Diagnostic Relevance of Overexpressed NOK mRNA in Breast Cancer

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Abstract. A novel oncogene with a kinase domain (NOK), a receptor protein tyrosine kinase, has been reported to cause proliferation of normal cells, suggesting its possible use as a diagnostic marker in human cancer. To determine the significance of NOK expression in cancer cells, the effect of NOK inhibition was first examined on cell proliferation in vitro. The degree of expression in 52 clinical breast cancer samples was then correlated with clinical features. The transduction of NOK small inhibitory (si) RNA in T47D breast cancer cells decreased NOK mRNA expression, thereby inhibiting growth. When the mean expression in non-cancerous tissues from the same breast resection specimens ±2SD was used as a cut-off value, 67.3% of breast cancers were positive for NOK expression – a higher positivity rate than that found for c-erbB2 (28.8%). NOK mRNA expression did not correlate with c-erbB2 expression, indicating the independence of NOK as a diagnostic marker. Furthermore, NOK mRNA was highly expressed even at early clinical stages. NOK mRNA might be an ideal target to support the diagnosis of breast cancer especially in tiny tumors in which the malignancy cannot be confirmed by other means.

Receptor protein tyrosine kinases (RPTK) are key molecules in cell proliferation, affecting cell cycle progression, as well as resistance to apoptotic stimuli in cancer cells (1). After RPTK are activated by self-phosphorylation, with or without ligand binding, they initiate the growth signals before most other molecules have acted (2, 3). Since many RPTK genes are overexpressed or mutated in various cancers (1), RPTKs can serve as markers for the diagnosis of cancer that conventional diagnostic methods cannot detect or confirm.

The expression profile of an epidermal growth factor receptor family member, c-erbB2/HER2/neu, has been well-investigated, confirming clinical significance in breast cancers. Amplification or overexpression of c-erbB2 in breast cancer was associated with a poor prognosis (4, 5). From this evidence, antibody therapy targeting c-erbB2/HER2/neu was carried out in breast cancer patients, not only with metastasis, but also at early stages. Expression of the c-erbB2 gene or protein was examined in tumors by fluorescence in situ hybridization or immunohistochemistry to determine whether such therapy is indicated (6, 7). However, amplification or overexpression of c-erbB2 and other RPTK was detected in only 20% to 30% of breast cancers, and many patients still have poor outcomes in the absence of overexpression.

The novel oncogene with a kinase domain (NOK), an RPTK-like protein recently identified by Liu et al., shares 20% to 30% homology with members of the fibroblast growth factor receptor (FGFR)/platelet-derived growth factor receptor (PDGFR) family (8). NOK has a single putative transmembrane domain and an intracellular tyrosine kinase domain, but lacks an extracellular domain. Overexpression of NOK gene resulted in growth factor-independent cell proliferation in BaF3 cells, and also surface adhesion-independent growth and colony formation in both NIH3T3 and BaF3 cell (8). Furthermore, the overexpression of NOK in BaF3 cells induced tumorigenesis and metastasis in nude mice. These observations indicate that NOK is a critical molecule in promoting cell proliferation and suggest that NOK expression could be up-regulated in cancer cells. NOK was hypothesized to serve as a novel marker for the genetic diagnosis of breast cancer. To date, however, the degree of importance of NOK expression in cancer cells and the differences in NOK expression between human cancer and non-cancerous tissues remain unclear. The effect of small inhibitory (si)
RNA against NOK (NOK-siRNA) on the proliferation of breast cancer cells was examined. The degree of NOK mRNA expression in breast cancer tissues was then correlated with clinical features and the prevalence of positivity in breast cancers for NOK and c-erbB2 mRNA expression was compared.

Materials and Methods

Patients and frozen tissue samples. Samples of breast cancer and paired samples of non-cancerous tissue from the same resected breast were obtained from patients undergoing surgery at Sapporo Medical University Hospital and Sapporo Breast Surgical Clinic (Sapporo, Japan). Before the acquisition of these tissues, informed consent was obtained explaining the investigational nature of the study. Tissues were immediately frozen and stored in liquid nitrogen. Tissues were also stained with hematoxylin/eosin and were reviewed by well-experienced pathologists. The clinicopathological factors and clinical stages were evaluated according to the criteria of the Japanese Society of Breast Cancer.

Cell culture. The human breast cancer cell lines, T47D and MCF7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HMC-1 and SKBR-3 were kindly provided from 1st Department of Pathology, Sapporo Medical University School of Medicine (9). All breast cancer cell lines were cultured in RPMI-1640 (BioWhittaker, Walkersville, MD, USA) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA) and grown at 37°C in a humidified atmosphere of 5% CO2.

Quantification of NOK and c-erbB2 mRNA expression. The expression of NOK and c-erbB2 mRNA was determined by a quantitative RT-PCR using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA). Isolation of total RNA were performed using ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. Gene-specific primers and fluorescent hybridization probes used in quantitative PCR were as follows: NOK forward primer, 5'-CAT CCT TCG AGC CAA TAT GAA CAC-3'; reverse primer, 5'-TGG AAT TGG ATT CGC CCT AA'; and probe, 5'-FAM CCA GCT GGG CTC CAT GAG GTA CA; c-erbB2 forward primer, 5'-AGG ATG TGC GCG TCG TAC AC-3'; reverse primer, 5'-TAA TTT TGA CAT GGT TGG GAC TCT T-3'; and probe, 5'-FAM CTT GGC CGC TCG GAA CGT GC (TAMRA)-3'. Quantitative RT-PCR was performed using TaqMan One Step RT-PCR Master Mix Reagents (Applied Biosystems). To compare amounts of mRNA encoding NOK and c-erbB2 in different samples, the quantity of specific mRNA was normalized as a ratio to the amount of 18S ribosomal RNA (18S rRNA), which was determined using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) according to the manufacturer's protocol.

Transduction of small inhibitory RNA (siRNA) for NOK. A siRNA was designed to target the coding region of the NOK gene (GenBank accession no. NM-018423, which was originally expressed as STYK1, nucleotides 224 to 244, relative to the start codon), and prepared by QIAGEN (Tokyo, Japan). Single-strand RNAs were annealed by incubating each strand in siRNA suspension buffer for 1 min at 90°C, followed by 1 h of incubation at 37°C. As a transduction control, non-silencing control RNA (QIAGEN,) was used. The siRNA duplexes used in this study were as follows: NOK; 5'-GCA GGA CAU GGA GAA AAU G TT, 3'-TT CGU CCU GUA CCU CCC UUA C-5'. The transduction of siRNA was performed using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's protocol. Briefly, 1x10^6 of T47D cells were cultured in 6-well culture plates (Costar, Tokyo, Japan) in 2.3 ml of RPMI supplemented with 10% FBS. After 24 h, 100 µl of HiPerFect Transfection Reagent with the addition of 20 nM of either RNA were added and the cells were incubated for an additional 72 h. After 72 h, the cells were harvested, silenced mRNA expression was estimated and cell number was also counted on hemocytometer chambers.

Results

Effect of transducing NOK-siRNA on cell proliferation. To determine the importance of NOK expression in cancer cell growth, NOK-siRNA was transduced into breast cancer cells. The extent of NOK mRNA expression was determined in four breast cancer cell lines to select cells for siRNA transduction. The highest expression was observed in the T47D cells (Figure 1A). Transduction of NOK siRNA into T47D cells decreased the expression of NOK mRNA compared to the cells transduced with non-silencing control RNA (NSC; Figure 1B). Accordingly, after 3 days of transduction, growth was inhibited by approximately 50% in NOK siRNA-transduced cells compared with NSC-transduced cells (Figure 1C).

C-erbB2 and NOK mRNA expression in breast cancer tissues. To compare NOK expression, the mRNA expression for c-erbB2 was first measured in the breast tissues. The expression of c-erbB2 mRNA was significantly higher in breast cancer tissues (mean±SD, 0.511±0.971) than in adjacent non-cancerous breast tissues (0.059±0.077, p<0.002; Figure 2). When a cut-off value was set as the mean±2SD of expression in non-cancerous breast tissues, c-erbB2 mRNA expression was positive in 28.8% of cancers (15 out of 52; Figure 2).

The degree of NOK mRNA expression compared to c-erbB2 was subsequently examined. As shown in Figure 3, NOK mRNA expression in breast cancer tissues (0.088±0.085) was six times higher than that in non-cancerous tissues (0.015±0.013, p<0.0001). With positivity determined using a definition for cut-off value analogous that for c-erbB2 mRNA expression, the positivity rate for NOK mRNA in cancer tissues was 67.3% (35 out of 52), much higher than that for c-erbB2 mRNA expression.

The relationships between clinicopathological factors and NOK mRNA expression in breast cancer tissues were further analyzed. No correlations were found between age, menopausal state, or the expression of estrogen progesterone receptors or NOK mRNA. As for expression at clinical stages determined by tumor size, invasion, and metastasis to lymph nodes and distant organs, NOK mRNA expression was high, even at an early stage (Figure 4).
Correlation between NOK and c-erbB2 mRNA expression in breast cancer tissues. The correlation between NOK and c-erbB2 mRNA expression was examined in breast cancer tissues. As shown in Figure 5, no correlation ($r=0.017$) was found between NOK and c-erbB2 mRNA expression, indicating that each mRNA was an independent marker. Further, the positivity rate of both molecules was analyzed in four groups defined by the cut-off values for these two molecules. The plot shows that most NOK-positive tumors were c-erbB2-negative, reflecting the difference in positivity rate between these molecules. Only 5 tumors (9.6%) were positive for c-erbB2 alone, while positivity for NOK alone showed a rate of 48.1% (25 out of 52). Both c-erbB2 and NOK were positive in 19.2% of tumors (10 out of 52). Accordingly, combining NOK with c-erbB2 resulted in a higher positivity rate (76.9%; 40 out of 52).

Discussion

This study was performed to investigate whether NOK could be a useful gene expression marker for the diagnosis of breast cancer. No previous studies have compared amounts of NOK expression in resected human cancers with adjacent non-cancerous tissues. Our comparison demonstrated NOK mRNA to be overexpressed in breast cancer tissues. The expression of mRNA encoding c-erbB2, a well known receptor protein tyrosine kinase (RPTK) targeted therapeutically in HER2 antibody therapy, was also found to be overexpressed in cancer tissues – but far less frequently than NOK mRNA. Previous studies using various cut-off values have indicated nearly the same positivity rate for c-erbB2 mRNA, 17% to 20% by quantitative RT-PCR, as we found (10, 11). The positivity for NOK mRNA among breast cancers was also quite high compared to mRNA expression of other RPTK, such as epidermal growth factor receptor, FGFR and PDGFR(12-14).

As previously reported, RPTK generally require ligand binding for tyrosine phosphorylation and growth signal transmission, with the exception of variant molecules, such as FGFR4 and hepatocyte growth factor receptor (Met) with alterations in the extracellular domain (15, 16). In contrast to these RPTK, NOK has been reported to lack an extracellular

![Figure 1. In vitro experiments. A) Expression of NOK mRNA in human breast cancer cell lines including T47D. NOK mRNA expression as measured by quantitative RT-PCR is reported relative to expression by MCF7 cells. Data are the means of triplicate measurements. B) NOK mRNA expression in T47D cells transduced with siRNA against NOK (NOK-siRNA). C) Proliferation of these cells. Cells were transduced with non-silencing control RNA (NSC) or NOK-siRNA. Total RNA was extracted at 72 h after transduction to measure mRNA expression. NOK mRNA expression in siRNA transfectants is reported relative to expression in NSC transfectants. Cells were counted in hemocytometer chambers at 72 h after transfection.](image)
domain. Instead, growth signals are thought to be transmitted by auto-activation of NOK (8, 17). Thus, the presence of NOK even without a specific ligand may lead to abnormal cell proliferation. Li et al. have reported that not only tumorigenesis but also metastasis were observed in mice transplanted with normal cells overexpressing NOK by gene transduction. However, the extent to which NOK expression by cancer cells participates in cell proliferation has been unclear. We, therefore, examined the significance of NOK expression in cancer cells, demonstrating that introduction of an siRNA against NOK leads to growth inhibition in breast cancer cells. Furthermore, the extent of growth inhibition was exceeded by the percentage decrease in mRNA expression. Taken together with the observation by Liu et al. that NOK gene overexpression led to progressive cell growth via the signaling pathway of phosphatidylinositol 3-kinase and mitogen activated protein kinase (8), NOK expression itself could be critical for the proliferation of breast cancer cells.

In addition to our observations in vitro, NOK mRNA was also found to be overexpressed even at early clinical stages of breast cancer. Amounts of NOK mRNA were elevated in 92.2% of tumor samples (47/51). Accordingly, NOK may play a significant role in the initial carcinogenesis of breast cancer. The quantitative measurement of NOK mRNA might be useful in supporting the diagnosis of breast cancer especially when only small numbers of cells can be obtained in tiny tumors in which the malignancy cannot be confirmed by other means.

It was found that mRNA expression of NOK did not correlate with that of c-erbB2, indicating that NOK could
be an independent marker for the diagnosis of breast cancer. A discrepancy in expression profiles between the molecules may reflect the structured difference that NOK lacks extracellular domain and does not require specific ligand binding for growth signal transmission. Tumors showing high c-erbB2 expression can be treated by Trastuzumab (Herceptin®), but some c-erbB2-negative cases also show poor outcomes. Since most of our c-erbB2-negative cases were NOK-positive, these patients might be treated in the future by inhibition of NOK expression.

References


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