The Human Homologue for the *Caenorhabditis elegans* cul-4 Gene Is Amplified and Overexpressed in Primary Breast Cancers

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**ABSTRACT**

Amplification is a key mechanism whereby a cancer cell increases the message level of genes that confer a selective advantage when they are overexpressed. In breast cancer, there are many chromosome regions present in multiple copies relative to overall DNA copy number (amplification), and their target genes are unknown. Using differential display, we have cloned and sequenced the full coding region of a candidate amplicon target gene located on chromosome 13. This candidate is the human homologue of the *Caenorhabditis elegans* cul-4 gene, cul-4A, a member of the novel cullin gene family, which is involved in cell cycle control of *C. elegans*.

cul-4A was amplified and overexpressed in 3 of 14 breast cancer cell lines analyzed, and it was overexpressed in 8 additional cell lines in which it was not amplified. The latter observation, indicating that its overexpression can occur by mechanisms other than gene amplification, suggests that cul-4A plays a key role in carcinogenesis. Moreover, cul-4A was found to be amplified in 17 of 105 (16%) cases of untreated primary breast cancers, and 14 of 30 cases analyzed (47%) were shown by RNA in situ hybridization to overexpress cul-4A. These results suggest that up-regulation of cul-4A may play an important role in tumor progression.

**INTRODUCTION**

Gene amplification can be defined as increased copy numbers of certain regions of the genome. Gene amplification often results in up-regulation of gene expression by increasing the number of templates available for transcription (1). Gene amplification is commonly found in tumor cells and is considered an important mechanism whereby tumor cells gain increased levels of expression of critical genes (1). Thus, identification and characterization of these genes should provide important insights into the pathobiology of cancer.

In breast cancer, cytogenetic evidence of increased DNA copy number is common (2–4). For example, homogeneously staining regions have been found in more than 50% of primary breast cancers (3, 4). Many genes amplified in breast cancers have been identified as oncogenes that were characterized previously in model systems. These include *erbB2* (17q12), c-myc (8q24), *cyclin D* (11q13), *FLG* (8p12), and *BEK* (10q24); each was amplified in ~10–30% of breast cancers (5–9). However, these known oncogenes are not sufficient to account for all of the genetic material present in large homogeneously staining regions in breast cancer (3). This indicates that other amplified genes might contribute to breast cancer progression.

Recently, several new amplified chromosomal regions have been identified in breast cancer by comparative genomic hybridization (10, 11), a method particularly suited to the study of breast cancer and other slow-growing tumors. These amplified regions often contain as many as 50–100 genes, which can be identified by different techniques, including differential display (12–14). However, it is possible that not all genes present in amplified regions contribute to carcinogenesis. Some may be amplified simply because of their proximity in the genome to amplicon target genes. To distinguish true target genes that are likely to be involved in carcinogenesis, we have used the following criteria. (a) A target gene (but not its unrelated neighbors) will always be amplified and overexpressed when the amplicon is present in a tumor cell. Because amplicons vary in size among different tumors, any unrelated gene might sometimes be excluded from the amplicon. (b) A target gene (but not an unrelated gene) should be overexpressed in some cases in which it is not amplified, because there are other mechanisms for inducing gene overexpression besides amplification. If the overexpression of the target gene is important for malignancy, other mechanisms should be involved in up-regulating the expression of the critical gene.

Using these criteria, we have isolated and characterized several genes that may be target genes for breast cancer amplicons. Here, we report characterization of one of the genes, which is the human homologue (Hs-cul-4A) of the *Caenorhabditis elegans* “cullin” gene (15).

**MATERIALS AND METHODS**

Human Mammary Epithelial Cells, Breast Cancer Cell Lines, and Tissues. All breast cancer cell lines except 600PE were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the donor’s instructions. The line 600PE was developed in our laboratory from the pleural effusion of a patient (16). Nonmalignant breast epithelial cells were derived from normal reduction mammoplasty specimens and cultured as described previously (17). Normal tissues and primary breast carcinomas were obtained at surgery from University of California Medical Center (San Francisco, CA).

Differential Display, Cloning, and Sequencing. Differential display of cDNA, recovery, and reamplification of cDNA fragments were performed essentially as described by Liang et al. (12–14). cDNA fragments were cloned into the PCR II vector using the TA cloning system (Invitrogen, San Diego, CA). The 2A12-1 cDNA reported here was isolated using TCGCGCGATAG and TTTTTTTTTTATG primers for PCR amplification. A BT747 cDNA library was constructed in λ GT10 and screened with the cloned cDNA fragment to isolate the longer cDNA clones. The 5' end of the cDNA was obtained by the rapid amplification of cDNA ends technique using the AmpliFinder kit from Clontech (Palo Alto, CA). The cDNA was then subjected to double-stranded sequencing (Retrogen, San Diego, CA).

Southern and Slot Blot Analysis. Tumor DNA samples were extracted from cultured cell lines and primary breast carcinomas as described (18). Normal DNA samples were extracted from 12 normal skin biopsies and 5 human placenta. For Southern hybridization, 10 μg of DNA were digested with EcoRI and subjected to electrophoresis on a 0.8% agarose gel (19). For slot blot analysis, 0.5 μg of DNA was loaded in each slot as described (20). The blots were then hybridized with a 32P-labeled 1.6-kb cul-4A cDNA fragment (corresponding to nucleotides 1551–3210 in Fig. 1). After autoradiogram was obtained, the probe was stripped, and the blot was reprobed using a reference probe to adjust for differences in sample loading. Chromosome 2 probe D2S6 and a chromosome 21 probe D21S16 (American Type
Fig. 1. The nucleotide sequence of human cul-4A cDNA and its deduced amino acid sequence. The predicted amino acid sequence of human cul-4A is shown below the nucleotide sequences using single letter code. Underlined letters, amino acids that are identical in human and C. elegans cul-4 genes. Boldface line, end of the shorter mRNA, at nucleotide position 3100.

Culture Collection) were used as reference probes on Southern blots, and human repeated sequences (Sigma Chemical Co., St. Louis, MO) were used as reference probe on slot blots.

Northern Blot Analysis. Total RNA was extracted from cultured cells and uncultured mammary epithelial tissues by guanidine isothiocyanate-CsCl step gradients (21) and stored in ethanol. Ten μg of total RNA was analyzed by Northern hybridization (22) using a 32P-labeled 1.6-kb cul-4A cDNA fragment as probe. β-Axin and 36B4 probes (American Type Culture Collection) were used as reference probe to adjust sample loading.

Chromosome Mapping. A human BAC clone cul-4A was isolated from a human BAC library (Research Genetics Inc.) according to the supplier’s protocol and used to determine the location on human chromosomes. DNA was extracted and labeled with digoxigenin-11-dUTP by nick translation. FISH was carried out in the presence of human Cot 1 DNA to suppress the background.

4The abbreviations used are: BAC, bacterial artificial chromosome; DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; UTR, untranslated region; pVHL, von Hippel-Lindau tumor suppressor gene product.
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Fig. 2. Metaphase mapping of cul-4A by FISH. cul-4A was mapped to human chromosome 13q34–qter in normal human lymphocytes using a BAC clone for cul-4A. Inset, localization of cul-4A (green) to 13q34–qter, based on DAPI banding of metaphase chromosome 13.

signal and hybridized to metaphase chromosomes overnight. The hybridization signal was detected by antidigoxigenin conjugated with FITC. The chromosomes were counterstained with DAPI. The location of the probe was determined by digital image microscopy following FISH and localized by DAPI banding (23).

Definition of Cutoff Points. To calculate the cutoff points for either gene amplification or overexpression, a set of nonmalignant (normal) samples was analyzed by Southern or Northern hybridization. Densities of the signals on the autoradiograms were obtained using a densitometer (Molecular Dynamics, Sunnyvale, CA). The density ratio between the cul-4A gene and the reference gene was calculated for each sample. Two steps were required to determine the cutoff point. First, the data for normal tissues were transformed so that it became normally distributed (i.e., followed a Gaussian distribution curve). Next, a table of tolerance bounds for a normal distribution was used to define cutoff points so that the confidence was 90% and so that a fraction of the distribution of no more than P would lie above the cutoff point. Each cutoff point is defined by cutoff point = mean + k(SD), where the mean and SD are based on values from normal tissues. Values of k are found in the table and depend on P and the number of normal tissue samples (n; Ref. 24). The cutoff point was then transformed back to the original measurement unit.

RNA in Situ Hybridization. Archival paraffin blocks of infiltrating breast cancer were obtained from 30 randomly selected patients from University of

A.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 3. Southern blot analysis of cul-4A in human breast cancer cell lines. Ten μg of genomic DNA from placenta (Lanes 1 and 2) and breast cancer cell lines BT474 (Lane 3), MCF7 (Lane 4), MDA-MB-157 (Lane 5), SKBR-3 (Lane 6), ZR-75-30 (Lane 7), CAMA-1 (Lane 8), Du4475 (Lane 9), 600PE (Lane 10), MDA-MB-453 (Lane 11), MDA-MB-231 (Lane 12), T47D (Lane 13), UACC812 (Lane 14), BT468 (Lane 15), MDA-MB-134 (Lane 16), and MDA-MB-435 (Lane 17) were digested with EcoRI, electrophoresed on a 0.8% agarose gel, and hybridized with 32P-labeled Hs-cul-4A (A). D2S6 (B) and D21S16 (C) were used as reference probes for loading control. The cell lines MDA-MB-157 (Lane 5), SKBR-3 (Lane 6), and 600PE (Lane 10) show increased cul-4A gene copy number.
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Fig. 4. Amplification of cul-4A in breast cancer cell lines. Slot blots of DNA from 15 samples of normal tissues and 16 breast cancer cell lines were quantitated by densitometer. The ratio of the density of cul-4A to the density of total DNA was calculated for both normal tissues and breast cancer cell lines and plotted as shown. The cutoff point was calculated as described in “Materials and Methods.” - - - - - - - , cutoff point for P = 0.01.

RESULTS

Isolation, Cloning, and Sequence Analysis of cul-4A. Using the differential display technique (12-14), we compared the cDNAs of the breast cancer cell lines BT474, SKBR3, and MCF7 with cDNAs of normal breast epithelial cells derived from reduction mammoplasties. Overabundant cDNAs in tumor cell lines were isolated and screened for overexpression (Northern analysis) and gene amplification (Southern analysis) in these and other breast cancer cell lines (see below). One of the cDNA fragments, 2A12-1, which showed overexpression and amplification in the SKBR3 cell line after the preliminary screening, was further characterized. Longer cDNA clones were isolated from a BT474 cDNA library using the original 2A12-1 cDNA fragment as a probe. Additional 5' sequences were cloned by rapid cDNA amplification technique and assembled to generate a nearly full-length cDNA (Fig. 1).

A BLAST search for sequence homologues in the GenBank database revealed that the 2A12-1 gene is the human homologue (Hs-cul-4A) of the C. elegans cul-4 gene (Ce-cul-4; Ref. 15). The human cDNA for Hs-cul-4A, as compiled from the expressed sequence tag database was incomplete, lacking 246 amino acids at the NH2 terminus, as judged by comparison with the C. elegans homologue (15). Fig. 1 shows the full-length cul-4A cDNA sequences of 3643 nucleotides, which encodes 659 amino acids and is 30% identical to Ce-cul-4.

The cDNA sequences shown in Fig. 1 are derived from the breast cancer cell line BT474. When compared with the sequences available from GenBank, which were assembled from the expressed sequence tag database, no amino acid differences were found. However, two species of mRNA with different lengths of 3'-UTR were isolated from the BT474 cDNA library. Sequencing results revealed that the 3.8-kb mRNA had a 3'-UTR of 1502 nucleotides, whereas the 3.5-kb mRNA had a shorter 3'-UTR of 960 nucleotides. Northern analysis showed that the 3.8-kb mRNA was more abundant than the 3.5-kb mRNA (see below).

Using human-rodent somatic cell hybrids (data not shown; Coriell Institute for Medical Research, Camden, NJ), we localized cul-4A to chromosome 13q. Subsequently, cul-4A was mapped to chromosome 13q34–qter by FISH using a cul-4A BAC clone isolated from a human BAC library (Fig. 2).

Amplification of cul-4A in Breast Cancer Cell Lines and Primary Breast Cancers. Fig. 3 illustrates the Southern analysis of cul-4A in breast cancer cell lines. It appeared that the cul-4A was present in increased gene copy in some cell lines as compared to normal DNA derived from placenta tissues. The additional 8-kb EcoRI fragment present in ZR-75-30, 600PE, and UACC812 (Fig. 3, Lanes 7, 10, and 14) was due to an EcoRI polymorphism. It is interesting to note that the amplification of cul-4A in 600PE cells seemed to occur in only one allele.

To achieve a statistically significant cutoff point for defining gene amplification, we used slot analysis, which allowed simultaneous analysis of many normal and cancer cell samples. Fig. 4 shows the relative gene copy number in normal and tumor cell line DNA samples, as measured by slot blot analysis. On the basis of the defined cutoff point, 3 (600PE, MDA-MB-157, and SKBR3) of the 15 (20%) cell lines analyzed showed a significantly increased gene copy number (P < 0.01). In addition, using the same methodology, cul-4A was found to be amplified in 17 of 105 (16%) untreated primary breast tumors analyzed (P < 0.01).

Expression of cul-4A in Breast Cancer Cell Lines. The expression level of cul-4A mRNA in both breast tumor cells and normal breast epithelial cells was measured by Northern hybridization, as compared to internal loading controls, β-actin, and 36B4 (Fig. 5). The blots were quantitated by densitometry and expressed as a density ratio of cul-4A mRNA to β-actin or 36B4 mRNA. On the basis of the defined cutoff point (see “Materials and Methods”), 10 of 14 breast
cull-4A as a Candidate Target Gene for the Amplicon on Chromosome 13q. Table 1 summarizes the results presented in Figs. 3–6. All three cell lines that amplified the cul-4A gene also overexpressed the gene. In eight of the cell lines (BT474, CAMA-1, T47D, MDA-MB-134, MDA-MB-231, MDA-MB-361, MDA-MB-435, and MDA-MB-468) the gene was overexpressed without amplification. This is consistent with the hypothesis that there are other mechanisms for gene overexpression besides amplification. Four cell lines (BT20, MCF7, DU4475, and UACC812) neither amplified nor overexpressed cul-4A. Most importantly, as predicted for a candidate target gene of an amplicon, we found no cell lines in which the cul-4A gene was amplified but not overexpressed.

Overexpression of cul-4A in Primary Breast Cancers. The expression of cul-4A RNA was evaluated by RNA in situ hybridization on formalin-fixed, paraffin-embedded sections in a panel of 30 cases of infiltrating breast carcinoma. Of the 30 cases, 15 also had adjacent nonmalignant breast epithelium. The slides were reviewed independently by at least two people. Fig. 7 shows the differential expression of cul-4A mRNA on paraffin-embedded sections. A negatively stained cell line (BT20) and a positively stained cell line (600PE) are shown in Fig. 7, a and b, respectively. This result is consistent with the data obtained by Northern hybridization. Fig. 7c shows a representative tumor that overexpressed the gene for cul-4A, whereas adjacent normal breast epithelium stained negatively, as shown in Fig. 7d. cul-4A mRNA was detected in 14 of 30 (47%) breast tumors. In contrast, in 14 of 15 (93%) cases, the adjacent normal breast epithelium stained negatively for cul-4A mRNA.

Tissue Expression. The expression of cul-4A gene in a variety of normal human tissues was analyzed by Northern blotting (Clontech, Palo Alto, CA; Fig. 8). Northern blot showed that cul-4A mRNA was expressed in multiple tissues. Although expression of cul-4A was most abundant in heart and skeletal muscle, it was almost undetectable in kidney and lung.
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**DISCUSSION**

Progression of human cancers is known to involve multiple genetic changes that lead to up-regulation or down-regulation of expression of critical genes (27). Gene amplification is a common mechanism whereby tumor cells increase expression of genes that are critical for malignancy. Positional cloning and differential display are convenient methods of identifying amplified genes in tumors. However, not all genes present in multiple copies are necessarily relevant to malignancy. Amplicons may contain irrelevant genes that are present by virtue of their physical proximity to target genes. Furthermore, how the results of differential display are interpreted is very dependent upon the cell substrates being used. However, we have shown that, by selecting proper substrates for differential display, followed by careful screening of gene amplification and overexpression, potential target genes for amplicons can easily be cloned.

Using this approach, we have identified the human homologue of the C. elegans cul-4 gene. We have shown that the gene is located on chromosome 13q and is a candidate target gene for an amplicon located there. Two (MDA-MB-157 and SKBR3) of the three cell lines in which cul-4A was amplified also revealed a small amplicon at chromosome 13q by comparative genomic hybridization analysis (11), whereas the third cell line, 600PE, was shown not only to have lost a copy of chromosome 13 but to have gained a marker chromosome of 1q:13q. The exact copy numbers of cul-4A gene in these cell lines remain to be determined.

We have also shown that the cul-4A gene is amplified in 16% of primary breast tumors examined. Because the tumor samples used for analysis were a mixture of tumor cells and the surrounding normal cells, the 16% positive cases may be an underestimate. Furthermore, in both cell lines and primary tumors, the number of cases with cul-4A overexpressed was greater than the number of cases with cul-4A gene amplified. This suggests that overexpression of this gene is an important event in carcinogenesis and not the result of a chance presence in an amplicon region.

cul-4A belongs to a conserved family of genes, known as the cullin family (15). There are at least six human, five nematode, and three yeast cullin genes. Several possible functions for cullin genes have been suggested. The rabbit orthologue of cul-5, known as vasopressin-activated calcium mobilizing receptor-1, has been shown to mobilize Ca2+ after arginine vasopressin induction, increasing the intracellular calcium concentration (28). The highest expression level of Hs-cul-5 was found to be in the heart and the skeletal muscle, contractile tissues that require a high level of calcium influx (29). We found that heart and skeletal muscle also expressed the highest level of Hs-cul-4A, suggesting it may also be involved in calcium mobilization. However, there is no obvious transmembrane sequence in Hs-cul-4A.

On the other hand, the yeast cul-1 gene, or Cdc53 (15), is part of a protein complex that targets cell cycle proteins for degradation by the ubiquitin-proteasome pathway (30, 31). The C. elegans cul-1 (Ce-cul-1) was shown to be required for cells to exit the cell cycle. Moreover, a null mutation of Ce-cul-1 causes hyperplasia in all tissues. Recently, human cul-2 gene product has been shown to form a stable complex with pVHL (32). Taken together, these results suggest that cul-1 and cul-2 gene products might be candidate tumor suppressors.

In contrast, our data suggest that overabundance of cul-4A contributes to malignancy; thus, cul-4A may function as an oncogene. For instance, Hs-cul-4A may target tumor suppressors or other proteins that negatively regulate the cell cycle. It is also possible that overexpression of Hs-cul-4A may have a profound effect on localization of the protein to the proper cellular compartment. Both Ce-cul-1 and Hs-cul-2 contain potential bipartite nuclear targeting signals (15, 32), and the latter is a cytosolic protein that can be translocated to the nucleus by binding to the pVHL complex (32). It has been shown that overexpression of Hs-cul-2 without pVHL or coexpression of Hs-cul-2 with a mutant pVHL results in localization of Hs-cul-2 to the cytosol exclusively (32). However, the Hs-cul-4 described here and the previously reported Hs-cul-5 were shown to contain a variant nuclear localization signal (29). The lack of conservation of the nuclear localization motif in some members of the cullin family suggest that this motif is not required for its functions.

In summary, we have shown that the cul-4A transcript is overexpressed in primary breast cancers. Preliminary data suggest that cul-4A is also overexpressed in other types of human cancers (data not shown). Because Hs-cul-4A may be the target for an amplicon on chromosome 13, it may be a potential predictive and prognostic indicator. It would be important to identify the interacting protein(s) for Hs-cul-4A, which might help in understanding the biological function of Hs-cul-4A. Moreover, the interacting proteins may themselves be good targets for therapeutic intervention.

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