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Detection of K-ras Gene Point Mutation's Style in Human Pancreatic Cancer Cell Line PANC-1 by PCR-SSP

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Abstract ***Objective:*** To detect the style of K-ras gene point mutation in human pancreatic cancer cell line PANC-1 and decide the bp sequence of Ras target position interfered by RNA. ***Methods:*** Three kinds of special sequence primers (SSP) for polymerase chain reaction (PCR) with regard to the mutation styles (GAT, CGT and GGT) at codon 12 of K-ras were used to study the human pancreatic cancer cell line PANC-1. The amplification products were studied with polyacrylamine gel electrophoresis to detect the style of point mutation. ***Results:*** The style of K-ras gene point mutation at codon 12 was GAT in human pancreatic cancer cell line. ***Conclusion:*** PCR-SSP is rapid, convenient and high specific. The results provide a basis for further gene therapy by RNA interference for pancreatic cancer.

Key words: pancreatic cancer; K-ras gene; point mutation; polymerase chain reaction

Pancreatic cancer is the fourth leading cancer of cancer death because of its extremely poor prognosis[1]. An early diagnosis of pancreatic cancer is rare, hence, the rate of cure of this disease is less than 10%. Furthermore, surgical therapy for pancreatic cancer is frequently not curative, most often as a consequence of this tumor's propensity to metastasize. Only a minority of cases is the diagnosis made at a very early stage, when curative surgery might significantly ameliorate the 5-year survive rate[2, 3]. Therefore, it is extremely important to have early detection of pancreatic cancer, possibly based on molecular markers rather than the size of the tumor. Alteration of oncogenes and tumor suppressor genes such as K-ras and p53 was highly specific for diagnosing pancreatic cancer. K-ras is involved in transducing growth-promoting signals. Point mutations of K-ras have been found in 80%–100% of pancreatic cancer, suggesting that mutant K-ras is a sensitive marker for detection of pancreatic cancer[4]. Further, K-ras mutations occur almost exclusively in three hot spots (codes 12, 13 and 61), most of them are concentrated at codon 12. Here we report the way of PCR-SSP (special sequence primer) to detect the K-ras mutation style in pancreatic cancer cell line PANC-1, approaching for the early detection of pancreatic cancer. We demonstrated that PCR-SSP could detect the point mutation of cancer cells with higher sensitivity and specificity. Our long-term goal is to develop a simple clinical procedure for early cancer detection.

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Materials and methods

Materials

Pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (CRL 1469), the gift of Professor Douglas, Cancer Research Center of Boston Medical College. The cells were grown in Dubecco's modified Eagle's medium (DMEM) complemented with 10% fetal calf serum, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed once every 3 days, and the cells were transferred by 1:3 if they had grown full.

Methods

Confluent cells growing on 75 mm dish were digested by 0.02% EDTA, then transferred to the 1.5 mL Eppendorf tube, and the cells collected were centrifugated at

1200 r/min for 10 min at 4 ℃. The supernatant was discarded and 1 mL PBS was added into the Eppendorf tube to wash. The cells were resuspended in 0.5 mL of TES solution (150 mmol/L NaCl, 10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L EDTA), added with 0.1 mL of 10% SDS to the tube. The tube was kept at room temperature for 5 min. The solution was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol and once with chloroform:isoamyl alcohol. The aqueous phase was tranferred to a fresh tube, and 1/10 volume of 3 mol/L NaAc and 2 volumes of 100% ethanol were added. After mixture well, the precipitate could be seen immediately. The tube was spinned at 10 000 r/min for 1 min and the supernatant was discarded. The pellet was dissolved in 0.1 mL of TE buffer and 5 μL of them used for amplification reaction of PCR.

The primers were designed and synthesized by BoJie Bio-Synthesis Inc., Wuhan (China). The sequences were as follows: R_1=5'-GTAGTTGGAGCTGA-3'; R_2=5'-GGTAGTTGGAGCTC-3'; R_3=5'-GTAGTTGGAGCTGT-3'; R_4=5'-TTGCTTCC TG TAGGAATCCT-3'.

The pairing of R_1–R_4 amplified GAT mutation with a 108 base pair fragment; the pairing of R_2–R_4 amplified CGT mutation with a 109 base pair fragment; the pairing of R_3–R_4 amplified GTT mutation with a 108 base pair fragment.

The PCR reactions were carried out in a final reaction volume of 50 μL containing 10×buffer 5 μL, 4dNTP 1 μL, 1 μL of each primer, 2 U of T*aq* polymerase (Gibco Inc., USA) and dH_2O 31.5 μL. A negative control was set up in every PCR amplification (not adding DNA), and the sample was performed by 3 PCR reaction systems. The PCR amplification protocol was as follows: pre-denaturing for 5 min at 95 ℃, then denaturing for 15 s at 95 ℃, annealing for 20 s at 58 ℃, extending for 30 s at 72 ℃, 30 cycles, followed by a final extension time of 6 min at 72 ℃.

Electrophoresis was performed using 8% polyacrylamine gel. 15 μL samples were loaded on the well of gel for 60 min run, with 110 V electric pressure, then stained with ethidium bromide for 20 min. The gel was observed under UV light.

Results

Detection of the codon 12 mutation in the K-ras oncogene in pancreatic cancer cell line PANC-1 was as the Fig. 1 showed: The pairing of R_1–R_4 had the amplification product of GAT mutation with a 108 base pair fragment, but the pairings of R_2–R_4 and R_3–R_4 had no amplification product of any mutation. Therefore, K-ras gene point mutation at codon 12 was found in human pancreatic cancer cell line PANC-1, and the mutation style was GAT, no other mutation styles were found.

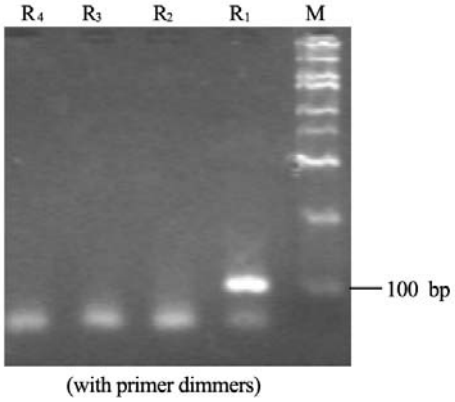

Fig. 1 Detection of the codon 12 mutation in the K-ras oncogene in pancreatic cancer cell line PANC-1 by PCR-SSP analysis. Ethidium bromide-stained polyacrylamine gel of PCR-SSP analysis showing samples of different PCR amplified products in pancreatic cancer cell line. R_4: Negative control; R_3: Negative product; R_2: Negative product; R_1: Positive product (108 bp GAT mutation); Marker (M): 100 DNA ladder

Discussion

Mutational activation of the K-ras at codon 12 has been demonstrated in 90%–100% of the cases of pancreatic cancer[5]. This very high prevalence of mutation has never been identified in other types of human tumors. Apparently, the substitution of a nucleotide at the first or second base of codon 12 may precede the development of pancreatic cancer[6]. These both suggest that point mutations in the K-ras gene might be used in future screening protocols for pancreatic cancer and it seems to be an important target for novel anti-cancer therapies.

In pancreatic cancer, K-ras mutation usually arises at codon 12, the mutation style focuses on CGT, GTT and GAT, and the mutations of TGT and AGT are rare. The ways of detecting the K-ras mutation include PCR-RFLP[7], PCR-RMCA[8], PCR-SSCP[9], PCR-SSP, PCR-DSM[10], PCR-ASO[11], Molecular Beacons[12] and so on. PCR-SSP has some superiorities compared with other ways as follows: it is rapid, convenient, specific and sensitive. The cycle of experiment is relatively simple and short, the determination of results does not need enzyme cut hybridization and autoradiography, not need expensive equipments and high level of experiment condition, the cost of finishing the protocol is cheap. Therefore, it may be an effective approach for the early diagnosis of pancreatic cancer, and may serve as a practical method for distinguishing chronic pancreatitis or pancreatic benign masses from malignant ones. The value of application may be better than the examination of cytology[13].

RAS genes are frequently mutated in human cancers, particularly in pancreatic cancer. The proteins encoded by the RAS genes (K-Ras, H-Ras, and N-Ras) are guanine nucleotide binding proteins that are associated with the inner plasma membrane and transduce external signals to the interior of the cells. They regulate a broad spectrum of cellular activities, including proliferation, differentiation, and cell survival. Mutant RAS oncogenes of-

ten contain point mutations that alter only a single amino acid, which locks the oncogenic RAS proteins in a persistently activated GTP-bound state. A complication in using RAS oncogenes as targets in anticancer therapy is that at present it is not possible to specifically inhibit only the oncogenic RAS alleles. This may be essential, since the wild-type K-ras gene appears to be required for viability, as evidenced by the embryonic lethal phenotype of mice nullizygous for K-ras[12]. Therefore, tools are required to effectively inhibit the activity of oncogenic K-ras, but not that of the wild-type K-ras protein in normal tissues.

We report here that oncogenic alleles of K-ras can be specifically and stably inactivated in human cancer through the use of RNA interference sequences, leading to loss of tumorigenicity. We are in the process of the research about the silencing K-ras gene expression by siRNA in pancreatic cancer cell line PANC-1. It is well known that the premise of suppression of K-ras point mutation is to find the mutation style in the pancreatic cancer cell line. Designing the special sequence primer of detecting the point mutation is based on the chief 3 mutation styles. The 3' terminal base of R_1, R_2, R_3 sense primer matches the wild type GGT, resulting in second G→A, first G→C, second G→T, respectively; and they have the same antisense primer R_4. If the base pair of sense and antisense primer have point mutation, the amplification product can be gotten by PCR; if not mutating, the 3' terminal base of sense primer can not match the template and PCR not amplify at higher temperature, it assures that no false positive happens. In our experiment, it was found that there was no false positive to happen when the temperature was controlled beyond 55 ℃ and false positive appeared as temperature below 52 ℃. DiGiuseppe *et al*[11] reported that the point mutation of K-ras gene in pancreatic cancer tissues was detected by PCR-SSP, but the amplified product was 88 base pair fragment as he designed the primer, which was difficult to display by electrophoresis, therefore, we designed the primer to make the amplified product 108 base pair fragment, which was easy to display by electrophoresis, make this method more convenient and useful. The result of detecting the K-ras gene point mutation in pancreatic cancer cell line PANC-1 is coincidence with the reported result through molecular beacons abroad[11]. We design and synthesize the siRNA sequences to suppress or silence the mutated base at 12 codon. We hope to conclude that K-ras gene is involved in maintenance of tumor growth of pancreatic cancer, and siRNA expression against K-ras expression is a powerful tool to dissect RAS-signaling pathways and may be used therapeutically against pancreatic cancer.

PCR-SSP is helpful to detect the point mutation of K-ras gene at codon 12. When we are not sure about the diagnosis of pancreatic cancers, it may serve as a practical method for distinguishing benign masses from malignant ones, and making an effective diagnosis of pancreatic cancer. It also is recommended to use in the research about mutation of oncogene, not only in pancreatic cancer.

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