ESTABLISHMENT OF DIFFERENTIAL DISPLAY mRNA AND ITS APPLICATION IN THE STUDY ON THE MECHANISM OF LUNG CANCER INDUCED BY RADIATION

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ABSTRACT

Objective: A method for separating mRNAs by means of the polymerase chain reaction (differential display mRNA), and identifying the genes related to radiation-induced lung cancer was introduced. Methods: The RNAs were isolated from two pairs of samples, SV40-immortalized human fetal tracheal fibroblast cell (SHTF) versus αSHTF cell (transformed SHTF cell induced by α particles) and lung cancer tissue versus normal lung tissue obtained from one miner, and amplified by RTPCR. The differential expressed gene fragments were displayed by auto-radiograph or silver nitrate stain. Results: The differential display mRNA method was established using both cell and tissue samples. The bands stained by silver nitrate were clearer than those on X-ray film. The rate of reamplification of differentially expressed gene fragments stained by silver nitrate is 80%, higher than that by autoradiograph, 50%. Conclusion: Differential display mRNA method was established successfully on both cell and tissue samples. The modified method for staining band increased the rate of reamplification and established the basis for confirming relative genes.

Key words: Differential display mRNA, Autoradiograph, Silver nitrate stain, Radiation induced cancer.

Differential display mRNA was developed as a method to identify and analyze altered gene expression at the mRNA level in any eukaryotic cell.[1] Regarding the molecular mechanism of radiation-associated cancer, the alteration and regulation of well known oncogenes and suppresser genes, such as ras, myc, p53 and Rb have been investigated extensively.[2] We used and modified the differential display mRNA to study the genes related to lung cancer induced by radiation.

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MATERIALS AND METHODS

Cell and Tissue Sample RNA Isolation

The cells used in the experiment were SHTF cells (SV40-immortalized human fetal tracheal fibroblast) and the αSHTF cells (SHTF cells forming clone on agar after irradiated by α particles emitted by ^{238}Pu). The tissues, a pair of surgical samples, lung cancer tissues (diagnosed as adenocarcinoma in the clinical) and the normal lung tissues, were taken from one patient who was a miner at Gejiu Tin Corporation, Yunnan, China. Total RNA was extracted by Trizol method.

Primers

The primers were purchased from Gene Hunter Company. The sequences of 3' anchored primers named HT11M (G, A, C) are: HT11G: 5'-AAGCTTTTTTTT-TTTM(G, A, C)-3'.

5' arbitrary primers used in SHTF and αSHTF cell are HAP17–24. The sequences are:

HAP17: 5'-AAGCTTACCAGGT-3'
HAP18: 5'-AAGCTTAGAGGCA-3'
HAP19: 5'-AAGCTTATCGCTC-3'
HAP20: 5'-AAGCTTGTTGTGC-3'
HAP21: 5'-AAGCTTTCTCTGG-3'
HAP22: 5'-AAGCTTTTGATCC-3'
HAP23: 5'-AAGCTTGGCTATG-3'
HAP24: 5'-AAGCTTCACTAGC-3'

5' arbitrary primers used in lung cancer tissues versus normal lung tissues are HAP1–8. The sequences are:

HAP1: 5'-AAGCTTGATTGCC-3'
HAP2: 5'-AAGCTTCGACTGT-3'
HAP3: 5'-AAGCTTTGGTCAG-3'
HAP4: 5'-AAGCTTCTCAACG-3'
HAP5: 5'-AAGCTTAGTAGGC-3'
HAP6: 5'-AAGCTTGCACCAT-3'

HAP7: 5'-AAGCTTAACGAGG-3'
HAP8: 5'-AAGCTTTTACCGC-3'
The upstream and downstream primers were combined randomly.

Differential Display mRNA

The differential display mRNA used in SHTF cells and αSHTF cells was the autoradiograph method.[3] The method of revised silver nitrate staining was used in the samples of lung tissues. The main method was as follows: The total RNA of tissue was isolated and the 1st strands of cDNA were composed by MMLV reverse transcriptase with 3' primer HT11M. The reaction system was: 2.0 μl of 2 μmol/L 3' primer HT11M, 0.2 μg total RNA, 1.6 μl of 250 μmol/L dNTP in volume of 20 μl for denaturation 5 min at 65°C, and the incubation for 10 min at 37°C was followed, then 100 uMMLV reverse transcriptase was added for 5 min at 37°C and 5 min at 75°C. Three independent reactions, using HT11M (G, A, C) as primers, were performed. The cDNA were then amplified by PCR. The α-^{32}P-dATP (1.1 GBq/mol) was only used for the autoradiography method. The PCR reaction mixtures (20 μl) included 2.0 μl of resultant cDNA, 2.0 μl of 10 × PCR buffer, 1.6 μl of dNTP (25 μmol/L), 2.0 μl of HAP primer (2.0 μmol/L), 2.0 μl of HT11M primer (2.0 μmol/L), 0.2 μl of AmpTaq, and dH_2O 10.2 μl. The samples were subjected to 40 cycles of PCR using the following parameters: 94°C for 30 s, 42°C for 2 min, and 72°C for 30 s; the last cycle was followed by a 5 min extension at 72°C.

The PCR products were fractionated by electrophoresis in denaturing 6% polyacrylamide gels. A gel temperature indicator was adhered to monitor the gel temperature during electrophoresis. At the end of electrophoresis, the gel were stained with silver nitrate staining solution or subjected to autoradiography (exposure time 24 h, -20°C) using an X-ray film.

The differentially expressed gene fragments were excised from denaturing 6% polyacrylamide gels, soaked in 100 μl dH_2O for 10 min, then boiled for 15 min. After centrifugation for 2 min at 12000 g to pellet any solid debris, the supernatant was removed to another tube. The cDNA was recovered by ethanol precipitation in the presence of 10 μl of 3 mol/L sodium acetate for overnight at –80°C, centrifugalized by spinning for 10 min at 4°C, 10,000 rpm/min, washed with 85% ethanol, dissolved in 10 μl of dH_2O, kept at –20°C. Reamplification was done using the same primer set and PCR conditions except that the dNTP concentration at 20 μmol/L instead of 2 μmol/L, and 4.0 μl of the cDNA instead of 2.0 μl.

RESULTS AND DISCUSSION

The banding autoradiographys of denaturing polyacrylamide electrophoresis for the radiolabeled cDNA products amplified from total RNA of SHTF and αSHTF cells by RTPCR using the HT11M/HAP17–24 primer set are showed in Figure 1a. Meanwhile the silver nitrate staining banding maps of PCR amplified products of cDNA 1st strands, which were reverse transcribed from the total RNA of lung cancer tissues and normal lung tissues with the action of HT11M primers paired by HAP1–8 primers, are showed in Figure 1b. Comparison of Figure 1a and 1b showed that there are about 70 bands, not regular, and some contacted closely to each other for over- radioactivity in the autoradiography method. There were 23 cDNA fragments expressed intensively in αSHTF cells only and not in SHTF cells. In the silver nitrate stain, there were 100–200 bands, thin, regular and clear intervals between two bands on a polyacrylamide gel. Thirty cDNA fragments that were differentially expressed were ascertained directly from the gel. Among these fragments, 16 were expressed in lung cancer tissues (C) and not in normal ones; 14 were expressed on the contrary (N). Twelve differentially expressed cDNA bands selected by autoradiography method were reamplified using the same conditions as the first PCR. Only 6 fragments were recovered. The rate of reamplification was 50%. Two of six cDNA, C23–1 and C17–5 were the special bands in all PCR. This may be related to the operational error in the autoradiography method. In the silver nitrate stain method, differentially expressed cDNA fragments could be recovered under the condition of staining which resulted in raising the specificity and accuracy of the recovered fragment and avoiding the pollution of the isotope. All 30 cDNA fragments differentially expressed in lung cancer tissues or normal lung tissues were reamplified. 24 of them were reamplified successfully with 80% recovery rate. Some longer DNA fragments were cloned, sequenced and showed 3', 5' primers in all DNA fragments.

The size of amplified cDNA bands from two methods was different. A shift was observed that the size of amplified cDNA (up to 500 –600 bp) in silver nitrate stain was longer than that of in autoradiography (less than 400 bp). There may be two reasons. One is that the dNTP labeled by some kind of isotope is poorly incorporated by Taq DNA polymerase[4] and the other is that related to the gel temperature at the time of polyacrylamide gel electrophoresis. In the silver nitrate stain method, the size of amplified cDNA bands could be up to 500–800 bp after electrophoresis for 3 h at 55°C of gel temperature. For example, using the 5' primers in combination with the 3' primer HT11C, two differentially expressed gene fragments were recognized respectively at 550 and 700 bp when the gel temperature reached 55°C at electrophoresis. But when the gel temperature was at 50°C, the length of fragments was usually below 300 bp after electrophoresis for 3 h. For example, using the 5' primers in combination with the 3' primer HT11G, the PCR products were 136 bp of CG2, 91 bp of CG5 and 270 bp of CG3. In the autoradiography, the dNTP labeled by isotopes is incorporated poorly by

Taq DNA polymerse, and the longest amplified cDNAs were only 300 bp or so. When the gel temperatures rise, the PCR products run more rapidly in the gel, but the head of gel appeared blank after electrophoresis for 3 h, and the size of amplified cDNA was not changed.

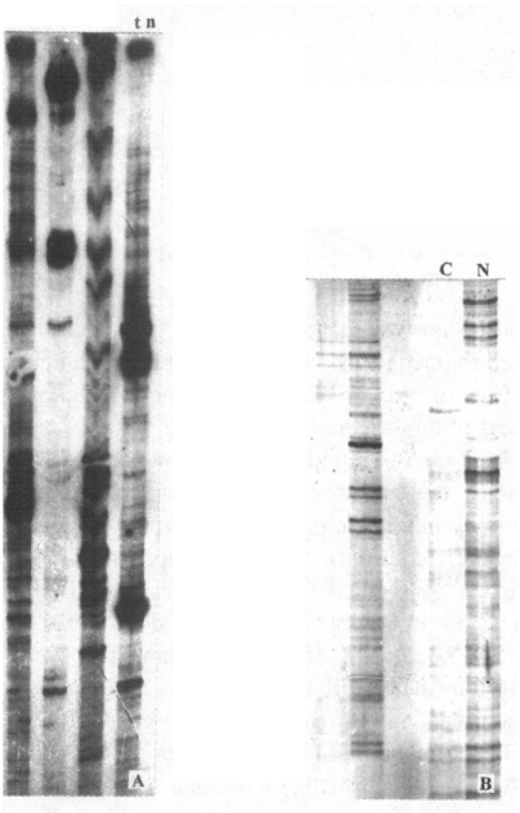

Fig. 1. Comparison of isotopic and non-isotopic differential display

A: αSHTF cells (t) versus SHTF cells (n)

B: lung cancer tissue (C) versus normal lung tissue (N)

Differential display mRNA, developed in the 1990s, is a powerful new tool for identifying and cloning differentially expressed genes. It has several technical advantages over the subtractive hybridization method, such as simplicity, high sensitivity, speed, and reproducibility. A lot of articles about differential display mRNA employed in the study on the mechanism of carcinogenesis were reported; most of them were used to compare differentially expressed genes of a pair of related cells, such as, normal cells versus metastatic human breast cancer cells,[5] or human prostate carcinoma cells.[6] Only a few reports were involved to the study of differential expressed genes in cancer tissues. Huang et al.[7] first searched for genes related to hepato-carcinogenesis with this technique. 20 paired tumor and non-tumor liver RNA samples were selected for this study. We studied differentially expressed genes of SHTF cells and α SHTF cells with differential display method which was developed by Liang P. The method was modified and improved by replacing radio-label and autoradiograph with silver nitrate staining and employed to study the differentially expressed genes of tin miner's lung cancer tissues versus normal lung tissue. The result showed that using the differential display mRNA for lung cancer tissues and normal lung tissues taken from one miner, the expression of most genes was the same; The number of differentially expressed gene fragments was similar to that of the SHTF cells versus α SHTF cells, between 20–40. The results described here demonstrated the suitability of mRNA differential display method to analyze differential gene expression in cancer and normal tissues from one sample.

In summary, the differential display of mRNA was established on both cell and tissue samples. The differentially expressed gene fragments between lung cancer tissues and normal lung tissues taken from one patient sample were studied. The rate of reamplification of differentially expressed genes fragments stained by silver nitrated was 80%, higher than that of autoradiography, 50%.

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