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Expression of Proto-Oncogenes in Xenografts of Human Renal Cell Carcinomas

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Summary. In a recent paper, we described the expression pattern of proto-oncogenes in primary human renal cell carcinoma [12]. To test the possibility of using xenografts as a useful alternative for such studies, we analyzed xenografts of a number of human renal cell carcinomas in nu/nu mice. Xenografts included RC2, RC14, RC21, RC43 and NC65. Northern blot analysis indicated that c-Ras was expressed in all these xenografts. The identity of the ras transcripts in the individual xenografts was further specified as c-Ha-ras, c-Ki-ras or N-ras. Expression of c-myc and the p53 gene was also found in a number of these tumors. Only RC21 failed to express the c-myc or the p53 gene. In all xenografts, a 3.0 kb c-fes/fps mRNA was present. In RC2, RC14, RC21 and RC43, low levels of the 4.8 kb abl transcript were detectable. Transcripts of myb and sis could not be detected in any of the xenografts. The results indicated that the expression pattern of a variety of proto-oncogenes in xenografts of human renal cell carcinomas was similar to that in the primary tumors.

Key words: Renal cell carcinoma, Proto-oncogenes, Xenografts.

Introduction

Molecular oncology has led to the discovery of genes whose malignant potential becomes apparent upon retroviral transduction of these genes. Because of this characteristic, the genes are also thought to be implicated in the onset and development of naturally ocurring tumors. Collectively, they are called proto-oncogenes. Up to this moment, more than fourty proto-oncogenes have been characterized [3, 4]. Recently, classification of proto-oncogenes in a nuclear and cytoplasmic group was proposed on the basis of the site of action of their gene products [27]. The nuclear group is the smallest one and includes c-myc, c-myb and the p53 gene. An important characteristic of these genes is their immortalizing capacity. In general, the nuclear group of proto-oncogenes weakly induces anchorage independent growth of fibroblasts in tissue culture [27]. There are indications that these genes are involved in regulation of gene expression and that disturbance in their own expression patterns (in place and/or in time) is a major factor in tumorigenesis [2, 15]. The gene products of c-myc and c-myb are structurally homologous [6, 20]. p53 is a phosphoprotein that is overexpressed in some transformed cells [11, 29]. The cytoplasmic group of proto-oncogenes is larger and its members are thought to be a major factor in processes involved in malignant transformation. Proto-oncogenes of this group include for instance the ras-gene family, c-fes/fps and c-abl. It has been suggested that in general their malignant activation is the result of mutations [15]. The gene products of c-abl and c-fes/fps are tyrosine-specific protein kinases [10, 22, 24] and the ras gene product resembles the G-protein and probably acts as a signal transducer [4, 27]. A proto-oncogene that does not belong to either of the groups described above, is the sis proto-oncogene. Its translation product is highly similar to the B-chain of the platelet-derived growth factor (PDGF) [17].

In a recent paper [12], we described proto-oncogene expression patterns in primary human renal cell carcinomas. We found that c-Ras and c-myc were clearly detectable in most of the tumors. Furthermore, expression of c-fes/fps was observed in a small percentage of these tumors. Since RNA isolated from primary human renal cell carcinomas was often of low quality probably due to necrosis in the tumor, we wanted to test xenograft tumor lines of renal cell carcinomas in nu/nu mice as a suitable alternative. The nu/nu mouse, described by Flanagan [7] and later found to be thymus deficient and lacking T-cell mediated immune response by Pantelouris [18], was chosen as an animal host for

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Table 1. DNA fragments used as molecular probes

Proto-oncogene	Fragment used as probe		Reference
N-ras	SalI/EcoRI	0.65 kbp	[9]
v-Ki-ras	EcoRI/EcoRI	1.15 kbp	[25]
c-Ha-ras	SmaI/SmaI	0.65 kbp	[25]
c-myc	SacI/SacI	1.20 kbp	[6]
c-myb	BamHI/XbaI	0.80 kbp	[19]
p53 gene	PstI/PstI	0.55 kbp	[11]
v-abl	PstI/PstI	0.70 kbp	[24]
c-fes	EcoRI/EcoRI	0.95 kb	[22]
c-sis	BamHI/BamHI	1.80 kbp	[17]

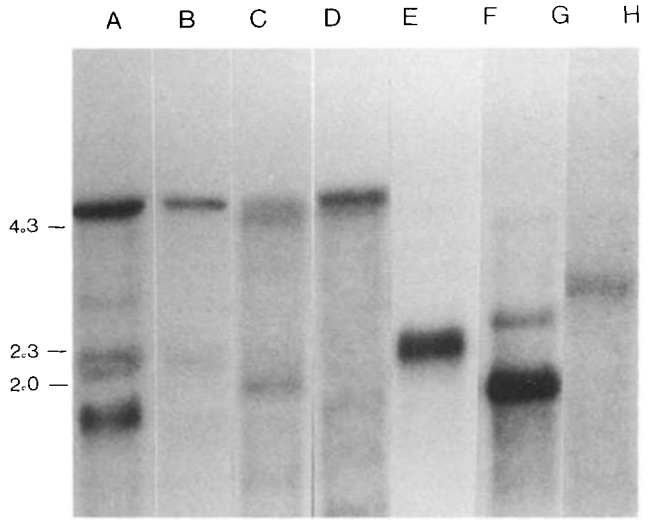

Fig. 1. Northern blot analysis of xenografts of human renal cell carcinomas. Poly-A selected RNA from RC2 (lane *E*, *F* and *H*), RC14 (lane *B* and *D*) RC43 (lane *C* and *G*) and Vero (lane *A*) was size fractioned by agarose gel electrophoresis and analyzed by Northern blot technique. Molecular probes used were N-ras (lane *A* and *B*), v-Ki-ras (lane *C*), c-Ha-ras (lane *D*), c-myc (lane *E*), p53 (lane *F*), c-fes (lane *G*) and v-abl (lane *H*). Molecular weight markers included are single strand HindIII digested lambda DNA molecules

this type of transplantation experiments, because of its usefulness for a wide range of human tumors [8]. We already described that, histologically and ultrastructurally, xenografts of human renal cell carcinomas in nu/nu mice resemble primary renal tumors [13].

In the present study, we studied expression of c-Ha-ras, c-Ki-ras, N-ras, c-myc, c-myb, c-abl, c-fes/fps, c-sis and the p53 gene by Northern blot analysis in xenografts of five different human renal cell carcinomas and an established kidney cell line of a monkey (Vero).

Materials and Methods

Tumor Materials

Xenografts of human renal cell carcinomas in nu/nu mice were obtained from the Department of Urology, Dijkzigt Hospital, Rotterdam. All xenografts were derived from primary tumors of patients with apparent metastases at the time of diagnosis. Xenografts showed no dedifferentation compared to the original tumors [14]. After removal, tumor material was immediately frozen in liquid nitrogen. The established kidney cell line (Vero), derived from an African green monkey, was also included in this study.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated according to the procedure described by Auffray and Rougeon [1]. Upon poly(A) selection by oligo(dT)-cellulose chromatography, RNA was dissolved in sodium phosphate buffer (10 mM, pH 7.0), which contained DMSO (50%) and glyoxal (1 M), and heated at 50 °C for 1 h. RNA was size fractionated by agarose gel electrophoresis (1.0% agarose gel) and transferred to nitrocellulose or Hybond-N membrane as described before [22].

Preparation of Molecular Probes

The characteristics of the molecular probes used in this study are summarized in Table 1. The probes were prepared as described before [14]. Radioactive labeling of the probes was performed according to Van den Ouweland et al. [17].

Hybridization and Dehybridization

Hybridization was performed under conditions as described by Church and Gilbert [5] and dehybridization according to the procedures as recommended by Amersham.

Results

Expression patterns of proto-oncogenes from the ras gene family are well documented. The human c-Ha-ras mRNA is 1.4 kb [25, 28], the human N-ras-specific transcript is 2.2 kb [9] and transcription of the human c-Ki-ras gene results in a 4.6 kb mRNA [25]. A complicating factor in the interpretation of results concerning ras expression is the fact that the ras genes are strongly homologous, therefore crosshybridization is often observed in Northern blot analysis. This is illustrated in Fig. 1 (lanes A to D), in which typical ras hybridization patterns are shown, using an N-ras (lane A–B), c-Ki-ras (lane C) and a c-Ha-ras (lane D) probe. In lane A (Fig. 1), it can be seen that the N-ras probe in addition the N-ras-specific mRNAs (doublet at 2.2 kb) also detects the c-Ki-ras (4.6 kb) and the c-Ha-ras transcript (1.4 kb). In the interpretation of the results described in this report, however, only specific transcripts are considered, namely 1.4 kb for c-Ha-ras, 2.2 kb for N-ras and 4.6 kb for c-Ki-ras. It is evident that because of crosshybridization ras expression cannot be studied unambiguously by a dot blot assay, but requires Northern blot analysis.

The results of the ras gene expression studies are summarized in Table 1. All tumors except RC21 showed detectable levels of N-ras transcripts. It appeared that both RC2 and RC43 contained the highest level of c-Ki-ras mRNA. It should be noticed that in these two tumor lines also a sec-

Table 2. Summary of proto-oncogene expression in xenografts of human renal cell carcinomas and the Vero cell line. Symbols: 0: no expression detected; + to +++: detectable to high levels of expression; NT: not tested

probe	mRNA	RC2	RC14	RC21	RC43	NC65	Vero
N-ras	2.2 kb	+	+	0	+	+	++
c-Ki-ras	4.6 kb	++	+	+	++	+	NT
c-Ha-ras	1.4 kb	0	+	++	+	0	NT
c-myc	2.3 kb	+++	++	0	+++	++	NT
c-myb	5.0 kb	0	0	0	0	0	0
p53	3.0 kb	++	0	0	+	0	++
c-fes	3.0 kb	++	+	+	++	+	0
v-abl	6.6 kb	0	0	0	0	0	++
	4.8 kb	+	+	+	+	0	+++
	2.9 kb	0	+	0	0	0	++
	0.8 kb	0	0	0	0	0	NT
v-sis	3.5 kb	0	0	0	0	0	NT

ond mRNA at approximately 5.3 kb was visualized using the v-Ki-ras probe. c-Ha-ras expression was the highest in RC21 and was not found in RC2 and NC65. From these data it appeared that in each of the tumors at least one of the ras genes was expressed.

Expression of c-myc, c-myb and P53

The cellular localization of proto-oncogene products is indicative for their mode of action and for their role in carcinogenesis. The c-myc, c-myb and p53 gene products are all localized in the cell nucleus. Expression of these oncogenes was also studied by a Northern blot analysis and the results are summarized in Table 2. In none of the xenografts were detectable levels of c-myb present, which is in agreement with the fact that c-myb expression is predominantly confined to hematopoetic malignancies [19]. High levels of c-myc expression were found in all xenografts, except in RC21. The possibility that in RC21 another member of the myc gene family (N-myc or L-myc) was expressed was not tested and can therefore not be excluded.

The human p53 specific transcript was found to be elevated in RC2 and RC43. These data indicate that in most of the xenografts tested, detectable levels of mRNA of a nuclear proto-oncogene were present.

Expression of c-abl and c-fes/fps

The c-abl and c-fes/fps proto-oncogenes both contain a domain that encodes a tyrosine-specific protein kinase [21]. The gene products of these two genes are localized in the cytoplasm and at least a fraction of them seems to be associated with membranes. Unlike ras and myc/p53, elevated

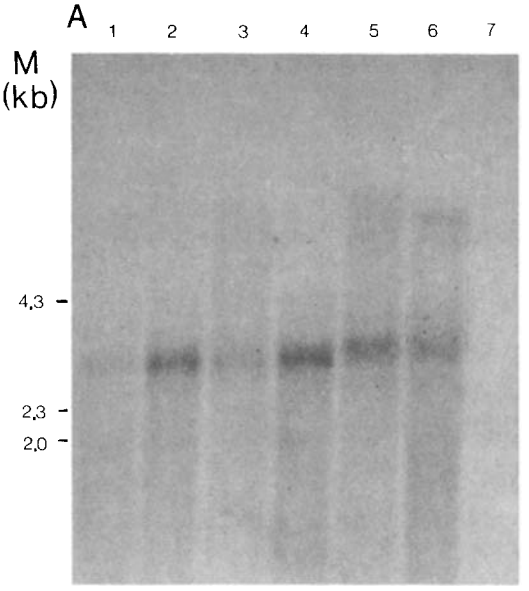

Fig. 2. Comparative Northern blot analysis of c-fes mRNA in xenografts RC2 (lane *2*), RC8 (lane *3*), RC14 (lane *5*), RC21 (lane *6*), RC43 (lane *4*) and NC65 (lane *1*). The c-fes EcoR1EcoR1 0.95 kb DNA fragment was used as a molecular probe. Molecular weight markers included are single strand HindIII digested lambda DNA molecules

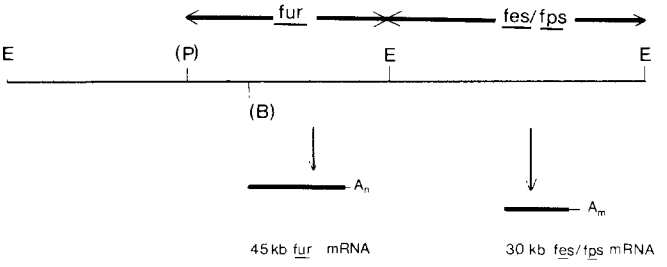

Fig. 3. Schematic representation of the human genomic region containing the fur gene and the fes/fps proto-oncogene. The putative promoter region is situated between the BamHI and PstI site. *E*, EcoRI; *B*, BamHI; *P*, PstI. The restriction endonucleases placed between brackets indicate that not all the cleavage sites for this enzyme are depicted

expression of these genes is not common in human malignancies [25]. Detectable levels of fes transcripts are sometimes found in haematopoetic malignancies [25], lung tumors [25], breast carcinomas and renal cell carcinomas [12, 25]. Expression of c-abl in primary tumors is only reported in the case of chronic myelogenous leukemia [25]. Northern blot analysis of RNA of the xenografts revealed that 4 out of 5 tumors exhibited abl expression. They included RC2, RC43, RC14 and RC21. The Vero kidney cell line also expressed the abl proto-oncogene. In contrast to the xenografts, a number of different abl transcripts were found in the cell line. As far as the expression of the fes/fps proto-oncogene was concerned, in all xenografts a 3.0 kb fes/fps specific mRNA was found (Fig. 2). Some fluctuation in the levels of the transcripts was observed. No fes/fps transcript

could be detected in the Vero kidney cell line. The fact that c-fes/fps expression was found in xenografts of human renal cell carcinomas is in agreement with previous studies on primary renal tumors [12, 25].

Expression of c-sis

The c-sis proto-oncogene contains sequences that could encode a protein similar to the B-chain of platelet-derived-growth factor (PDGF). A consensus is emerging that in fact the c-sis locus encodes the B-chain of PDGF. It was of interest to see whether in renal cell carcinomas autocrine growth stimulation as a consequence of c-sis activity was involved. Northern blot analysis revealed no c-sis transcripts and, therefore autocrine growth stimulation as a consequence of c-sis activation can be ruled out.

Discussion

Proto-oncogene expression patterns in xenografts of human renal cell carcinomas were studied to determine whether xenografts could be used as an alternative for primary renal cell carcinomas. A major problem in studying mRNA from primary human renal cell carcinomas is degradation of mRNA. In contrast to primary tumors high amounts of intact mRNA could be isolated routinely from xenografts of human renal cell carcinomas. This was probably due to the fact that the time period elapsing between excision of the primary tumor and its freezing in liquid nitrogen was much longer in the clinical setting than under the conditions of a laboratory experiment. It is well established that tumor devitalization starts when the renal vascular pedicle in radical nephrectomy is clamped. The better presentation of tumor tissue in xenografts was confirmed by microscopic examination of the xenografts revealing homogenous tumor structures and almost no necrosis.

Our results indicated that as far as proto-oncogene expression patterns are concerned, xenografts of human renal cell carcinomas provide a good alternative for primary tumors. Expression of all three members of the ras gene family was found in the xenografts. This is in accordance with earlier observations in primary renal tumors in our laboratory (Schalken, unpublished observation) and in agreement with results from other groups [25].

Similarly, expression of c-myc in xenografts also agrees with studies on primary tumors. The fact that in one of the xenograft tumor lines no c-myc transcripts could be found does not necessarily mean that this tumor does not contain a transcript that is related to c-myc. In recent studies [16], the discovery of N-myc and L-myc genes were described and they are related to the c-myc gene. However, limited nucleotide sequence homology could explain the failure to detect transcripts of them with a c-myc probe, especially under the stringent hybridization condition employed.

Expression of the c-fes/fps proto-oncogene in renal cell tumors is a matter of interest. In contrast to primary human renal cell carcinomas in which c-fes/fps expression was found in only 10% of the cases, the xenografts exhibited in all cases tested a clearly elevated level of fes/fps expression. At the moment it is not clear how to explain this observation. It is well established that in normal cells expression of c-fes/fps is restricted to cells of hemopoietic origin and mainly to those of the myeloid lineage [28]. The possibility that many myeloid cells were present in the xenografts and that the observed fes/fps expression was due to such cells in the tumor specimens could be ruled out on the basis of histopathological analysis. The fact that all xenografts exhibited fes/fps expression suggests that xenograft-specific factors are involved and may be selected for, during in vivo propagation of the tumor cells. In situ hybridization or immunofluorescence analysis could shed light upon this matter by identifying in xenografts as well as in primary tumors the cell types that express the proto-oncogene.

The high levels of fes/fps expression in xenografts of renal cell carcinomas is also remarkable. In specimens of normal kidney, the fes/fps DNA region seems transcriptionally silent. The genetic region immediately upstream of the proto-oncogene, however, is transcriptionally active in tissue specimens of normal kidney [22]. This region, which we have designated fur (for fes/fps upstream region) contains a gene, which is located very closely (less than 1 kbp) to the proto-oncogene [22]. For a schematic representation of this genetic region in the human genome, see Fig. 3. The fur gene, which seems to encode a membrane associated protein with a recognition function, has a relatively strong promoter [23]. The promoter of the proto-oncogene remains to be identified. It is tempting to speculate that in renal cell carcinomas the promoter region of the fur gene is involved in expression of the proto-oncogene. The fact that xenografts of human renal cell carcinomas resemble primary renal cell carcinomas and that mRNA of high quality can be isolated from them enables characterization of the fes/fps transcription unit in these tumor cells by cDNA analysis and S1 nuclease protection experiments. By this approach, the hypothesis mentioned above now can be tested.

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References

1. Auffray C, Rougeon F (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur J Biochem 107.303–314
2. Berridge MJ (1985) The molecular basis of combination within the cell. Sci Am 253:124–134
3. Bishop JM, Varmus H (1982) Molecular biology of RNA tumor viruses. Weiss R, Teich N, Varmus HE, Coffin J (eds), p 999–1108

4. Bishop JM (1985) Viral oncogenes. Cell 42:23–38
5. Church GM, Gilbert W (1984) Genomic Sequencing. Proc Natl Acad Sci USA 81:1991–1995
6. Colby WC, Chen EY, Smith DH, Levinson AD (1983) Identification and nucleotide sequence analysis of the human locus humologous to the c-myc oncogene of avian myelocytomatosis virus MC29. Nature 301:722
7. Flanagan SP (1966) Nude, a new hairless gene with pleiotropic effects in the mouse. Genet Res 8:295–309
8. Giovanella BC, Stekkir JS, Williams LJ Jr, Shih-Shun Lu, Shepard RC (1978) Heterotransplantation of human cancer into nude mice. A model system for human cancer chemotherapy. Cancer 42:2269–2281
9. Hall A, Marshall CJ, Spurr NK, Weiss RA (1983) Identification of transforming gene in two human sarcoma cell lines as a new member of the ras-gene family located on chromosome 1. Nature 303:396–400
10. Hunter T (1984) The proteins of oncogenes. Sci Am 251:60–69
11. Jenkins JR, Rudge K, Chumakor P, Currie GA (1985) The cellular oncogene p53 can be activated by mutagenesis. Nature 317:816–818
12. Karthaus HFM, Schalken JA, Feitz WFJ, Debruyne FMJ, de Haan PI, Bloemers HPJ, Van de Ven WJM (1986) Expression of the human fes cellular oncogene in renal cell tumors. Urol Res 14:123–127
13. Karthaus HFM, Feitz WFJ, Schalken JA, Bloemers HPJ, Van de Ven WJM, Debruyne FMJ (1986) Multiparameter analysis of four human renal cell carcinoma xenografts in nude mice. (Submitted)
14. Kurth KH, v. Dongen JM, Romijn JC, Lieber MML, Schröder FH (1984) Assay evaluability of drug testing systems determined with human renal carcinoma cell lines. World J Urol 2: 146–155
15. Land H, Parada LF, Weinberg RA (1983) Cellular oncogenes and multistep carcinogenesis. Science 22:771–777
16. Nau MM, Brooks BJ, Battey J, Sausville E, Gazdar AF, Kirsch IR, McBride OW, Bertness V, Hollis GF, Minna JD (1985) L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318:69–72
17. Ouweland van den AMW, Breuer ML, Steenbergh PH, Schalken JA, Bloemers HPJ, Van de Ven WJM (1985) Comparative analysis of the human and feline c-sis proto-oncogenes: identification of 5' human c-sis coding sequences that are not homologous to the transforming gene of simian sarcoma virus. Biochim Biophys Acta 825:140–147
18. Pantelouris EM (1968) Absence of thymus in a mouse mutant. Nature 217:370–371
19. Pelicci PG, Lanfrancone L, Brathwaite MD, Wolman SR, Dalla Favera R (1984) Amplification of the c-myb oncogene in a case of human acute myelogenous leucemia. Science 224:1117 to 1121
20. Ralston R, Bishop JM (1983) The protein products of the oncogenes myc, myb and adenovirus E1a are structurally related. Nature 306:803–806
21. Roebroek AJM, Schalken JA, Verbeek JS, Onnekink C, Bloemers HPJ, Van de Ven WJM (1985) The structure of the human c-fes/fps proto-oncogene. EMBO J 11:1197–1203
22. Roebroek AJM, Schalken JA, Bussemakers MJG, van Heerikhuizen H, Onnekink C, Debruyne FMJ, Bloemers HPJ, Van de Ven WJM (1986) Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. Molec Biol Rep 11:117–125
23. Roebroek AJM, Schalken JA, Leunissen JAM, Onnekink C, Bloemers HPJ, Van de WJM (1986) Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. EMBO J 5:2197–2202
24. Schalken JA, Van den Ouweland AMW, Bloemers HPJ, Van de Ven WJM (1985) Characterization of the feline c-abl proto-oncogene. Biochim Biophys Acta 824:104–112
25. Slamon DJ, deKernion JB, Verma IM, Cline MJ (1984) Expression of cellular oncogenes in human malignancies. Science 224:256–262
26. Steenbergh PH, Höppener JWM, Zandberg J, Jansz HS and Lips CJM (1984) Calcitonin gene related peptide coding sequence is conserved in the human genome and is expressed in medullary thyroid carcinoma. J Clin Endocrinol Metab 59: 358–367
27. Weinberg RA (1985) The action of oncogenes in the cytoplasm and nucleus. Science 230:770–776
28. Westin EH, Wong-Staal F, Gelmann EP, Dalla Favera R, Papas TS, Lautenberger JA, Eva A, Reddy EP, Tronick SR, Aaronson SA, Gallo RC (1982) Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc Natl Acad Sci USA 79:2490–2494
29. Wolf D, Harris N, Rotter V (1984) Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transjection of a junctional p53 gene. Cell 38:119–126

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