Involvement of C12orf32 overexpression in breast carcinogenesis

JUNG-WON KIM[1], CHIKAKO FUKUKAWA[1], KOJI UEDA[2], TOSHIHIKO NISHIDATE[1], TOYOMASA KATAGIRI[1,3] and YUSUKE NAKAMURA[1]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo; [2]Laboratory for Biomarker Development, Center for Genomic Medicine, RIKEN, Yokohama; [3]Division of Genomic Medicine, Institute for Genomic Research, The University of Tokushima, Tokushima, Japan

Received April 19, 2010; Accepted June 16, 2010

DOI: 10.3892/ijo_00000737

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 the development of molecular-targeted therapeutic drugs, such as tamoxifen, aromatase inhibitors, and trastuzumab have contributed to a reduction of mortality rate and improving the quality of life of women with breast cancer. However, these drugs have been shown adverse reactions such as the increase in the risk of endometrial cancer with long-term tamoxifen administration and severe cardiac toxicity with trastuzumab treatment, as well as the risk of bone fracture due to osteoporosis in postmenopausal women with aromatase inhibitor prescription (3-7). Therefore, it is necessary to search for novel anticancer-drugs with the lower risk of adverse reactions.

Gene-expression profiles obtained by cDNA microarray analysis have been proven to provide detailed characterization of individual cancers, and such information should contribute to improve clinical strategies for neoplastic diseases through development of novel drugs as well as provide the basis of personalized treatment (8,9). Through the genome-wide expression analysis of a large number of microdissected clinical cancer materials, we have identified dozens of genes that function as oncogenes in the process of development and/or progression of breast cancer (10-17), bladder cancer (18,19), synovial sarcoma (20,21), testicular seminoma (22), and renal cell carcinoma (23,24). Such molecules are considered to be candidate molecular targets for development of new therapeutic modalities. Because cytotoxic drugs often cause severe adverse reactions, careful selection of novel molecular targets on the basis of well-characterized mechanisms of action is essential for development of effective anticancer drugs with the minimum risk of adverse reactions. Toward this goal, we previously analyzed the genome-wide expression profile of 81 breast cancers using cDNA microarray representing 23,040 cDNAs/ESTs in combination with laser microbeam microdissection (25).

Among many overexpressed genes in breast cancers, we in this study focused on the characterization of a novel gene, *chromosome 12 open reading frame 32* (*C12orf32*), which was up-regulated in a great majority of breast cancer cases. Although C12orf32 protein was highly conserved among other species such as chimpanzee (99% in amino acid sequences), cow (71%), mouse (67%), rat (67%), and chicken (35%), its pathophysiological roles in growth of human cancer cells have not been investigated. We demonstrate evidence that implies a critical role of C12orf32 overexpression in mammary carcinogenesis.

Materials and methods

Breast cancer cell lines and clinical breast cancer samples. Human breast cancer cell lines, BT-549, HCC1937, MCF-7, MDA-MB-231, MDA-MB-435S, SK-BR-3, T47D, YMB-1,

Correspondence to: Dr Yusuke Nakamura, Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
E-mail: yusuke@ims.u-tokyo.ac.jp

Key words: breast cancer, chromosome 12 open reading frame 32, molecular target

ZR-75-1 and BSY-1 were purchased from American Type Culture Collection (ATCC, Rockville, MD), and cultured under their respective depositors' recommendations. HBC4 and HBC5 cells were kind gifts from Dr Takao Yamori of Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. HBC4, HBC5, BT-549, HCC1937, T47D, YMB-1, ZR-75-1 and BSY-1 cell lines were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) (with 2 mM L-glutamine). MDA-MB-231 and MDA-MB-435S cell lines were cultured in L-15 (Roche, Basel, Switzerland). SK-BR-3 cell line was cultured in McCoy's 5A (Sigma-Aldrich) (with 1.5 mM L-glutamine). MCF-7 cell line was cultured in EMEM (Sigma-Aldrich) supplemented with 10 μg/ml insulin. Each medium was supplemented with 10% fetal bovine serum (FBS; Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). MDA-MB-231 and MDA-MB-435S cell lines were maintained at 37˚C in atmosphere of humidified air without CO_2, and other cell lines were maintained at 37˚C in atmosphere of humidified air with 5% CO_2. Tissue samples from surgically resected breast cancers and their corresponding clinical information were obtained from Department of Breast Surgery, Cancer Institute Hospital, Tokyo after obtaining written informed consent.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Microdissection of breast cancer cells from cancer tissue sections was carried out as described previously (10). Total RNA was extracted from each of the microdissected breast cancer clinical samples, microdissected normal breast ductal cells, and breast cancer cell lines using RNeasy Micro kits (Qiagen, Valencia, CA, USA) and polyA$^+$ RNA isolated from mammary gland (Takara Clontech, Kyoto, Japan). Subsequently, T7-based amplification and RT were carried out as described previously (10). We prepared appropriated dilutions of each single-stranded cDNA for subsequent PCR by monitoring ß-actin as a quantitative control. The sequences of each primer set were as follows; 5'-TTTTAG AGAATCCTGCTTCCATCAG-3' and 5'-TTTGACTGG GGAAGTCCTTCTG-3' for *C12orf32* (GenBank accession number; NR_027363), and 5'-GAACGGTGAAGGTGACA GCA-3' and 5'-ACCTCCCCTGTGTGGACTTG-3' for ß-actin.

Northern blot analysis. 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-^{32}P]-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'-TTTTAGAA TCCTGCTTCCATCAG-3' and 5'-CAATCCTAAAGAACT CATCTATGTC-3'. It was radioactively labeled with the megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK).

Gene-silencing 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'-AAGCTGACTGCCATCAGT AAT-3' for si-#2, 5'-AACAGTTCAGTTTAGTGTCAT-3' for si-#3, 5'-AACCTGACTGCGATCTGTAAA-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'-CTCATT CACCGGTTGATGCC-3' and 5'-GCTTTTCACAAGGAAT TGGCT-3' for *C12orf32*; 5'-GAACGGTGAAGGTGACA GCA-3' and 5'-ACCTCCCCTGTGTGGACTTG-3' for ß-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 (Wako, Osaka, Japan) according to manufacturer's recommendations. Absorbance at 570-nm wavelength was measured with a Microplate Reader 550 (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 observe the knockdown-effect of *C12orf32*. The sequences targeting C12orf32 (si-C12orf32) or EGFP (si-EGFP) were as follows: si-C12orf32; 5'-GCUGACUGCCAUCAGUAA UTT-3', si-EGFP (control); 5'-GCAGCACGACUUCUUC AAG-3'. T47D cells (1x10^6 cells in 10 cm dish for FACS analysis) cells were transfected with those siRNAs using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in OptiMEM (Invitrogen) medium according to the instructions of the manufacturer.

Fluorescence-activated cell sorting (FACS) analysis. T47D breast cancer cells, which were used for siRNA experiments as indicated above, were harvested at 48 h after transfection with siRNA-oligonucleotides. Cells were collected and fixed with 70% ethanol, and maintained at 4˚C before use. Cells were incubated with 1 mg/ml RNase I in PBS (-) at 37˚C for 30 min and stained with 50 μg/ml of propidium iodide (PI) at

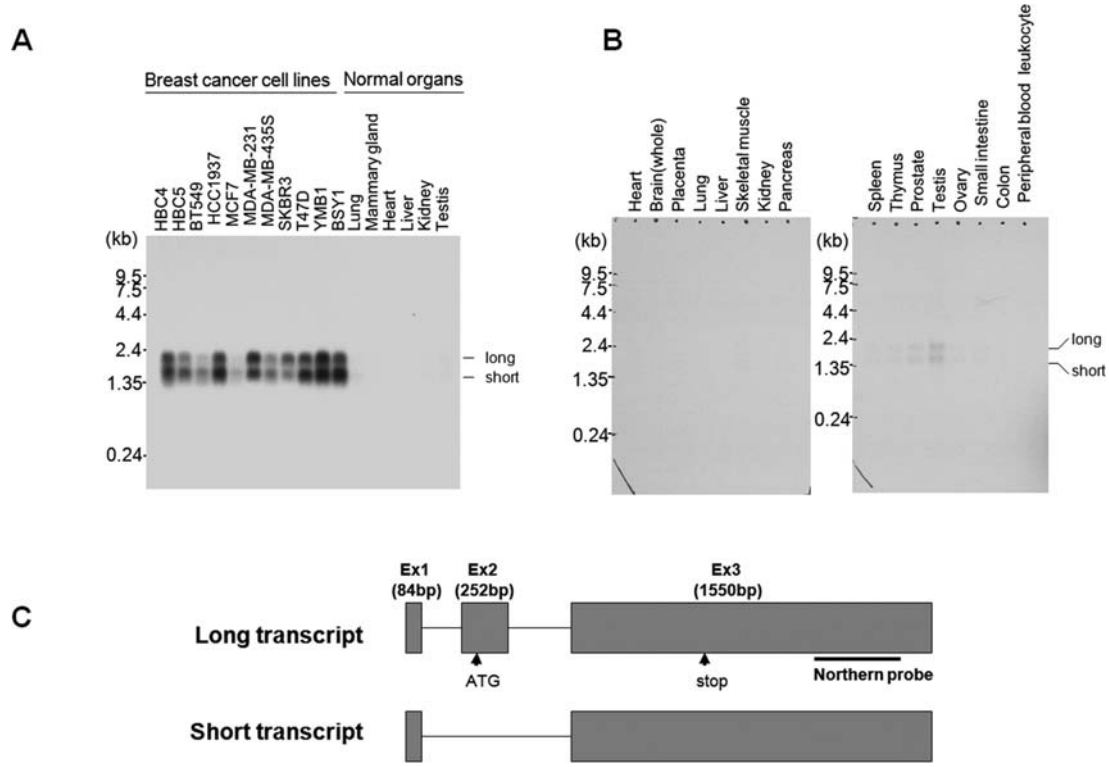

Figure 1. Overexpression of chromosome 12 open reading frame 32 (C12orf32) in breast cancer cells. (A) Northern blot analysis of the *C12orf32* transcripts in 11 breast cancer cell lines (HBC4, HBC5, BT549, HCC1937, MCF7, MDA-MB-231, MDA-MB-435S, SKBR3, T47D, YMB1 and BSY1) and lung, mammary gland, heart, liver, kidney and testis. Long and short transcriptional variants are indicated. (B) Northern blot analysis of the *C12orf32* transcripts in various normal human tissues. (C) Schematic diagram of *C12orf32* transcripts.

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'-CCGGAATTCCTCATTCACCGGTTGATGCC-3' and 5'-CCGCTCGAGGCTTTTCACAAGGAATTGGCT-3' (underlines indicate recognition sites of restriction enzymes). The PCR product was inserted into the *EcoR*I and *Xho*I 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).

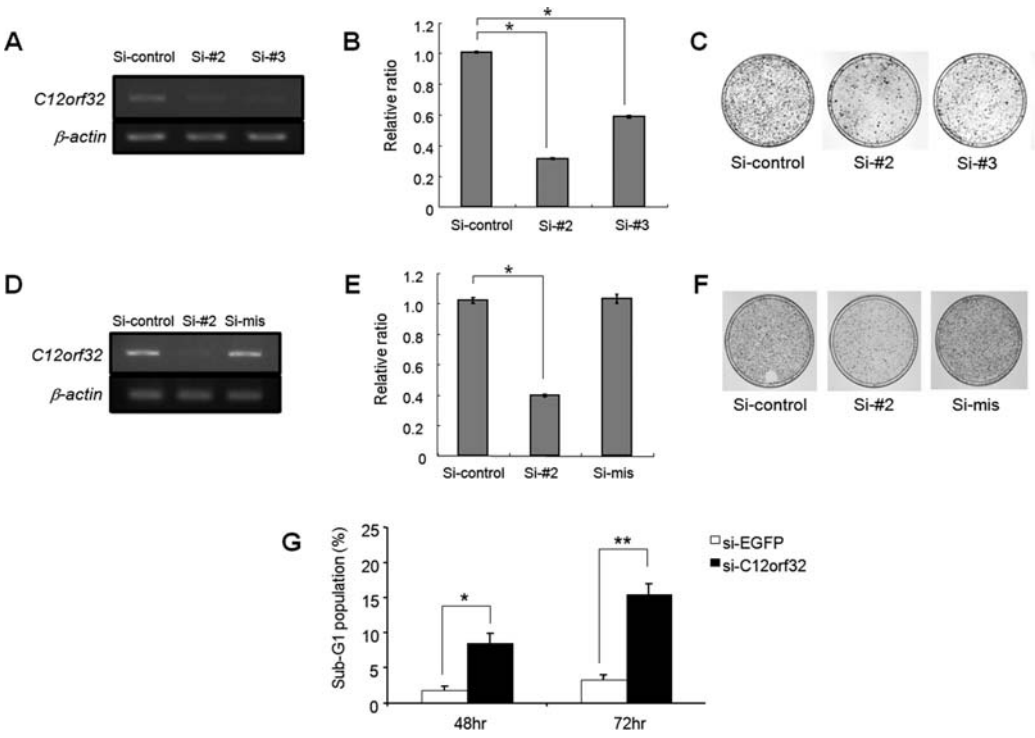

Figure 2. Growth-inhibitory effects of C12orf32 by small interfering RNA (siRNA) on breast cancer cells, HBC4 (A-C) and T47D (D-F). (A) Semi-quantitative RT-PCR shows the expression of endogenous *C12orf32* in HBC4 cells transfected with control siRNA and siRNA targeting C12orf32 (Si-#2 and Si-#3); expression of ß-actin is served as an internal quantitative control. (B) MTT assay represents the number of living cells 10 days after transfection: bars, ± SE. *P<0.0001; unpaired t-test. (C) Colony-formation assay visualizes the living cells. (D) Semi-quantitative RT-PCR shows the expression of endogenous *C12orf32* in T47D cells transfected with control siRNA, Si-#2 and its 4-base substituted mismatch siRNA, si-mis; expression of ß-actin is served as an internal quantitative control. (E) MTT assay represents the number of living cells 10 days after transfection: bars, ± SE. *P<0.0001; unpaired t-test. (F) Colony-formation assay to visualize the living cells. (G) Flow cytometry analysis showed the increase of sub-G1 population in T47D cells treated with si-#2 (*P=0.0245 at 48 h and **P=0.009 at 72 h; unpaired t-test) compared with the cells treated with si-EGFP control.

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 1:1000 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,

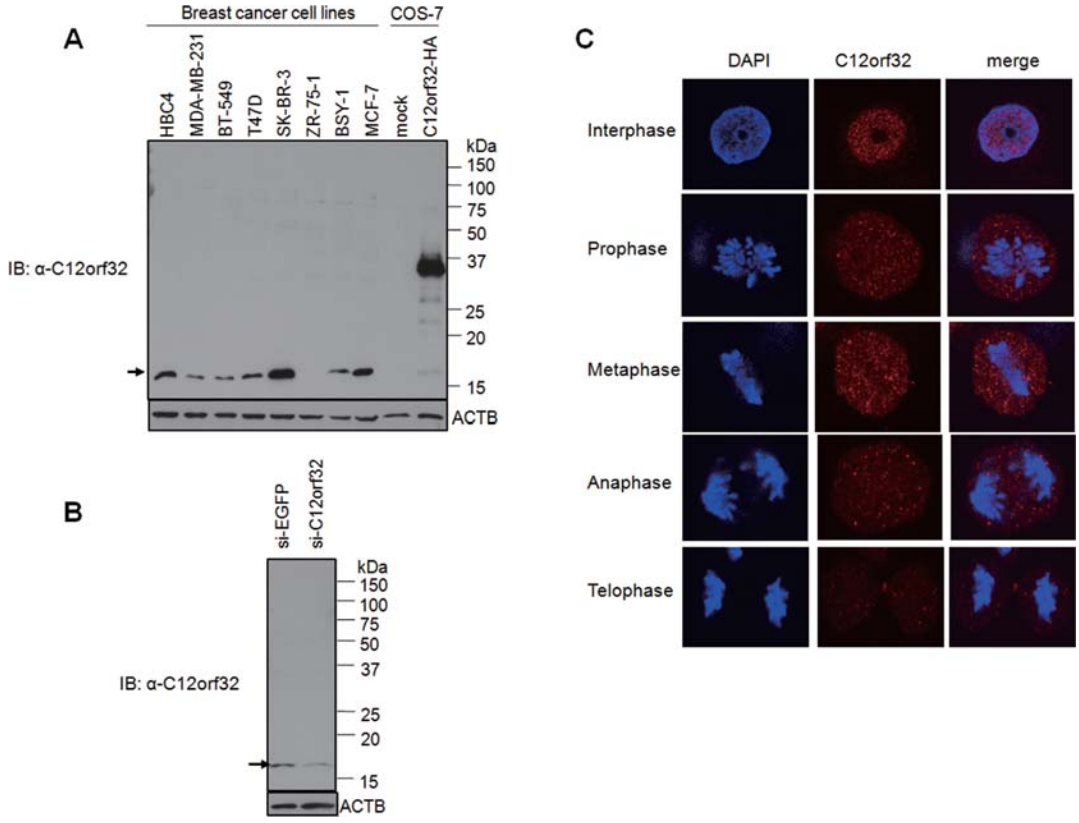

Figure 3. C12orf32 protein expression in breast cancer cell lines. (A) Expression of C12orf32 protein in eight breast cancer cell lines (HBC4, MDA-MB-231, BT-549, T47D, SK-BR-3, ZR-75-1, BSY-1 and MCF-7) as well as COS-7 cells that were exogenously transfected with mock or C-terminal HA-tagged C12orf32 construct (C12orf32-HA) by Western blot analysis using the affinity purified anti-C12orf32 polyclonal antibody. Arrow indicates the 16-kDa protein. ß-actin (ACTB) served as a loading control. (B) Western blotting of HBC4 cells transfected with either siEGFP or si-C12orf32. (C) Subcellular localization of endogenous C12orf32 protein at various cell cycle stages; T47D cells were stained using affinity-purified anti-C12orf32 polyclonal antibody (red). Nucleus were counterstained with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI; blue).

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

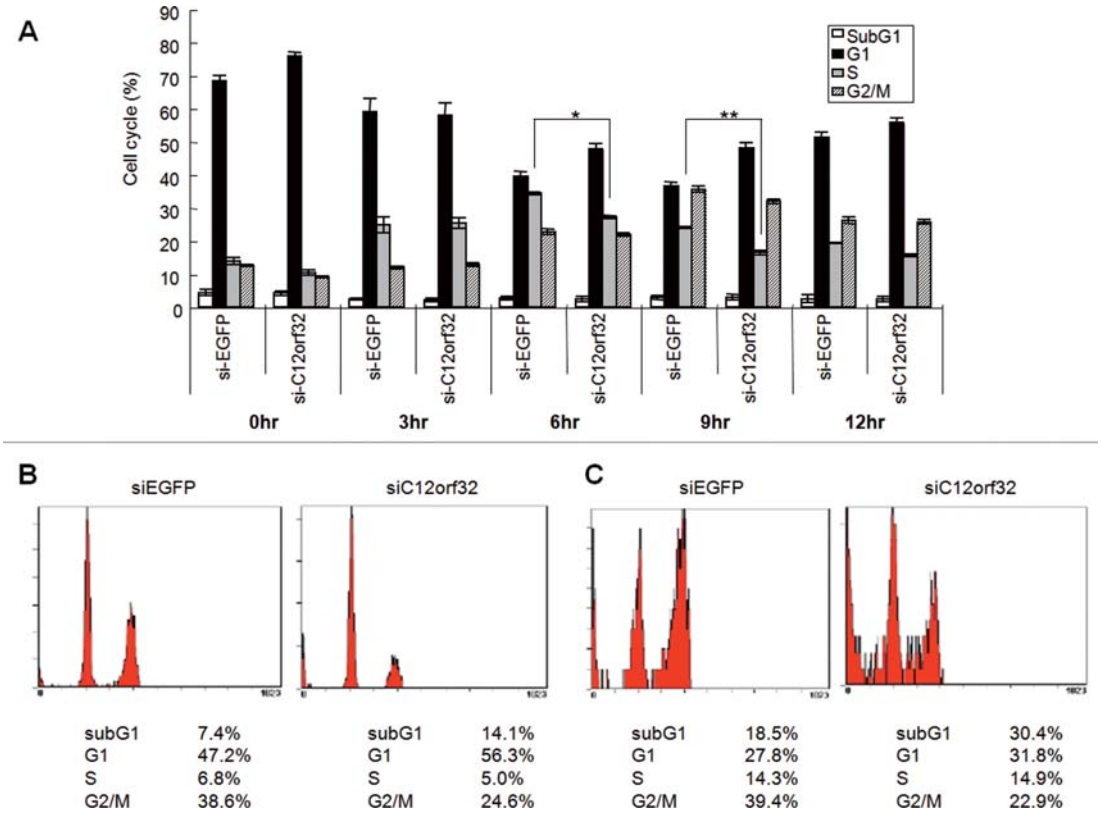

Figure 4. Effect of C12orf32-knockdown on cell cycle. (A) Knockdown effect of C12orf32 on the cell cycle progression of T47D cells. Forty-eight hours after transfection with si-EGFP or si-C12orf32, T47D cells were synchronized by aphidicolin (2 μg/ml) for 18 h. After releasing from the cell cycle arrest by replacement of the media, cells were collected every 3 h up to 12 h for flow cytometry analysis ; bars, ± SE. *P=0.004, **P=0.003; unpaired t-test. (B and C) T47D (B) or HBC4 (C) cells were transfected with si-EGFP or si-C12orf32 for 48 h, followed by treatment with 0.3 μg/ml of nocodazole for 18 h, and cells were analyzed by flow cytometry.

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.

Acknowledgements

We thank Ms. Kie Naito, Ms. Yoshiko Fujisawa, Ms. Aya Sasaki, Ms. Kyoko Kijima, Ms. Mami U and Mr. Yasuo Mochizuki for excellent technical assistance.

References

1. McCracken M, Olsen M, Chen MS Jr, *et al*: Cancer incidence, mortality, and associated risk factors among Asian Americans of Chinese, Filipino, Vietnamese, Korean, and Japanese ethnicities. CA Cancer J Clin 57: 190-205, 2007.
2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C and Thun MJ: Cancer statistics, 2006. CA Cancer J Clin 56: 106-130, 2006.
3. Berry DA, Cronin KA, Plevritis SK, *et al*: Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 353: 1784-1792, 2005.
4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. Lancet 365: 1687-1717, 2005.
5. Romond EH, Perez EA, Bryant J, *et al*: Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 353: 1673-1684, 2005.
6. Bilancia D, Rosati G, Dinota A, Germano D, Romano R and Manzione L: Lapatinib in breast cancer. Ann Oncol 18: 26-30, 2007.
7. Adamo V, Iorfida M, Montalto E, *et al*: Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. Ann Oncol 18: 53-57, 2007.
8. Petricoin EF III, Hackett JL, Lesko LJ, *et al*: Medical applications of microarray technologies: a regulatory science perspective. Nat Genet 32 (Suppl.): S474-S479, 2002.
9. Bange J, Zwick E and Ullrich A: Molecular targets for breast cancer therapy and prevention. Nat Med 7: 548-552, 2001.
10. Park JH, Lin ML, Nishidate T, Nakamura Y and Katagiri T: PDZ-binding kinase/T-LAK cell-originated protein kinase, a putative cancer/testis antigen with an oncogenic activity in breast cancer. Cancer Res 66: 9186-9195, 2006.
11. Shimo A, Nishidate T, Ohta T, Fukuda M, Nakamura Y and Katagiri T: Elevated expression of protein regulator of cytokinesis 1, involved in the growth of breast cancer cells. Cancer Sci 98: 174-181, 2007.
12. Lin ML, Park JH, Nishidate T, Nakamura Y and Katagiri T: Involvement of maternal embryonic leucine zipper kinase (MELK) in mammary carcinogenesis through interaction with Bcl-G, a pro-apoptotic member of the Bcl-2 family. Breast Cancer Res 9: R17, 2007.
13. Shimo A, Tanikawa C, Nishidate T, *et al*: Involvement of kinesin family member 2C/mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. Cancer Sci 99: 62-70, 2008.
14. Ueki T, Nishidate T, Park JH, *et al*: Involvement of elevated expression of multiple cell-cycle regulator, DTL/RAMP (denticleless/RA-regulated nuclear matrix associated protein), in the growth of breast cancer cells. Oncogene 27: 5672-5683, 2008.
15. Lin ML, Fukukawa C, Park JH, *et al*: Involvement of G-patch domain containing 2 overexpression in breast carcinogenesis. Cancer Sci 100: 1443-1450, 2009.
16. Ueki T, Park JH, Nishidate T, Kijima K, Hirata K, Nakamura Y and Katagiri T: Ubiquitination and downregulation of BRCA1 by ubiquitin-conjugating enzyme E2T overexpression in human breast cancer cells. Cancer Res 69: 8752-8760, 2009.
17. Park JH, Nishidate T, Nakamura Y and Katagiri T: Critical roles of T-LAK cell-originated protein kinase in cytokinesis. Cancer Sci 101: 403-411, 2009.
18. Kanehira M, Katagiri T, Shimo A, *et al*: Oncogenic role of MPHOSPH1, a cancer-testis antigen specific to human bladder cancer. Cancer Res 67: 3276-3285, 2007.
19. Kanehira M, Harada Y, Takata R, *et al*: Involvement of upregulation of DEPDC1 (DEP domain containing 1) in bladder carcinogenesis. Oncogene 26: 6448-6455, 2007.
20. Nagayama S, Iiizumi M, Katagiri T, Toguchida J and Nakamura Y: Identification of PDZK4, a novel human gene with PDZ domains, that is upregulated in synovial sarcomas. Oncogene 23: 5551-5557, 2004.
21. Nagayama S, Fukukawa C, Katagiri T, *et al*: Therapeutic potential of antibodies against FZD10, a cell-surface protein, for synovial sarcomas. Oncogene 24: 6201-6212, 2005.
22. Okada K, Hirota E, Mizutani Y, *et al*: Oncogenic role of NALP7 in testicular seminomas. Cancer Sci 95: 949-954, 2004.
23. Togashi A, Katagiri T, Ashida S, *et al*: Hypoxia-inducible protein 2 (HIG2), a novel diagnostic marker for renal cell carcinoma and potential target for molecular therapy. Cancer Res 65: 4817-4826, 2005.
24. Hirota E, Yan L, Tsunoda T, *et al*: Genome-wide gene expression profiles of clear cell renal cell carcinoma: identification of molecular targets for treatment of renal cell carcinoma. Int J Oncol 29: 799-827, 2006.
25. Nishidate T, Katagiri T, Lin ML, *et al*: Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. Int J Oncol 25: 797-819, 2004.
26. Shimokawa T, Furukawa Y, Sakai M, Li M, Miwa N, Lin YM and Nakamura Y: Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. Cancer Res 63: 6116-6120, 2003.
27. Navolanic PM and McCubrey JA: Pharmacological breast cancer therapy (review). Int J Oncol 27: 1341-1344, 2005.