Construction of Human Tongue Squamous Cell Carcinoma Cell cDNA Library by *in vivo* Recombination in AH109

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Objective: *To construct a cDNA library of human tongue squamous cell carcinoma cell line (Tca8113) for further study of protein-protein interaction in oral squamous cell carcinoma cells for exploring the mechanisms of carcinogenesis.*
Methods: *Total RNA was extracted and reverse-transcribed into cDNA, followed by long distance PCR. The product was co-transformed into the competent AH109 with Sma I-linearised pGADT7-Rec. After growing on SD/-Leu plates, these transformants were harvested and the quality of the constructed library was analysed.*
Results: *The transformation efficiency was 1×10^6 transformants/3 μg pGADT7-Rec. The library size was 4×10^7 cfu/ml. The inserts were from 500 bp to 3 kb. With the antibody of HA-tag of pGADT7-Rec, the corresponding fusion proteins expressing in AH109 were detected.*
Conclusion: *A two-hybrid cDNA library from Tca8113 has been constructed. Analysis of the quality of this two-hybrid cDNA library indicated that this library would be useful for identifying protein–protein interactions in oral squamous cell carcinoma.*
Key words: *cDNA library, squamous cell carcinoma cell line, yeast two-hybrid*

Squamous cell carcinoma of the head and neck (HNSCC) is a major public problem. It is estimated that there are more than 500,000 new cases worldwide every year and two-thirds of patients present with locally advanced lesions and/or regional lymph node involvement[1].

Due to our limited understanding of oral cancer aetiology and lack of effective therapeutic targets, the patients' five-year-survival rate is at present only about 65%. In this regard, identification of those proteins closely involved in the progression of HNSCC is urgent, and will finally result in the discovery of promising therapeutic targets. The yeast two-hybrid system is a powerful molecular genetic approach for studying protein– protein interactions and regulatory pathways, which play pivotal roles in physiological status. Using this method, some special therapeutic targets have already been identified and these have greatly facilitated our understanding of carcinogenesis[2-10].

Compared with other cancers, some special biological behaviours exist in oral cancer cells. To apply this

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technology in identifying the potential therapeutic targets in oral cancer, a two-hybrid cDNA library that covers the entire transcripts of oral cancer is required. In this study, the established human tongue squamous cell carcinoma (Tca8113) cells were chosen to construct a yeast two-hybrid cDNA library of oral squamous cell carcinoma, and the high quality of this library was demonstrated by randomly sequencing seven clones.

Materials and Methods

Cell culture

Tca8113 cell, a human tongue squamous cell carcinoma cell line, was established in Affiliated Ninth People's Hospital, Shanghai Jiao Tong University, and maintained in RPMI 1640 10% foetal bovine serum supplemented with 1 mmol/l L-glutamine, 100 U/ml of penicillin plus 100 μg/ml of streptomycin at 37 ºC under 5% CO_2.

RNA isolation

Total RNA was isolated from Tca8113 cells by Trizol (Invitrogen), and purified with Mini-Rneasy Isolation Kit (Qiagen). The quality of total RNA was examined on a denaturing formaldehyde agarose gel. mRNA was further isolated with mRNA purified kit (Qiagen).

Synthesis of first-strand cDNA

First-strand cDNA was synthesised with MMLV Reverse Transcriptase with a modified oligo (dT) primer (CDS III/primer: 5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)30VN-3'). When MMLV reverse transcriptase encountered a 5'-terminus on the template, the enzyme's terminal transferase activity added a few additional nucleotides, primarily deoxycytidine, to the 3'end of the cDNA. The SMART III oligonucleotide, which had an oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, created an extended template. Reverse transcriptase then switched templates and continued replicating to the end of the oligonucleotide. The resulting first-strand cDNA contained SMART III and CDS III sequences on both sides.

Long distance PCR

Two 100 μl PCR reactions were set up with Advantage 2 PCR Kit (Clontech) with 3' primer (5'-GTA TCG ATG CCC ACC CTC TAG AGG CCG AGG CGG CCG ACA-3') and 5' primer (5'-TTC CAC CCA AGC AGT GGT ATC AAC GCA GAG TGG-3'). With 0.318 μg Poly A+ RNA used in the first-strand synthesis, 22 cycles were used to decrease the nonspecific PCR products. When the cycling was completed, a 7 μl aliquot of the PCR products was analysed alongside 0.25 μg of a 1 kb DNA size marker on a 1% agarose gel. A CHROMA SPIN + TE-400 Column (Qiagen) was used to remove those DNA molecules less than 200 bp. The products were then condensed into 20 μl of deionised H_2O.

Transformation of yeast strain AH109

Fresh competent yeast cells were prepared in the standard manner. Double strand cDNA (20 μl), 6 μl pGADT7-Rec (Sma I-linearised) and 20 μl Herring Testes Carrier DNA (denatured) was combined and transformed into competent AH109. The transformants were selected on SD/-Leu plates, and incubated upside down at 30 ºC until colonies appeared. To check the transformation efficiency, a small aliquot of the transformation mixture was removed and diluted by 1,000 fold, and then 150 μl of this dilution was spread on a 150 mm SD/-Leu plate. The number of colonies growing on the dilution plate was counted: colonies × 1,000 = transformants/3 μg pGADT7-Rec.

Harvest of transformants

The plates were chilled at 4 ºC for 4 hr; 5 ml of freezing medium was added to each plate. The colonies were carefully scraped into the liquid and combined together. One ml of aliquot was stored at -80 ºC; 100 μl of 1:100, 1:1,000, and 1:10,000 dilutions were spread on 100 mm SD/-Leu plates, and incubated at 30ºC until colonies appeared. The colonies (cfu) were counted and the size of this library was calculated.

Quality analysis

In order to verify the quality of the newly constructed two hybrid cDNA library, 7 random colonies were retrieved and seeded into 10 ml of SD/-Leu liquid, and incubated overnight at 30 ºC. The liquid was collected, treated with lyticase, and the transformed plasmid was extracted with phenol/chloroform. The inserts were amplified with 3' primer (GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG ATT) and 5' primer (CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA). The products were run on 1% agarose gel, and sequenced with the primer 5'-CTA TTC GAT GAA GAT ACC CCA CCA AAC CCA-3'.

Western blot

Since a HA epitope tag existed in pGADT7-Rec, with the polyclonal antibody (anti-HA, Clontech), the protein products were examined with Western blot. Seven single colonies were inoculated into 15 ml SD/-Leu liquid medium. At an optical density (A_{600}) around 0.7, the culture was centrifuged at 2,500 rpm for 5 min, the pellet was washed in distilled water and boiled for 3 min in

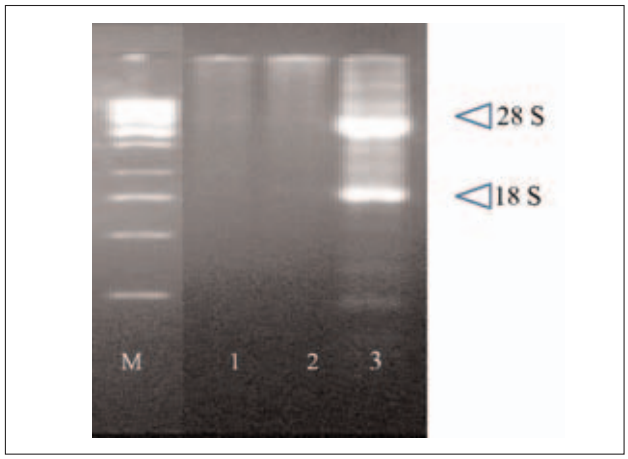

Fig 1 Total RNA and mRNA extracted from Tca8113 cells. Lanes 1 and 2 were mRNA purified from total RNA, and appear as a smear with faint 28S and 18S rRNA. Lane 3 was total RNA extracted from Tca8113, appear as two bright band (28S and 18S rRNA, indicated by arrows).

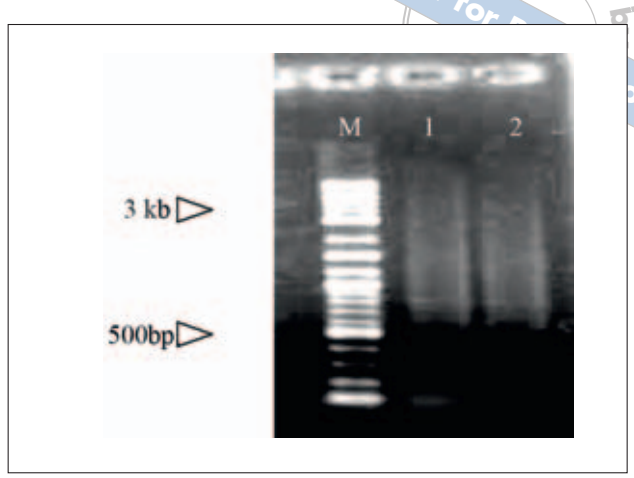

Fig 2 Result of long distance-PCR. The double strand cDNA was analysed on a 1% agarose gel after column purification. Lanes 1 and 2 show the length of PCR product was from 500 bp to approximately 3 kb. Lane M is the 1 kb marker.

200 µl of 2 × SDS loading buffer. Of this sample, 50 µl was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Amersham Life Sciences). Detection of the expressed fusion proteins was performed with anti-HA and peroxidase-conjugated anti-rabbit antibody using ECL™ (enhanced chemiluminescence, Amersham Life Sciences).

Results

Total RNA appeared as two bright bands, with the ratio of intensities of the 28S and 18S rRNA bands 1.5–2.5:1. mRNA obtained from the total RNA was 3.2 µg, appeared as a smear with faint 28S and 18S rRNA bands (Fig 1).

According to the amount of RNA (0.318 µg) used in first-strand synthesis, the thermal cycle was optimised to 22 cycles. The products appeared as a moderately strong smear from 100 bp to 5 kb (Fig 2). To avoid nonspecific PCR products, no more cycles were taken. CHROMA SPIN+TE-400 Columns were used to remove DNA molecules smaller than 200 bp, to maintain the library quality.

The competent AH109 was prepared and transformed with double strand cDNA, sma I-linearised pGADT7-Rec, and herring testes carrier DNA. Exploiting the SMARTIII and CDSIII sequences-incorporated into the double strand cDNA by reverse transcriptase and long distance PCR, the double strand cDNA was recombined with pGADT7-Rec *in vivo* to yield a complete GAL4 AD expression vector (Fig 3). The transformation efficiency was 1×10^6 transformants/3 µg pGADT7-Rec, and the library size was 4×10^7 cfu/ml.

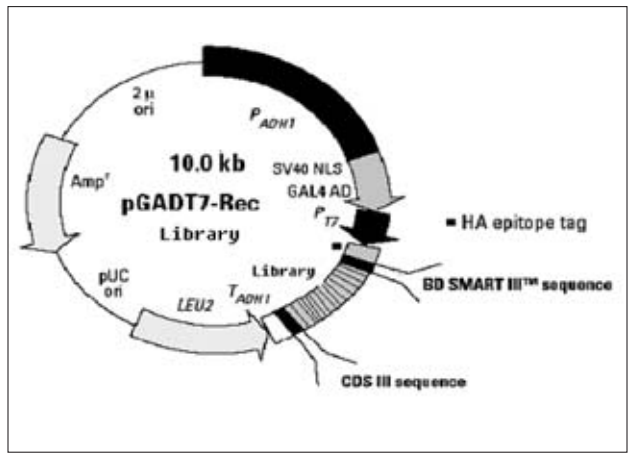

Fig 3 Scheme of pGADT7-Rec-Library. A GAL4 AD fusion library was produced by co-transforming yeast with SMART double strand cDNA and pGADT7-Rec. SMART double strand cDNA recombined with pGADT7-Rec in vivo (between CDSIII sequence and SMWRT III sequence) to yield a complete GAL4 AD expression vector, that was pGADT7-Rec-Library.

Seven colonies were randomly picked, and the corresponding pGADT7-Rec-Library plasmids and the encoding proteins were extracted and tested. The PCR results showed the size of inserts was from 500 bp to 3 kb,

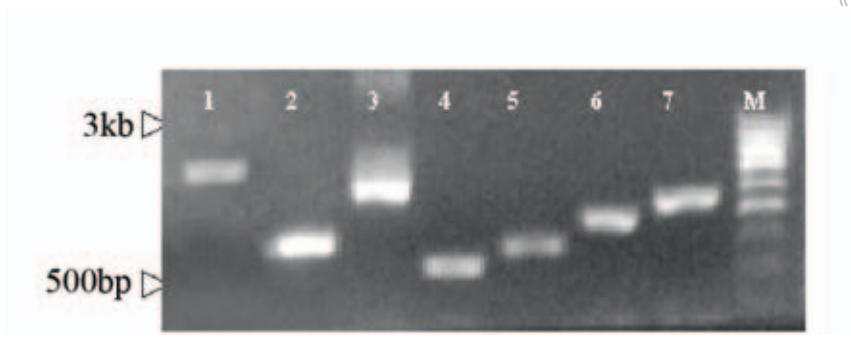

Fig 4 Quality Analysis with PCR. In order to verify the quality of the newly constructed two-hybrid cDNA library, we retrieved plasmid from 7 randomly chosen colonies. Standard PCR was performed to test the insert fragments, whose sizes varied from 500 bp to 3 kb (Lane 1 to Lane 7).

Fig 5 Western blot. Total protein was extracted from 7 randomly chosen colonies and examined with the anti-HA antibody. As the results show, different protein was synthesised in these 7 colonies (Lane 1 to Lane 7).

while most were approximately 1 kb (Fig 4). The sequencing results showed that these inserts were 99–100% homologous to AC 025164.37, AL353578.18, AF078845, BC065293.1, BC072683.1, AP003392.2, and AK096787.1. The corresponding fusion proteins were all detected with the HA-tag (Fig 5).

Discussion

In the present study, a two-hybrid cDNA library was constructed from Tca8113. The quality was analysed, and indicated that the cDNA library could be used for identifying protein–protein interactions in oral squamous cell carcinoma. To screen a mammalian cDNA library until saturation, more than 5–10 × 10^6 yeast transformants needed to be screened[11,12]. Our library size was approximately 4 × 10^7 and the sequencing and Western blot results of the seven clones assured the library for the next screening application.

Protein–protein interactions are intrinsic to virtually every cellular process[13] and make up biological machines that are like intricate three-dimensional jigsaw puzzles, forming arrays of interlocking protein components that assemble and disassemble over time and in response to complex signals. Our primary goal was to

identify the interacting proteins that might significantly be involved in the malignant oral epithelium, and possibly causal in the development of HNSCC with this cDNA library. Approximately 10% of the total number of genes are suspected to be expressed in a given cell type and maybe directly related to disease, including cancer[14]. Determining their identity and interactions is an important first step towards understanding the patterns of gene expression that mediate normal cellular physiology and disease process, and will lead to global genomics information for further studies[15]. Yeast two-hybrid technology is one of the top choices for large-scale analysis of protein–protein interaction[16]. To carry out the screening procedure, a library prepared from a special resource in which the target protein was known to be biologically relevant was necessary. According to our limited knowledge, there was no commercially available two-hybrid cDNA library of oral cancer. In this study, a yeast two-hybrid library of Tca8113 was constructed with an *in vivo* homologous recombination-mediated approach in the GAL4 activation domain-based vector, pGADT7-Rec. This would be suitable for following detecting protein–protein interactions in oral squamous cell carcinoma in a high-throughput manner. Previous studies have reported on libraries constructed from some types of squamous epithelium. However, these libraries often used a bacterial host, where not all post-translational modifications needed for the protein interaction might occur, and could not be efficiently used in detection of protein interactions. Traditionally, the tools available to analyse protein–protein interactions had been restricted to biochemical approaches or some physical methods, such as protein affinity chromatograph, affinity blotting, immunoprecipitation and cross-linking[17]. During these analyses, all combinations of protein–protein interactions assayed, including those that might never occur *in vivo*, so the possibility of identifying artifactual partners existed and was a typical disadvantage of most exhaustive screening procedures.

Yeast two-hybrid technology, since developed by Fields and Song[18] in 1989, has been modified greatly for a rapid and high-throughput screening procedure[16]. It provided an easy and rapid *in vivo* binding assay in eukaryotic cells; the genes encoding the interacting partners are readily available; it embodied an *in vitro* technique using the yeast host cell as a live test tube[19]. This yeast system brings the higher eukaryotic reality closer than most *in vitro* approaches or techniques based on bacterial expression. In general, in any two-hybrid experiment a protein of interest is fused to a DNA-binding domain and transfected in a yeast host cell bearing a reporter gene controlling this DNA-binding domain. When this fusion protein cannot activate transcription on its own, it can be used as 'bait' or as a 'target' to screen a library of cDNA clones that are fused to an activation domain. The cDNA clones within the library that encode proteins capable of forming protein–protein interactions with the bait are identified by virtue of their ability to cause activation of the reporter gene. After extracting the corresponding AD-library plasmid form the positive colonies, and sequencing the inserts, the interacting protein is identified. So the yeast two-hybrid is devised to identify genes encoding proteins that are physically associated with a given protein *in vivo*.

An appealing feature of the MatchMaker library construction method we chose was the minimal requirement for starting materials, as little as 25 ng mRNA or 100 ng total RNA. SMART III and CDS III sequences were incorporated into double strand cDNA using Clontech's SMART technology, which was particularly well suited for two-hybrid library construction because it consistently delivered high yields of cDNA while maintaining a high complexity and good representation of low abundance cDNA. The pGADT7-Rec vectors contained T7 promoters and HA epitope tags, which would be convenient for the following confirmation[20]. After the long distance PCR, the fragments smaller than 200 bp were removed with TE-400 column, as it would be preferable to have long inserts in the library. With this expression library, we could acquire the global information of the protein–protein interactions in a two-hybrid library. If any valuable interactions were identified, the corresponding inserts could be easily subcloned in later stages for additional interaction controls and analyses outside the two-hybrid system.

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