Research

Reduction in PSA messenger-RNA expression and clinical recurrence in patients with prostatic cancer undergoing neoadjuvant therapy before radical prostatectomy

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Abstract

Background: We assessed the incidence of micro-metastases at surgical margins (SM) and pelvic lymph nodes (LN) in patients submitted to radical retropubic prostatectomy (RP) after neoadjuvant therapy (NT) or to RP alone. We compared traditional staging to molecular detection of PSA using Taqman-based quantitative real-time PCR (qrt-PCR) never used before for this purpose.

Methods: 29 patients were assigned to NT plus RP (arm A) or RP alone (arm B). Pelvic LN were dissected for qrt-PCR analysis, together with right and left lateral SM.

Results: 64,3% patients of arm B and 26.6% of arm A had evidence of PSA mRNA expression in LN and/or SM. 17,2% patients, all of arm B, had biochemical recurrence.

Conclusions: Qrt-PCR may be more sensitive, compared to conventional histology, in identifying presence of viable prostate carcinoma cells in SM and LN. Gene expression of PSA in surgical periprostatic samples might be considered as a novel and reliable indicator of minimal residual disease after NT.

Background

Prostatic carcinoma has an unpredictable clinical behavior. This cancer is widespread in males with 1/5 of men being affected throughout life. Every year 209,900 new cases are diagnosed in the USA. However, only 20% of them will lead to demise of the patient with an approximately 9-year reduction in expected survival. Thus, prostate cancer remains the second cause of oncologic death in males in Europe and in the USA [1].

Because of patient-to-patient heterogeneity in the clinical behavior of this disease, prognostic markers that may help tailor therapeutic strategies to individual clinical situations are continuously re-assessed. This frequent reassessment leads to modifications of clinical criteria utilized for the selection of therapeutic strategies which, in turn, make difficult the comparison and interpretation of clinical results among different therapeutic strategies.

In particular, there is a well known discrepancy between post-surgical progression after radical prostatectomy and pathological staging. Often local or systemic progression is observed in patients theoretically at low risk such those staged as pT2N0 [2,3]. Thus, current pathological staging cannot comprehensively evaluate neoplasm extent, aggressiveness and risk of progression. Clinical recurrence after surgical excision could be ascribed to microscopic nodal metastases and/or micro-invasion at the margins of resection missed by pathological staging. To complement pathological staging, new diagnostic and prognostic markers (SHBG [4], p53, bcl2 Human Callicrein-2, PSMA, and so forth) are under evaluation. Reverse Tanscriptase Polymerase Chain Reaction (RT-PCR) for determination of prostate-specific antigen (PSA) can detect a single prostate cancer cell in a tissue sample [5,6]. The high sensitivity of this technique may increase the accuracy of staging in low tumor burden cases and, consequently, be a more appropriate molecular marker for the determination of the effectiveness of a neoadjuvant therapy such as Maximum Androgenic Blockage (MAB) before radical prostatectomy. In fact, this therapeutic approach is believed to induce a down staging of the disease. However, it has been questioned whether pre-operative hormonal blockade may introduce a bias in pathological staging due to the iatrogenically induced tissue changes[3,7].

In this study, we assessed the incidence of micro-metastases at surgical margins and pelvic lymph nodes in patients submitted to radical prostatectomy after neoadjuvant MAB or to radical prostatectomy alone. We compared traditional histopathological staging to molecular detection of PSA using Taqman-based quantitative real-time PCR (qrt-PCR) never used before for this purpose.

Materials and methods
Study design and patients' population
This is a report about an open randomized monocentric pilot study with parallel arms, approved by the Ethics Committee of our Institution. Thirty-three < 80 year-old patients were accrued from July 2000 to June 2002. All were clinically staged as having a previously untreated, histologically confirmed T1-T2 prostate cancer. Patients were randomly assigned to neoadjuvant hormonal therapy plus radical prostatectomy (arm A) or radical prostatectomy alone (arm B). Clinical and pathological stages were determined according to the 1997 American Joint Committee-International Union Against Cancer TNM System. Pre-treatment evaluation included clinical history, digital rectal examination, serum PSA determination using Hybritech technique, routine blood tests, trans-rectal ultrasound, confirmation of out-side diagnosis, abdominal computerized tomography and radioisotope bone scan.

Patients in both arms underwent pelvic lymphadenectomy and radical retro-pubic prostatectomy 55 to 70 days from randomization. Neoadjuvant therapy consisted of: Nilutamide 150 mg daily until surgery and subcutaneous administration of Buserelin Depot 9,9 mg on the day of randomization.

Surgical procedure
All patients underwent radical retro-pubic bladder neck sparing prostatectomy with or without nerve sparing. Bilateral pelvic (iliac and obturator) node dissection was consistently performed and one right and one left node were taken proximal to the iliac bifurcation, and preserved in liquid nitrogen for subsequent qrt-PCR analysis. In addition, the right and left lateral margins of resection were submitted for pathological evaluation after dissection of a small wedge for cryostatic preservation (at least 0,5 cm of lenght). In all cases surgeons and pathologists have checked the integrity of prostatic capsule in order to be sure that these strips had been removed outside the capsule (non containig prostatic tissue).

Pathological staging and molecular analysis
Pathological staging was traditionally assessed by immunohistochemistry. In addition, the expression of the tumor associated antigen PSA was quantified by means of qrt-PCR in surgical margins and lymph nodes.

Tissue biopsies were immediately stored in liquid nitrogen until use. Once thawed, samples were minced with a mechanical shredder. Total RNA was then isolated using RNeasy Mini kits (Qiagen, Santa Clarita, CA) following manufacturer's guidelines. One μg of total RNA was reverse transcribed into cDNA using 1 μl of oligo-dT primers (1 μg/μl, GibcoBRL), 5 μl first strand buffer (Gibco-BRL), 2 μl 0.1 M DTT, 2 μl magnesium sulfate (GibcoBRL), 1 μl of 10 mM dNTP and 1 μl Superscript-II (SS-II, GibcoBRL). The reaction mixture was heated to 65°C for 10 minutes before adding SS-II, then synthesis was continued with 1 hour at 42°C. cDNA was stored at -20°C until use.

The expression of PSA and of a housekeeping gene (i.e., β-actin) was assessed by qrt-PCR as performed by the ABI Prism 7700 Sequence Detection System (Perkin Elmer, Foster City, CA) [8,9]. Probes and primers (See Table 1) were designed to span intron-exon junctions in order to avoid genomic DNA amplification.

To generate a standard curve for each gene, 1 μl of cDNA obtained from normal prostate was added to the following reagents: 1 μl Taqman primers (10 μM, Perkin Elmer), 5 μl 10 × PCR buffer (Perkin Elmer), 11 ml MgCl$_2$ (25 mM), 1 ml dNPT (10 mM) and 0.5 μl AmpliTaq Gold (Perkin Elmer) in a final volume of 50 μl. The thermal

Table 1: Probes and primers used

	Probe	Forward	Reverse
PSA	6FAM-CAGCATTGAACCAGAGGAGTTCTTGACCC-TAMRA	5'-GACCACCTGCTACGCCTCA-3'	5'-GGAGGTCCACACTGAAGTTTC-3'
β-actin	6FAM-TCAAGATCATTGCTCCTCCTGAGAGCGC-TAMRA	5'-GGCACCCAGCACAATGAAG-3'	5'-GCCGATCCACACGGAGTACT-3'

cycler conditions for this PCR reaction were as follows: after 10 minutes at 95°C, 15 seconds at 95°C followed by 1 minute at 60°C were repeated 40 times. The equation describing the standard curves was used to extrapolate the absolute copy number of PSA and β-actin. Quantitative real time PCR of sample cDNA and standard amplified cDNA was conducted in a 25 µl final volume mixture containing primers, probe and 1 × Taqman Master Mix (Perkin Elmer) at optimized concentrations (probe: 200 nM, primers: 400 nM). The TaqMan ABI Prism 7700 Sequence Detection System allows running 96 samples at a time. Thermal cycler parameters included 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles involving denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. To ensure that probes and primers in use did not amplify genomic DNA, we extracted genomic DNA from a normal prostate biopsy and tested it with the Taqman primers. Given the small size of the amplicons (<100 bp), a FluorImager 595 (Molecolar Dynamics, Sunnydale, CA) scanning system was used to detect the fluorescence of the Vistra Green fluorescent intercalator, which was adopted instead of ethidium bromide to visualize the bands of the agarose gel after electrophoresis. An Argon laser for excitation (488 nm) and an emission band-pass filter 530 DF30 were applied.

For qrt-PCR purposes, probes were labeled with a fluorescent reporter dye at the 5'-end (6-carboxyfluorescein, 6-FAM; emission λ_{max} = 518 nm) and a quencher dye at the 3'-end (6-carboxytetramethylrhodamine, TAMRA; emission λ_{max} = 582 nm). Probe and primers were designed to obtain amplicons less then 100 bp in length in order to enhance PCR efficiency. Furthermore, they were designed to span an intro-exon junction in order to avoid genomic DNA amplification. Since both the amount of total RNA added to each reverse transcription reaction tube (based on waive length absorbance) and its quality (i.e. degradation) are not reliable parameters as measures of starting material, transcripts of a housekeeping gene were quantified as endogenous control. Beta-actin (β-actin) is one of the most used aspecific housekeeping genes, but according to the experimental conditions, other internal references can be adopted (e.g. leukocyte markers). For each experimental sample the value of both the target and the housekeeping gene are extrapolated from the respective standard curve. The target value is then divided by the endogenous reference value to obtain a normalized target value independent from the starting material amount. The results were reported as number of PSA copies per 100,000 β-actin copies.

Follow up
Patients were seen for follow up 50 days after surgery and every 3 months thereafter during the first year, every 6 months during the subsequent four years and then annually. At each visit serum PSA was performed. Imaging studies or biopsies were performed as appropriate.

Statistical analysis
Statistical analysis was performed on 29 valuable patients randomized and included in the study. For each patients (arm A or B) qrt-PCR results from lateral surgical margins and pelvic nodes were analyzed. Statistical evaluation and data elaboration was made using SAS's software (Statistical Analysis System). Continuity Adjust Chi-Squared test was used to assess differences in qrt-PCR results between arm A and B. P values of = 0.05 were considered statistically significant. P-values are given as two-tailed.

Results
A total of 33 eligible patients were enrolled and randomized. Of these, 29 were evaluated for analysis. Four patients did not complete the protocol. One dropped out for noncompliance, one for pneumonia, one for allergy to Nilutamide and one because the sample was inadequate to perform qrt-PCR analysis. Of 29 patients, 15 (51,7%) underwent neo-adjuvant MAB treatment (arm A) and 14 (48,3%) received prostatectomy alone (arm B). Five of the 29 patients (17,2%) experienced biochemical recurrence after a mean follow-up of 19 months, all of these patients belonged to arm B.

Pathological and molecular findings
In the neo-adjuvant arm A 5/15 (33,3%) patients were staged as pT2a-G2. All of them were negative at qrt-PCR analysis for PSA expression. One of 15 patients (6,6%) was staged as pT2a-G3 and resulted positive at qrt-PCR. Four of the 15 patients (26,8%) were pT2b-G2: 2 resulted negative and 2 positive at qrt-PCR. Five of the 15 (33,3%) patients were pT3b-G2 and 4 resulted negative and 1 positive to qrt-PCR (See Table 2).

The ratio of PSA mRNA expressing patients according to qrt-PCR in arm A was of 26,6% (4/15).

Table 2: Pathological and qrt-PCR data of arm A patients.

N° pts	%	Path. staging	qrt-PCR (-)	qrt-PCR (+)
5	33,3	T2a-G2	5	0
I	6,6	T2a-G3	0	I (surgical margins)
4	26,8	T2b-G2	2	2 (surgical margins)
5	33,3	T3b-G2	4	I (surgical margins)

Table 3: Pathological and qrt-PCR data of arm B patients.

N° pts	%	Path. staging	qrt-PCR (-)	qrt-PCR (+)
4	28,6	T2a-G2	3	I (surgical margins)
6	42,8	T2b-G2	I	5 (2 pts positive nodes)
4	28,6	T3b-G2	I	3 (2 pts positive nodes)

Table 4: Disease recurrence

Pathological staging	qrt-PCR result
T2a-G2	(-)
T2b-G2	(+)
T2b-G2	(+)
T2b-G2	(-)
T3b-G2	(+)

In arm B, 4/14 (28,6%) patients were pT2a-G2 of this 3 negative and one positive at qrt-PCR analysis; 6/14 (42,8%) patients were pT2b-G2, one negative and 5 positive to qrt-PCR; 4/14 (28,6%) patients were pT3b-G2, one negative and 3 positive for PSA expression in periprostatic tissues (See Table 3)

Overall 64,3% (9/14) of the patients had evidence of PSA mRNA expression in arm B, while 26.6% (4/15) resulted molecularly positive in arm A (Fisher exact test = 0.04).

Pathological and molecular finding of the patients who had biochemical recurrence are reported in Table 4.

Statistical analysis
Analysis was performed on patients from arm A or B whether they had or had not cumulative evidence of PSA mRNA expression by qrt-PCR in lymph nodes and/or lateral surgical margins. Nine of 14 (64,29%) patients expressed PSA in arm B and four of 15 (26,67%) in arm A (See Table 5).

Continuity Adjust Chi-Square (a Chi-Square modified for not continuous variable) was calculated and resulted in *0.0965*, this value trend to statistical significance. This results suggests a difference between arm A and B, although not statistically significant (See Table 6). In Table 7 are reported assessment of Relative Risk and his confidence limits. Value founded show the risk to be positive if not treated with neoadjuvant therapy respect to treated arm; the risk to have positive surgical margins or nodes to qrt-PCR is 2,41 higher in not treated patients (arm B).

Discussion
Radical Prostatectomy is widely performed in early prostate cancer and it may lead to cure as long as the cancer is confined to the prostate and all neoplastic cells are removed [10].

Clinical staging is inaccurate and a significative number of patients with prostate cancer defined as cT1-2 are found to have positive surgical margins. This in turn is associated with increased chances of cancer recurrence and

Table 5: Treatment Response

	Negative	Positive	Total
Arm B	5	9	14
%	17.24	31.03	48.28
Row Pct	35.71	64.29	
Col Pct	31.25	69.23	
Arm A	11	4	15
%	37.93	13.79	51.72
Row Pct	73.33	26.67	
Col Pct	68.75	30.77	
Total	16	13	29
	55.17	44.83	100.00

Table 6: Statistics for Table of Treatment by Response

Statistic used	DF	Value	Prob
Continuity Adj. Chi-Square	1	2.7621	0.0965

Table 7: Estimates of the Relative Risk

Type of Study	Value	95% Confidence Limits
Cohort Sample Size = 29	2.4107	0.9554

progression within 5 years from the surgical resection of the primary tumor [11].

Neoadjuvant hormonal therapy may reduce the rate of margin positiveness, therefore, decreasing the chance of observing a local recurrence after Radical Prostatectomy [12]. However, it has been suggested that after neoadjuvant hormonal therapy residual foci of atrophic glands can be difficult to identify with hematoxylin and eosin staining, increasing the possibility that pT may be under staged [13-15].

Clinical randomized studies adopting neoadjuvant therapy before radical prostatectomy have demonstrated a reduction of the risk of leaving positive surgical margins in pT1-2 patients at the time of the surgical procedure and this reduced risk is proportional to the length of treatment.

The timing and schedule of administration of neoadjuvant therapies are still not well defined [7]. Several groups investigated the use of hormonal therapy before radical

prostatectomy. The primary end point of these studies was to assess whether neoadjuvant therapy can increase overall survival. Indeed, there is no published evidence that patients undergoing MAB before radical prostatectomy have a clear benefit [16]. At the same time there is no compelling evidence that neoadjuvant therapy is not effective [17].

The use of PCR methods for determination of PSA gene expression in surgical periprostatic samples has suggested new staging strategies based on higher sensitivity. It is now possible to obtain information about presence of prostatic tissue in extra glandular structures with more sensitivity compared with conventional histology [5,6].

Traditional methods to study protein or gene expression in tumor biopsies include mainly immunohistochemistry and polymerase chain reaction (PCR). These techniques are not quantitative and allow only a semi quantitative evaluation based on the subjective judgment of the observer, which makes the comparison between samples inaccurate (unreliable). Flow cytometry is highly reliable

to determine the levels of expression of a given antigen. However, it requires tissue desegregation procedures, which can damage cell surface antigens, and can not be performed in case of hypo cellular specimens. To measure of the amount of gene expression several molecular methods have been described such as competitive PCR and SAGE. These techniques are cumbersome, time consuming and require multiple manipulations of the samples, increasing the risk of carrying over contamination. Quantitative real-time PCR (qrtPCR) allows a highly sensitive measurement of the transcriptional levels of the gene of interest in a few hours with minimal handling of the samples. In addition, qrt-PCR is stringently sequence dependent because of the labelled probe. Therefore, it yields indirect sequence confirmation that the amplified product is the gene of interest. Finally, the utilization of intro-exon junction spanning probes avoids mis-calculation of gene level expression due to genomic DNA contamination. This technique has been mainly used for viral load measurement [18], but recently it has been employed for eukaryotic studies and in particular analysis of human mRNA expression in various clinical and therapeutic conditions [19]. In this study, we adopted qrt-PCR for the sensitive identification of PSA mRNA level in areas where it should not be present such as positive surgical margins and/or draining lymph nodes.

Conclusions

In our experience, neoadjuvant therapy for 3 months before surgical intervention appears to affect disease staging. In fact, according to qrt-PCR results, the rate of lymph nodes and/or surgical margins in which PSA mRNA could be detected was significantly lower (26.6%) in arm A (MAB plus surgery) as compared to that found in arm B (surgery alone) (64,3%). All the 5 patients that experienced biochemical evidence of recurrence (17,2%) belonged to arm B.

Although, PSA mRNA was detected by qrt-PCR in 60% of patients who experienced biochemical recurrence six of the remaining 8 patients in arm B had also detectable PSA mRNA.

This finding suggests that identification of PSA mRNA expression at the surgical margin or draining lymph-nodes is more a marker of the ability of the neoadjuvant therapy to sterilize the patient before surgical intervention than an independent predictor of recurrence. In fact, the most significant difference in PSA mRNA expression was noted between the two randomization arms where in the neo-adjuvant protocol only 4 of 15 patients were positive while 9 of 14 were positive in the surgical treatment alone arm (Fisher test p_2 value = 0.02). Thus, qrt-PCR appears to yield useful information about the effectiveness of a given therapy and it may be more sensitive, compared to con-

ventional histology, in identifying presence of viable prostate carcinoma cells in surgical resection margins and draining lymph nodes. Finally, this study seems to confirm that neoadjuvant therapy with Nilutamide 150 mg + Buserelin Depot for 3 months before surgical intervention induces clinically relevant down staging, at least in the short term. If these results were confirmed in a larger series of patients, we believe that gene expression of PSA in surgical periprostatic samples might be taken into consideration as a novel and reliable indicator of minimal residual disease after neoadjuvant therapies. In this case, the molecular detection of cancer cells might represent an indication to adjuvant treatments in order to sterilize microscopic foci of tumor possibly responsible for local recurrence.

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