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CRISP3 glycoprotein: a good biomarker for prostate cancer?

Glicoproteína CRISP3: um bom biomarcador para câncer de próstata?

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ABSTRACT

Introduction: Cysteine-rich secretory protein 3 (CRISP3) is expressed at low levels in normal human prostate but often overexpressed in prostate cancer (PCa). The relevance of this overexpression for the malignancy of PCa is still unclear. The prognostic value of the currently used prostate specific antigen (PSA) test can be misleading under certain circumstances, resulting in overtreatment of indolent tumors. New biomarkers are needed to reduce overtreatment and improve quality of life of men. **Objective:** Evaluate if CRISP3 expression could be a good biomarker for PCa. **Methods:** CRISP3 expression was determined by immunohistochemistry in tissue sections of prostate cancer from twenty-five patients subjected to radical prostatectomy. Gleason grading system was used as prognostic indicator and the staging of PCa was defined using the TNM system. Clinical parameters and PSA levels before and after surgery were determined. **Results:** CRISP3 expression was strong in 14 (56%), moderate in four (16%) and weak in seven (28%) specimens. There was no correlation between the intensity of CRISP3 expression and pre- and post-treatment PSA levels. Fifteen (60%) of PCa biopsies showed extension of the primary tumor pT2. Seven patients (28%) showed Gleason score higher than 7; thirteen (52%) equal to 7, and five (20%) lower than 7. There were no significant statistical differences between Gleason score and CRISP3 expression. **Conclusion:** CRISP3 is expressed in prostate cancer at different levels. Additional studies are required to better evaluate if CRISP3 could be used as a biomarker.

Key words: prostate specific antigen; immunohistochemistry; prostatic neoplasms; prostatectomy; pathology molecular.

RESUMO

Introdução: *A proteína CRISP3 é expressa em baixos níveis na próstata humana normal, mas superexpressa no câncer de próstata (CaP). Contudo, sua relevância em pacientes com CaP ainda não está clara. O teste de antígeno específico da próstata (PSA) pode proporcionar interpretações erradas em determinadas circunstâncias, resultando em excesso de tratamento para tumores indolentes. Novos biomarcadores são fundamentais para evitar tratamentos desnecessários e melhorar a qualidade de vida do paciente.* ***Objetivo:*** *Avaliar se a expressão de CRISP3 poderia ser um bom biomarcador para CaP.* ***Métodos:*** *A expressão de CRISP3 foi determinada por imuno-histoquímica em seções de tecido de CaP de 25 pacientes submetidos a prostatectomia radical. O sistema de classificação Gleason foi utilizado como indicador prognóstico, e o estadiamento foi determinado pelo sistema TNM. Parâmetros clínicos e níveis de PSA antes e pós-cirurgia foram determinados.* ***Resultados:*** *A expressão de CRISP3 foi forte em 14 (56%) amostras; moderada em quatro (16%) e fraca em sete (28%). Não houve correlação entre a expressão de CRISP3 e PSA pré e pós-tratamento. Quinze (60%) biópsias de CaP apresentaram extensão do tumor primário pT2. Sete pacientes (28%) mostraram escore de Gleason maior que 7; treze (52%), igual a 7; e cinco (20%), menor que 7. Não houve diferenças estatísticas significativas entre o escore de Gleason e a expressão de CRISP3.* ***Conclusão:*** *CRISP3 é expresso no CaP em diferentes níveis. Estudos adicionais são necessários para avaliar se CRISP3 realmente pode ser usado como biomarcador.*

Unitermos: *antígeno prostático específico; imuno-histoquímica; neoplasias da próstata; prostatectomia; patologia molecular. .*

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RESUMEN

Introducción: *La proteína rica en cisteína secretora 3 (CRISP3) se expresa en bajos niveles en la próstata humana normal, pero está mayormente expresada en el cáncer de próstata (CaP). Todavía, su relevancia en pacientes con CaP aún no está clara. La prueba de antígeno prostático específico (PSA) puede generar interpretaciones erróneas bajo ciertas circumstancias, acarreando sobretratamiento de tumores indolentes. Nuevos biomarcadores son fundamentales para evitar tratamientos innecesarios y mejorar la calidad de vida del paciente.* ***Objetivo:*** *Evaluar se la expresión de CRISP3 podría ser un buen biomarcador de CaP.* ***Métodos:*** *Se determinó la expresión de CRISP3 por inmunohistoquímica en cortes de tejido de CaP de 25 pacientes sometidos a prostatectomía radical. La clasificación Gleason fue utilizada como indicador pronóstico, y la estadificación fue determinada por el sistema TNM. Parámetros clínicos y niveles de PSA antes y después de la cirurgía fueron determinados.* ***Resultados:*** *La expresión de CRISP3 fue fuerte en 14 (56%) muestras; moderada en cuatro (16%) y débil en siete (28%). No hubo relación entre la expresión de CRISP3 y PSA pre y post-tratamiento. Quince (60%) biopsias de CaP tuvieron extensión del tumor primario pT2. Siete pacientes (28%) demostraron escala de Gleason mayor que 7; trece (52%), igual a 7; y cinco (20%), menor que 7. No hubo diferencias estadísticas significativas entre la escala de Gleason y la expresión de CRISP3.* ***Conclusión:*** *CRISP3 se expresa en CaP en diferentes niveles. Se necesitan estudios adicionales para evaluar se CRISP3 puede realmente ser usado como biomarcador.*

Palabras clave: *antígeno prostático específico; inmunohistoquímica; neoplasias de la próstata; prostatectomía; patología molecular.*

INTRODUCTION

Prostate cancer (PCa) is the second most common type of cancer among men around the world and the fifth leading cause of death[1]. It is estimated that 1,356,176 new cases of PCa will be reported worldwide in 2020[2]. In Brazil, 65,840 new cases of this type of cancer per 100 thousand habitants are estimated for the year of 2020 with approximately 15,391 deaths[3].

Although several studies have been focused on understanding the etiology of PCa, the pathogenesis of the disease remains unknown[4]. At the same time, genetic, environmental, and behavioral factors have been associated with an increased risk of PCa[5]. PCa may have often an indolent course and require minimal or even no treatment; nevertheless, more advanced stages of this cancer may become metastatic. For this reason, follow up, active surveillance and surgery, chemotherapy and radiotherapy are needed[6].

The early detection of PCa is based on rectal digital examination and detection of elevated plasmatic levels of prostatic specific antigen [(PSA) > 4 ng/ml]. However, since men without cancer also may present elevated PSA, prostate tissue biopsy is used to confirm the diagnosis[7-9].

The results of this analysis help in the pretreatment prognosis parameters that include Gleason score, assessment of tumor extension, pre-surgery PSA and clinical parameters[6]. Many studies have questioned the use of PSA as a biomarker for PCa diagnosis and the increase of unnecessary radical treatment such as radiotherapy and prostatectomy[10, 11]. Additionally, the need for better systems able to discriminate between indolent and aggressive tumors has been often emphasized, besides the search for novel and more reliable biomarkers[12].

Cysteine-rich secretory protein 3 (CRISP3) is a protein expressed in the male reproductive tract, where it plays a role in sperm function and fertilization, and in the female reproductive tract, where it plays a role in endometrial receptivity for embryo implantation[13, 14]. It has been shown that CRISP3 is able to inhibit growth of subsets of PCa cell lines[15]. In addition, overexpression of CRISP3 combined with ETS-related gene (ERG) and phosphatase and tensin homologue (PTEN) expression has been associated to poor prognosis and it has been suggested to act as a prognostic marker for PCa[16]. CRISP3 knockdown in LNCaP cells did not affect cell viability however reduced invasiveness[12]. Nevertheless, several controversies arise concerning the putative use of this protein as a biomarker for PCa.

OBJECTIVE

Herein we aimed to investigate the level of expression of CRISP3 in a panel of PCa and try to correlate this expression with clinical and pathological parameters, such as PSA values, Gleason score and TNM staging.

METHODS

Tissue samples

Twenty-five prostate biopsies from patients submitted to radical prostatectomy diagnosed with prostatic adenocarcinoma in the period of 2009 to 2013 were provided by the Laboratory of Pathology and Cytopathology from Jataí Prevention Center, Goiás State, Brazil. Experimental protocol used in this work was approved by the ethical committee for human research from Universidade Federal de Goiás, protocol number 1.500.280.

Tissue preparation and processing

Macroscopic examination and determination of size and weight were performed on surgical specimens consisting of prostate tissue, seminal vesicles and left and right obturator lymph nodes. The tissues were fixed in 10% formaldehyde and slices of 4-5 mm thickness were obtained with a disposable Leica scalpel. Embedding and sectioning of paraffin blocks prostate tissues were performed according to standard protocol described by Michalany (1980)[17]. Sections of 4 µM thickness were obtained using an American Optical 820 microtome and transferred onto Dako Flex IHC microscope slides. Deparaffinization was performed at 50ºC for 30 minutes, followed by rehydration and then slides were stained with hematoxylin and eosin (HE) or submitted to immunohistochemistry. Slides were mounted with glass cover slips and examined under Olympus C×21 optical microscopy at 40×, 200× and 400× magnification to confirm the clinical hypothesis according to the histopathological findings and to determine the Gleason score and staging.

Immunohistochemistry

Immunohistochemistry was performed on a Dako automated autostainer Link 48 system following manufacturer instructions. Polyclonal CRISP3 antibody (rabbit, Abcam) was used for detection of CRISP3 expression. Bound primary antibody was visualized using the Dako EnVision Kit (Dako, Denmark) and staining was evaluated according to two parameters: staining intensity and the fraction of positive tumor cells recorded for each tissue spot. The intensity was classified following a 4-tiered system: no staining (0); only visible at high magnification or weak staining (1+); visible at low magnification, or moderate staining (2+); and striking staining at low magnification or strong staining (3+). The final score built based on these two parameters was: negative – absence of staining; weak – intensity of 1+ in ≤ a 70% of tumor cells or staining intensity of 2+ in ≤ 30% – of tumor cells; moderate – intensity of 1+ in > 70% of tumor cells, or staining intensity of 2+ in > 30% but ≤ 70% of tumor cells or staining intensity of 3+ ≤ 30% of tumor cells; strong – staining intensity of 2+ in > 70% of tumor cells or staining intensity – of 3+ in > 30% of tumor cells. Analysis was performed by a qualified medical pathologist.

Clinical parameters

Medical records were used to assess demographic description and clinical parameters, such as PSA levels before surgery and after surgery, as well as histological information from prostatectomy biopsies.

Statistical analysis

Differences in CRISP3 expression and clinicopathological variables were determined by Pearson's chi-squared test. Association between CRISP3 expression and PSA levels in the same samples was analyzed by One Way analysis of variance (Anova). Statistically significant differences were considered for a level $p < 0.05$.

RESULTS

The mean age of patients analyzed was 65.8 (± 6.5) years old (range 54-80). Most PSA values pre-treatment varied from 4 to 10 ng/ml (36%), followed by PSA levels between 10-20 ng/ml (24%). These values presented no statistically significant difference in relation to Gleason score (**Table 1**). Post-treatment follow-ups varied from 2-80 months, with a mean of 30 months, and most of the patients (60%) showed PSA values below the reference value for disease recurrence.

TABLE 1 – Clinical epidemiological parameters of patients subjected to radical prostatectomy in the period of 2009 to 2013 analyzed in this study

Clinical-epidemiological parameters	*n* (%)
Age (years)	
< 60	5 (20)
60 to 70	15 (60)
> 70	5 (20)
Pre-treatment PSA levels (ng/ml)	
< 4	3 (12)
4 to 10	9 (36)
10 to 20	6 (24)
> 20	2 (8)
Post-treatment PSA levels (ng/ml)	
< 0.2	15 (60)
≥ 0.2	7 (28)

PSA: prostate specific antigen.

Tissue sections from all 25 patients showed expression of CRISP3 protein determined by immunohistochemistry. Fourteen (56%) showed strong staining; four (16%), moderate staining; and seven (28%), weak staining. **Figure 1** shows the representative images of HE stained prostate tissue and the standardized parameters used in the classification of CRISP3 expression. There was no correlation between PSA levels and intensity of CRISP3 staining in pre- or post-treatment (R = 0.09, p = 0.4593 and p = 0.4072, respectively) (**Figure 2**).

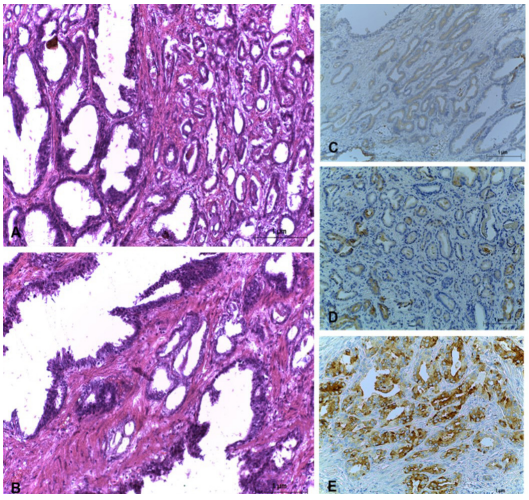

FIGURE 1 – *Histological and immunohistochemical analysis of prostate cancer tissue*

A and B) HE staining; C) weak staining of CRISP3; D) moderate staining of CRISP3; E) strong staining of CRISP3.
200× magnification. Photomicrography was taken using a Leica® camera, model DS750.

HE: hematoxylin and eosin; CRISP3: cysteine-rich secretory protein 3.

The histology analysis of the tissue specimens showed that the majority 24/25 (96%) was usual acinar adenocarcinoma and one was mucinous (colloid) type. Only eight patients (32%) presented seminal vesicle involvement and 12 (48%) were positive at the surgical margin. Among prostatectomized patients 13 (52%) had a Gleason score equal to 7. For seven (53.3%) out of these thirteen patients, this score was the result of the sum of the score 3 plus 4 (grade group 2). The TNM staging system showed that 15 (60%) patients presented extension of the primary tumor (pT2) (**Table 2**). There was no significant difference between CRISP3 expression level and the clinical-pathological parameters of the PCa patients analyzed. Seven patients had a Gleason score lower than 7, among them two patients (28.57%) showed low CRISP3 expression and five (71%) high expression. From the thirteen patients who had a Gleason score equal to 7, three (23.08%) showed a weak expression of CRISP3, three (23.08%) moderate expression and seven (53.3%) high CRISP3 expression. Five patients had Gleason score higher than 7, among them two (40%)

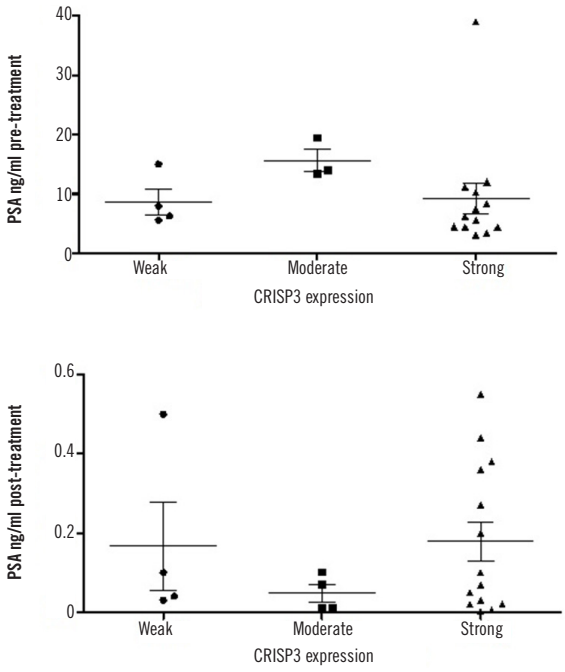

FIGURE 2 – *Comparison of pre- and post-radical prostatectomy PSA levels with intensity of CRISP3 expression determined by immunohistochemistry. No significant differences were observed between groups*

PSA: prostatic specific antigen; CRISP3: cysteine-rich secretory protein 3.

TABLE 2 – Clinical pathological parameters of tumor specimens obtained from patients subjected to radical prostatectomy and association with intensity of CRISP3 positive staining

Clinical-pathological parameters	CRISP3				
	n (%)	Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)	*p* value
Histology evaluation					
Usual acinar	24 (96)	6 (25)	4 (16.66)	14 (58.33)	$p = 0.7471$
Mucinous (colloid)	1 (4)	1 (100)	0	0	
Seminal vesicle invasion					
Present	8 (32)	2 (25)	2 (25)	4 (50)	$p = 0.3128$
Absent	19 (76)	5 (29.41)	2 (11.76)	10 (58.83)	
Surgical edge invasion					
Present	12 (48)	1 (48)	4 (33.33)	7 (58.4)	$p = 0.2896$
Absent	13 (52)	6 (52)	0	7 (58.9)	
Gleason score					
< 7	7 (28)	2 (28.57)	0	5 (71.43)	$p = 0.1297$
= 7	13 (52)	3 (23.08)	3 (23.08)	7 (53.84)	
3 + 4	6 (24)	1 (16.6)	1 (16.66)	4 (66.66)	
4 + 3	7 (28)	2 (28.57)	2 (28.57)	3 (42.86)	
> 7	5 (20)	2 (40)	1 (20)	2 (40)	
pT category (AJCC)					
pT1	2 (8)	1 (50)	0	1 (50)	$p = 0.3852$
pT2	15 (60)	4 (26.66)	2 (13.33)	9 (60)	
pT3	7 (28)	2 (28.57)	2 (28.57)	3 (42.86)	
pT4	1 (4)	0	0	1 (100)	

AJCC: American Joint Committee on Cancer 2010.

showed weak CRISP3 expression; one (20%), moderate expression; and two (40%), high expression. Statistical analysis showed no significant differences between Gleason score and expression of CRISP3 in the PCa samples analyzed (Table 2, **Figure 3**).

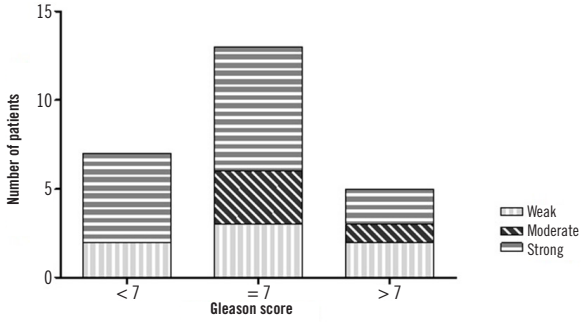

FIGURE 3 – *Distribution of patients according to the Gleason scores and CRISP3 positive immunohistochemistry staining intensity*

Pearson Chi-square test ($\chi 2$) statistical analysis was performed using GraphPad Prism version 5.0. No significant difference was found considering $p < 0.05$.

CRISP3: cysteine-rich secretory protein 3.

DISCUSSION

CRISP3 immunohistochemical labelling of prostate tissue has been associated with PCa[7, 18]. Our results showed that all PCa samples analyzed were positive for the presence of this glycoprotein, although some reports in the literature have shown positivity rates ranging between 19% and 96% in prostatic neoplastic tissue[8, 9, 19, 20].

The high CRISP3 positivity observed in the present work was not due to possible technical artifacts since the appropriate controls were used in each staining procedure. In addition, internal control represented by lack of staining in normal epithelial tissue adjacent to tumor tissue was evident in each tissue slice analyzed. The antibody used in our study was able to detect CRISP3 at a good level of sensitivity. The positive labelling of cancer cells suggests the possibility to use CRISP3 as a diagnostic biomarker for PCa. However, the level of expression could not be associated to the aggressiveness of the tumor in this study probably due to the small number of patients analyzed. Interestingly, Volpert *et al.* (2018)[21] demonstrated that CRISP3 can induce migration and invasion of PCa cells *in vitro*. Deletion of the CRISP3 encoding gene delayed the transition from prostatic intraepithelial neoplasia to carcinoma *in situ* blocking the transition to the invasive disease.

Additionally, the results shown here are not enough to support the hypothesis that CRISP3 could be used to differentiate indolent and metastatic tumors. Further studies including a large cohort comprised by aggressive and indolent types of PCa are needed. Nevertheless, CRISP3 expression detection could be an additional tool to be used in the active surveillance, since it can help in the evaluation of the tumor tissue, especially at the moment of needle biopsy and the time for the choice of the best appropriated treatment, considering each patient clinical-pathological situation[22]. This could avoid patients be exposed to unnecessary radical treatments such as prostatectomy, which can result in serious side effects such as urinary incontinence, erectile dysfunction and morbidity associated to anesthesia and surgical complications[23, 24]. Zhang *et al.* (2016)[25], aiming to identify potential biomarkers for accurate diagnostic of PCa, demonstrated that a panel of three proteins including urinary CRISP3, besides serum PF4V1 and PSA, was able to differentiate PCa from benign prostate hyperplasia (BPH). This panel showed greater discriminatory ability than PSA alone.

In the last decades prostate neoplasia has been diagnosed earlier due to recommended increased screening of the disease based on PSA blood test. Thus, the detection of latent tumors that may never progress to an aggressive phenotype also increased significantly. Therefore, it increased the diagnostic and treatment of men that would not necessarily needed to be treated since they would not have a lethal disease, but on the other hand suffer the side effects and the worsen in their quality of life[4]. For these reasons, there is an urgent need for risk-assessment system that incorporates novel biomarkers to better determine the risk of recurrence and help patients to make informed decisions about treatment.

There is controversy about using PSA blood test to diagnose PCa in men with no symptoms of the disease. However, PSA test is useful for detecting early stage PCa, particularly in men with many risk factors associated. On the other hand, the result of this test may indicate other conditions that are not cancer in addition to very slow-growing cancers that would not be a life threatening for men. Therefore, PSA screening may sometimes make men to decide to have surgery and other treatments that may not be needed[11]. In this context, a novel biomarker that could differentiate indolent slow-growing PCa from aggressive metastatic one would be of great interest. Pre- and post-treatment PSA values did not directly correlate to CRISP3 expression in our study. However, Noh *et al.* (2016)[26] demonstrated that high expression of CRISP3 associated with low expression of PTEN, a tumor suppressor protein, characterize a subgroup of patients with a poor prognosis for short biochemical recurrence. Also, it has been shown that CRISP3 is able to regulate the expression of PSA, as well as genes related to cell invasion. Moreover, *CRISP3* gene promoter has

been demonstrated to be epigenetically regulated by androgen receptor[12]. It is known that prostate differentiation and function, as well as PCa, are dependent upon androgen receptor. Therefore, better understanding of the interaction between androgen receptor and CRISP3 could shed light on the role of CRISP3 in the progression and malignancy of PCa.

Finally, although CRISP3 is highly expressed in neoplastic prostate tissue, additional studies still need to be performed before it could be used as a biomarker for diagnosis of PCa, either by itself or associated with other proteins. Alternatively, CRISP3 could possibly be a tool in the active surveillance to help in the screening of prostate cancer if it is proved that expression levels can differentiate between indolent and aggressive tumor.

CONCLUSION

CRISP3 protein is expressed in PCa, but our data are not able to support its use as biomarker for stratification of patients with indolent and aggressive PCa. Additional studies with larger group of patients need to be performed to better evaluate its prognostic value as biomarker.

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