

The application of the RT-PCR method for the staging of the prostate cancer progression

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Abstract. Molecular biology seems to bring more convincing markers for the detection of prostate cancer as well as the development of metastases than immunohistochemistry. The main goal of present work was to detect the expression of prostate specific antigen (PSA) and prostate-specific membrane antigen (PSM) genes in the micrometastases by the RT-PCR to assess the progression of prostate cancer. We analyzed 50 patients: 28 patients with clinically localized or locally advanced prostate cancer who underwent radical prostatectomy, 7 patients with clinically proven metastases, 8 patients with benign prostatic hyperplasia, and 7 healthy young men. The results of RT-PCR in the first group of 28 patients varied, however, they were in good correlation with the health status of the patients. Positive results of PSA and notably for PSM were good predictors of beginning metastasizing process. Seven patients with metastatic disease had positive RT-PCR results both for PSA and PSM. All of the patients with benign prostatic hyperplasia and healthy young men had negative RT-PCR results for PSA and PSM. The study showed that positive RT-PCR results for PSA and especially for PSM correlated well with the progression of the disease and negative results reflected good health status of the patients.

Key words: Prostate cancer — Micrometastases — RT-PCR — Prostate specific antigen — Prostate specific membrane antigen

Introduction

Prostate cancer is now recognized as one of the most important medical problems facing the male population. In Europe, prostate cancer is the most common solid neoplasm, with an incidence rate of 214 cases *per* 1000 men, outnumbering lung and colorectal cancer (Boyle and Ferlay 2005). Furthermore, prostate cancer is currently the second most common cause of cancer death in men (Jemal et al. 2008). It is predominantly a disease of older males, with a peak incidence after the age of

50 (Fletcher 2007). Therefore, it is a bigger health concern in developed countries with their greater proportion of elderly men (Parkin et al. 2001). There are still many unanswered questions about the prostate cancer, and that is the reason why prostate gland carcinoma belongs to one of the most studied malignant diseases.

This disease is characterized by a wide range of clinical, pathological, histological, genetic, and molecular features regarding specific treatment and monitoring. Sometimes it is even clinically silent for many years (occult carcinoma) and diagnosed when progressive metastatic process has began (Kumar et al. 2007). The biggest attention today is dedicated to the detection of the disease at the earliest possible stage, the assessment whether the cancer is significant or non-significant, and last but not least the staging of the progression

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of the disease and the selection of appropriate therapy. This is especially difficult since the symptoms of prostate cancer and the rapidity of the progression of the disease can vary greatly from patient to patient. In some men, prostate cancer is life threatening, while in many others, it can exist many years without causing health problems (Rubin and Strayer 2007). Standardly used prognostic parameters of prostate cancer, as the determination of the extent of the disease (staging), the degree of glandular differentiation (grading, Gleason score), prostate specific antigen (PSA) and its dynamics in blood seem to be insufficient in the prediction of the behaviour of the disease in many cases (Haas et al. 2005; Zhang et al. 2008). The tendentious to impose molecular-genetic methods to assess the degree of the progression of the disease grow stronger; especially, for the detection of the early metastatic process. Seeking for new parameters enabling better prediction of the biological behavior of the tumor is the only way that leads to improved and individualized approach to the treatment of particular patients.

We attempted to do one of the first analyses of chosen markers in Slovak republic. We introduced nested Reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of circulating tumor cells, so called micrometastases, in the peripheral blood of patients with prostate cancer. This method is being used in many countries for the assessment of the progression of mammary cancer, renal cell carcinoma, or bladder cancer. The other goal of the present study was to verify the reliability of this approach. During 5-year study we used a group of 28 patients with verified diagnosis of localized or locally advanced prostate cancer who underwent radical prostatectomy, and our ambition was to check the correlation between the presence of the circulating tumor cells in peripheral blood of these patients and the clinical stage of the disease.

Materials and Methods

During the five-year period, from 2004 to 2009, we studied a group of 28 patients with localized or locally advanced prostate cancer in the age between 50 and 80. In analyzed patients no metastases were diagnosed by standardly used clinical methods. These patients underwent radical prostatectomy and their health status was regularly monitored, especially by the detection of PSA level in their blood. The levels of PSA antigen in these patients were measured before radical prostatectomy, proximately after the operation, one year after the operation, and two years after the operation. Nested RT-PCR method was used to detect possible micrometastases in peripheral blood of these patients. These patients with localized or locally advanced prostate cancer were diagnosed by RT-PCR method before they underwent radical prostatectomy and two years after the operation.

Peripheral blood of healthy young women in age between 18 and 24 was used as a negative control. The malignant tissue obtained from prostate gland was used as a positive control. Then we examined 7 patients with clinically diagnosed metastases of prostate cancer, 8 patients with benign prostatic hyperplasia, and seven healthy young men using RT-PCR method. Informed consent was obtained from all of the studied subjects.

The first step of our work was the isolation of cell elements from the peripheral blood. The second step was the isolation of RNA. Then the RT-PCR method was used to transcript the RNA to the complementary DNA (cDNA) and its subsequent multiplication. Primers that bound to the RNA sequences complementary to a short section of PSA (prostate-specific antigen) and PSM (prostate-specific membrane antigen) gene were used in this step. However, these primers were not specific enough. In order to avoid this problem, we applied nested PCR, using primers complementary to sequences that were nowhere else in the genome. To realize our study we had to determine the sequence of primers, establish suitable algorithm of programs for our thermal cycler, and modify all of the reactions according to our laboratory conditions.

The acquisition of sufficient amount of cell elements from peripheral blood was an unavoidable step before follow-up analysis. The isolation was assured by the sedimentation reagent Ficoll-Paque Plus (Amersham Pharmacia Biotech, UK). We collected 6 ml of peripheral blood from all of the patients and isolated cell fraction from it. Prostatic epithelial cells were suspected to be part of this cell fraction.

The whole RNA was isolated from cells by using Trizol reagent (Gibco, USA), following the method published by Baffa et al. (1996). Supernatant with the RNA was then transferred into another test-tube, and isopropanol was used to precipitate RNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to assess mRNA integrity (GAPDH forward primer: TCT-GCCCCCTCTGCTGATGC and GAPDH reverse primer: CCACCACCCTGTTGCTGTAG).

The Platinum Quantitative RT-PCR Thermoscript One-Step System (Invitrogen, USA) was used for the RT-PCR (Brawer et al. 1992). Primers used for the analysis of PSA system were PSA forward primer: CGGAAGTGGAT-CAAGGACA and PSA reverse primer: AACGTGATGATA – 16XTV. For the analysis of PSM system the following pair of primers was used: PSM forward primer GGGAGTCAT-TCCCAGGAAT'T and PSM reverse primer: AACGTGATGATA – 16XTV.

RT-PCR was performed at thermal cycler PTC¹⁰⁰ Programmable Thermal Controller (MJ Research Inc., USA) according to algorithm published earlier (Katz et al. 1994). The products of the reaction were 741 bp PSA and 630 bp PSM.

The products of the former RT-PCR reaction served as a substance for nested PCR reaction, which was performed according to Repiská et al. (2005). In the nested PCR technique, the amplified DNA was used as a template for another set of inner primers for selective amplification of shorter DNA sequences. One of each pair of primers for nested PSA or nested PSM system was used. N PSA forward primer: TTGGAACCTTGGAATGAC, N PSA reverse primer: GACAACCCATCCTATCTGTG, N PSM forward primer: GTGGACCCTTCCAAGGC and N PSM reverse primer: GACATACCACACAAATTCAATACGG. After the rough mixing of all of the components of nested PCR reaction, short centrifugation was realized. The products of the nested PCR procedure were 231 bp PSA and 142 bp PSM. Agarose gel electrophoresis was used for the detection of PCR product.

Results

The results of our study and their comparison with usually studied parameters are shown in the Table 1. Nine out of twenty-eight patients with localized or locally advanced prostate cancer examined by the RT-PCR method before they underwent radical prostatectomy showed positive result both for PSA and PSM. Six of these patients showed positive RT-PCR reaction for PSA and PSM also two years after surgical operation. Their health status worsened, metastases occurred in a short time and 4 of them already died up to this date. One of the nine patients, who had positive RT-PCR results for PSA and PSM before operation, had negative results of RT-PCR reaction both for PSA and for PSM two years after the operation. His health status shows no progression of the disease. The last two of the nine patients with positive PSA and PSM results before radical prostatectomy had negative result for PSA and positive result for PSM using RT-PCR method two years after the operation. They show the progression of the disease in means of metastases in bones.

Six out of twenty-eight patients had negative result for PSA and positive result for PSM using RT-PCR reaction before radical prostatectomy. Independently to the results of RT-PCR for PSA after the operation, five of these patients had positive results for PSM also two years after the operation and one of these five patients have already died and in two patients metastases have developed. The last out of the six patients had negative result of RT-PCR for PSM two years after the operation. In this patient no metastases were diagnosed. We assume that RT-PCR for PSM has better predicting value of the progression of the disease than RT-PCR for PSA. The remaining two of the patients with positive RT-PCR result for PSM after the operation have not experienced health problems up this time; however, we suggest that continuous monitoring is especially important in these patients.

Finally, thirteen out of twenty-eight patients with localized or locally advanced prostate cancer had negative results of RT-PCR for PSA and also for PSM before the operation. Nine of them had negative result for PSA and negative result for PSM also two years after the operation, and two of them had positive result for PSA and negative result for PSM after the operation. The health conditions of all of these eleven patients are still very good; they show no progression of the disease. The last two of the thirteen patients had negative result for PSA and positive result for PSM two years after the operation and their health status is getting worse, the disease is progressing, and metastases in bones and in some other organs appeared too. This may also speak for better sensitivity of RT-PCR for PSM.

Then we diagnosed seven patients with clinically diagnosed metastases of prostate cancer. RT-PCR reaction for PSA and also RT-PCR reaction for PSM was positive in all of these patients. The results of RT-PCR reaction of the eight patients with benign prostatic hyperplasia were negative both for PSA and PSM. Likewise, RT-PCR reaction of the seven healthy young men was negative for both parameters.

For better evaluation of the relevance of RT-PCR reaction in prediction of the progression of prostate cancer, we have made some statistical analysis. In 28 patients with clinically localized or locally advanced prostate cancer, we correlated pre-operative and then post-operative RT-PCR results with final health conditions of the patients. First, we evaluated the predicting role of RT-PCR for PSA and for PSM separately, and then we evaluated its role when considering the two parameters together. The results are presented in the Tables A1–A6 (see Appendix). Seeing Tables A1–A6 and supplementary Chi-Square Tests, especially Fisher's exact test, we can observe good significance of predicting value of RT-PCR reaction, both for PSA and notably for PSM. Preoperative PSA RT-PCR had an odd ratio of 22.4 (95% confidence interval, 2.2–227; $p = 0.04$) and preoperative PSM RT-PCR had an odd ratio of 15.12 (95% confidence interval, 2.3–100; $p = 0.03$) in predicting the progression of the disease.

We also evaluated the significance of PSA antigen, standardly used prognostic parameter. Since all of the patients had PSA values higher than upper limit of normal (4.0 ng/ml) before undergoing radical prostatectomy, we correlated the values of PSA antigen in blood one year and two years after the operation with health conditions of the 28 patients. The value 0.2 ng/ml of PSA antigen in blood in patients after radical prostatectomy was considered marginal, according to international consensus defining recurrent cancer (Boccon-Gibod et al. 2004; Moul 2000). The results proved significance of this prognostic parameter (see Table A7, A8 and supplementary Chi-Square Tests).

From these results we can see that RT-PCR reaction both for PSA and especially for PSM has significant prognostic value. Admittedly, additional analyses need to be done.

Table 1. Summary of results

Case No.	Age	Gleason	cTNM	PSA - before RP	PSA1 RT-PCR	PSM1 RT-PCR	pTNM	PSA 3 weeks after RP	PSA 1 year after RP	PSA 2 years after RP	PSA2 RT-PCR	PSM2 RT-PCR	Health conditions 4 years after RP
1	55	5 [2+3]	pT1aN0M0	7	negat.	negat.	pT2aN0M0	0	0.1	0.12	negat.	negat.	good
2	62	5 [3+2]	pT1cN0M0	12	negat.	negat.	pT2aN0M0	0	0.08	0.14	negat.	negat.	good
3	64	5 [2+3]	pT3aN0M0	14	negat.	posit.	pT3aN0M0	0	0.03	0.1	negat.	posit.	progression
4	53	5 [3+2]	pT2bN0M0	8	negat.	negat.	pT2cN0M0	0	0.1	0.12	negat.	negat.	good
5	61	6 [3+3]	pT2bN0M0	11	negat.	negat.	pT2bN0M0	0	0.06	0.08	posit.	negat.	good
6	48	6 [4+2]	pT2bN0M0	9	negat.	posit.	pT2cN0M0	0	0.05	0.09	negat.	negat.	good
7	59	6 [3+3]	pT2cN0M0	10	posit.	posit.	pT3aN0M0	0	0.1	0.15	negat.	negat.	good
8	70	6 [3+3]	pT2bN0M0	12	negat.	negat.	pT2bN0M0	0	0.08	0.12	negat.	negat.	good
9	63	6 [3+3]	pT1cN0M0	19	posit.	posit.	pT3bN0M0	0	0.5	0.7	posit.	posit.	progression
10	62	6 [4+2]	pT1cN0M0	8	negat.	negat.	pT2aN0M0	0	0.04	0.04	negat.	negat.	good
11	66	7 [3+4]	pT2cN0M0	12	negat.	posit.	pT2cN0M0	0	0.09	0.12	posit.	posit.	good
12	58	7 [4+3]	pT3aN0M0	11	negat.	negat.	pT3bN0M0	0	0.1	0.18	posit.	negat.	good
13	57	7 [4+3]	pT3aN0M0	9	posit.	posit.	pT3cN0M0	0	0.08	0.1	negat.	posit.	progression
14	61	7 [3+4]	pT1bN0M0	13	negat.	negat.	pT2bN0M0	0	0.12	0.19	negat.	negat.	good
15	64	7 [3+4]	pT2cN0M0	14	posit.	posit.	pT3aN0M0	0	0.1	0.16	posit.	posit.	progression
16	72	8 [4+4]	pT3aN0M0	17	posit.	posit.	pT3bN0M0	0.2	1.3	3.9	posit.	posit.	progression (ex.)
17	59	8 [5+3]	pT1bN0M0	12	negat.	negat.	pT2cN0M0	0	0.07	0.1	negat.	negat.	good
18	60	8 [3+5]	pT1cN0M0	7	negat.	posit.	pT2aN0M0	0	0.04	0.06	negat.	posit.	good
19	54	8 [4+4]	pT1cN0M0	8	negat.	negat.	pT2bN0M0	0	0.09	0.16	negat.	posit.	progression
20	52	9 [5+4]	pT2bN0M0	18	negat.	negat.	pT2bN0M0	0	0.1	0.15	negat.	negat.	good
21	56	9 [4+5]	pT2cN0M0	14	posit.	posit.	pT3aN0M0	0	0.07	0.12	negat.	posit.	progression
22	64	9 [5+4]	pT3aN0M0	10	negat.	posit.	pT3bN0M0	0.15	0.3	5.6	posit.	posit.	progression (ex.)
23	72	9 [4+5]	pT2bN0M0	9	posit.	posit.	pT2cN0M0	0	0.1	0.3	posit.	posit.	progression (ex.)
24	77	9 [5+4]	pT2cN0M0	16	negat.	negat.	pT2cN0M0	0	0	0.25	negat.	posit.	progression
25	56	10 [5+5]	pT2aN0M0	13	posit.	posit.	pT2aN0M0	0.15	2	8.9	posit.	posit.	progression (ex.)
26	61	10 [5+5]	pT2cN0M0	12	negat.	negat.	pT3aN0M0	0.08	0.8	5.2	posit.	posit.	progression
27	78	10 [5+5]	pT2cN0M0	15	negat.	negat.	pT2cN0M0	0	0.1	0.17	negat.	negat.	good
28	54	10 [5+5]	pT2bN0M0	11	posit.	posit.	pT2bN0M0	0	0.4	3.2	posit.	posit.	progression (ex.)

RP, radical prostatectomy; cTNM, clinical TNM; pTNM, pathological TNM; ex, exitus letalis; PSA1 RT-PCR, PSM1 RT-PCR, the results of RT-PCR reaction before radical prostatectomy; PSA2 RT-PCR, PSM2 RT-PCR, the results of RT-PCR reaction two years after radical prostatectomy; PSA antigen, the values are indicated in ng/ml.

However, we assume that this method can greatly improve the choice of appropriate management of patients with prostate cancer, using and evaluating it together with up to now used prognostic parameters.

Discussion

Prostatic specific antigen, one of the most standardly used screening and prognostic parameters used by clinical patriicians, has too many limitations. Traditionally, a PSA value of 4.0 ng/ml has been used as the upper limit of normal. The level of the PSA is usually higher in men with prostate cancer than in men without cancer (Chang et al. 2005). Cancer cells usually produce more PSA, however also any other conditions that disrupt the normal architecture of the prostate, including nodular hyperplasia, prostatitis, prostatolithiasis, may also cause an elevation in serum levels of PSA (Underwood and Cross 2009). Similarly, transrectal or transperineal biopsy of prostate, massage of prostate, cystoscopy, and transurethral resection of the prostate elevate the serum levels of PSA (Rubin and Farber 1999). Moreover, in a minority of cases of prostate cancers, especially those confined to the prostate, serum PSA levels are not elevated. In addition, the source of pathologically elevated production of PSA in prostate cancers and its bone metastases are most commonly well-differentiated adenocarcinoma structures in particular, and the production of the PSA in a general way diminishes with the decrease of the glandular differentiation (Kolombo et al. 2007). Finally, PSA was initially considered specific product of the prostatic tissue. However, it is not organ specific; by means of immunohistochemic methods minimal quantities were detected in malignant tissue of mammary gland, lungs, endometrium, and periurethral glands (Narita et al. 2005, 2008). Because of these restrictions in both specificity and sensitivity, PSA is of limited use when used as an isolated screening parameter for prostate cancer. Some better application of PSA screening seems to be in monitoring patients after radical prostatectomy, but there appear some limitations, too. Several refinements in the interpretation of PSA values have been introduced to further enhance its diagnostic utility. These include rate of changes of PSA values within time, especially PSA velocity and PSA doubling time, determination of the ratio between the serum PSA and volume of the prostate gland (PSA density), the measurement of free *versus* bound forms of circulating PSA (PSA ratio), and obviously age-specific PSA (Kataja and Bergh 2005).

The target of the molecular biology research is to find more persuasive markers of biological character of prostate cancer in individual patients that would be helpful in setting the stage of the disease and assessing an adequate strategy and aggressiveness of the treatment of these patients. The

circulating tumor cells that can sometimes be detected in patients with prostate cancer are originally epithelial prostatic cells that are characterized by the expression of the genes for PSA and PSM (Altamari et al. 2008). It is suspected that their presence in the peripheral circulation of patients with prostate cancer can be helpful in predicting the progression of the disease. Normal immunological reaction of the human organism does not anticipate the survival of the non-malignant prostate cells in blood, so their presence in the circulation is regarded as one of the first steps in the cascade of metastasing process (Ghossein et al. 1995). The micrometastases are detectable by RT-PCR reaction, which is an extremely sensitive method. Experimental data indicate that RT-PCR can detect a single PSA/PSM expressing prostate cancer cell in up to 100 million other ambient cells *in vitro*. Using this method, one PSA/PSM positive cell can be detected in 5 ml of peripheral blood. The presence of 1000 PSA/PSM expressing cells in peripheral circulation eventuates in the positive result of RT-PCR (Gala et al. 1998). Hence, the sensitivity of this method is high enough to detect smaller amount of circulating tumor cells than is needed for the formation of metastases. From the experiments made on laboratory animals, it is assessed that approximately 10 000 circulating tumor cells in peripheral blood result in the formation of metastases. The result of the RT-PCR method *in vivo* thus depends on the ongoing process of the dissemination of tumor cells in circulation and on the efficiency of the immunological reactions of the organism in eliminating these micrometastases (Katz et al. 1994).

The results of our study showed that RT-PCR reaction for PSA and PSM correlates well with health status of patients. When we compare it with standard prognostic factors, such as Gleason score, staging by TNM classification and preoperative PSA serum level, we see a good correlation and we can say that this approach seems to have relevant prognostic value. In many cases, the detection of micrometastases in peripheral blood of patients using RT-PCR reaction even seems to have better predicting value of the extracapsular extention than monitoring the serum level of PSA. Positive RT-PCR reaction for both PSA and PSM in majority of cases meant progression of the disease and the formation of metastases. The patients with negative PSA and positive PSM prevailingly also showed the worsening of their health status. We noticed that patients with positive RT-PCR reaction for PSA and negative RT-PCR reaction for PSM showed no progression of the disease; their health conditions were optimal. From these results we can imply higher significance of RT-PCR for PSM than for PSA. Patients with negative RT-PCR reaction for PSA and also for PSM generally did not show any progression of the cancer. This means that at the time of the realization of RT-PCR reaction, they did not have detectable amount of epithelial prostate cells – micrometastases in peripheral blood. Obvi-

ously, repetitive RT-PCR testing of the series of gradually taken blood samples has better clinical value.

Up to this time, much research has been done that proven a high sensitivity of RT-PCR reaction. Almost all of them have showed that RT-CR positive patients were at high risk for recurrence of prostate cancer after radical prostatectomy. It showed that RT-PCR reaction could be considered early staging modality for radical prostatectomy candidates. Other researches and detailed analyses need to be done to come to definite conclusions. Obviously, repetitive testing is one of the conditions. Nevertheless, researches that have been done up to this time show that RT-PCR method seems to be a commitment to future predictions.

Conclusion

To summarize, we can claim that by our study we confirmed a concord between the result of RT-PCR reaction and the

clinical status of the disease. RT-PCR reaction can be used for the prediction of the progression of prostate cancer. In many cases, the detection of serum PSA levels seems to be less reliable than the detection of PSA/PSM encoding gene in circulating prostate cells by RT-PCR reaction. Obviously, continual monitoring of patients by RT-PCR reaction is essential for more accurate deductions. Searching for new parameters allowing better assessment of the biological behavior of the tumor is necessary for setting an appropriate strategy and aggressivity of the treatment of individual patients. Molecular biology plays a big role in this. RT-PCR reaction and the detection of circulating tumor cells in peripheral circulation of patients with prostate cancer seem to be a step forward. However, the impact of this technique in the management of patients with prostate cancer requires continued investigation.

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Appendix

Table A1. PSA1 RT-PCR health crosstabulation

		health2		Total	
		1 good	2 bad		
PSA1 RT-PCR	Count	14	5	19	
	0 negat	% within PSA1 RT-PCR	73.7%	26.3%	100.0%
		% within health	93.3%	38.5%	67.9%
		Adjusted residual	3.1	-3.1	-
	1 posit	Count	1	8	9
		% within PSA1 RT-PCR	11.1%	88.9%	100.0%
	% within health	6.7%	61.5%	32.1%	
	Adjusted residual	-3.1	3.1	-	
Total	Count	15	13	28	
		% within PSA1 RT-PCR	53.6%	46.4%	100.0%
		% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	9.614 ^b	1	.002	.004	.003	-
Continuity correction ^a	7.263	1	.007	-	-	-
Likelihood ratio	10.494	1	.001	.004	.003	-
Fisher's exact test	-	-	-	.004	.003	-
Linear-by-linear association	9.270 ^c	1	.002	.004	.003	.003
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 x 2 table; ^b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.18; ^c the standardized statistic is 3.045; df, degree of freedom; Asymp. Sig., asymptotic significance; Exact Sig., exact significance.

Table A2. PSM1 RT-PCR health crosstabulation

		health2		Total
		1 good	2 bad	
PSM1 RT-PCR	Count	11	2	13
	% within PSM1 RT-PCR	84.6%	15.4%	100.0%
	% within health	73.3%	15.4%	46.4%
	Adjusted residual	3.1	-3.1	-
	Count	4	11	15
	% within PSM1 RT-PCR	26.7%	73.3%	100.0%
	% within health	26.7%	84.6%	53.6%
Total	Adjusted residual	-3.1	3.1	-
	Count	15	13	28
	% within PSM1 RT-PCR	53.6%	46.4%	100.0%
	% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2- sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)	Point Proba- bility
Pearson Chi-Square	9.403 ^b	1	.002	.003	.003	-
Continuity correction ^a	7.217	1	.007	-	-	-
Likelihood ratio	10.113	1	.001	.003	.003	-
Fisher's exact test	-	-	-	.003	.003	-
Linear-by- linear association	9.067 ^c	1	.003	.003	.003	.003
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 × 2 table; ^b 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.04; ^c the standardized statistic is 3.011. Other abbreviations see Table A1.

Table A3. PSA1 + PSM1 RT-PCR health crosstabulation

		health2		Total
		1 good	2 bad	
PSA1- PSM1	Count	11	2	13
	% within PSA1- PSM1	84.6%	15.4%	100.0%
	% within health	73.3%	15.4%	46.4%
	Adjusted residual	3.1	-3.1	-
	Count	4	11	15
	% within PSA1- PSM1	26.7%	73.3%	100.0%
	% within health	26.7%	84.6%	53.6%
Total	Adjusted residual	-3.1	3.1	-
	Count	15	13	28
	% within PSA1- PSM1	53.6%	46.4%	100.0%
	% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2- sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)	Point Proba- bility
Pearson Chi-Square	9.403 ^b	1	.002	.003	.003	-
Continuity correction ^a	7.217	1	.007	-	-	-
Likelihood ratio	10.113	1	.001	.003	.003	-
Fisher's exact test	-	-	-	.003	.003	-
Linear-by- linear association	9.067 ^c	1	.003	.003	.003	.003
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 × 2 table; ^b 0 cells (0%) have expected count less than 5. The minimum expected count is 6.04. ^c the standardized statistic is 3.011. Other abbreviations see Table A1.

Table A4. PSA2 RT-PCR health crosstabulation

		health2		Total	
		1 good	2 bad		
PSA2 RT-PCR	0 negat	Count	12	5	17
		% within PSA2 RT-PCR	70.6%	29.4%	100.0%
		% within health	80.0%	38.5%	60.7%
	1 posit	Adjusted residual	2.2	-2.2	-
		Count	3	8	11
		% within PSA2 RT-PCR	27.3%	72.7%	100.0%
		% within health	20.0%	61.5%	39.3%
Total	Adjusted residual	-2.2	2.2	-	
	Count	15	13	28	
	% within PSA2 RT-PCR	53.6%	46.4%	100.0%	
		% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	5.038 ^b	1	.025	.051	.031	-
Continuity correction ^a	3.447	1	.063	-	-	-
Likelihood ratio	5.185	1	.023	.051	.031	-
Fisher's exact test	-	-	-	.051	.031	-
Linear-by-linear association	4.858 ^c	1	.028	.051	.031	.027
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 × 2 table; ^b 0 cells (0%) have expected count less than 5. The minimum expected count is 5.11. ^c the standardized statistic is 2.204. Other abbreviations see Table A1.

Table A5. PSM2 RT-PCR health crosstabulation

		health2		Total	
		1 good	2 bad		
PSM2 RT-PCR	0 negat	Count	13	0	13
		% within PSM2 RT-PCR	100.0%	.0%	100.0%
		% within health	86.7%	.0%	46.4%
	1 posit	Adjusted residual	4.6	-4.6	-
		Count	2	13	15
		% within PSM2 RT-PCR	13.3%	86.7%	100.0%
		% within health	13.3%	100.0%	53.6%
Total	Adjusted residual	-4.6	4.6	-	
	Count	15	13	28	
	% within PSM2 RT-PCR	53.6%	46.4%	100.0%	
		% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	21.031 ^b	1	.000	.000	.000	-
Continuity correction ^a	17.691	1	.000	-	-	-
Likelihood ratio	26.893	1	.000	.000	.000	-
Fisher's exact test	-	-	-	.000	.000	-
Linear-by-linear association	20.280 ^c	1	.000	.000	.000	.000
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 × 2 table; ^b 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.04. ^c the standardized statistic is 4.503. Other abbreviations see Table A1.

Table A6. PSA2+PSM2 RT-PCR health crosstabulation

		health2		Total	
		1 good	2 bad	1 good	
PSA2- PSM2	Count	11	0	11	
	% within PSA2- PSM2	100.0%	.0%	100.0%	
	% within health	73.3%	.0%	39.3%	
	Adjusted residual	4.0	-4.0	-	
	PSA2 or PSM2 posit.	Count	4	13	17
		% within PSA2- PSM2	23.5%	76.5%	100.0%
% within health		26.7%	100.0%	60.7%	
	Adjusted residual	-4.0	4.0	-	
Total	Count	15	13	28	
	% within PSA2- PSM2	53.6%	46.4%	100.0%	
	% within health	100.0%	100.0%	100.0%	

Chi-Square Tests

	Value	df	Asymp. Sig. (2- sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)	Point Prob- ability
Pearson Chi-Square	15.702 ^b	1	.000	.000	.000	-
Continuity correction ^a	12.778	1	.000	-	-	-
Likelihood ratio	20.123	1	.000	.000	.000	-
Fisher's exact test	-	-	-	.000	.000	-
Linear-by- linear association	15.141 ^c	1	.000	.000	.000	.000
N of valid cases	28	-	-	-	-	-

^a computed only for a 2×2 table; ^b 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.11. ^c the standardized statistic is 3.891. Other abbreviations see Table A1.

Table A7. PSA antigen 1 year after RP health crosstabulation

		health2		Total	
		1 good	2 bad	1 good	
PSA antigen- 1 year after RP	Count	15	7	22	
	% within PSA antigen 1	68.2%	31.8%	100.0%	
	% within health	100.0%	53.8%	78.6%	
	Adjusted residual	3.0	-3.0	-	
	0 less than 0.2	Count	0	6	6
		% within PSA antigen 1	.0%	100.0%	100.0%
% within health		.0%	46.2%	21.4%	
	Adjusted residual	-3.0	3.0	-	
Total	Count	15	13	28	
	% within PSA antigen 1	53.6%	46.4%	100.0%	
	% within health	100.0%	100.0%	100.0%	

Chi-Square Tests

	Value	df	Asymp. Sig. (2- sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)	Point Prob- ability
Pearson Chi-Square	8.811 ^b	1	.003	.005	.005	-
Continuity correction ^a	6.283	1	.012	-	-	-
Likelihood ratio	11.152	1	.001	.005	.005	-
Fisher's exact test	-	-	-	.005	.005	-
Linear-by- linear association	8.497 ^c	1	.004	.005	.005	.005
N of valid cases	28	-	-	-	-	-

^a computed only for a 2×2 table; ^b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.79. ^c the standardized statistic is 2.915. Other abbreviations see Table A1.

Table A8. PSA antigen 2 years after RP health crosstabulation

		health2		Total	
		1 good	2 bad		
PSA antigen 2 years after RP	0 less than 0.2	Count	15	5	20
		% within PSA antigen 2	75.0%	25.0%	100.0%
		% within health	100.0%	38.5%	71.4%
		Adjusted residual	3.6	-3.6	-
	1 and more	Count	0	8	8
		% within PSA antigen 2	.0%	100.0%	100.0%
		% within health	.0%	61.5%	28.6%
Total		Adjusted residual	-3.6	3.6	-
		Count	15	13	28
		% within PSA antigen 2	53.6%	46.4%	100.0%
		% within health	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square		1	.000	.000	.000	-
Continuity correction ^a	10.084	1	.001	-	-	-
Likelihood ratio	16.180	1	.000	.000	.000	-
Fisher's exact test	-	-	-	.000	.000	-
Linear-by-linear association		1	.000	.000	.000	.000
N of valid cases	28	-	-	-	-	-

^a computed only for a 2 × 2 table; ^b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.71. ^c the standardized statistic is 3.530. Other abbreviations see Table A1.

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