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Rapid Isolation of Cell-Type-Specific Protein Tyrosine Kinases by Degenerate Polymerase Chain Reaction Combined with Differential Hybridization Technique

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Summary: To identify protein tyrosine kinase (PTK) genes preferentially expressed in renal cell carcinoma cell line, we screened a PTK-cDNA-enriched library constructed from RNA of an renal cell carcinoma cell line with a PTK probe, each produced from renal cell carcinoma, gastric cancer or esophageal cancer cell lines by degenerate polymerase chain reaction. Two cDNA fragments of PTK genes, *FRK* and *FLT-3*, were isolated from the PTK-cDNA-enriched library of the renal cell carcinoma cell line by differential hybridization technique. The *FRK* cDNA clone represented 15.8% of the PTK-cDNA-enriched library from the renal cell carcinoma cell line, while the *FLT-3* cDNA clone was 2.8% of the same library. Both of the two PTK genes were expressed preferentially in renal cell carcinoma cell lines. This method, described here, is useful for the rapid isolation of PTK cDNA fragments, including a low abundant cDNA, preferentially expressed in a specific cell line.

In understanding the development and progression of cancer, not only signal transduction pathways common to all cancer cells but also those specific to cell types should be elucidated. With the

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introduction of polymerase chain reaction (PCR) (1) and other technical advances in molecular biology, increasing numbers of genes are being isolated and characterized. However, we frequently encounter difficulty in the identification of protein tyrosine kinase (PTK) genes responsible for a cell type specific signal transduction pathway.

We report here the establishment of an efficient method to rapidly isolate cell type-specific PTK by degenerate polymerase chain reaction with differential hybridization. We constructed a PTK-cDNA-enriched library from a renal cell carcinoma cell line, RCC23, using rapid amplification of cDNA 3' ends (3'RACE) with primers from PTK consensus sequences (2), and screened it by the differential hybridization technique with probes produced from various cell lines by 3' RACE. Using this strategy, we could rapidly isolate cDNA clones for two PTK genes, *FRK* and *FLT-3*, preferentially expressed in the renal cell carcinoma cell line, without laborious procedures.

MATERIALS and METHODS

Cells and culture conditions

Seventeen cancer cell lines were used in the present study (Table 1 and 2). KATO-III, MKN45, MKN74 and Lu-140 cells were maintained in RPMI 1640 supplemented with 10% FCS, while TE-1, TE-10, TE-13, A-172, HT-1080, RD, G-402 were cultured in RPMI 1640 with 7% FCS, and SMKTR-1, SMKTR-2, SMKTR-3 and SMKTR-4 cells were maintained in MEM with D-valine modification medium with 10% FCS (3).

Construction of PTK-cDNA-enriched library and probes

PTK cDNA fragments were amplified by PCR with primers corresponding to these consensus sequences: PTK I, 5'-GGA ATT CAT CCA C(AC)G (AGCT)GA (CT)(CT)T-3' ; PTK II, 3'-CT GCA GAC CAG GAA ACC TTA AGG-5' of PTK (2). Total RNAs were extracted from the RCC 23 cells using the guanidinium-isothiocyanate/cesium-chloride method and mRNAs were prepared. The first strand was synthesized by reverse transcriptase (BRL: Superscript) with DT7 primer, 5'-TAATACGACTCACTATAGGGATTTTTTTTTTTTTTT-3' (4). First round PCR was performed with PTKI and T7 primer, 5'-AATACGACTCACTATAG-3'. The first-round PCR products were diluted 1:20 and amplified again by PCR with the PTK I and PTK II primers. The major second-round PCR products of approximately 200 bp were purified and ligated to λgt10 to construct RCC23 PTK-cDNA-enriched library. The second-round PCR products with approximate 200bp, prepared by exactly the same method, each from RCC23 cells, TE-1 cells and MKN45 cells, were used as a probe on differential hybridization.

Screening with differential hybridization technique

One thousand plaques of the RCC23 PTK-cDNA-enriched library were transferred to Hybond-N$^+$ Nylon filters (Amersham), and hybridized with a probe, each prepared from RCC23, MKN45 or TE1 cells. Hybridization was performed at 42° C for 12 hours in a solution of 5xSSC, 0.1% SDS, 5mM EDTA, 50% formamide, 5x Denhardt's solution, 100mg/ml of denatured salmon sperm with 10^6cpm/ml of [^{32}P]dCTP labeled probes. After hybridization, filters were washed in 0.1xSSC, 0.1%SDS at 65°C, and then were exposed to X-ray film (X-OMAT, Kodak) for 12 hours at -70°C. After screening by differential hybridization, plaques, which hybridized preferentially with RCC23 probe, were selected and futher analyzed.

Northern blot analysis

Twenty μg of total RNAs or 2μg of poly (A)$^+$ RNAs were loaded onto 1% agarose gels containing formaldehyde, electrophoresed and transferred to Nitro plus (Micron Separation, Inc). Hybridization and washing were done under the same conditions as the screening procedure.

Sequencing analysis

Sequencing analysis of the selected clones was done using Sequencenase Version 2.0 Kit (USB, Inc), and sequence data of nucleotides and deduced amino acids were analyzed in GenBank Nucleotide Sequence Database.

RESULTS

After screening the RCC23 PTK-cDNA-enriched library for selection of the clones expressed preferentially in the RCC23 cells by differential hybridization, we randomly selected 76 clones hybridized strongly with the probe from RCC23 cells. We further selected 20 clones, which hybridized with the RCC23 probe but not with MKN45 and TE1 probes (Fig. 1). Twenty clones were classified into two groups after cross hybridization analysis. By sequencing analyses, nineteen out of 20 clones were revealed to be identical to human src-like tyrosine kinase gene, *FRK*, and one clone to a human *FLT-3* tyrosine kinase gene. We performed Northern blot analysis to investigate the expression level of *FRK* and *FLT-3* in RCC23 cells. The representative data are shown in Fig. 2. The *FRK* mRNAs with sizes of 3.9kb and 9.5kb were detected in total RNA of the RCC23 cells. But even in poly(A)$^+$ RNAs of gastric cancer cells (MKN45 and MKN74), and esophageal cancer cells (TE1, TE10 and TE13) cells, the *FRK* mRNAs were not detected (Fig. 2). The *FRK* mRNAs were also not found in total RNAs of embryonal rhabdomyosarcoma (RD), fibrosarcoma (HT-1080), malignant melanoma (G361), renal leiomyoblastoma (G402),

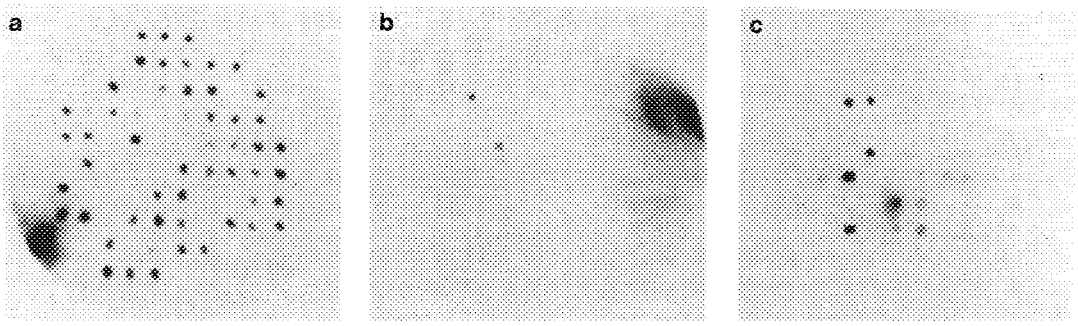

Figure 1. Differential hybridization. After the first screening by differential hybridization, 76 plaques were transferred and hybridized with probes, amplified fragments from **a.** renal cell carcinoma cell line (RCC23), **b.** stomach cancer cell line (MKN45) and **c.** esophageal cancer cell line (TE-1), respectively. Twenty clones hybridized only with RCC23 probe were picked up randomly.

glioblastoma (A172) and lung cancer (Lu140). *FLT-3* mRNA with the size of 3.7 kb was detected only in poly(A)$^+$ RNA of the RCC23 cells. The amount of *FLT-3* mRNA was small, so that the bands, corresponding to *FLT-3* mRNA, could not be detected in total RNA from the RCC23 cells. *FLT-3* mRNA was not detected in total RNA samples or poly(A)$^+$ RNA samples from other types of cell lines, TE1, TE10, TE13, MKN45, MKN74, RD, HT-1080, G361, G402, A172 and Lu140 (Fig. 2).

Sensitivity of the isolation method for PTK genes preferentially expressed in the RCC23 cells was estimated by plaque hybridization. Hybridization with an *FRK* probe gave us 315 positive signals in 2000 plaques of the RCC23 PTK-cDNA-enriched library (15.8%) and 13 positive signals in 2000 plaques of the TE1 PTK-cDNA-enriched library (0.7%). The results with the *FLT-3* probe were 56 positive signals out of 2000 plaques of the RCC23 PTK-cDNA-enriched library (2.8%), showing that the number of the *FLT-3* clones was much lower than that of the *FRK* cDNA clones. No *FLT-3* clones were found in 2000 plaques of the TE1 PTK-cDNA-enriched library (Table 1).

On the contrary, c-*src* and *FGFR2*, which were commonly expressed not only in RCC23, but also in TE1 and MKN45 cells, were eliminated completely by the differential hybridization (data not shown). The other *fms*-like tyrosine kinase gene, *FLT-1* not expressed in RCC23 cells (5), was not included in the RCC23 PTK-cDNA-enriched library (data not shown).

Northern blot analysis was performed to investigate the preferentiality for expression of *FRK* and *FLT-3* in renal cell

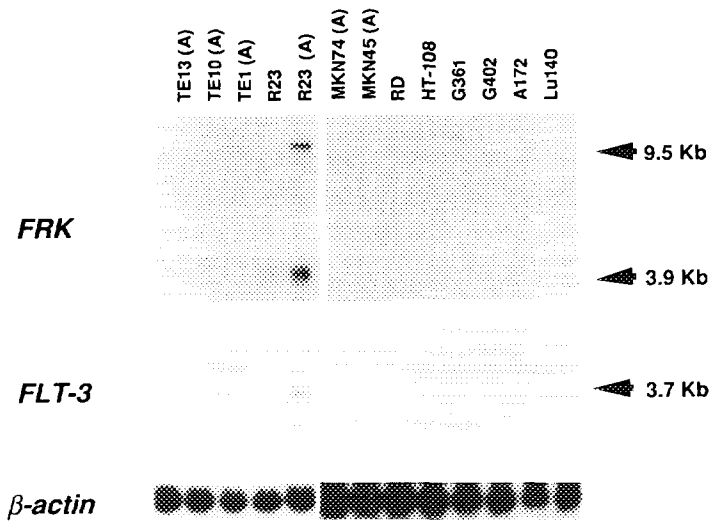

Figure 2. Northern blot analysis of isolated genes in various cell lines. The amounts of mRNAs were analyzed in embryonal rhabdomyosarcoma (RD), fibrosarcoma (HT-1080), malignant melanoma (G361), renal leiomyoblastoma (G402), glioblastoma (A172), lung cancer (Lu140), stomach cancer (MKN45 and 74), esophageal cancer (TE1, 10 and 13) and renal cell carcinoma (RCC23) cell lines. The transcript sizes of *FRK* are 3.9Kb and 9.5Kb and that of *FLT-3* is about 3.7Kb.
β-actin was used as internal control.
'(A)' means poly $(A)^+$ RNA.

carcinoma cells. The results are summarized in Table 2. *FRK* mRNAs were expressed in all 5 renal cell carcinoma cell lines, but not in other types of 11 cancer cell lines except for a gastric cancer cell line, KATOIII cells. *FLT-3* mRNAs were detected in all 3 renal cell carcinoma cell lines tested, which were not detected in all the other types of cancer cell lines.

Table 1. *Estimation of sensitivity of the differential hybridization*

	RCC23	TE1
FRK	15.8%(315/2000)	0.7%(13/2000)
FLT-3	2.8%(56/2000)	0%(0/2000)

Filters from RCC23 and TE1 PTK-cDNA-enriched libraries were hybridized with probes of *FRK* and *FLT-3*.
Number of clones hybridized with each probe out of number of recombinant PTK clones examined (/).

Table 2. *Tumor cell lines used for analysis for presence of FRK and FILT-3 transcripts*

Cell	*FRK*[a]	*FLT-3*
Renal cell carcinoma		
SMKTR-1	+	ND
SMKTR-2	+	ND
SMKTR-3	+	+
SMKTR-4	+	+
RCC23	+	+
Gastric cancer		
MKN45	-	-
MKN74	-	-
KATOIII	+	-
Esophageal cancer		
TE1	-	-
TE10	-	-
TE13	-	-
Sarcoma		
RD	-	-
HT-1080	-	-
G-361	-	-
G-402	-	-
A-172	-	-
Lung cancer		
Lu140	-	-

[a]The presence of *FRK* and *FLT-3* transcripts was analyzed by northern blot; +, presence of the transcripts; -, absence of the transcripts.
ND: not determined.

DISCUSSION

Amplification and overexpression of the K-*sam* gene in the poorly differentiated type of gastric cancer and those of c-*erb*B-2 in the differentiated type and those of c-*met* in both types are good examples of usage of the cell type specific protein tyrosine kinase (PTK) in carcinogenesis (6-10). Isolation of genes expressing specifically or predominantly in a specific cancer cell provides us with an important tool to understand the molecular characteristics of the cancer cells. The introduction of the subtractive or differential cDNA cloning technique (11) resulted in the successful identification of a gene specifically expressed in a certain type of cancer cell. These two cDNA cloning techniques demand that we repeat screening

procedures many times and analyze many clones before we can isolate PTK-cDNA clones, which play important roles in signal transduction in both normal and cancer cells. In this report, we presented an efficient and simple method to isolate the PTK genes preferentially expressed in renal cell carcinoma. Using the degenerate primers to specifically amplify PTK cDNAs from RNA of RCC23 cells, we first constructed a RCC23 PTK-cDNA-enriched library. Through this strategy, a cDNA library containing a large number of target cDNAs was obtained, enabling us to increase the chances of detection of cDNAs of even low abundancy. We screened the PTK-cDNA-enriched library by the differential hybridization technique with PTK-enriched cDNA probes made by 3'RACE under high stringent conditions. This procedure made it possible to rapidly isolate PTK-cDNA clones expressed preferentially in a specific type of cell. Hybridization of the PTK-cDNA-enriched libraries with the probes of *FRK* and *FLT-3* showed the preferential presence of *FRK* and *FLT-3* cDNA in the RCC23-derived library but not in the other libraries derived from the other cell lines (Table 1). These results of relative abundancies of *FRK* or *FLT-3* cDNA clones were consistent with the results of Northern blot analysis (Fig. 2).

It should be noted that the membrane type of PTK, *FLT-3*, was less than 3% in the proportion of plaques (Table 1), which means that it is very difficult to isolate this gene from the cDNA library by the previously reported method of cDNA subtraction (12). Our strategy can be applied to rapid cloning of new PTK genes or isolation of PTK genes expressed preferentially in normal or malignant cells without laborious procedures.

Human src-like tyrosine kinase, *FRK*, was cloned from lymphoid tissue (Gene Bank Data, not published), and belongs to the src family of non-receptor tyrosine kinase. The src family of genes encodes highly conserved non-receptor type PTKs, which are implicated in signal transduction processes, because they physically associate with certain membrane receptors and functionally respond to the binding of cognate ligands or receptor cross linking (13, 14). The *FRK* gene was conserved between human and rodent, and was expressed preferentially in the kidney, prostate and small intestine (data not shown). It was not expressed in the heart, brain, placenta, pancreas, spleen, thymus and leukocyte.

Flt-3, encoding receptor tyrosine kinase, was cloned as *Flk-2* (15, 16) from mouse, and human homolog was cloned as *FLT-3* from the cDNA library of human testis (17) and pre-B cell line (18) or as

STK-1 from $CD34^+$ hematopoietic stem cell-enriched library (19). The murine Flt-3 ligand has been isolated (20). The ligand stimulates the proliferation of both mouse and human primitive hematopoietic cells.

The fact that both of the genes were preferentially expressed in renal cell carcinoma cells suggested their possible role in renal cell carcinoma cell-specific signal transduction pathways. Further work should be done to reveal the potential functions or roles of *FRK* and *FLT-3* in the carcinogenenisis and progression of renal cell carcinoma.

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