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Expression of the Human *fes* Cellular Oncogene in Renal Cell Tumors *

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Summary. Renal cell tumors were screened for expression of the cellular oncogenes c-abl, c-fes, c-fms, c-myc, c-ras, and c-sis in dot blot hybridization analysis. Expression of c-ras and c-myc was clearly detectable in most of the 15 tumors that were studied. The c-fes oncogene appeared to be expressed in only two of them. Comparative Southern blot analysis of molecularly cloned human c-fes DNA and genomic DNA of the 15 renal tumors revealed no major genetic differences. Northern blot analysis of poly(A)-selected RNA from the fes-positive tumors with the complete viral v-fes oncogene of the Gardner-Arnstein strain of feline sarcoma virus as a molecular probe revealed hybridization of RNA species of 3.0 and 4.5 kb, respectively. The 3.0 kb c-fes transcript has also been reported in RNA from patients suffering from acute myelogenous leukemia [13]. The 4.5 kb transcript, however, has not been described before and represents either a c-fes-related splicing intermediate or, more likely, a completely processed transcript. The results of this study could imply that human c-fes coding sequences are more extensive than was previously assumed.

Key words: Renal cell tumors, Oncogene expression, c-fes.

Introduction

Based upon morphological and histochemical features, renal cell tumors are thought to be adenocarcinomas and to originate from the proximal convoluted tubule [2]. In a recent study, Herman et al. [10] reported expression of vimentin and keratin within the same cell. These intermediate filament types are generally biological markers of both sarcomatous and carcinomatous tumors. Additional biological markers are required to enable further characterization of renal tumors. Potentially useful in this respect are the cellular oncogenes. These genes have been implicated in the induction of malignant transformation in general and their elevated levels of expression in a number of human tumor cell lines as well as fresh human tumors is documented [13]. Sometimes, malignant activation of cellular oncogenes seems to involve point mutations or more extensive genetic changes such as chromosomal rearrangements or translocations [21]. In many tumors, cellular oncogenes such as c-H-ras, c-K-ras, c-myc and c-fos seem to be involved simultaneously [13]. Other cellular oncogenes, such as for example the fes cellular oncogene, show a more restricted expression pattern. Expression of this cellular oncogene was mainly found in lung and hematopoietic malignancies and, sporadically, in renal tumors [13].

To test the relevance of expression patterns of cellular oncogenes as markers in renal tumors, we set up a dot blot screening assay to determine the expression of a number of cellular oncogenes in a single experiment. To assess the potential implications of the expression of the cellular fes oncogene in renal tumors, we studied its genetic organization by Southern blot analysis and analyzed fes-related RNA transcripts by Northern blot analysis. The discovery of a new c-fes-related transcript is discussed.

Materials and Methods

Renal Cell Tumors

Renal cell tumors were obtained from the Department of Urology of the St. Radboud University Hospital. Preoperative diagnostic procedures including intravenous urography, selective renal arteriography, cavography, lung tomography, computer scanning tomography and bone scanning were performed. By this means, a valid T.N.M. classification of the tumors could be made. The renal tumors from patients including those with positive lymphnodes (N^+) and those with metastases (M^+) were removed by a paramedian transabdominal radical nephrectomy. Tumor material was frozen in liquid nitrogen for biochemical analysis.

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Table 1. DNA fragments used as molecular probes

Source DNA fragments	Fragment used as probe	Reference
c-abl (mouse)	1.2 kb PstI-PstI (cDNA)	19
c-fes (human)	1.3 kb EcoRI-BglII	8
v-GA-fes (feline)	3.8 kb BglII-HindIII	9
v-fms (feline)	1.45 kb PstI-PstI	5
c-myc (human)	1.2 kb EcoRI-ClaI	3
c-H-ras-1	5.2 kb BamHI-BamHI	7
v-H-ras (rat)	0.96 kb HindIII-HindIII	6
c-sis (human)	1.7 kb BamHI-BamHI	19
actin (hamster)	1.2 kb PstI-PstI (cDNA)	4
feline leukemia virus proviral DNA	5.0 kb XhoI-XhoI	11
vimentin (hamster)	0.5 kb PstI-PstI (cDNA)	4

Gel Electrophoresis and Hybridization

Restriction endonucleases were purchased from either New England Biolabs or Bethesda Research Laboratories (BRL) and used according to the specification of the manufacturers. High molecular weight DNA was isolated from tumor samples frozen in liquid nitrogen. Molecularly cloned human v-fes homologous sequences were isolated from bacterial strain HB101 transformed with recombinant plasmid pPH2. pPH2 is derivative of pSP64 containing a 12 kb EcoRI DNA insert that includes all human v-fes homologous sequences detectable by Southern blot analysis. Analysis of restriction endonuclease digested high molecular weight DNA from renal cell tumors was essentially as described by Southern [14]. DNA fragments were size fractionated by electrophoresis through 0.8% agarose (Seakem) gels, transferred to Gene Screen Plus (New England Nuclear) and hybridized to ^{32}P-nick-translated DNA probes. Molecular probes were nick-translated to specific activities of about $2-5 \times 10^8$ cpm/μg. Hybridization analysis was performed under conditions of low stringency at 42 °C for appropriate periods of time in 40% formamide (deionized, Mixed Bed Resin AG501-X8D, Biorad), 1% SDS, 1 M NaCl, 10% dextran sulphate and $2-5 \times 10^5$ cpm/ml nick-translated DNA. DNA probes were labeled according to the method of Rigby et al. [12] using ^{32}P-dCTP (3,000 Ci/mmole) from Amersham, DNAse I from Sigma and E. coli DNA polymerase I from Boehringer. cDNA was labeled to high specific activity by reverse transcription of poly(A)-selected RNA (25 μg/ml) in 100 mM Tris-HCl (pH 8.3), $MgCl_2$ (8 mM), NaCl (20 mM), dithiothreitol (10 mM), a mixture of dATP, dCTP, dGTP and dTTP (5 μM each), especially treated calf thymus DNA [15] (0.25 μg/ml) and reverse transcriptase (20 U) for 20 min at 42 °C. Using ^{32}P-dCTP, specific activities of the cDNA probes were about $1-2 \times 10^8$ cpm/μg. Upon hybridization, filters were washed twice in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na-citrate) at room temperature for 5 min and twice in 2 x SSC and 1% SDS at 50 °C for 30 min and autoradiography was performed by exposing XAR-2 film (Kodak) for up to four days at −70 °C with Dupont lightning plus intensifying screens.

Preparation of DNA Probes

Characteristics of the molecular probes used in this study are summarized in Table 1. DNA probes were prepared by digestion of 50–100 μg of DNA with appropriate restriction endonucleases followed by electrophoresis through low melting point agarose (BRL). The desired bands were excised from gels and, upon melting by heating at 65 °C for 15 min as described [20], agarose was removed by two extractions with phenol, equilibrated with 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA and one extraction with phenol/chloroform/isoamylalcohol (25:24:1). After concentration using butanol-2, DNA was precipitated in ethanol and 0.1 M NaOAc (pH 5.2).

RNA Isolation and Northern Blotting

Total cellular RNA was isolated according to the lithium-urea method described by Auffray and Rougeon [1]. Upon poly(A) selection by oligo-dT-cellulose chromatography, 10 μg RNA was dissolved in sodium phosphate buffer (10 mM, pH 7.0), which contained DMSO (50%) and glyoxal (1.0 M), and heated to 50 °C for one hour. RNA was size fractionated by agarose gel electrophoresis (1.0% agarose gel) and transferred to Gene Screen Plus for hybridization analysis as described above.

Results

To detect expression of the cellular oncogenes abl, fes, fms, myc, ras and sis in a single experiment, a dot blot hybridisation assay was performed in which increasing amounts of DNA homologous to those cellular oncogenes were bound to Gene Screen Plus paper. Upon denaturation of the DNA, hybridization was performed with a ^{32}P-cDNA probe synthesized in reverse transcriptase reactions with poly(A)-selected RNA isolated from renal cell tumors as templates. Synthesis of ^{32}P-cDNA was performed with random oligodeoxynucleotides from DNAse-digested calf thymus DNA as primer (see under Materials and Methods). A typical example of such a dot blot hybridization analysis is shown in Fig. 1. A clear hybridization signal is observed in places where DNA is spotted homologous to v-H-ras, c-H-ras-1 and c-myc(human), indicating the presence of relatively high levels of related transcripts in this particular tumor. A weaker hybridization signal is observed in places where v-Ga-fes and c-fes (human) is spotted. It should be noted that in only 2/15 of the tumors that were tested expression of the cellular fes oncogene could be detected in this way. Expression of c-myc and c-H-ras was found in most of the 15 renal cell tumors that were analyzed. Transcripts homologous to the cellular oncogenes abl, fms and sis were not detectable in the tumors by this procedure. To control the assay conditions and the quality of the RNA preparations, actin and vimentin genes were included in the experiments (Fig. 1). It should be noted that the experimental approach does not allow the identification of the cell types that express the various cellular oncogenes.

The renal cell tumor whose expression pattern of some cellular oncogenes is shown in Fig. 1, was obtained from a 41 year old male patient. The tumor was found in the left kidney following an intravenous urogram indicated in the investigation of renal stone disease. The tumor was staged $T_2N_0M_0$ (clear cell). However, it should be noted that histological analysis revealed papillary structures in the tumor as well as multiple intracytoplasmatic inclusions. The inclusions were identified by electronmicroscopy as crystalline protein bodies. Expression of vimentin intermediate filament protein was observed in a immunofluorescence study (Ramaekers, personal communication) which was in agreement with the hybridization data. Furthermore, the observation that in some cells of a renal tumor both vimentin and keratin were expressed, was also made in this particular tumor (data not shown). In a soft agar assay, the renal tumor cells showed no colony formation.

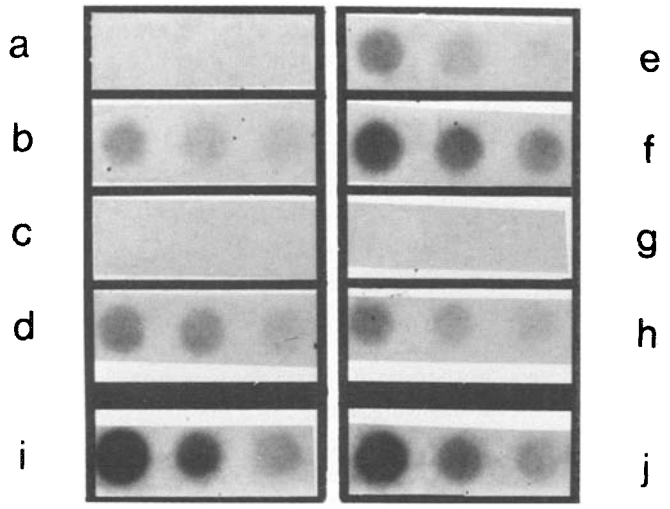

Fig. 1. Analysis of expression of cellular oncogenes in renal cell tumor # 63 by dot blot hybridization analysis. Increasing amounts of DNA samples (8, 40 and 200 ng) representing molecularly cloned sequences of viral or cellular oncogenes were spotted onto Gene Screen Plus and analyzed as described in the text. DNA samples included c-abl (mouse) (A), c-fes (human) (B), v-fms (feline) (C), c-myc (human) (D), v-H-ras (rat) (E), c-H-ras-1 (human) (F), c-sis (human) (G) and v-GA-fes (feline) (H). Actin (hamster) (I) and vimentin (hamster) (J) were included as controls. As a molecular probe, ^{32}P-cDNA synthesized with poly(A)-selected RNA from tumor # 63 as template was used

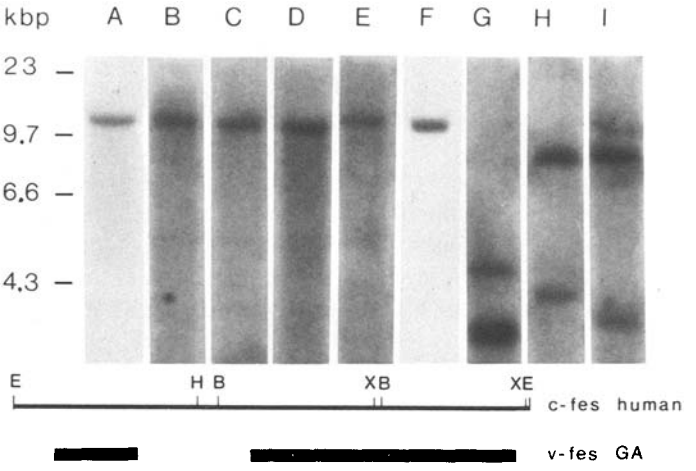

Fig. 3. Comparative Southern blot analysis of the c-fes locus in human renal cell tumors and molecularly cloned human c-fes sequences. Ten μg of high molecular weight DNA of normal human liver (*lane A*), renal cell tumor # 63 (*lane B*), tumor # 87 (*lane C*), tumor # 148 (*lane D*), tumor # 160 (*lane E*) and 0.5 ng of pPH2 DNA (*lane F*) or mixtures of DNA from tumor # 63 (10 μg) and pPH2 DNA (0.5 ng) (*lanes G, H, and I*) were digested with restriction endonuclease EcoRI (*lanes A, B, C and D*) or combinations of EcoRI and BamHI (*lane G*), EcoRI and HindIII (*lane H*) or EcoRI and XhoI (*lane I*), transferred to Gene Screen Plus and hybridized to the same molecular probe as described in the legend to Fig. 2. Molecular weight markers included are HindIII-digested λ DNA fragments. In the lower part of the figure, a schematic restriction map of the human c-fes 12 kb EcoRI DNA fragment is depicted. *B*, BamHI; *H*, HindIII; *E*, EcoRI; *X*, XhoI

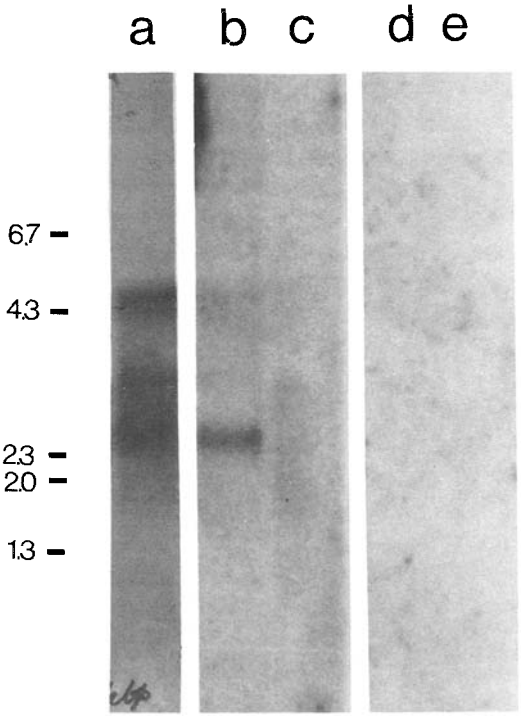

Fig. 2. Northern blot analysis of expression of the fes cellular oncogene in renal cell tumors. Poly(A)-selected RNA from tumor # 160 (*lane A*), tumor # 63 (*lane B*), normal kidney (*lane C*), tumor # 87 (*lane D*) and tumor # 148 (*lane E*) were size fractionated by agarose gel electrophoresis and analyzed by Northern blot analysis. The complete viral oncogene v-GA-fes was used as a molecular probe. Molecular weight markers included are single strand HindIII-digested λ DNA fragments

In a further analysis of the fes expression in renal tumors, we studied the fes transcripts by Northern blot analysis. As a molecular probe, the complete viral oncogene of the Gardner-Arnstein strain of feline sarcoma virus was used. Two fes-related RNA species were detected in a poly(A)-selected RNA sample from the two renal tumors. Their lengths were 4.5 and 3.0 kb (Fig. 2, lanes A and B). A difference in the relative amounts of the two RNA species was observed. No fes-related RNA transcripts were detected in poly(A)-selected RNA samples of two other renal tumors (Fig. 2, lanes D and E) or from normal kidney (Fig. 2, lane C). Since the DNA probe contained some genetic sequences of feline leukemia virus (FeLV), hybridization analysis was also performed with a large portion of the proviral DNA of FeLV as a molecular probe but no hybridization was observed (data not shown).

A 3.0 kb fes-related RNA species was found in RNA isolated from myeloblasts of patients suffering from acute myelogenous leukemia [13]. Our observations suggest that expression of the c-fes locus in renal cell tumors differs from that in acute myelogenous leukemia. To investigate whether or not this could be explained by chromosomal rearrangement, we analyzed the genetic organization of the c-fes locus in renal cell tumors by Southern blot analysis and compared the data with those obtained with molecularly cloned human c-fes DNA (Fig. 3). With the complete

v-GA-fes oncogene as a molecular probe, all human v-GA-fes homologous sequences were found in a 12 kb DNA fragment in control human genomic DNA digested with restriction endonuclease EcoRI (Fig. 3, lane A). Similar analysis of genomic DNA isolated from the 15 renal tumors revealed the same 12 kb EcoRI DNA fragment in each case (Fig. 3). The results of only four tumors are shown (Fig. 3, lanes B, C, D and E). Southern blot analysis of mixtures of genomic DNA of a fes-positive renal tumor (tumor # 63) and cloned human c-fes DNA digested with combinations of restriction endonuclease EcoRI with BamHI, HindIII or XhoI (Fig. 3, lanes G, H, and I, respectively) did not reveal any differences. These results indicate that there are no major genetic changes in the parts of the c-fes loci in the renal tumors that can be detected with the complete viral v-fes oncogene as a probe.

Discussion

The present study indicates that expression of the fes cellular oncogene occurs in some renal cell tumors and that it involves two RNA species of different sizes. The 3.0 kb RNA has a similar molecular weight as the transcript found in myeloblasts of patients with acute myelogenous leukemia (AML) [13]. The other transcript is 4.5 kb. The novel appearance of a 4.5 kb fes transcription product in some renal tumors could be explained in different ways. The 4.5 kb RNA species is probably not the result of a chromosome translocation nor of other major chromosomal changes in the v-fes homologous region of renal cell tumors since no abnormalities were seen in restriction endonuclease analyses of tumor DNA. A minor genetic change, on the other hand, such as a point mutation, a small deletion or insertion is usually not detected by the Southern blot analysis. It could be assumed that such a minor genetic change would affect the processing of c-fes pre-mRNA in such a way that two different products are being formed from one gene. In that case, it may be that one particular step in the splicing process is limited in renal tumors since no other fes-related splicing intermediates were detected. Differential gene splicing is a well documented phenomenon in tumor viruses. There is no reason why this mechanism might be limited to viral genes.

Alternatively, the new transcript could be due to a secondary mutation in a tumor cell which grew out to a subpopulation of cells within the tumor. Finally, the possibility that both RNAs constitute independent and naturally occurring transcripts cannot be excluded. Clearly, more experimental work is needed to discriminate between these explanations.

While the 3.0 kb transcript is approximately of the same size as the viral gene, v-fes, used as a molecular probe in these studies, the newly discovered occurrence of a 4.5 kb transcript in two different tumors raises the possibility that the human c-fes locus is larger than the 12 kb EcoRI restriction endonuclease fragment that contains all sequences homologous to v-fes. It is accepted that the retroviral oncogenes which have been generated in a process in which RNA tumor viruses have captured cellular genetic sequences from their natural hosts, often represent only a portion of the cellular locus [9, 19]. It is, therefore, possible that the v-fes oncogene of the Gardner-Arnstein strain of feline sarcoma virus encodes only the tyrosine-specific protein kinase domain of a more complex protein and that parts of the cellular locus involved are not represented in any of the other fes-related viral oncogenes. The epidermal growth factor receptor [17] and the insulin receptor [16] are examples of such complex proteins with a tyrosine-specific protein kinase domain as an intrinsic structural component. The 4.5 kb fes-related RNA species could represent a transcript of a larger yet to be defined c-fes locus. Further studies are required to resolve this matter.

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