Invasion Activating Caveolin-1 Mutation in Human Scirrhous Breast Cancers

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Abstract

We looked for mutations in the caveolin-1 gene, encoding a critical molecule for membrane signaling to cell growth, in 92 primary human breast cancers, and we report here the identification of a mutation in caveolin-1 at codon 132 (P132L) in 16% of cases. The mutation-positive cases were mostly invasive scirrhous carcinomas. In cell lines expressing the same mutant of caveolin-1, we observed that the mutant Caveolin-1 expression seemed to induce cellular transformation and activation of mitogen-activated protein kinase-signaling pathway and to promote invasiveness as well as altered actin networks in the cells. These results provide, for the first time, genetic evidence that a functioning Caveolin-1 mutation may have a role in the malignant progression of human breast cancer.

Introduction

Molecular cloning had identified three distinct caveolin genes (1–4) caveolin-1, caveolin-2, and caveolin-3. Alignment of the protein sequences encoded by these caveolin genes is shown in Fig. 1a. Note that most of the predicted functional domains of the caveolins are well-conserved among the family members, including the scaffolding domain and the membrane-spanning domain. It is well-known that one of the conserved amino acids in caveolin-3, the expression of which is muscle-specific, was found to be a disease-involved site (5–8). This site is evolutionarily conserved from worms to man, providing evidence that this region of the membrane-spanning domain is critical for Caveolin function. From these exciting findings, we surmised that the mutation of the amino acid in this site might function in an alternative physiological role among the caveolins. Then, we targeted the site to survey mutations in caveolin-1 in human tumors, because previous reports suggest identifying caveolin-1 as a candidate tumor suppressor gene (9–11).

Materials and Methods

Preparation and Analysis of DNA and RNA. We extracted DNA from primary tumors, corresponding noncancerous tissues, cultured cells, and blood leukocytes, as described before (12). Total RNAs were also isolated with Trizol Reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Five μl (1–3 μg/μl) of mRNA were reverse transcribed to generate cDNA using Superscript II and random hexamers (Life Technologies, Inc.) according to manufacturers’ protocols.

PCR-RFLP Analysis. The Ucav (sense, 5’-TTGGAAGGCCCAGCT- TCAC-3’) and Deav (antisense, 5’-GATAGGAACCTTACAGT-3’) primers were designed specifically to amplify the caveolin-1 sequences. The amplified caveolin-1 DNA fragments from genomic DNAs or cDNAs were digested at 37°C for 5 h with the indicated restriction endonucleases. The digested DNAs were electrophoresed in 0.8% agarose gel or in 6% polyacrylamide gel before the UV-photos.

Tissue Samples, Cell Lines, and Antibodies. We obtained tumors and corresponding normal breast tissues with informed consent from 92 patients who had undergone mastectomy. All tumors were diagnosed histopathologically as carcinomas. Parental NIH3T3 cells were transfected with the mutant or wild-type caveolin-1 genes introduced into pcDNA3 expression vector using a lipofection protocol. Resistant clones were selected with G418, as described before. Antibodies used were as follows: (a) antihuman caveolin-1, antihuman, and antinouse caveolin-1 (Transduction Laboratories); and (b) anti-Ras, antiphosphoMAPK, anti-ERK2, anti-phopho-p38, anti-p38, anti-phophoAKT, and anti-AKT (New England Biolaboratories).

Sequence Analysis. We purified aberrant PCR products detected by PCR-RFLP study. The DNA sequences of each aberrant sample and some normal samples were determined (13) using an Applied Biosystems DNA sequencer with a Dye-terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). We also confirmed all mutations by repeated experiments (at least three times) using DNAs extracted from the tumor and corresponding tissues.

Cell Migration Assay. Cells were grown in the presence of 10% fetal bovine serum until confluence. A wound area was generated by scraping with a plastic scraper. After 2 days, cells in the wounded monolayer were counted randomly at multiple fields.

Invasion Assay. Cells were assayed for their invasiveness by a modified Boyden chamber method. Briefly, conditioned media obtained from NIH3T3 were placed in the lower compartment of the chamber. Cells suspended in serum-free DMEM were seeded onto Matrigel-coated filters. After 12 h of incubation, cells that had invaded to the lower surface of the filter were fixed, stained, and counted.

Immunofluorescence and Western Blotting. To visualize polymerized actin, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 10 min, and incubated with 1 μg/ml FITC-labeled phalloidin (Sigma Chemical Comp.) for 1 h. Antibody against actin (Sigma Chemical Co.) also was used for indirect immunofluorescence. For the immunofluorescence, cells were also fixed with methanol/acetic acid for 10 min and permeabilized with 0.5% Triton X-100 for 10 min. They were incubated with the indicated antibody for 1 h at 37°C before a 1-h incubation with fluorescein isothiocyanate-conjugated goat anti-IgG antibody (Sigma Chemical Co.). Cells were viewed on a Nikon microscope and photographed. Western blotting was carried out according to the methods described before (12).

Results and Discussion

To assess the potential for caveolin-1 mutation in cases of breast cancer, we constructed PCR primers to specifically amplify a DNA fragment of human caveolin-1 sequences from genomic DNA of the tissue. We used conventional PCR-RFLP mutational screening because RFLP is a reliable and easy method for mutation screening if a proper enzymatic site exists in the target region (14). As can be seen in Fig. 1b, the mutation in codon 132 eliminated Rsa-I and/or Nla III sites; then we were able to develop a rapid screen for the caveolin-1 mutation using Rsa-I- and Nla III-based RFLP assay. After purifying genomic DNAs derived from paired normal and tumor samples, we amplified caveolin-1 DNA fragments using the specific primers and...
performed PCR-RFLP assays (Fig. 2, a–c). First, we screened DNA from 92 primary breast cancers for alterations of caveolin-1 and found 15 genetic alterations (7 scirrhous carcinomas, 3 solid tubular carcinomas, 2 papillo-tubular carcinomas, 2 invasive-lobular carcinomas, and 1 unknown carcinoma) among them. Almost all of the mutation-positive cases were invasive and/or scirrhous carcinomas. Mutations were confirmed by manual sequencing analyses (Fig. 3). Pilot studies using the PCR-RFLP identified two cases (scirrhous carcinoma) of double mutations in codon 132 and 133 that were confirmed by manual sequencing (Fig. 3, a and b), but we were unable to determine
whether the mutations were allelic, because RNA from these samples was not available. Direct DNA sequencing also confirmed the mutation (Fig. 3c). We could not observe loss of heterozygosity of the normal caveolin-1 gene in any of the breast cancer specimens, and none of the tumors harboring the mutation demonstrated microsatellite instability (data not shown). Although we screened 92 primary human breast cancer specimens with this assay, we were unable to successfully identify the P-L mutation in any of these tumors in DNA derived from normal, adjacent, matched tissue samples from patients with tumors harboring the codon 132 mutation. Furthermore, the genomic DNA analyses of the 26 normal healthy volunteers, from whom DNA was obtained from their peripheral blood leukocytes, failed to show that mutation at the site of caveolin-1 (Fig. 2, d–f). In addition, none of the other eight normal healthy volunteers had such mutations in the mRNA from their normal tissues (data not shown). We concluded that all of those mutations found in the breast carcinomas occurred as somatic events.

Taken together, our results and previous findings prompted us to hypothesize that the mutation at this site in caveolin-1 might lead to an advantage for tumor growth in the cells. Then, to determine the transformation potential of caveolin-1, NIH3T3 cells stably transfected with the mutant caveolin-1 (P132L) or wild-type caveolin-1 were derived using a mammalian expression vector. As shown in Fig. 4a, clones C13, C14, C15, and C16 were derived, and they expressed exogenous mutant caveolin-1 at various expression levels, whereas Ras and SHPS-1 (15) levels were not affected by the expression of the mutant caveolin-1. Examination of NIH3T3 cells harboring the caveolin-1 mutant by microscopy reveals that these cells have an overall altered morphology compared with that of cells expressing wild-type caveolin-1 or parental NIH3T3 (Fig. 4b). All independent caveolin-1 mutant cells had similar morphology, whereas untransfected NIH3T3 cells have an elongated, flattened, spindle-shaped, and stretched morphology. Furthermore, fiber-like phalloidin staining was not observed with NIH3T3 cells harboring the caveolin-1 mutant, in contrast with the typical fiber-like appearance of actin cytoskeleton networks with parental NIH3T3 cell controls (Ref. 16; Fig. 4c). Phalloidin staining of the other mutant cells of caveolin-1 (C14 and C15) were similar to that seen in the C13, and staining with anti-actin antibody also showed the disruption of actin cytoskeleton in the mutant cells (data not shown). We also examined effects of the caveolin-1 mutant expression on colony-formation in soft agar to assess anchorage-independent cell growth and found that these NIH3T3 cells harboring the caveolin-1 mutant exhibited growth in soft agar (parental NIH3T3 and NIH3T3 transfected with vector only showed no colony formation, whereas NIH3T3 with the caveolin-1 mutant showed >150 colonies/µg DNA; n = 2), in agreement with the previous report showing the dominant effect of the caveolin-1 mutant (17).

It seemed that the state of tyrosine phosphorylation was not altered by the expression of the caveolin-1 mutant. We next used a variety of phospho-specific antibodies (12) that have been generated against the activated forms of well-known signal transducers. Fig. 4d shows that both intracellular MAPKs and p38-MAPK were constitutively activated in the caveolin-1 mutant clones. On the other hand, the status of phosphorylation of AKT was not changed, although v-Src-transformed cells (SR3Y1) showed increased phosphorylation of AKT.
Again, these experiments demonstrated the capacity for the mutant to drive cell transformation. These observations are compatible with previous reports showing that alteration of caveolin-1 function could be involved in the cellular transformation as a dominant negative effect of caveolin-1.

The mutation-positive cases of breast cancer were mostly involved in the pathologically invasive types such as scirrhous carcinomas (18). Hence, we suspected that the expression of the caveolin-1 mutant may affect the invasive ability of the cells.

The invasiveness of cells was then evaluated by the modified Boyden chamber method as described (19). As can be seen in Fig. 4, e and f, the mutant cells could penetrate through the reconstituted membrane to a level similar to that of SK3Y1, whereas the wild-type caveolin-1 cells and parental NIH3T3 could not. In addition, rapid in vitro cell motility evaluated using a wound healing assay (20) showed that the transfectants of the mutant exhibited high motility-potential compared with the parental cells or with the clones of wild-type caveolin-1 (data not shown).

Caveolin-1 has been reported to participate in oncogenic processes in vitro, yet no genetic evidence had been presented that implicated this gene in the development or the progression of human cancer. Although Hurlstone et al. (9) reported previously that there was no mutation in the caveolin-1 gene in human cancers, we sought the mutation more intensively, focusing on human breast cancers. Here we have provided evidence for the existence of at least one naturally occurring mutant form of caveolin-1 that appears to have a role in human cancer. The results presented in this paper revealed that the mutation of caveolin-1 had a dominant negative effect on cell transformation and invasiveness. In addition, these findings indicate that caveolin-1 is likely to function as a tumor suppressor. We speculate that other effective caveolin-1 mutations, which we have not found yet, might exist, because there are other consensus sites for caveolin family members that were found to be at least critical sites in the scaffolding domain for caveolin-3 in limb-girdle muscular dystrophy. At this time, the study demonstrated in this paper may provide an experimental basis for additional analysis of caveolin-1 mutation in human diseases. In addition, investigation of signaling pathways affected by caveolins should provide additional insights into the molecular pathological action of caveolea disorders.

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References
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