Original Article

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Construction of *C35* gene bait recombinants and T47D cell cDNA library

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Summary **C35 is a novel tumor biomarker associated with metastasis progression. To investigate the interaction factors of C35 in its high expressed breast cancer cell lines, we constructed bait recombinant plasmids of *C35* gene and T47D cell cDNA library for yeast two-hybrid screening. Full length *C35* sequences were subcloned using RT-PCR from cDNA template extracted from T47D cells. Based on functional domain analysis, the full-length $C35_{1\text{-}348bp}$ was also truncated into two fragments $C35_{1\text{-}153bp}$ and $C35_{154\text{-}348bp}$ to avoid auto-activation. The three kinds of *C35* genes were successfully amplified and inserted into pGBKT7 to construct bait recombinant plasmids pGBKT7-$C35_{1\text{-}348bp}$, pGBKT7-$C35_{1\text{-}153bp}$ and pGBKT7-$C35_{154\text{-}348bp}$, then transformed into Y187 yeast cells by the lithium acetate method. Auto-activation and toxicity of C35 baits were detected using nutritional deficient medium and X-α-Gal assays. The T47D cell ds cDNA was generated by SMART™ technology and the library was constructed using *in vivo* recombination-mediated cloning in the AH109 yeast strain using a pGADT7-Rec plasmid. The transformed Y187/pGBKT7-$C35_{1\text{-}348bp}$ line was intensively inhibited while the truncated Y187/pGBKT7-C35 lines had no auto-activation and toxicity in yeast cells. The titer of established cDNA library was 2×10^7 pfu/mL with high transformation efficiency of 1.4×10^6, and the insert size of ds cDNA was distributed homogeneously between 0.5-2.0 kb. Our research generated a T47D cell cDNA library with high titer, and the constructed two C35 "baits" contained a respective functional immunoreceptor tyrosine based activation motif (ITAM) and the conserved last four amino acids Cys-Ile-Leu-Val (CILV) motif, and therefore laid a foundation for screening the C35 interaction factors in a BC cell line.**

Keywords: *C35* gene, bait construction, T47D cDNA library, breast carcinoma, yeast strain

1. Introduction

Breast carcinoma (BC) is the most common cancer and the first killer of women's health in both the developed and developing countries. According to the statistics from World Health Organization published in 2013 (*1*), BC was responsible for approximately 508,000 female deaths in 2011 in the world. In China, the incidence of BC has been increasing in recent years, more than 1.6 million patients were diagnosed and 1.2 million death cases occurred each year, among those the newly diagnosed cases accounted for 12.2% (*2*). C35 is a novel identified tumor biomarker for prediction and early diagnosis of either breast carcinoma (*3*) or other cancers such as colorectal and prostate cancer (*4,5*). By comparing 38 kinds of human normal tissue cells including brain cerebellum, endothelium and skeletal muscle, Evans *et al*. revealed that almost all normal cells were negative for C35 expression, with the exception of human leydig cells (*6*). Parallel experiments were also conducted in 10 kinds of human mammary carcinoma cell lines such as BT-20, MCF7 and T47D, and the *C35* gene was abundantly expressed in 7 kinds of mammary carcinoma cells, but no obvious

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expression was detected in adjacent normal breast epithelium (*6*). In particular, the expression level of *C35* gene in T47D cell line was 10 to 70 times more than in the H16N2 normal mammary epithelial cell line, and more frequent in infiltrating ductal carcinoma and invasive lobular carcinoma (6). Moreover, Kun *et al*. reported that overexpression frequency of C35 was associated with clinical Tumor Node Metastasis staging and Scarff-Bloom-Richardson grade ($p < 0.05$), indicated that C35 may take part in transforming normal cells into tumor progression and lymph node metastasis (*3*).

C35 gene is located on the minus region of human chromosome 17q12 (*7*). The full length of C35 transcript consists of 776 nucleotides and 4 exons, encoding a 12 kDa membrane-anchored protein. *In-situ* hybridization by Evans *et al*. indicated that gene expression and regulation of C35 was correlated with chromosomal amplification, especially overexpression of C35 was accompanied by amplification of the *ERBB2* gene, consistent with the fact that *C35* location is bounded by the oncogene *ERBB2* and growth factor receptor-bound protein 7 (*GRB7*) (*6*). The C-terminal end of C35 contains a conserved canonical immunoreceptor tyrosine based activation motif (ITAM) and a tiny functional domain made up of the last four amino acids Cys-Ile-Leu-Val (CVIL), these two domains were proved to have an important role in cancer progression and metastasis (*5,8*). By prediction of the functional sites and conserved domain of C35, Dasgupta *et al*. speculated that C35 may participate in four important physiological pathways including apoptosis, cell transformation, cytoskeleton remodeling and vesicle trafficking (*5*), yet the molecular mechanisms and interaction factors of C35 in regulation of those pathways are still unknown.

In this study, we have constructed 3 bait recombinant plasmids of C35 according to its functional domains and a T47D cell cDNA library in order to conduct a yeast-two hybrid system, for screening the interaction factors of C35 in its over-expressed cell line. The extensive detection of auto-activation and toxicity for the three baits were also performed to evaluate their application value. Our study laid a foundation for the following screening research.

2. Materials and Methods

2.1. *Cell culture conditions*

Human T47D breast cancer cell line (supplied by the Institute of Basic Medicine, Shandong Academy of Medical Sciences, Ji'nan, China) was cultured in DMEM (Gibco, Grand Island, NY, USA), supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL of penicillin (Hyclone, Logan, UT, USA), and 100 U/mL of streptomycin (Hyclone, Logan, UT, USA), in an incubator with 5% CO_2 at 37°C. For maintenance and subculture, T47D cells in exponential phase were treated with 0.25% trypsin solution containing 0.02% EDTA. After reaching 80% confluence, the cells were collected.

2.2. *Extraction of T47D cells mRNA*

The T47D cells (1×10^7) were harvested for mRNA extraction using the Oligotex Direct mRNA kit (Qiagen, Hilden, Germany). Extraction of T47D cells mRNA was conducted according to the kit instructions. The amount and quality of product was analyzed by using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Then it was first reverse transcribed into cDNA using a MulV reverse transcriptase kit (Fermentas, Burlington, Ontario, Canada) according to the protocol.

2.3. *Amplification of C35 gene*

The entire open reading frame of *C35* gene was amplified using a DNA thermal cycler (Applied Biosystems, San Diego, CA, USA). The primers sequences corresponded to the registered C35 gene sequence in GeneBank (ID: 84299): C35-1bp-*EcoR* I-5': 5'-CGGAATTCATGAGCGGGGAGCCGG-3'; C35-348bp-*Sal* I-3': 5'-ACGCGTCGACTCACAGGA TGACGCAGGGA-3' (the underlined were sequences of restriction sites *EcoR* I and *Sal* I respectively). PCR was performed in a 50 μL reaction volume containing 37 μL ddH_2O, 5 μL 10× Buffer, 2 μL deoxyribonucleoside triphosphate (dNTP), 1 μL each of C35 primer (50 mmoL/L), 2 μL DMSO (Sigma, USA), 1 μL cDNA template, and 1 μL Pfu DNA polymerase (Takara, Japan). The PCR reaction condition was: 95°C for 5 min, 30 cycles of 94°C for 1min, 56°C for 30 s, 72°C for 1min, with a final extension at 72°C for 5 min. PCR products were detected using 1.2% agarose gel electrophoresis with ethidiumbromide staining, and visualized under an ultraviolet transilluminator (Bio-Rad, USA).

2.4. *C35 bait plasmids construction*

Analysis of C35 functional and conserved domain was performed by the online software PROSITE (*http://www.expasy.org/prosite*) and the DNAStar Lasergene software v7.1 respectively. Two kinds of truncated *C35* fragments were designed as $C35_{1\text{-}153bp}$ and $C35_{154\text{-}348bp}$. The primers of *C35* truncated fragments were as follows: C35-153bp-*Sal* I-3': 3'-ACGCGTCGACCG GATACTGCTCCTTCACA; C35-154bp-*EcoR* I-5': 5'-CGGAATTCGGCATCGAGATCGAGTC. PCR amplification $C35_{1\text{-}348bp}$, $C35_{1\text{-}153bp}$ and $C35_{154\text{-}348bp}$ were performed and detected as described previously (section 2.3). All of the three PCR products were digested with *EcoR* I and *Sal* I (Takara, Japan), then inserted

into the corresponding sites of the bait expression vector pGBKT7 using T4 DNA ligase (Takara, Japan) to construct recombinant plasmids pGBKT7-$C35_{1\text{-}348bp}$, pGBKT7-$C35_{1\text{-}153bp}$ and pGBKT7-$C35_{154\text{-}348bp}$ respectively. The three plasmids were transformed into *E. coli* DH5α competent cells respectively and the positive clones were identified by colony's PCR and double digested with *EcoR* I and *Sal* I enzymes, then further verified by a commercial sequencing service (BGI Corporation, Beijing, China). The sequencing results were analyzed using Chromas 2.2 Software (Technelysium Pty Ltd, Australia).

2.5. *Yeast strain transformation*

Preparation of the Y187 and AH109 yeast competent cells and chemical transformation using the lithium acetate method were performed according to the Matchmaker™ Library Construction & Screening Kits User Manual (Cat # PT3955-1) and Yeast Protocols Handbook (Cat # PT3024-1) by Clontech Company (Palo Alto, CA, USA). A total of 5 kinds of plasmids pGBKT7-$C35_{1\text{-}348bp}$, pGBKT7-$C35_{1\text{-}153bp}$, pGBKT7-$C35_{154\text{-}348bp}$, pGBKT7-53 (positive control, Clontech) and pGBKT7-Lam (negative control, Clontech) were transformed into the Y187 competent cells respectively.

2.6. *Autoactivation and toxicity detection of C35 baits*

After transformation, five kinds of transformants were spread onto SD/-Trp/X-α-Gal plates respectively and cultivated at 30°C for 3-5 d, then the colony with the largest diameter was transferred on SD/-His/-Trp/X-α-Gal and SD/-Ade/-Trp/X-α-Gal plates (Sigma, USA). To estimate the optimal concentration of 3-AT (Sigma, USA), the largest colonies were also cultivated on SD/-His/-Trp/-3-AT plates respectively at 30°C for 3-5 d, with a 3-AT concentration gradient of 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 mM. The positive colonies with more than 2 mm diameter were picked out and inoculated at 250-270 rpm/min in 50 mL SD/-Trp/-Kan media (20 μg/mL) for 16-24 h at 30°C to measure the A_{600} values.

2.7. *Generation of T47D cells ds cDNA*

The first strand cDNA of T47D cells was synthesized with SMART™ technology by using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and CDS III primer (Clontech, Palo Alto, CA, USA) according to the manual's instructions. Then the LD-PCR was used to amplify ds cDNA using the first-strand cDNA as template and the advantage 2 polymerase mixture. The reaction condition was performed according to the instruction of PT3955-1, and detected using 1.0% agarose gel electrophoresis. The ds cDNA products were purified by CHROMA SPIN™+TE-400 columns to remove small DNA molecules less than 200 bp. The amount of ds cDNA was assessed by a NanoDrop 2000 spectrophotometer.

2.8. *Library construction and titer detection*

20 μL of ds cDNA (2-5 μg) and 6 μL of pGADT7-Rec plasmid (0.5 μg/μL) were co-transformed into competent AH109 yeast cells. The transformed cells were resuspended in 15 mL of 0.9% NaCl. Every 100 μL of a 1/10, 1/100, 1/1,000 and 1/10,000 dilution of transformed cells was spread on a 100 mm SD/-Leu/plate and grown at 30°C for 3-4 days to determine independent colonies (Formula: Number of cfu/mL on SD/-Leu × 15 mL) which indicated transformation efficiency. The remainder of the transformed cells were spread on 150 mm SD/-Leu plates (a total of 100 plates) with 150 μL cells per plate and incubated at 30°C for 3-4 d. The transformants in each 150 mm plate were harvested in 5 mL of freezing YPDA medium containing 25% glycerol and isolated using sterile glass beads, the cell density was calculated with a hemocytometer. After that, all liquids of the library were divided into 1 mL aliquots for library screening or stored at −80°C. To determine the library titer (cfu/mL), every 100 μL of a 1/100, 1/1,000 and 1/10,000 dilution of the aliquot was spread on a 100 mm SD/-Leu/ plate (Formula: number of colonies/ plating volum (mL) × dilution factor). 20 colonies were randomly picked up from the plates to check the insert size using Matchmaker™ Insert Check PCR Mix 2 Kit (Clontech, Palo Alto, CA, USA).

3. Results

3.1. *Amplification of C35 and its truncated fragments*

The mRNA isolated from T47D cells was identified by spectrophotometer and the OD_{260}/OD_{280} value was 1.944, indicating that the quality of the extracted mRNA met the requirement for being the template of the following RT-PCR and LD-PCR. The full-length sequences of *C35* were amplified by RT-PCR of the cDNA. Analysis of the RT-PCR products using 1.2% agarose gel electrophoresis showed positive bands with the expected 348 bp size (Figure 1A). Based on the results of conserved domain analysis, C35 protein contains two functional motifs, ITAM and CVIL. The ITAM motif in sequence $_{36}$EATYLELASAVKEQYPGIEI$_{53}$ conforms to a prototypical immunoreceptor tyrosine-based activation consensus sequence with the Tyr39 as a tyrosine kinase activity site. The COOH-terminal "CVIL" sequence fits the prenylation motif CAAX and may take part in the membrane combining process. Since the kinase domain usually induces an auto-activation activity in the yeast system, we designed two truncated fragments of $C35_{1\text{-}153bp}$ and $C35_{154\text{-}348bp}$. These two truncated fragments were amplified and verified by the same protocols as the full-length one. The

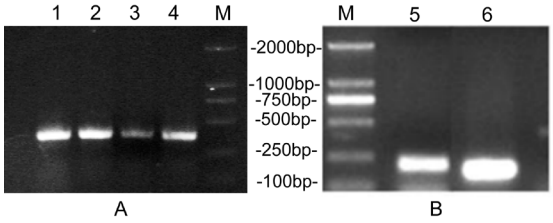

Figure 1. PCR amplification results of C35 gene. (A): PCR products of full-length *C35* gene. 1-4: $C35_{1\text{-}348bp}$; M: DL 2000 DNA marker. **(B):** PCR products of truncated C35 fragments. 5: $C35_{154\text{-}348bp}$; 6: $C35_{1\text{-}153bp}$.

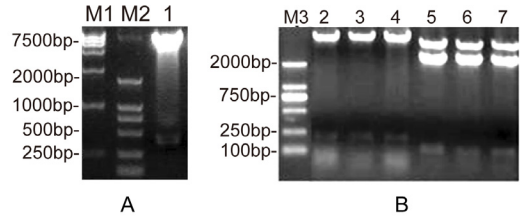

Figure 2. Identification of the *C35* bait recombinant plasmids pGBKT7-C35 by restriction enzyme digestion with *EcoR* I and *Sal* I. (A): Gel electrophoresis of double digested pGBKT7-$C35_{1\text{-}348bp}$. M1: DL15000 DNA marker; M2: DL2000 DNA marker; 1: pGBKT7-$C35_{1\text{-}348bp}$. **(B):** Gel electrophoresis of double digested truncated pGBKT7-C35. M3: DL2000 DNA marker; 2-4: pGBKT7-$C35_{154\text{-}348bp}$; 5-7: pGBKT7-$C35_{1\text{-}153bp}$.

corresponding electrophoresis results also showed clear positive bands with 153 bp for $C35_{1\text{-}153bp}$ and 195 bp for $C35_{154\text{-}348bp}$ respectively (Figure 1B).

3.2. *Construction and identification of C35 bait recombinant plasmids*

The three kinds of C35 fragments preliminarily amplified by PCR were digested with *EcoR* I/*Sal* I enzymes, then ligated in-frame into the corresponding sites of the bait expression vector pGBKT7 respectively. The three generated recombinants were named as pGBKT7-$C35_{1\text{-}348bp}$, pGBKT7-$C35_{1\text{-}153bp}$ and pGBKT7-$C35_{154\text{-}348bp}$. After transformation, the plasmids were extracted from positive colonies and identified by double digestion with *EcoR* I and *Sal* I. The 1% agarose gel electrophoresis results displayed the approximately 7,300 bp empty pGBKT7 and the expected 348 bp (Figure 2A), 153 bp and 195 bp *C35* fragments (Figure 2B). All of the three sequencing results were verified to be correct by conducting BLAST database homology searches referred to published *C35* sequences in GenBank (data not shown), and proved that the recombinant *C35* bait plasmids were successfully established.

3.3. *Determination of auto-activation and toxicity of C35 baits*

The recombinant *C35* bait plasmids were transformed

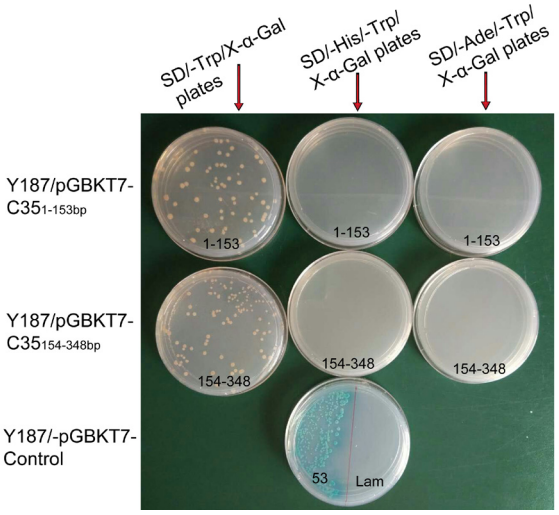

Figure 3. Auto-activation activity detection results of two truncated *C35* bait plasmids. The transformation of truncated Y187/pGBKT7-C35 lines showed white colonies on SD/-Trp/ X-α-Gal plates but could not grow on SD/-His/-Trp/X-α-Gal and SD/-Ade/-Trp/X-α-Gal plates, indicating the "baits" had no Auto-activation activity and self transcriptional activity. Colonies of Y187/pGBKT7-53 positive control turned blue on SD/-His/-Trp/ X-α-Gal plates.

into yeast strain Y187 using the LiAc-mediated method to produce the Y187/pGBKT7-C35 lines. Surprisingly, no visible colony of Y187/pGBKT7-$C35_{1\text{-}348bp}$ appeared on SD/-Trp plates, suggesting that the full-length C35 protein had intensive toxicity to the Y187 yeast strain (data not shown). White colonies of Y187/pGBKT7-$C35_{1\text{-}153bp}$, Y187/pGBKT7-$C35_{154\text{-}348bp}$ and Y187/pGBKT7-Lam appeared on SD/-Trp/ X-α-Gal plates in 3-5 d (Figure 3). At the same time, after being transferred the largest colonies of the two truncated Y187/pGBKT7-C35 lines to SD/-His/-Trp/ X-α-Gal and SD/-Ade/-Trp/X-α-Gal plates respectively, showed no background growth on these two kinds of nutritional deficiency plates (Figure 3), suggesting that none of them had an auto-activation effect and were not required for 3-AT inhibition. To determine the baits' toxicity to the yeast strain, the largest colonies of Y187/pGBKT7-$C35_{1\text{-}153bp}$ and Y187/pGBKT7-$C35_{154\text{-}348bp}$ on SD/-Trp/X-α-Gal plates were picked out and inoculated respectively in 50 mL SD/-Trp/-Kan (20 μg/mL) media for 20 h, the A_{600} value was 0.859 for Y187/pGBKT7-$C35_{1\text{-}153bp}$ and 0.807 for Y187/pGBKT7-$C35_{154\text{-}348bp}$ respectively, confirming that both of them were nontoxic and thereby the recombinant plasmids pGBKT7-$C35_{1\text{-}153bp}$ and pGBKT7-$C35_{154\text{-}348bp}$ were eligible to be used as "baits" for yeast two-hybrid analysis.

3.4. *Synthesis of T47D cells ds cDNA*

The 1.0% agarose electrophoresis results showed that 7 μL LD-PCR product of ds cDNA fractions appeared mainly as a 0.25-2.0 kb smear (Figure 4). More than 3

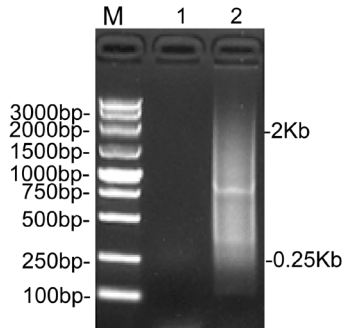

Figure 4. Gel electrophoresis results of the purified ds cDNA fractions of T47D cells by LD-PCR. M: DL 5000 DNA marker; 1: empty lane; 2: ds cDNA. The ds cDNA fractions appeared mainly as a 0.25-2.0 kb smear.

μg of ds cDNA were obtained from the remainder of the 93 μL LD-PCR product after purification on the CHROMA SPIN™+TE-400 Columns. The amount and quality of the amplified ds cDNA were satisfactory for constructing the yeast two-hybrid cDNA library.

3.5. *Construction and titering of the T47D cDNA library*

After co-transforming the ds cDNA product and pGADT7-Rec into AH109 competent cells, a total of 1.4 × 10^6 independent colonies which contained the T47D cell cDNA library were harvested on the 100 mm SD/-Leu plates were which spread with gradient dilution transformed cells, and the transformation efficiency was calculated as 1.4 × 10^6 transformants/3 μg pGADT7-Rec, indicating that the transformation efficiency in this study could yield a satisfactory library with high complexity. Further, after harvesting colonies on all of the 150 mm SD/-Leu plates, the cell density was about 8.2 × 10^7 – 1.0 × 10^8 cells/mL and sufficient enough for screening. The titer of the T47D cell cDNA library was 2 × 10^7 cfu/mL calculated by colony numbers on the SD/-Leu plates spread with a gradient diluent library aliquot. The electrophoresis results of 20 random colonies showed that the insert size of the ds cDNA was distributed randomly between 0.5 kb-2.0 kb (Figure 5), indicating that our library was homogeneous and the recombination rate of the library was 95% (19/20).

4. Discussion

The *C35* gene, also termed as C17orf37/MGC14832, which has a 95% identity compared with the migration and invasion enhancer 1 (MIEN1) protein, is a novel oncogene discovered in various kinds of cancers. Expression of C35 has been reported to positively relate to grade and stage of cancer progression, for example, BC, ovarian cancer, prostate cancer, and colorectal cancer. Yet studies on the function of over-expressed C35 and its interaction factors are still in the initial stage. The known interaction factor of C35 was

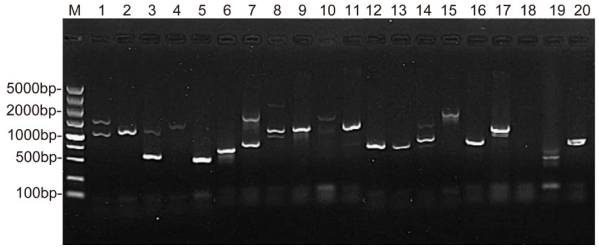

Figure 5. Identification results of insert size of T47D cell cDNA library by PCR. M: DL 5000 DNA marker; 1-20: Gel electrophoresis results of PCR from library colonies selected randomly.

discovered by a Leung *et al*. report (*9*), in that research, C35 was identified as a potential protein partner of p73 by yeast two-hybrid screen using a Hela cDNA library and a p73 bait vector. The co-expression of C35 and ΔNp73, a dominant-negative isoform of p73, which lacks the transactivation domain, could induce greater cisplatin treatment resistance in ovarian cancer cells by activating AKT and nuclear NF-κB p65. Consistently, Dasgupta *et al*. (*5*) reported that downregulation of C35 in prostate cancer cells suppressed PKB/Akt phosphorylation and NF-κB activity was also reduced by down-regulating downstream target genes MMP-9, uPA and VEGF, suggesting C35 takes part in the regulation of the NF-κB signaling pathway. In addition, Rajendiran *et al*. (*10*) demonstrated that overexpression of MIEN1 in DOK cells also increased the Akt/NF-κB effectors MMP-9, uPA and VEGF, and thereby further confirmed the correlation between C35 overexpression and the activation of Akt/NF-κB. However, Katz *et al*. (*8*) discovered that the expression levels of NF-κB markers MMP-9, uPA and VEGF were not correlated with overexpression of C35 in BC cells. These findings suggest that C35 may participate in different signaling pathways in different cancer cell lines. At the same time, they also discovered that high levels of C35 protein expression in primary BC cells could induce transformation in 3D cell cultures by activating Syk kinase, a downstream mediator of signaling from the ITAM motif. Similarly, inhibition of MIEN1 protein expression in BC cells decreased the expression level of matrix metallopeptidase 9 by downregulating the expression of AKT kinase (*11*). The correlation between C35 and kinase activity suggests that the application value of C35 may not only be the biomarker gene for genetic diagnosis and prognosis, but also be a potential drug target for BC therapy or other cancers. At present, there are a series of reports regarding the inhibition effects of C35. Liu *et al*. (*12*) have designed two kinds of C35 siRNAs with significant inhibition effects for both mRNA and protein expression levels of C35, and could induce apoptosis of T47D cells *in vitro*. Rajendiran *et al*. (*13*) proved that inhibiting the expression of MIENI protein in prostate cells by microRNA-940 could suppress the migratory and invasive potential of prostate cells. Li *et al*. (*14*)

reported that microRNA-26b suppressed the metastasis of non-small cell lung cancer by targeting MIEN1 *via* NF-κB/MMP-9/VEGF pathways. Therefore C35 is a promising novel diagnostic and therapeutic tool in various kinds of cancers, exploring and screening its interaction factors may elucidate the signaling pathways it takes part in, reveal it mechanisms in inducing cell migration and invasion in different kinds of cancers, and may provide the relevant targets for the development of new drugs.

Notably, the COOH-terminal amino acid sequence "CVIL" of C35 fits the prenylation CAAX motif, and Dasgupta *et al*. (*15*) demonstrated that this region could be post-translationally modified by protein geranylgeranyltransferase-I (GGTase-I). This C-terminal geranylgeranylation was essential for its membrane, which enhanced cells migratory phenotype by inducing increased filopodia formation, and thus potentiates directional migration. In particular, this "CVIL" motif is completely conserved among all species. Additionally, the ITAM motif was identified to determine the phosphorylation-dependent activation of MIEN1, which regulated filopodia generation, migration and invasion of breast cells (*16*). These studies indicated that the C-terminal "CVIL" motif and the ITAM domain act as the dominant functional motif of C35. Furthermore, the ITAM-phosphorylation of MIEN1 could be significantly impaired in isoprenylation-deficient MIEN1 mutants, and imply that the post-translational modification of MIEN1 was required for cross-phosphorylation of tyrosine residues (*16*).

Based on these studies, it is deduced that the functional domains of C35 are mutually independent and functionally interacting in regulating metastatic progression of cancers. Therefore in this study, we have designed three different fragments of C35 for constructing bait vectors for yeast two-hybrid screening according to the results of conserved domain analysis. After transforming the full-length pGBKT7-$C35_{1\text{-}348bp}$ into Y187 competent cells, we found that the plasmid intensely inhibited yeast strain Y187 and no visible colony appeared on SD/-Trp/X-α-Gal plates under optimized transformation and recovery conditions, indicating that the full-length of C35 protein has a strong toxicity effect on the yeast strain and potential unknown significant roles on yeast cells. On the contrary, we found that the two C35 truncations ($C35_{1\text{-}153bp}$ and $C35_{154\text{-}348bp}$) eliminated the toxicity effects completely. The recombinant baits pGBKT7-$C35_{1\text{-}153bp}$ contains the ITAM domain and the pGBKT7-$C35_{154\text{-}348bp}$ contains the C-terminal "CVIL" motif, with no observed auto-activation or toxicity effect, and thus could be used for following the screening process in order to figure out their corresponding interaction factors respectively. At the same time, we also constructed the cDNA library of T47D cell lines in which with the *C35* gene insert is over-expressed. The size distribution of T47D ds cDNA in the present study was homogeneous and the amount of independent clones on SD/-Leu was sufficient enough for generating library cells with satisfactory density and high titer. Altogether, our study laid a promising foundation for screening the C35 interaction factors in BC cell lines and exploring the accurate mechanisms of C35 in regulating cancer metastasis and invasion.

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