivirus system, we created CD74-NRG1–expressing SAECs, which are immortalized normal lung epithelial cells (30). We analyzed the efficiency of sphere formation by these cells in serum-free medium without EGF, bFGF, or B27 supplement. We found that CD74-NRG1–expressing SAECs formed spheres, but control SAECs formed no spheres. These results support the notion that CD74-NRG1 initiates CSCs in lung tissues without requiring paracrine factors such as growth factors, indicating that this fusion gene is a strong driver gene (Fig. 6B and C).

Discussion

In this study, we provide evidence that the CD74-NRG1 fusion gene appears to play critical roles in the initiation and maintenance of CSC properties. Moreover, we clarified the signaling pathways controlled by the CD74-NRG1 protein. The CD74-NRG1 protein activates the ErbB/PI3K/NF-κB pathway, which leads to activation of the IGF2-autocrine/paracrine circuit. Thus, oncogenesis may occur at the level of CSCs. It is reasonable to hypothesize that the CD74-NRG1 protein confers CSC properties on a few immature, progenitor-like cells rather than on the terminally differentiated cancer cells. This finding is important, because therapy targeting a single molecule or several molecules in combination in this pathway may eradicate tumors and prevent recurrence (Fig. 6D).

IMAs of the lung constitute 2% to 10% of all lung adenocarcinomas in Japan and the United States, and are regarded as more malignant than other more common types of lung adenocarcinomas (31–33). The KRAS mutation was the only driver aberration found in IMAs. However, to improve clinical outcomes, it is necessary to identify novel driver aberrations in IMAs. Following much effort, the CD74-NRG1 fusion gene was identified in IMAs in 2014 (12, 13). This fusion gene is mutually exclusive with KRAS mutations. In CD74-NRG1 fusion–positive tumors, enhanced expression of NRG1 is observed (13). CD74-NRG1 is regarded as a driver gene aberration in the development of IMA. As it has been recently reported that the fusion genes that include a part of NRG1 are found in ovarian cancer (15), the gene alteration involving NRG1 fusion may also occur in other types of tumors.

In breast cancer cells and tissues, breaks within NRG1 are frequently detected and may be responsible for NRG1 gene fusion (16, 17). In fact, DOC4-NRG1 fusion gene is observed in the MDA-MB-175 breast cancer cell line (18). In this cell line, NRG1 expression is enhanced because of the promoter activity of DOC4 and NRG1 is produced in the culture medium. It is, thus, reasonable that some breast cancers are caused by NRG1 gene fusion in a manner similar to that observed in CD74-NRG1 fusion–positive IMAs. We are currently trying to identify important gene

graft model. We injected $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ cells subcutaneously into the right flank of nude mice and observed tumorigenesis. CD74-NRG1–expressing H322 cells induced tumor formation more efficiently than control cells (Table 1). However, the tumor growth rate was not significantly increased by CD74-NRG1 expression (Fig. 6A). These results indicate that the CD74-NRG1 fusion protein has tumor-initiating ability in vivo.

The CD74-NRG1 protein induces sphere formation of normal lung epithelial cells

Figure 5. Secreted IGF2 is crucial for sphere formation. A and B, sphere formation assay with vector- or CD74-NRG1–transduced H322 (A) and BT20 (B) cells. Cells were incubated with 10 μg/mL anti-IGF2–neutralizing antibody (NAb; N.T., not treated) or 5 μg/mL DHMEQ for 24 hours. Phosphorylation levels of IGF1R were determined by immunoblotting. DHMEQ was reduced by DHMEQ treatment. These results suggest that the CD74-NRG1 protein induces sphere formation by activating the PI3K/Akt/NEK-xB/IGF2 signaling pathway and the IGF2 autocrine/paracrine circuit.

The CD74-NRG1 fusion gene enhances the tumor-initiating ability

We next examined whether CD74-NRG1 expression alters the tumor-initiating ability using a limiting dilution assay in a xenograft model. We injected $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ cells...
aberrations including gene fusions in breast cancer by using next-generation sequencing.

In this study, we showed that expression of the CD74-NRG1 protein not only induces sphere-forming ability in vitro but also enhances the tumor-initiating ability in vivo. These findings indicate that this fusion gene is involved in tumor development by inducing CSC properties and will be an effective therapeutic target for these tumors. Moreover, we showed that the CD74-NRG1 protein activates the PI3K/Akt/NF-κB signaling pathway, leading to IGF2 autocrine/paracrine circuit to initiate and maintain cells with CSC properties. Our findings provide a rationale for developing alternative treatment options, despite the emergence of

Table 1. Results of limiting dilution assay of vector- or CD74-NRG1–transduced H322 cells

<table>
<thead>
<tr>
<th>Cells (per site)</th>
<th>Empty vector</th>
<th>CD74-NRG1 (C6;N6)</th>
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<tbody>
<tr>
<td>10^2</td>
<td>1/6</td>
<td>4/6</td>
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<tr>
<td>10^3</td>
<td>3/6</td>
<td>5/6</td>
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<tr>
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<tr>
<td>10^5</td>
<td>5/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Tumor-initiating cell frequency estimate</td>
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<td>1,507</td>
</tr>
<tr>
<td>Probability</td>
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<td>4.25E–03</td>
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Figure 6.
The CD74-NRG1 fusion protein enhances the tumor-initiating ability of cancer cells. A, tumor growth curves of vector- or CD74-NRG1–transduced H322 cells when injected with 1 × 10^4 cells (n = 6). B and C, sphere formation assay with vector- or CD74-NRG1–transduced SAECs. (N.T., not treated; n = 4, ***P < 0.001). Scale bar, 100 μm. D, The CSC maintenance signals. The CD74-NRG1 protein activates PI3K/Akt/NF-κB pathway to induce IGF2 autocrine/paracrine circuit for maintenance of CSCs. Blocking one or several molecules in the pathways would be effective for eradicating tumors.
acquired resistance. Because the major acquired resistance mechanisms have been reported to be additional mutations in the tyrosine kinase domain of the fusion gene, effective drugs targeting the mutant tyrosine kinases have been developed (34). Our findings suggest that targeting other molecules in this pathway, rather than the mutant tyrosine kinases themselves, for the initiation and maintenance of CSCs, may be equally effective.

It does not seem that expression of the CD74-NRG1 protein strongly stimulates cell growth in vitro and in vivo. It is thought that cells with CSC properties grow rather slower than other differentiated cancer cells (1). It is thus reasonable that the CSC properties conferred by expression of the CD74-NRG1 are not strongly associated with stimulation of cell growth. As the important characteristic of CSCs is the resistance to stressful conditions, the CD74-NRG1-expressing cancer cells may be more resistant to conventional chemotherapeutics than those without expression of the CD74-NRG1 protein.

Expression of CD74-NRG1 increased the percentage of CD44high/CD24low CSC-enriched cells from 1.9% to approximately 9.5%. The fact that CD44high/CD24low CSC-enriched cells are still a minor population indicates that expression of the CD74-NRG1 protein is not sufficient for conferring CSC properties on all cells. As the parental BT20 cells do not express the CD74-NRG1 gene, it is possible that the intrinsic CSC properties carried by a subpopulation of BT20 cells are conferred by other gene alterations. However, the increase in the population of BT20 cells with CSC properties by enforced CD74-NRG1 expression may indicate that the intrinsic CSC properties are conferred on more cells by the IGF2 autocrine/paracrine circuit. It is known that even within a cancer cell line, there are immature cell populations with CSC properties and other differentiated cancer cell populations (27, 35). It is thus possible that expression of the CD74-NRG1 protein shifts the cell population toward more cells with CSC properties than differentiated cells.

The mechanisms underlying the production of IGF2 by NF-kB are unclear. We recently found that NRG stimulates the transcription of IGF2 mRNA in an NF-kB-dependent manner (Tominaga K, Murayama T, and colleagues; unpublished data). Several binding site motifs for NF-kB are present in the IGF2 promoter sequence. Thus, increased NF-kB activity may lead to production of IGF2 at the transcriptional level.

In conclusion, our results suggest that PI3K/Akt/NF-kB/IGF2 signaling activated by the CD74-NRG1 fusion protein is involved in CSC maintenance and tumor initiation. Therefore, development of mutant inhibitors or antibodies targeting the molecules in this pathway is anticipated to improve the prognosis of IMA patients with the CD74-NRG1 fusion gene. Furthermore, establishment of effective diagnostic methods capable of detecting this gene aberration is necessary. A therapeutic strategy that targets cells with CSC properties by inhibiting PI3K/Akt/NF-kB may be useful in other types of cancers caused by NRG1 gene fusions, besides IMAs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T. Murayama, T. Nakaoka, N. Gotoh

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Murayama, N. Gotoh

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Murayama, T. Nakaoka, T. Nishimura, K. Tominaga, A. Nakata, N. Gotoh

Writing, review, and/or revision of the manuscript: T. Murayama, N. Gotoh

Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): M. Enan, A. Tojo, S. Sugano

Study supervision: S. Sugano, T. Kohn

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Oncogenic Fusion Gene CD74-NRG1 Confers Cancer Stem Cell-like Properties in Lung Cancer through a IGF2 Autocrine/Paracrine Circuit

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