Increased Incidence of Luteinizing Hormone-releasing Hormone Receptor Gene Messenger RNA Expression in Hormone-refractory Human Prostate Cancers

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ABSTRACT

There are few options for treating hormone-refractory prostate cancer (PC). Various studies indicate that luteinizing hormone-releasing hormone (LHRH) agonists may have a direct inhibitory effect on prostate tumors mediated by specific LHRH receptors. One study evaluated LHRH receptors in hormone-dependent PC tissue, but no data have thus far been obtained on the presence of LHRH receptors in benign prostatic hyperplasia (BPH) and especially hormone-refractory PC in patients. Thus, it is not yet clear whether LHRH receptors indicate tumor-related differentiation or even hormone-refractory dedifferentiation or are likewise associated with BPH. The aim of this study was to determine the rate of LHRH receptor mRNA expression in BPH and in primary, potentially androgen-dependent and in hormone-refractory PC with clinical progression.

Multiplex reverse transcription-PCR was used to simultaneously detect the expression of mRNA for LHRH receptors and β-actin in 48 patients with BPH, 14 with a primary, possibly hormone-dependent, prostate carcinoma (PPC), and 18 with a hormone-refractory prostate carcinoma (HRPC).

Sixteen of 18 samples with HRPC showed intact RNA and expressed mRNA for LHRH receptors (100%). However, the RNA-intact PPC and BPH showed significantly lower expression of mRNA for LHRH receptors (46.2 and 55.3%, respectively; variance analysis: $P = 0.0017$).

The significantly higher expression of mRNA for LHRH receptors in HRPC indicates that therapeutic concepts should be developed that target this site of action. In addition to possible direct effects of LHRH agonists or antagonists demonstrated previously *in vitro*, it seems useful to apply targeted cytotoxic LHRH analogues or monoclonal antibodies.

INTRODUCTION

PC[2] is the second most common cause of death among male cancer patients in Western industrialized nations (1). Metastases are already found at the initial diagnosis of PC in 30% of cases and subsequently develop despite treatment in another 20%. Although organ-confined PC can be managed surgically or by radiotherapy (2), metastatic PC is initially treated by androgen withdrawal. However, hormone-refractory tumor progression usually occurs after a few years and can then be managed only by palliative therapy. In the past few years, LHRH agonists have been increasingly used for the palliative treatment of PC. Hypothalamic decapeptide LHRH plays a key role in the regulation of reproduction. This hormone formed in the hypothalamus stimulates the gonadotropic hormones LH and FSH in the anterior pituitary lobe, which in turn control the gametogenic and hormonal function of the gonads. Normally, the antitumor effect of LHRH agonists is based on the inhibition of the pituitary-gonadal axis (3, 4). LHRH agonists and antagonists are increasingly used in the treatment of advanced PCs (5–7). Various findings point to an *in vitro* effect on LHRH receptors demonstrated in both androgen-sensitive and -independent PC cell lines (8–15). In one study, the expression of mRNA for LHRH receptors was found in a larger number of human PC tissue samples. Here, both radioligand binding and RT-PCR detection revealed (the presence of) LHRH receptors in >80% of the cases (16).

However, the above study only examined BPH tissue in a very small number of cases ($n = 3$). Thus, it is not yet known whether the presence of LHRH receptors in PPC tissue is more frequent, equal, or even reduced compared with BPH, which would suggest carcinoma-related differentiation. In this study, we examined tumor samples from patients with HRPC, because, to the best of our knowledge, no data are yet available on this topic. Discussions have already focused on the possibility of tumor dedifferentiation even with an increased incidence of mRNA for LHRH receptor expression at the stage of hormone-refractory progression. Some authors have already suggested antitumor therapy of HRPC using agonists or antagonists acting on the LHRH receptor (17). The mere detection of mRNA for

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[2] The abbreviations used are: PC, prostate cancer; LHRH, luteinizing hormone-releasing hormone; BPH, benign prostatic hyperplasia; FSH, follicle-stimulating hormone; PPC, primary, possibly hormone-dependent prostate carcinoma; HRPC, hormone-refractory prostate carcinoma; TURP, transurethral resection of the prostate gland; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR.

LHRH receptor expression in HRPC cell lines is not sufficient to confirm this hypothesis made by other authors.

The aim of this study was to detect the expression of mRNA for LHRH receptors in hormone-sensitive PC, HRPC, or BPH tissue.

MATERIALS AND METHODS

Tissue Samples. Thirty-two PC tissues and 48 BPH samples were collected from patients who underwent surgery in our department. Examinations were conducted in agreement with the Helsinki Declaration. Institutional Review Board approval was obtained for this study. Each patient signed a consent form approved by the Committee on Human Rights in Research of our institution.

Fourteen tissue samples were taken from patients with primary, untreated PC (patient ages, 54–73 years; mean age, 62.1 ± 1.7) during radical prostatectomy in the following manner. Immediately after removing the gland from the operation site, a 7–9-mm-thick slice was cut out of the sample in the area of the positive punch biopsy, divided into 12 blocks of approximately the same size, and stored at −80°C; the remainder was put in formalin for conventional histology. Only those samples containing almost exclusively, *i.e.*, a minimum of >90%, pure carcinoma tissue were included for further processing. With this method, the 12 blocks from each of the 42 different biopsy specimens had to be processed to obtain 14 different samples that fulfilled the above-mentioned criteria. Five 30-μm-thick slices were cut from the sample blocks in the cryostat (Kryostat 2800; Leica Instruments, Nussloch, Germany) and submitted to further processing (see below). We applied the above procedures, because the RNA quality of the tumor material obtained by laser microdissection was not adequate for RT-PCR because of extensive sample processing.

Tissue slices from 42 BPH patients (patient ages, 48–87 years; mean age, 67.8 ± 1.4), who underwent TURP, were initially stored at −80°C. These frozen tissue slice blocks were subsequently prepared with the cryostat as described above.

Tissue slices from 18 patients with a HRPC (patient ages, 57–88 years; mean age, 72.1 ± 2.4), who underwent palliative TURP, were also processed by the same sampling method. All of these patients showed progression in their clinical status and PSA values; 17 of them had had orchiectomy and were still on secondary hormone therapy in most cases (aminoglutethimide, antiandrogens, and estramustine-17β-dihydrogen phosphate); one patient received only hormone withdrawal therapy.

RNA Extraction and RT-PCR. RNA was prepared from tissue samples using RNAClean (Hybaid, Heidelberg, Germany) according to the manufacturer's instructions. RNA quality was exclusively evaluated electrophoretically (presence of 28/18S RNA) and RT-PCR for β-actin (see below). All reagents for RT-PCR and the protocol were obtained from the Preamplification kit (Life Technologies, Inc., Karlsruhe, Germany).

Initially, 1 μl of oligo(dT)$_{12–18}$ (0.5 μg/μl) was annealed to 1 μl (corresponding approximately to 0.5–1.0 μg) RNA by incubation at 70°C for 10 min in a reaction volume of 12 μl of cDNA. Synthesis was performed after adding 2 μl of 10× PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 2 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of deoxynucleotide triphosphate mix (10 mM each dATP, dCTP, dGTP, and dTTP), 1 μl of SuperScript II RNase H$^-$ reverse transcriptase (200 units/μl) for 50 min at 42°C. Finally, the enzyme was inactivated at 65°C for 15 min; remaining RNA was degraded by incubation with 1 μl of *Escherichia coli* RNase H (2 units/μl) for 20 min at 37°C.

For multiplex PCR, 2 μl of cDNA were placed into an 18-μl reaction volume containing 2 μl of 10× PCR buffer (see above), 1.2 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM deoxynucleotide triphosphate mix (see above), and 0.2 μl of Taq DNA polymerase (Roche, Mannheim, Germany). Primers for LHRH receptor were: sense, 5′-GAC CTT GTC TGG AAA GAT CC-3′; antisense, 5′-CAG GCT GAT CAC CAC CAT CA-3′ (18); and for β-actin: sense, 5′-GTG GGG CGC CCC AGG CAC CA-3′; and antisense, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. Thirty-five cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min) were run on a 9600 thermal cycler (Applied Biosystems, Ueberlingen, Germany) for LHRH receptors and β-actin. Negative controls were concomitantly run to confirm that the samples were not cross-contaminated. A sample with 1 μl of diethylpyrocarbonate-treated water, instead of reverse transcriptase, was concomitantly examined for each of the reaction units described above. Two control assays were performed from each tissue. Subsequently, the PCR products were subjected to gel electrophoresis in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. Variables were examined for Gaussian distribution and by ANOVA, and the results were given as mean ± SE. The significance of differences ($P \leq 0.05$) between the three groups was assessed by the least squared differences test using the Statistical Package from STATISTICA (StatSoft, Tulsa, OK).

RESULTS

Thirteen of 14 PPC samples (93%) expressed the "housekeeping gene" β-*actin* as proof of RNA integrity, and 6 expressed (46.2%) mRNA for LHRH receptors, although there was no statistically significant correlation between the presence of mRNA for LHRH receptors and the Gleason score (mean, 6.9 ± 0.5), pathological staging or grading, the preoperative PSA value, or age (Table 1). There was no significant variation in β-*actin* gene mRNA expression between the three groups. β-*actin* was expressed in 38 of 48 patients after TURP as proof of intact RNA; expression of mRNA for LHRH receptors was detected in 22 cases (55.3%). No significant correlation was found between the expression rate of mRNA for LHRH receptors and patient data such as PSA values or age (Table 1). Tissue from HRPC patients expressed mRNA for β-actin in 88.9% of the cases (16 of 18). mRNA for LHRH receptors were expressed in all of the HRPC cases (100%; 16/16; $P = 0.0017$; Table 1). Again, there were no significant correlations between age, PSA values, and antihormone therapy. Examples of typical electrophoresis patterns after RT-PCR analyses are given in Fig. 1 for each group of patients.

DISCUSSION

LHRH receptors and direct antiproliferative effects of LHRH analogues on tumor growth were experimentally and clinically demonstrated not only in PC but also in other malig-

Table 1 General features and RT-PCR data

The data are given as mean values (SE) for general features and as total numbers/% for RT-PCR results. See text for details.

	HRPC (n = 18)	PPC (n = 14)	BPH (n = 48)	ANOVA P
General features				
Age of patients (yr)	72.1 (2.4)	62.1[a] (1.7)	67.3 (1.4)	0.013
PSA value, ng/ml	119.8 (73.2)	33.1 (11.3)	8.0[a] (1.3)	0.029
Gleason Score		6.9 (0.5)		
Tumors = T3 (%)		78.6		
RT-PCR				
Total number of β-actin-positive samples	16	13	38	
β-*actin*-positive samples from total number of samples, %	88.9	92.9	79.2	0.39
Total number of LHRH receptor-positive samples	16	6	21	
LHRH receptor-positive from β-*actin*-positive samples, %	100	46.2[a]	55.3[a]	0.0017

[a] $P \leq 0.05$ *versus* HRPC, respectively, by ANOVA and LSD test.

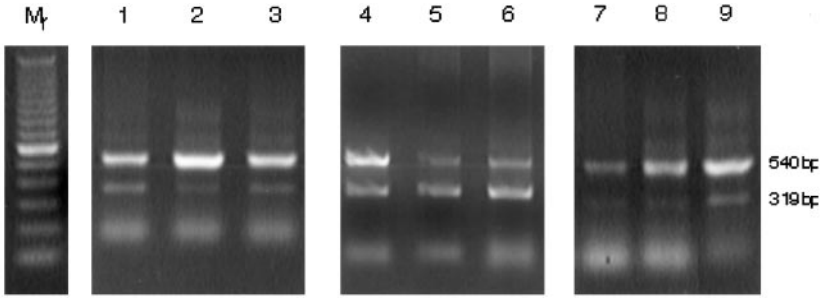

Fig. 1 The typical RT-PCR product pattern after electrophoresis in 1.5% agarose gels for each patient group (540-bp product for β-actin mRNA and 319-bp product for LHRH receptor mRNA). *Lanes 1–3,* PPC; *Lanes 4–6,* HRPC; *Lanes 7–9,* BPH. The 100-bp ladder (Life Technologies, Inc.) was used as a size standard.

nant tumors (19–26). The efficacy of LHRH analogues after long-term application is usually based on a down-regulation of pituitary receptors and thus suppression of LH and FSH release, which in turn dramatically reduces testosterone release. Therapeutic administration of LHRH antagonists rapidly decreases LH and FSH and thus testosterone. Several groups have shown the presence of local LHRH receptors and a possible direct antitumor effect of LHRH agonists and antagonists on human PC cell lines (9, 11–13, 27, 28). Experimental studies demonstrating a direct antitumor effect of LHRH antagonist in HRPC cell lines seem particularly interesting with regard to the limited therapeutic options in HRPCs. In one study, LHRH receptors were only detected in HRPCs (16). The number of positive specimens detected by radioligand binding was nearly the same as the number of mRNA for LHRH receptor expression by RT-PCR; however, the relatively high number (86%) of LHRH receptor-positive patient samples was not obtained in our study in which only 6 of the 13 patients (46%) with β-*actin*-positive RT-PCR (*i.e.,* intact RNA) were LHRH receptor positive. A possible explanation may be the complete lack of neoadjuvant hormone therapy in our PPC patients. The authors of the above-mentioned study only commented on the use of hormone therapy in a small group (n = 2) with longer treatment times. It is unclear whether the tissue samples were also obtained during radical prostatectomies (16). If they were taken during TURP, this may mean that only patients were considered who had symptomatic progression, especially obstructive symptoms. In particular, it is still unclear whether human HRPCs even possess LHRH receptors or whether they are lost during tumor dedifferentiation or occur more frequently. Data on the expression of mRNA for LHRH receptors in BPH tissues have likewise been inadequate until now. According to our data, there was no significant difference in the expression rate of mRNA for LHRH receptors between BPH (55.3%) and PPC (46.2%), but there was a significantly higher occurrence (100%) in HRPC as compared with the other two groups. Some authors have suggested treatment with antitumor agents, such as targeted cytotoxic LHRH analogues (29, 30) or monoclonal antibodies (31). An antitumor effect has only been shown *in vitro* in some hormone-dependent cell cultures. Studies evaluating this hypothesis have not yet been performed in patients with HRPC but appear to be justified in the light of the present results.

In the past, the potential mechanisms acting on LHRH receptors in prostate carcinoma have been identified as possible antitumor strategies for treating specific HRPCs. This study is the first to show that HRPC involving clinical progression has a higher incidence of LHRH receptor mRNA expression than in hormone-dependent prostate carcinomas. Our results may indicate that HRPC could be managed by a novel antitumor therapy that targets mechanisms acting on the LHRH receptors.

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