Involvement of C12orf32 overexpression in breast carcinogenesis

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Abstract. Through genome-wide gene expression profile analysis of breast cancer, we identified a gene, chromosome 12 open reading frame 32 (C12orf32), to be involved in mammary carcinogenesis. Semiquantitative RT-PCR and Northern blot analysis confirmed C12orf32 overexpression in breast cancer cells and its almost undetectable level of expression in normal human tissues. Immunocytochemical staining analysis using breast cancer cell lines revealed a cell cycle-dependent subcellular localization of endogenous C12orf32 protein. Depletion of C12orf32 expression by small-hairpin RNA interference significantly suppressed the growth of breast cancer cell lines possibly due to the inhibition of G1/S transition and subsequent cell death. Western blot analysis indicated that a C12orf32 protein of 35 kDa predicted from the cDNA sequences was processed to a 16-kDa protein of (C12orf32-p16) which was accumulated in most of breast cancer cell lines examined. Our data suggest that C12orf32 is a promising molecular target for the development of novel anticancer drugs such as peptide vaccines and siRNA drugs.

Introduction

Breast cancer is the most common cancer among women worldwide. Incidence of breast cancer is increasing in most countries including the USA and Japan, and the increasing rate is much higher in countries where its incidence was previously low (1,2). Early detection with mammography and a reduction of mortality rate is much higher in countries where its incidence was previously low (1,2). Early detection with mammography and subsequent breast cancer cell lines revealed a cell cycle-dependent subcellular localization of endogenous C12orf32 protein. Depletion of C12orf32 expression by small-hairpin RNA interference significantly suppressed the growth of breast cancer cell lines possibly due to the inhibition of G1/S transition and subsequent cell death. Western blot analysis indicated that a C12orf32 protein of 35 kDa predicted from the cDNA sequences was processed to a 16-kDa protein (C12orf32-p16) which was accumulated in most of breast cancer cell lines examined. Our data suggest that C12orf32 is a promising molecular target for the development of novel anticancer drugs such as peptide vaccines and siRNA drugs.

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sequences of each primer set were as follows; 5'-TTTAGGAA-3' for si-AAT-3' for si-#2, 5'-AACAGTTGTTGGTGCAT-3' for si-#3, 5'-AACCTGACTGCGATCGTAA-3' for si-mis (underlined letters indicate the nucleotides mismatched in si-#2). All of the constructs were confirmed by DNA sequencing with ABI3700 DNA sequencer (PE Applied Biosystems). Human breast cancer cell lines, HBC4 and T47D, were plated onto 10-cm dishes (1x10^6 cells/dish) and transfected with 8 μg each of psiU6BX3.0-EGFP and psiU6BX3.0-C12orf32 (si-#2, si-#3 and si-mis including four-base substitutions in #2) using FuGENE6 transfection reagent (Roche) according to the manufacturer's instructions. Twenty-four hours after the transfection, cells were re-seeded for colony formation assay (1x10^6 cells/10 cm dish), RT-PCR (1x10^6 cells/10 cm dish) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (2x10^5 cells/well). We selected psiU6BX3.0-introduced HBC4 or T47D cells with culture medium containing 0.4 mg/ml or 0.8 mg/ml of neomycin (Geneticin; Invitrogen), respectively. We changed culture medium twice a week. Total RNAs were extracted from the cells after 5-day incubation with neomycin, and then the knockdown effect of siRNAs was examined by semi-quantitative RT-PCR using specific primer sets: 5'-CTCAGTGGCTGGAGCTC-3' and 5'-ACCTCCCCCTGTTGGACCTG-3' for β-actin as an internal control. HBC4 or T47D cells expressing siRNA were grown for 4 weeks in selective media containing 0.4 mg/ml or 0.8 mg/ml of neomycin, and then fixed with 4% paraformaldehyde at 4°C for 30 min before staining with Giemsa's solution (Merck, Whitehouse Station, NJ) to assess the colony number. To quantify cell viability, MTT assays were performed with cell counting kit-8 (Bio-Rad). These experiments were performed in triplicate.

We used siRNA oligonucleotides (Sigma Aldrich Japan KK, Tokyo, Japan) due to its high transfection efficiency to human multiple-tissue Northern-blot membrane. Breast cancer Northern blot membrane was prepared as described previously (10). Human multiple-tissue Northern-blot membranes (Takara Clontech, Kyoto, Japan) were hybridized with the [α-32P]-dCTP-labeled PCR products of C12orf32 prepared by RT-PCR (see below). Prehybridization, hybridization, and washing were carried out according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80°C for 14 days. Specific probe for C12orf32 (343bp) was prepared by RT-PCR using the following primer set; 5'-TCTTAAAGAGGCTGCTGAT-3' and 5'-CTCCCACTGACCTGATC-3'. It was radioactively labeled with the megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK).

Genesilencing effect by siRNA. We previously established a vector-based RNAi (RNA interference) expression system using psiU6BX3.0 siRNA expression vector as described (26). The siRNA expression vectors against C12orf32 (psiU6BX3.0-C12orf32) were prepared by cloning of double-stranded oligonucleotides into the BbsI site in the psiU6BX3.0 vector. The target sequences of synthetic oligonucleotides for siRNAs were as follows; 5'-AAAGCTGACTGCGATCGTAC-3' for si-#2, 5'-AAGCAGTTGTTGGTGCAT-3' for si-#3, 5'-AACCTGACTGCGATCGTAA-3' for si-mis for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA-3' for C12orf32 (GenBan...
room temperature for 30 min. Cell suspensions were analyzed for DNA content by flow cytometer (FACS Calibur; Becton-Dickinson, San Diego, CA). The data were analyzed by CellQuest software (BD Biosciences, Sparks, MD). Assays were done in duplicate independently.

Construction of C12orf32 expression vectors. To construct full-length protein of C12orf32 expression vector, the coding sequence was amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Primer sets were 5'-C CGGAATTCCTCATTCACCGTTGATGCC-3' and 5'-CCGCTCGAGGCTTTTCACAAGGAATTGGCT-3' (underlines indicate recognition sites of restriction enzymes). The PCR product was inserted into the EcoRI and XhoI sites of pCAGGSnHC expression vector in frame with a hemagglutinin (HA) tag at the C-terminus or pCAGGSn3FH expression vector in frame with a Flag-tag at the N-terminus. DNA sequences of the construct were confirmed by DNA sequencing.

Generation of anti-C12orf32 specific polyclonal antibody. A plasmid designed to express a fragment of C12orf32 (amino acids 1-208) using pET21a (+) vector in frame with a T7 tag at the N-terminus and a His tag at the C-terminus (Novagen, Madison, WI). The recombinant peptide was expressed in Escherichia coli, BL21 codon-plus strain (Stratagene, La Jolla, CA), and purified using Ni-NTA resin agarose (Qiagen) according to the supplier's protocols. The purified recombinant protein was mixed together and then used for immunization of rabbits (Medical and Biological Laboratories, Nagoya, Japan). The immune sera were subsequently purified on antigen affinity columns using Affigel 15 gel (Bio-Rad Laboratories, Hercules, CA) according to supplier's instructions. We confirmed that this antibody specifically recognized endogenous C12orf32 protein in breast cancer cell lines, T47D and HBC4 by comparison of cells with or without C12orf32 using siRNA-oligonucleotides.

Western blot analysis. To examine the expression of endogenous C12orf32 protein in breast cancer cell lines (HBC4, MDA-MB-231, BT-549, T47D, SK-BR-3, ZR-75-1, BSY-1 and MCF-7), the cells were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40) including 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). After homogenization, cell lysates were incubated on ice for 30 min and centrifuged at 17,800 x g for 5 min to separate supernatant from cell debris. The amount of total protein was measured by protein assay kit (Bio-Rad), and then the proteins were mixed with SDS-sample buffer and boiled for 5 min before loading at 12% SDS-PAGE gel. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (GE Healthcare). The membrane was blocked by blocking solution overnight, and then incubated with purified anti-C12orf32 polyclonal antibody for 1 h to detect endogenous C12orf32 protein. Finally, the membrane was incubated with HRP conjugated secondary antibody for 1 h and protein bands were visualized by ECL detection reagents (GE Healthcare).
Immunocytochemical staining. To examine the subcellular localization of endogenous C12orf32 protein in breast cancer cells, T47D cells were seeded at 1x10^5 cells per well (Lab-Tek II Chamber Slide System; Nalgen Nunc International, Naperville, IL). After 24 h of incubation, cells were fixed with PBS (-) containing 4% paraformaldehyde at 4˚C for 30 min and rendered permeable with PBS (-) containing 0.1% Triton X-100 at 4˚C for 2 min. Subsequently, the cells were covered with 3% BSA in PBS (-) for 1 h to block non-specific hybridization followed by incubation with anti-C12orf32 polyclonal antibody diluted at 1:100 for another 1 h. After washing with PBS (-), cells were stained by Alexa 488-conjugated anti-rabbit secondary antibody (Molecular Probe, Eugene, OR) diluted at 1:100 for 1 h to block non-specific hybridization followed by incubation with anti-C12orf32 polyclonal antibody diluted at 1:100 for another 1 h. After washing with PBS (-), cells were stained by Alexa 488-conjugated anti-rabbit secondary antibody (Molecular Probe, Eugene, OR) diluted at 1:1000 for 1 h. Nuclei were counter-stained with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under TCS SP2 AOBS microscope (Leica, Tokyo, Japan).

Characterization of a processed form of the C12orf32 protein. N-terminal-HA-tagged C12orf32 was expressed exogenously in HEK293 cells. C12orf32 protein was immunoprecipitated with anti-HA antibody from the cells, separated on SDS-polyacrylamide gel electrophoresis gels, transferred to a nitrocellulose membrane (GE Healthcare), and stained with Coomassie Brilliant Blue (CBB, Invitrogen). The nitrocellulose membrane was used for immunoblot analysis with anti-HA antibody to detect the C12orf32 protein. We excised the band corresponding to the small-size protein of C12orf32 from the CBB-stained gel and subjected to Peptide Mass Fingerprint (PMF) analysis using MALDI TOF-MS (Shimadzu Biotech, Tsukuba, Japan).

Statistical analysis. Statistical significance was determined by Student’s t-test using Statview 5.0 software (SAS Institute, Cary, NC). P<0.05 was considered to be statistically significant.

Results

Overexpression of C12orf32 in breast cancer cells. We identified C12orf32 to be up-regulated in the majority of breast cancer clinical samples through the genome-wide expression analysis (25) and verified its up-regulation by semi-quantitative RT-PCR. Subsequently, we performed Northern blot analysis using a C12orf32 cDNA fragment as a probe and detected overexpression of approximately 1.9-kb and 1.6-kb transcripts of C12orf32 in all of 11 breast cancer cell lines examined, although its expression was hardly detectable in normal tissues including mammary gland (Fig. 1A). Furthermore, we performed multiple-tissue Northern blot analysis and found it is hardly detectable in any of normal organs except testis, prostate, ovary, thymus and small intestine with very low expression level in concordance with the results of cDNA microarray analysis (Fig. 1B). There are transcript variants of C12orf32 deposited in NCBI database, namely,
NR_027363 (1896 bp) and NR_027365 (1644 bp), thought to correspond to longer and shorter transcripts, respectively. Short transcript lacked exon 2, which contains a start codon of the open reading frame in the long transcript (Fig. 1C), and we did not find any long open reading frame in the short transcript. Therefore, we focused further analysis on the protein product of the long transcript.

Knockdown effect of C12orf32 on growth of breast cancer cell lines.

To assess a role of C12orf32 in the growth of breast cancer cells, we knocked down the expression of endogenous C12orf32 in breast cancer cell lines, HBC4 and T47D, which expressed C12orf32 at high level, by means of the mammalian vector-based RNA interference (RNAi) technique (26). We examined the expression level of C12orf32 by semi-quantitative RT-PCR analysis. Two shRNAs (si-#2 and si-#3) significantly suppressed the expression of C12orf32 compared with a control shRNA construct, psiU6BX-EGFP (si-control) (Fig. 2A). In concordance with the knockdown effect, MTT assay (Fig. 2B) and colony formation assay (Fig. 2C) revealed significant suppressive effects of si-#2 and si-#3 (P<0.0001; unpaired t-test) on the growth of HBC4 cells. We also generated shRNA that contained 4 substitutions in si-#2 sequence [si-C12orf32-mismatch (si-mis), see ‘Materials and methods’], and found no suppressive effect on the expression of C12orf32 or on cell growth of T47D cells (Fig. 2D-F, P>0.0001; unpaired t-test). These observations suggest that C12orf32 has an important role in the growth of breast cancer cells. In order to analyze the mechanism of growth suppression induced by knocking down of c12orf32 expression, we performed flow cytometry of the cells transfected with siRNA-oligonucleotides against C12orf32 and found a significant increase of the sub-G1 population by the treatment with siRNA-oligonucleotides of C12orf32 (si-C12orf32) compared with that with si-EGFP (*P=0.0245, **P=0.009; unpaired t-test) (Fig. 2G), suggesting that suppression of C12orf32 expression induces cell death.

Endogenous expression of C12orf32 protein in breast cancer cell lines.

To further investigate expression levels of endogenous C12orf32 protein in breast cancer cells, we generated a polyclonal antibody against C12orf32 protein (α-C12orf32), and performed Western blot analysis using cell lysates from eight breast cancer cell lines as well as COS-7 cells that was transfected with mock or C-terminal HA-tagged C12orf32 construct (C12orf32-HA) (Fig. 3A). Unexpectedly, we observed the smaller-size protein (approximately 16 kDa) than the predicted one (35 kDa) in a majority of breast cancer cell lines examined while we detected C12orf32-HA at the predicted molecular weight (35 kDa). To investigate whether this 16-kDa protein corresponds to the endogenous C12orf32 protein, we performed Western blot analysis of HBC4 cells that were transfected with either control siEGFP or siC12orf32, and found that the protein level of the 16-kDa protein was...
suppressed by siC12orf32 (Fig. 3B). Because we did not find any transcriptional variants that might produce the 16-kDa protein, we thought this smaller-size protein is probably generated as a product of post-translational processing of full-length C12orf32 protein. To characterize this 16 kDa protein, we immunoprecipitated an N-terminal HA-tagged C12orf32 protein from HEK293T cells and subjected to SDS-PAGE. The 16-kDa protein was excised from the gel and analyzed by MALDI-TOF MS. We finally identified the 16-kDa protein corresponds to amino acids 1-144 of the full-length C12orf32 protein.

Then, the subcellular localization of the endogenous C12orf32 protein in breast cancer cells was examined by immunocytochemical analysis using anti-C12orf32 polyclonal antibody (Fig. 3C). The endogenous C12orf32 was mainly localized in the nucleus of cells at interphase and detected diffusely in the cells at mitosis.

**C12orf32 expression and cell cycle progression.** To investigate the biological role of C12orf32 in breast cancer cell growth, we assessed the cell cycle progression of T47D cells in which the expression of C12orf32 was knocked down by siRNA. T47D cells transfected with control siRNA (siEGFP) or siC12orf32 were synchronized at the G1/S boundary by aphidicolin treatment, and then, cell cycle progression was monitored by flow cytometry at various time-points after releasing from the arrest. At 6 and 9 h after the release, the proportion of the cells at S phase was significantly less in the T47D cells transfected with siC12orf32 than those transfected with siEGFP (Fig. 4A), indicating that the delay of G1-S progression was induced by knocking down of C12orf32 expression. Furthermore, the proportion of the cells at G2/M phase under the nocodazole treatment was significantly smaller for the T47D and HBC4 cells transfected with siC12orf32 than those with siEGFP (Fig. 4B and C). These data also suggest an important role of C12orf32 in G1-S transition.

**Discussion**

Significant advances in development of molecular-targeting drugs for cancer therapy have been achieved in the last two decades. However, the proportion of patients showing good response to presently-available treatments is still very limited and some proportion of patients still suffer from severe adverse reactions without any benefit (27). Therefore, it is critically essential to develop new anticancer agents that are highly specific to malignant cells and have a minimum risk of adverse reactions. Through our whole genome expression profile analysis of clinical breast cancers after the careful enrichment of breast cancer cells with the microbeam microdissection method (25), we identified C12orf32 to be significantly up-regulated in the great majority of clinical breast cancer specimens. Northern blot analyses clearly demonstrated overexpression of C12orf32 in breast cancer cell lines, and...
its undetectable or low expression level in normal human tissues. Furthermore, depletion of C12orf32 expression by siRNA treatment drastically suppressed the growth of breast cancer cells. At protein level, an approximately 16-kDa protein was detected in breast cancer cell lines by Western blotting using anti-C12orf32 polyclonal antibody (Fig. 3), which was considerably smaller than the size estimated from cDNA sequences. Mass spectrometry analysis revealed that the 16-kDa protein is likely to be a processed form of full-length C12orf32 protein, which corresponds to amino acids 1-144. Cell cycle analysis revealed that the knocking down of C12orf32 resulted in retardation of the transition from G1 to S phase, indicating that C12orf32 has an important role in G1-S progression and its depletion inhibits cell cycle progression and subsequent cell death.

In conventional drug screening approaches, the great majority of compounds that enter into clinical trials fail in development due to the adverse reactions or the insufficient efficacy. To reduce the failure risk during drug-development processes, selection of target molecules that are applicable for screening of small molecular compounds, therapeutic antibodies or peptide vaccines are critically important. In this regard, our data suggest that C12orf32 is a promising molecular target for the development of novel anticancer drugs such as peptide vaccines and siRNA drugs. Moreover, if an interacting partner(s) with C12orf32 proteins that have crucial roles in cell growth is identified, the inhibitor for their interaction would be a possible valuable target to develop agents against breast cancer. It is notable that our cDNA microarray data identified up-regulation of C12orf32 in clinical cancer materials in other organs, including bladder, lung and testis. These results show that this gene should serve as a valuable target for the development of anticancer agent for various types of cancer in addition to breast cancer.

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References


