Differential Display and Cloning of Messenger RNAs from Human Breast Cancer versus Mammary Epithelial Cells

Peng Liang, Lidia Averboukh, Khandan Keyomarsi, Ruth Sager, and Arthur B. Pardee

Materials and Methods

Cell Culture. Both normal (76N) and tumor (21MT-2) cells were cultured in D medium (6) until reaching about 70% confluency. Their polyadenylated RNAs were extracted using the Quickprep mRNA purification kit from Pharmacia-LKB Biochemical Co.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PCR, polymerase chain reaction; cDNA, complementary DNA; poly(A), polyadenylate.

Differential Display of mRNA. Five-tenths µg of the purified polyadenylated RNA was reverse transcribed using either T\textsubscript{1}CA (5'-TTTTTTTTTTTTTTTC-3') or T\textsubscript{y}GC (5'-TTTTTTTTTTTTTGGC-3') and subsequently amplified by PCR using 3 different arbitrary primers; Ldd1 (5'-CTGATCCATG-3'), Ltk3 (5'-CTTGGATTCGC-3') and Ldd3 (5'-CTGCTCTCA-3'), essentially as described (5), except that the annealing temperature was 40°C instead of 42°C. The recovery and reamplification of cDNA fragments from a dried DNA sequencing gel were described previously (5).

Cloning and DNA Sequencing. The cDNA fragments S1, S2, and M1 (refer to Ref. 5, Fig. 3.) were PCR reamplified and cloned into plasmid PCR1000 using the TA cloning kit from Invitrogen, Inc. Both strands of the cDNA fragments were sequenced with the Sequenase kit from USB Biochemicals Co. using M13-40 and T7 promoter primers. The S1 cDNA fragment was purified by Geneclean (Biol01) as a BamHI-HindIII fragment from the plasmid and labeled with [α-32P]dCTP (NEN) using a random Prime DNA labeling kit from Boehringer Mannheim Biochemicals. Independent plaques (106) were screened from a 76N cDNA library constructed with Stratagene's ZAP-cDNA Synthesis kit.

Total Cellular RNA Isolation and Northern Blot Analysis. These procedures were carried out essentially as described previously (7). The cDNA probe of S1 isolated from the cDNA library was purified as a BamHI-XhoI fragment from pBluescript SK and labeled by [32P]dCTP as described above.

Results and Discussion

The ability to culture normal and tumor-derived mammary epithelial cells under identical conditions was essential and provides an excellent system for comparative studies of this disease (6). mRNA populations from a normal (76N) and a metastatic breast cancer cell line (21MT-2) (8) were compared by the differential display technique. Six different combinations of primer sets made of 2 anchored oligodeoxynucleotidyldate primers and 3 short arbitrary primers were used to obtain differential displays (see Ref. 5, Fig. 3). Patterns of amplified cDNA species between normal and tumor cells were largely identical, providing a reproducible uniform background over which specific differences could be observed. Some bands like S1 and S2 were seen only in the normal cell display while others like M1 were present only in the display of metastatic breast cancer cell. The differential display method, unlike subtractive hybridization, simultaneously identifies both groups of selectively expressed genes.

These differentially expressed bands S1, S2, and M1 were chosen for further characterization. They were recovered from the dried denaturing polyacrylamide gel and reamplified using the corresponding primer sets (Fig. 1A). The cDNA products are consistent in size with the bands in the original display gel. They were cloned into the pCR1000 plasmid vector. Then their nucleotide sequences were determined. They were all flanked by the sequences of the primer set used (Fig. 1B). Moreover, they all appear to be AT rich, some even with a putative

Identification of the genes that are specifically expressed in tumor cells but not in normal cells (oncogenes), or vice versa (tumor suppressor genes), is important for understanding the molecular basis of cancer. The differential display technique was applied to compare mRNAs from normal and tumor-derived human mammary epithelial cells, cultured under the same conditions. Complementary DNA fragments corresponding to several apparently differentially expressed mRNAs were recovered and sequenced. They exhibit characteristics of the 3' end of eukaryotic mRNA, as predicted by the method. A complementary DNA fragment seen only in the normal cell was used as a probe to isolate its corresponding complementary DNA clone from a library. Northern analysis confirmed its differential expression. Thus, this method can be used for detecting, cloning, and sequencing of genes that are unique to a host of biological and disease processes.

Introduction

Cancer is a result of cumulative multiple genetic aberrations, resulting in both activation of oncogenes, which override cellular growth regulatory commands, and inactivation of tumor suppressor genes, which render cells free of growth restraints (1, 2). It is the differential expression of these critical genes and subsets of genes which are governed by them that enables a cell to grow out of control and to become cancerous. A pressing problem is, therefore, to identify and characterize as many of these genes as possible in order to understand the nature of the disease and to devise rational therapies. Subtractive hybridization has been used for this purpose and several important genes implicated in tumorigenesis have been isolated (3, 4). However, this method gives incomplete recovery and selects only for either under- or overexpressed genes, and screening is laborious. To speed up these gene hunts, a method was developed that we call differential display (5). This method involves the reverse transcription of the mRNAs followed by the PCR reaction. The amplified cDNA subpopulations of 3' termini of mRNAs are distributed on a DNA sequencing gel. Here this method is applied to identify and then isolate a small subset of genes that are differentially expressed in normal versus tumor mammary human epithelial cells.
polyadenylation signal (9) located close upstream of the sequences complementary to the anchored oligodeoxythymidy- to be present only in either normal or cancer cells (see Fig. 3 of ref. 5) were partially expressed cDNA fragments. A, three bands, S1, S2 and M1 that appeared late primers, suggesting that these cDNA fragments correspond to the putative poly(A) tail, as in most eukaryotic mRNAs. This allows them to amplify a significantly greater number of mRNA species than theoretical (5).

These fragments can also be used as probes to isolate their original target cDNA (AAGCTCCATG) and the original target cDNA (AAGCTCCATG). This finding supports our earlier prediction that under the PCR conditions used 10-mers not only have a high enough melting temperature for efficient priming but also can exhibit some degree of degeneracy. This allows them to amplify a significantly greater number of mRNA species than theoretical (5).

Using a S1 cDNA clone as probe, Northern analysis confirmed its differential expression and showed that indeed the full length S1 mRNA is about 4 kilobases, much larger than any S1 cDNA clone isolated (Fig. 3). Further efforts will be required to isolate the full length cDNA. Moreover, Northern blot analysis of S1 using other different breast cancer cell lines showed that only 3 of 14 had lost the S1 gene expression. Characterization of this gene may provide clues as to whether it functions in providing normal growth control in these small subset of breast cancer cells. This raises an important question as to whether a gene identified by any method to be differentially expressed is a normal growth factor or a cancer gene.

Fig. 2. Nucleotide sequence of the S1 cDNA clone and position of the S1 polyadenylation sites (locations indicated at by lower case letters) or in the number of Ts found at the 5' end. The location of the S1 cDNA fragment sequence relative to the original cDNA clone is underlined, with mismatches in bold lower case letters.
mRNAs IN BREAST CANCER AND MAMMARY EPITHELIAL CELLS

In conclusion, this brief report shows the possibility of cDNA cloning of a differentially expressed gene by the differential display technique. Nucleotide sequence comparison between the cDNA probe identified by differential display method and its partial cDNA clone isolated from a cDNA library revealed mismatches at the 5' end of the arbitrary primer. The sequence of the probe was shown to be located right at the beginning of the poly(A) tail of the corresponding mRNA as predicted by the method. These results taken together further validate the differential display technique. Because of its simplicity, sensitivity, and reproducibility, this powerful method should speed up the gene hunt in the broad field of biological research.

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Fig. 3. Northern blot analysis of S1 mRNA. 20 μg per lane of each total RNA was analyzed. Lane 1, normal cell 76N; Lane 2, breast cancer cell 21MT-2. As a RNA loading control, the same blot was reprobed with 36B4 cDNA (12).

expressed by comparing only a pair of cells can be extended to be a general phenomenon for a specific biological process such as breast cancer. This often must be verified after isolation of the gene. Conventional methods such as subtractive hybridization will often fall into this pitfall because only two mRNA populations can be compared at a time. The differential display method that we developed circumvents this problem by comparing more than two RNA samples simultaneously and thus is able to identify differentially expressed genes which are unique for a process instead of just a particular cell line.4

It is worth noting that cDNA probes made from cloned PCR amplified cDNA fragments like S1 and M1 may fail directly to detect any mRNA on a Northern blot. Possibly these were poor probes because they are short and AT rich, and their mRNAs are of low abundance. Thus, isolation of a longer cDNA clone as a probe may be necessary for Northern blot confirmation.

4 P. Liang, unpublished data.

References

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