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Animal Model

Development of Seven New Human Prostate Tumor Xenograft Models and their Histopathological Characterization

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Seven human prostate tumor models were established by transplanting tumor fragments in NMRI athymic nude mice. Once established, the tumors were serially transplantable in both NMRI and BALB/c nude mice. The xenografts originated from primary prostatic carcinomas (prostatectomy specimens), transurethral resection material, and metastatic lesions (pelvic lymph nodes and scrotal skin). Histological examination revealed that, in the course of several mouse passages (8 to 23), tumors retained their resemblance to the original patient material. The PC-295, PC-310, PC-329, and PC-346 tumors are dependent on androgens for their growth. The PC-324, PC-339, and PC-374 tumors are androgen independent, although growth of PC-374 tumors still seemed androgen sensitive. All tumors are diploid, except for the PC-374, which is tetraploid. The diploid PC-295 tumor has an additional small population of tetraploid cells. All xenografts displayed a heterogeneous expression pattern of the androgen receptor except for the PC-324 and PC-339 tumors in which the androgen receptor could not be detected. Prostatic acid phosphatase and prostate-specific antigen were retained during serial transplantation in all tumors but the PC-324 and PC-339. This panel of permanent human prostate tumor models comprises tumors representing both the androgen-dependent and -independent stages of human prostate cancer with various degrees of differentiation and, therefore, is of great value for the study of many aspects of growth and progression of human prostate cancer. (Am J Pathol 1996, 149:1055–1062)

Prostate tumor research has been hampered by a limited availability of appropriate tumor models representing the various stages of clinical prostate cancer. A relatively small number of *in vitro* human prostatic tumor cell lines has been established.[1] For years our laboratory has put much effort in the establishment of *in vivo* models from human prostate tumor specimens. Generally, transplantation of human prostatic tumor tissue in athymic nude mice has shown a very poor take rate of less than 5%. Until recently, only three lines could be developed from more than 150 heterotransplantations carried out in our laboratory. In 1977, the first androgen-dependent *in vivo* prostate tumor model, designated PC-82, was established in our laboratory,[2] followed by the development of two androgen-independent *in vivo* lines, PC-133 and PC-135 (unpublished results). In 1984, a second androgen-dependent *in vivo* model, PC-EW, was established by Hoehn and associates.[3] More recently, Fridman et al[4,5] reported a greatly enhanced take rate of (human) tumor cells transplanted into nude mice when cells were co-injected

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with laminin or a reconstituted basement membrane gel (Matrigel). Using the same technique, Pretlow et al[6] succeeded in growing 6 of 10 injected primary prostate carcinomas in athymic nude mice. These tumors were reported, however, to have a highly variable take and growth rate.[7] Recently, we were able to establish seven new prostate tumor lines by conventional subcutaneous transplantation techniques, without any pretreatment of the tumor tissue or host animal. This paper describes the establishment of these human prostatic xenograft models and reports on the resemblance of the tumor models with the corresponding patient tumor tissue with regard to morphology, secretion of prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA), and expression of the androgen receptor (AR).

Materials and Methods

Patient Material

Prostatic carcinoma specimens were obtained from radical prostatectomies (ie, untreated primary carcinomas), transurethral resections of the prostate (TURP; ie, hormone-refractory primary carcinomas, except for the PC-346, which was an untreated primary tumor), lymph node dissections, and metastatic lesions. The tumor was dissected from the surrounding tissue and transferred aseptically to the laboratory. Tumor tissue was cut into small pieces for transplantation into nude mice. Adjacent tumor tissue was processed for histological examination, and the remaining tissue was snap-frozen in liquid nitrogen and stored at −80°C.

Heterotransplantation

Transplantation of human prostate specimens was carried out in BALB/c and NMRI athymic nude mice. Athymic nude mice with the BALB/c background were obtained from Harlan Netherlands, Zeist, The Netherlands. NMRI nude mice, initially supplied by Harlan Netherlands, were obtained from the breeding colony of the Erasmus Animal Facilities. The NMRI nude mouse strain originates from the U.S. Naval Marine Research Institute, Bethesda, MD, and was introduced in Europe (Germany) in 1960.[8]

Small pieces of solid tumor tissue (approximately 30 mm^3) were subcutaneously implanted at both shoulders of intact males or male and female mice supplemented with testosterone (t) or 5α-dihydrotestosterone. Testosterone or 5α-dihydrotestosterone was supplemented by subcutaneous implantation of silastic tubing of 1.0 cm length filled with crystalline steroid (Steraloids, Pawling, NY) and sealed with silastic adhesive.[9] The animals were anesthesized with ether. Tumors were routinely propagated in intact or testosterone-supplemented male nude mice. To assess androgen-dependent behavior of the tumor, tissue pieces were also transplanted in intact female mice.

Tumor Characteristics

Tumor growth was followed weekly by caliper measurements. Tumor volume was calculated from the formula $V = (\pi/6)(d_1 \times d_2)^{3/2}$ with d_1 and d_2 being two perpendicular tumor diameters. Doubling times were estimated from a growth curve with the tumor volume on a semilogarithmic scale. After sacrifice of tumor-bearing mice, plasma was sampled. The xenografts were dissected, and part of the tumor tissue was fixed in buffered formalin and embedded in paraffin. These samples were used for routine histological examination (hematoxylin and eosin (H&E) staining) and for immunohistochemical staining for PAP, PSA, and AR. PSA and AR were detected by the monoclonal antibodies ER-Pr1 and F39.4.1, developed at the Department of Pathology, Erasmus University Rotterdam.[10-12] PAP was detected using a polyclonal antibody from Dakopatts, Glostrup, Denmark. Immunoreactivity was visualized by incubation with the appropriate biotinylated second antibodies and subsequent incubation with streptavidin-biotin-peroxidase complex (Dakopatts) and 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Basel, Switzerland) as described previously.[10,12] Each transplant generation tumor tissue was examined for its human origin. To confirm the nature of human tumor cells, a bisbenzimide (Hoechst H-33258) staining, which allows the discrimination between mouse (stromal) cells and human (prostate epithelial) cells, was performed.[13] The remainder of tumor tissue was snap-frozen in liquid nitrogen and stored at −80°C. Plasma and tissue homogenates were used for analysis of PSA. PSA analyses were carried out at the Department of Endocrinology, University of Utrecht, Utrecht, The Netherlands (Dr. M. A. Blankenstein) using an automated enzyme immunoassay (IMx-MEIA, Abbott, IL). Tumor ploidy was assessed using the method described by Vindelov et al[14] with some minor modifications. Chicken red blood cells were used as internal standard. The population with the lowest DNA content was assumed to represent the diploid cell population. Cell populations were considered tetraploid if the second peak had a DNA index between 1.9 and 2.1 and the fraction contained more than 10% of the nuclei measured.

Table 1. *Establishment of Human Prostate Tumor Lines Grown in NMRI Athymic Nude Mice*

Tumor model	Origin	Prior treatment of patient	Hormonal status of host animal	Growth in females	Lag phase (months)	T_d (days)
PC-295	LN	None	♂ + T*	−	2–3	18
PC-310	PC	None	♂ + DHT	−	4	13
PC-324	TURP	Bilateral orchiectomy	☿ + T	+	1–2	10
PC-329	PC	None	♂	−	4–5	ND
PC-339	TURP	LHRH	♂ + DHT	+	1–2	20
PC-346	TURP	None	♂	−	1–2	7
PC-374	SSM	LHRH + RT + AA	♂	+	2	12

T_d, tumor doubling time; LN, lymph node metastasis; PC, primary prostate tumor; SSM, scrotal skin metastasis; LHRH, LHRH agonist therapy; AA, anti-androgen; RT, radiotherapy; T, testosterone; DHT, dihydrotestosterone; ND, not determined.
*First mouse passage in Balb/c athymic nude mice.

In addition, the expression of PSA mRNA was determined by Northern blot analysis. Total RNA was isolated from approximately 200 mg of tumor tissue according to the method of Chomczynski.[15] PSA mRNA was hybridized with a 600-bp *Eco*RI-*Cla*I fragment derived from the PSA cDNA clone PA 75.[16] PSA-expressing LNCaP cells served as control.[17]

Results

Tumor Take

Heterotransplantation of more than 150 clinical prostate tumor specimens in our laboratory over the last 20 years resulted in the establishment of three *in vivo* models, PC-82, PC-133, and PC-135, permanently growing in BALB/c athymic nude mice. From 1988 to 1990 we continued to transplant 35 prostatic cancer specimens in BALB/c nude mice. Only 1 of 10 grafted pelvic lymph node metastases had a positive take resulting in a tumor line, designated PC-295, which was serially transplanted in NMRI nude mice from the second mouse passage onwards. No tumors could be established from 21 primary adenocarcinomas and 4 TURP specimens. This result underlines the low take rate of human prostatic tissues generally observed in BALB/c athymic nude mice. Strikingly, the primary take rate of human prostate tumor tissue in NMRI athymic nude mice was approximately 10 times higher (35%) than the previously observed take in BALB/c nude mice. From 1991 to 1992 we were able to establish 6 permanent tumor lines from a total of only 16 heterotransplantations using NMRI instead of BALB/c nude mice: 2 (PC-310 and PC-329) of 7 transplanted prostate-confined, untreated primary prostate adenocarcinomas, 3 (PC-324, PC-339, and PC-346) of 6 TURP specimens of relapsed tumors (except for the PC-346, which was an untreated tumor), and 1 (PC-374) from a scrotal skin metastasis. In Table 1, the origin and the conditions of transplantation of the established tumor lines are summarized. Although the number of animals is small, it seemed that tumor take and growth at onset did not relate to androgen levels in the host animal as tumors developed in either intact male mice or in male and female mice supplemented with testosterone or 5α-dihydrotestosterone. Once established in NMRI nude mice, all tumors could be serially propagated in BALB/c athymic nude mice as well.

Tumor Growth

Growth of the androgen-dependent tumor lines PC-295, PC-310, and PC-329 is relatively slow with a tumor doubling time of more than 12 days and a lag phase (time from transplantation to tumors reaching a volume of 50 mm^3) of more than 3 months. In contrast, the PC-346 tumor is a fast growing tumor line with an estimated doubling time of less than 10 days and a relatively short lag phase of 1 to 2 months. The androgen-independent tumor lines PC-324 and PC-374 have a moderate growth rate with a tumor doubling time of approximately 10 days and a lag phase of approximately 1 to 2 months. In contrast, the androgen-independent tumor PC-339 has a slow growth rate (tumor doubling time of 20 days) in addition to a lag phase of approximately 1 to 2 months (Table 1).

Of all tumor lines, four (PC-295, PC-310, PC-329, and PC-346) could not be grown in female nude mice, and they are therefore considered to be androgen dependent. The PC-295, PC-310, and PC-329 tumors are propagated in testosterone-supplemented male mice because the take rate seemed to be improved in these animals. Tumor growth was not affected by testosterone supplementation. The PC-324 and PC-339 tumor models grew equally well in male and female mice, and thus their growth is considered as independent of androgen. The PC-374 tumor could be grown in male and female mice

(Table 1), but the take and growth seemed to be retarded in female animals (data not shown).

Tumor Histology

The histology of the original patient material and the corresponding tumor line after several (7 to 21) mouse passages is shown in Figure 1. Tumor line PC-295, originating from a pelvic lymph node metastasis, represents a poorly differentiated tumor with cells organized in solid sheets and microacini. The PC-310 and PC-329 tumors, established from primary prostate cancers, are both moderately differentiated. Only the PC-329 tumor exhibits a cribriform growth pattern. The PC-324, PC-339, and PC-346 lines, developed from TURP material, are undifferentiated tumors lacking glandular structures. Except for the PC-346 tumor, these latter tumors are characterized by a small-cell phenotype. Strikingly, the PC-339 also exhibits foci of squamous cell differentiation, which could not be detected in the material of the patient. The PC-374 tumor originating from a scrotal skin metastasis shows areas with moderate differentiation (glandular and partly cribriform growth pattern) and solid areas lacking differentiation. Although most prostate tumors are known to be composed of a heterogeneous mixture of various growth patterns, the figures show that, in general, the original morphology of the tumors is largely retained and remains stable during serial passages of the tumor in nude mice.

Tumor Characteristics

All but one (PC-374) of the tumor models consist of DNA-diploid tumor cells. In addition to a diploid population, the PC-295 tumor appears to have a small population of tetraploid cells (>10% of the second peak with a DNA index of 1.9 and 2.1). The PC-374 tumor is the only DNA-tetraploid tumor. Glandular differentiation and expression of the AR and prostate-specific markers PAP and PSA were used as functional parameters to demonstrate the resemblance of the tumor lines at mouse passages 5 to 8 with the original tumor tissue (Table 2). AR expression (Table 2) correlated with androgen-dependent growth as is indicated in Table 1. Of all tumor lines, only the androgen-independent tumor lines PC-324 and PC-339 had lost the expression of PAP and PSA during heterotransplantation. In all other tumor lines, the expression of PAP and PSA corresponded with its expression in the original tumor, although PSA expression in most cases was less intense as that observed in the original patient material. Correspondingly, PSA could also be detected in the plasma of tumor-bearing mice and in homogenates of tumor tissue (data not shown). Finally, a PSA mRNA message was detected in all but the PC-324 and PC-339 tumor lines (Figure 2).

Discussion

Notwithstanding the importance of rat prostate (tumor) models, such as the normal rat ventral prostate, the spontaneously arisen Dunning, the chemically induced Pollard, and the hormonally induced Noble rat prostate tumor models in the understanding of growth-regulating mechanisms of normal and malignant prostatic tissue, the nonhuman origin of these models has been a considerable drawback. Unfortunately, the number of representative *in vivo* and *in vitro* models of human prostate cancer has been very limited, and as in almost all cases, these concern the hormone-refractory stage of human prostate cancer. So far, only the *in vitro* cell line LNCaP[17] and the *in vivo* models PC-82 and PC-EW[18,19] showed hormone-responsive growth.

Although in the past it has become evident that human prostatic tumor tissue is very difficult to grow as xenotransplant in athymic nude mice,[20] the present study describes the establishment of seven new hormone-responsive and unresponsive human prostate tumor lines *in vivo* in NMRI athymic nude mice without any pretreatment of the tumor tissue or the host animals. Although the comparison of the take rate of BALB/c *versus* NMRI nude mice is historical and not made with the same tumors, the increase in the success rate of human prostate tumors in the NMRI nude mouse strain is significant ($P <$ 0.01, χ^2 test). However, after establishment of the tumors in NMRI nude mice, all tumor lines could also be propagated in BALB/c nude mice, suggesting that, for the establishment of a tumor, special conditions are required that are obviously not present in BALB/c nude mice and that are not essential for propagation of the tumor. These special requirements and the underlying mechanisms are still unknown but seem to be more supportive in NMRI nude mice than in BALB/c nude mice. Host-derived (stromal) components are assumed to play a crucial role; they may determine the immunological response of the host, the vascularization of the graft, and the induction or suppression of stromal and epithelial growth factors. Reports of an increased take rate of (human) tumor tissue when injected in Matrigel in athymic nude mice likewise suggest an important role for components of the basement membrane.[5,6]

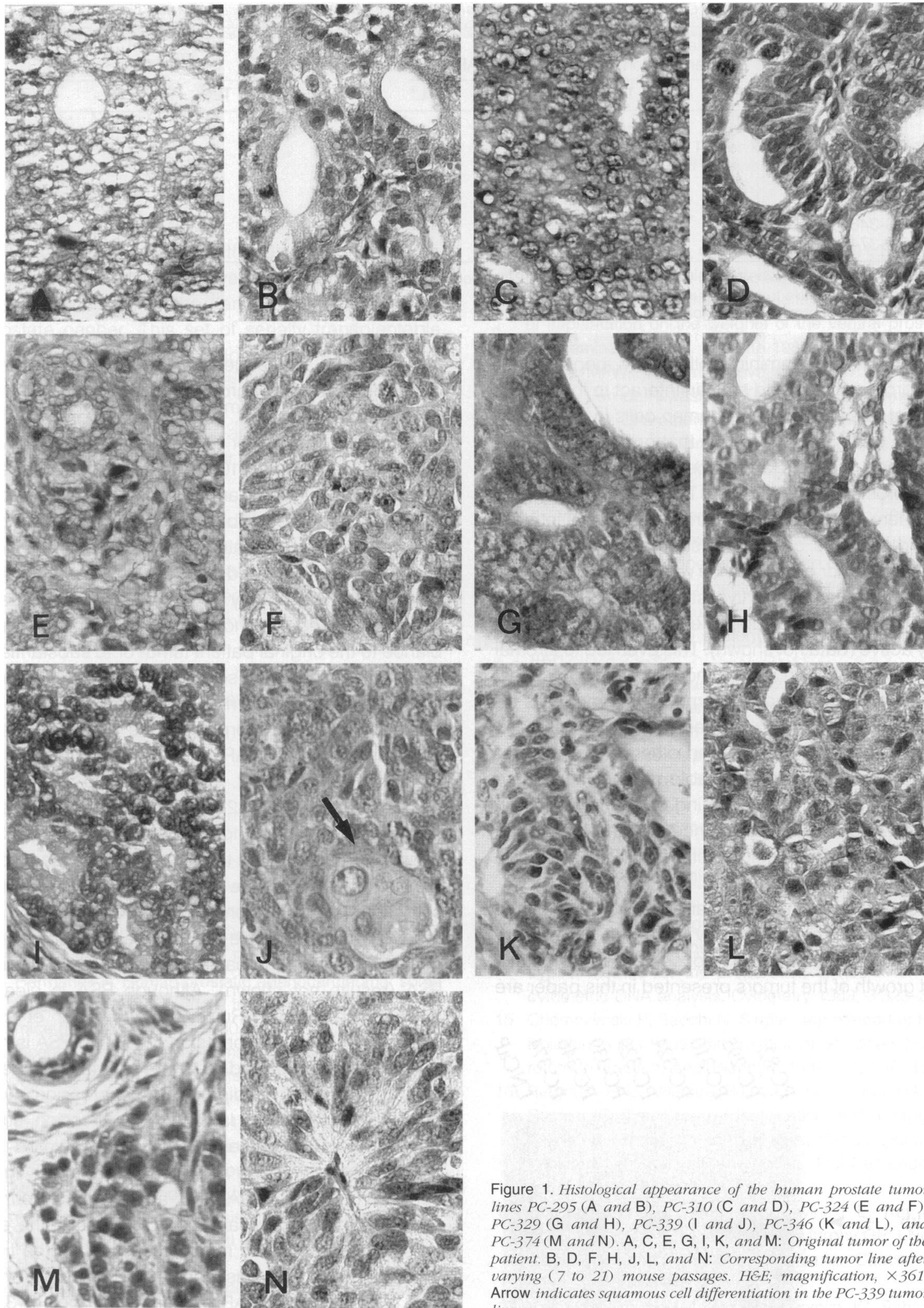

Figure 1. *Histological appearance of the human prostate tumor lines PC-295* (A *and* B), *PC-310* (C *and* D), *PC-324* (E *and* F), *PC-329* (G *and* H), *PC-339* (I *and* J), *PC-346* (K *and* L), *and PC-374* (M *and* N). A, C, E, G, I, K, *and* M: *Original tumor of the patient.* B, D, F, H, J, L, *and* N: *Corresponding tumor line after varying (7 to 21) mouse passages. H&E; magnification,* ×*361.* Arrow *indicates squamous cell differentiation in the PC-339 tumor line.*

Table 2. *Comparison between Characteristics of the Original Tumor Tissue and the Derived Tumor Line*

	Glandular differentiation		PAP[1]		PSA[2]		AR[3]	
Tumor model	PT	TL	PT	TL*	PT	TL*	PT	TL*
PC-295	+	±†	+	+	+	+	+	+
PC-310	+	+	+	+	+	+	+	+
PC-324	−	−	±	−	±	−	±	−
PC-329	+	+	+	+	+	+	+	+
PC-339	−	−	+	−	+	−	±	−
PC-346	−	−	+	±	+	+	±	+
PC-374	±	±	+	+	±	±	+	+

PAP, PSA, and AR were detected immunohistochemically. PT, patient tumor, TL, tumor line.
*Expression after five to eight mouse passages.
†± indicates a heterogeneous expression pattern.

Components like laminin, collagen IV, heparan sulfate-proteoglycan, and enactin interact to form a matrix that seems to support human cells in the host.[21] Gleave et al[22,23] have shown that prostate and bone fibroblasts induced and accelerated growth of human prostate cancer cells. Their findings suggest that there is a bidirectional stromal-epithelial interaction in prostatic cancer; ie, stromal cells affect the epithelial tumor cells and *vice versa*. Indeed, the morphology of the supportive stromal cells within the transplanted tumors suggested that these cells were very active (data not shown). This even led to stromal overgrowth of the human tumor cells in some cases. Moreover, transformation of murine stromal cells after transplantation of human epithelial cells has been observed by our group[24] and others[25] and has resulted in serially transplantable murine mesenchymal tumors. Regular monitoring of the human origin of the tumor by a fluorescence staining procedure using bisbenzimide (Hoechst dye 33258) is, therefore, strongly recommended.[13]

Depending on the take and growth rate of the various lines, the seven new xenograft models are propagated up to the 25th mouse passage. The take and growth of the tumors presented in this paper are fairly constant and range from 60% in the androgen-dependent, very slow growing tumors to 100% in the androgen-independent, relatively fast growing tumors. In all of our tumor lines, tumors can be grown up to sizes exceeding 1000 mm^3. This is in contrast to the report of Pretlow and co-workers,[26] who experienced an irreproducible take and growth of four newly developed prostate xenograft models of which the cell line designated CWR22 was described in more detail separately.[27] Unfortunately, no data were presented to show the phenotypic resemblance to the original patient tumor or to substantiate its reported androgen sensitivity. The newly established xenografts presented in this paper continue to retain their original morphology as well as the expression patterns of AR and the prostate-specific markers PSA and PAP. Only in the two androgen-independent lines PC-324 and PC-339 could expression of these markers not be detected. The PC-339 tumor expressed AR in the first mouse passage but did not have detectable levels of PAP and PSA (results not shown). In contrast, the PC-324 tumor already lacked detectable AR expression after the first mouse passage, whereas it still expressed PAP and PSA (results not shown). Although PSA is clearly androgen regulated, Ruizeveld de Winter et al[28] have shown that co-expression of AR and PSA is not absolute. They reported that, in 41 of 49 prostatectomy specimens studied, PSA expression was present in tumor areas lacking AR expression, suggesting that PSA expression can be regulated by factor(s) other than the AR. As all other tumor lines continue to express PSA, PAP, and AR in the course of several mouse passages and the loss of the expression has occurred relatively fast within the first two to three mouse passages, it is concluded that loss of PSA, PAP, or AR expression in the PC-324 and PC-339 xenograft models may be due to an *in vivo* selection of primarily PSA-, PAP-, and AR-negative tumor cells.

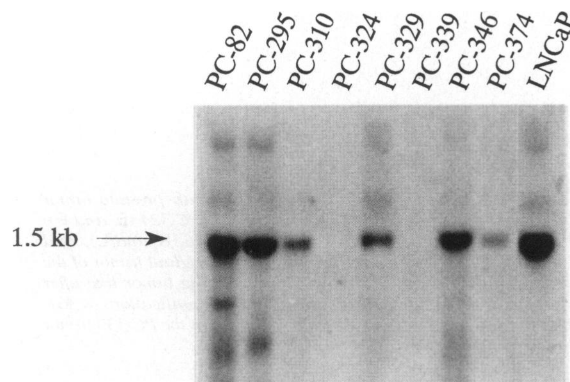

Figure 2. *Northern blot of PSA mRNA expression of the human prostate tumor xenografts. LNCaP cells served as a positive control.*

It is concluded that all of these xenografts can be considered as representative of the original prostate tumor tissue and of the various stages of clinical prostate cancer. This set of serially transplantable prostate tumor models is highly relevant for human prostate cancer research as it provides the unique possibility to study and compare many aspects of both androgen-dependent and androgen-independent human prostate cancer.

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