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CD63 AND DNA MISMATCH REPAIR PROTEIN EXPRESSION IN PROSTATE CANCER

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Protein expression levels in immunohistochemistry and molecular biomarkers have been reported for their ability to predict recurrence, progression, development of metastases, and patient survival. The molecular features in low- and high-grade prostate cancer can differ and influence treatment decision and prognosis. The objective of the current study was to compare the expression of exosomal biomarkers CD63 and mismatch repair proteins (MSH2, MSH6, MLH1, and PMS2) by immunohistochemistry (IHC) in tissue of patients with prostate cancer and benign hyperplasia. Altogether, 62 patients with prostate acinar adenocarcinoma and 20 patients with prostate benign hyperplasia were enrolled in this retrospective study. CD63, MSH2, MSH6, MLH1, and PMS2 expression was analysed by immunohistochemistry. The obtained results showed that CD63 expression was significantly higher in patients with Grade III–V prostate cancer compared to Grade I–II, respectively; 2.23 (1–3) vs 0.92 (0–2) score, p *= 0.001. In addition, a significant positive correlation between CD63 expression and grade groups was revealed (Rho = +0.54;* p *< 0.0001). Furthermore, progression-free survival was significantly higher in patients with low CD63 expression, compared to high CD63 expression (*p *= 0.0007). MMR expression was absent in 14 patients (four patients with Grade I–II cancer and 10 patients with Grade III–cancer). MMR was present in all cases of benign prostate hyperplasia (mild to moderate staining). The conclusion was that high grade prostate cancer (Grade groups III–V) was characterised by increased CD63 expression, which correlated with progression-free survival.*

Keywords: *CD63 exosomal biomarker, mismatch repair protein biomarkers, mismatch repair pathway deficiency, immunohistochemistry, prostate acinar adenocarcinoma, benign prostate hyperplasia.*

INTRODUCTION

Prostate cancer (PCa) remains the second most commonly diagnosed cancer in men, with an estimated 1.1 million diagnoses worldwide in 2012, accounting for 15% of all cancers diagnosed (Jahn *et al*., 2015; Siegel *et al*., 2019).

The Gleason score and Gleason grade groups are assigned by pathologists based on prostate cancer morphology to describe the loss of tissue structure and order, which are strongly correlated with disease aggressiveness and patient outcome. The Gleason grade is determined by pathologists based on the architectural growth patterns of the tumour in hematoxylin and eosin (H&E) stained tissue specimens (Siegel *et al*., 2019).

Gleason scoring categorises tumour tissue into patterns from 1 (low risk) to 5 (high risk). Therefore, Gleason score Grade groups have long been recognised as being strongly associated with risk of prostate cancer recurrence and metastasis (Siegel *et al*., 2019).

In many cases, successful treatment of prostate cancer is difficult due to the late detection and rate of metastasis (Kim and Kim, 2011). Importantly, the tumours of many patients with prostate cancer become refractory to androgen

therapy and progress to metastatic castration-resistant disease (Ingrosso *et al*., 2018).

An effective treatment course of prostate cancer patients requires predictive biomarkers in metastatic castration-resistant prostate cancer that support individual therapy (Kretschmer and Tilki, 2017).

Different risk classification tools have been developed to distinguish patients with early PCa according to the prognosis, including the D'Amico classification system, the Cancer of the Prostate Risk Assessment score, and the National Comprehensive Cancer Network (NCCN) risk groups classification (D'Amico *et al*., 2000; Kretschmer and Tilki, 2017; Braitbord *et al*., 2017; Filela *et al*., 2018). All these systems recognise a low risk of progression for patients with a biopsy Gleason score ≤ 6.

Liquid biopsies, circulating tumour cells, exosomes and circulating nucleic acids have been developed as minimally invasive assays to monitor PCa patients (Cullen *et al*., 2015).

Furthermore, a four-kallikrein panel comprised of total PSA, fPSA, iPSA has been proposed for risk stratification of disease progression (Filella *et al*., 2018).

In addition, novel tissue-based genomic biomarkers have been used to help in the post-biopsy decision, offering additional information in risk stratification, aiding to personalise therapies (Blume-Jensen *et al*., 2015; Cullen *et al*., 2015).

Exosomes are membranous nanovesicles (extracellular vesicles (EVs) with 30–150 nm diameter) of endocytic origin and are secreted by most cell types from diverse organisms. Depending on the cell of origin and the conditions for secretions, exosomes appear to contribute to a diverse range of biological processes and play a pivotal role in mediating intercellular and distant communication by transferring various functional biomolecules including RNAs, DNA, lipids, and proteins (Lu *et al*., 2019).

Exosomal membranes are enriched in endosome-specific tetraspanins (CD9, CD63, CD81) (Lu *et al*., 2019). They have been shown to carry a variety of lipids, proteins, mRNAs and non-coding RNAs that can be taken up by recipient cells, where they trigger intracellular signalling resulting in diverse physiological and pathological responses (Pant *et al*., 2012).

Currently there is great interest in understanding the role of exosomes in cancer progression, including of prostate cancer (PCa), and whether exosomes could be used as a potential source of biomarkers in PCa (Azmi *et al*., 2013; Soekmedji *et al*., 2013).

Exosomes have been shown to be crucial for the development of drug resistance in patients with prostate tumour (Tarhan *et al*., 2005). Exosome-derived microRNAs also contribute to PCa chemoresistance and can act as surrogate biomarkers of tumour response to taxanes (Kharaziha *et al*., 2015; Del Re *et al*., 2017).

The value of exosomal biomarkers CD63 and CD9 in patients with prostate carcinoma in blood serum, plasma and urine has been previously demonstrated and the significant correlation of disease prognosis with the level of exosomes in blood plasma has been observed (Tarhan *et al*., 2005; Thompson, 2006; Azmi *et al*., 2013; Soekmadji *et al*., 2013; Kharaziha *et al*., 2015; Del Re *et al*., 2017). However, the expression of exosomal biomarkers in the tissue assessed by immunohistochemical is still poorly understood.

Failure of effective DNA damage repair is a hallmark of cancer. The mismatch repair (MMR) pathway recognises and repairs insertions, deletions and base–base mismatches that occur on single-stranded DNA during replication (Li, 2007; Pritchard *et al*., 2014). However, previous studies showed that the prevalence of mismatch repair pathway deficiency in prostate cancer cases is about 15% (Kretschmer and Tilki, 2017). The most commonly altered MMR genes are MSH2 and MSH6, which can be inactivated by intronic structural rearrangements that are undetectable by exon-limited sequencing approaches (Pritchard *et al*., 2014).

However, it is not yet clear whether MMR defects are enriched in metastatic disease relative to localised disease.

A recent study of MSH2 protein expression in 1133 primary prostatic adenocarcinomas identified its loss in 1.1% of cases, which is similar to metastatic disease, but it was significantly enriched in Gleason score 9–10 tumours, which implies an association with disease progression (Pritchard *et al*., 2014; Guedes *et al*., 2017).

The aim of our study was to evaluate the expression of CD63 and DNA mismatch proteins (MMR)-MSH2, MSH6, MLH1, and PMS2 by immunohistochemistry in patients with prostate benign hyperplasia and adenocarcinoma tissue and their influence on progression-free survival.

PATIENTS AND METHODS

The study was retrospective. Altogether, 62 patients with prostate acinar adenocarcinoma who underwent radical prostatectomy and 20 patients who underwent fine needle biopsy with prostate benign hyperplasia during 2013–2015 were enrolled in the study.

The study was performed in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Institute of Cardiology and Regenerative Medicine, Rīga, Latvia. Tissue samples were collected from the Biobank of Rīga East University Hospital and the Institute of Clinical and Preventive Medicine, University of Latvia. All patients gave written consent to participate in scientific research.

Histology. The histopathological evaluation of prostate cancer tissue was performed according to the guidelines of the current WHO classification of Tumours of the Urinary System and Male Genital Organs and CAP (College of American Pathologist) prostata cancer protocol. Briefly, the

tumour type, Gleason grading, grade group and cancer invasiveness were assessed.

The tumour TNM staging was performed according to the 8th AJCC (American Joint Committee on Cancer) Cancer Staging Manual.

Tissue processing and immunohistochemistry. Paraffin-embedded tissue specimens were retrieved from the Biobank of Rīga East University Hospital. Specimens were cut in 3-μm-thick sections and the slides were stained with haematoxylin and eosin to evaluate histopathological changes. Antigen retrieval was achieved by incubating the slides with Tris/EDTA buffer at pH = 9.0 for 30 min in a scientific microwave. The slides were then incubated overnight at 4 ^{o}C with mouse monoclonal CD63 (AbCam, ab, ab215891, rabbit monoclonal MSH2 (AbCam, ab227941, dilution 1 : 500), rabbit monoclonal MSH6 (AbCam, ab273076), rabbit monoclonal MLH1 (AbCam, ab23844, dilution 1 : 500), and rabbit monoclonal PMS2 (AbCam, ab110630, dilution 1 : 100. Antibody binding was detected using the EnVision reagent following the manufacturer's instructions (DAKO).

Immunostained slides of each histology sample were scanned with a Pannoramic Midi slide scanner (3D Histech Hungary) at magnification ×20. The whole area of each slide scanned was analysed using an Image Analysis QuantCenter (3DHistech).

CD63, MSH2, MSH6, MLH1, and PMS2 expression was evaluated by intensity of staining and percentage of stained cancer cells and stromal cells respectively: intensity was given scores 0–3 (0 = no, 1 = weak, 2 = moderate, 3 = intense), and the percentage of immunopositive cells for CD63 was given scores 0–3 (0 = 0%, 1 = 10%, 2 = 20–30%, 3 = 40–100%).

Statistical analysis. Values were expressed as means (range). The Fisher exact test or chi-square test was used to evaluate the association between categorical variables.

Associations between CD63, MSH2, MSH6, MLH1, and PMS2 expression and clinicopathological findings were analysed using the chi-square test. Overall survival (OS) was defined as the time from operation to death from any cause. OS curves were estimated by the Kaplan–Meier method and compared using the log-rank test. Multivariate analysis was carried out using the Cox proportional hazard model. p values less than 0.05 were considered to be statistically significant. SPSS 21. version software was used for the statistical analysis.

RESULTS

Overall clinical characteristics. Table 1 demonstrates the general characteristics of patients. Altogether, 82 patients were enrolled in the study. 62 patients had prostate acinar adenocarcinoma and 20 patients had benign prostate hyperplasia.

Table 1. Characteristics of patients with prostate cancer

Age	64.5 (43–85) years
Grade group I	22 patients
Grade group II	10 patients
Grade group III	12 patients
Grade group IV	10 patients
Grade group V	8 patients
pT2	48 patients
pT3a	10 patients
pT3b	4 patients
pN0	54 patients
pN1	8 patients

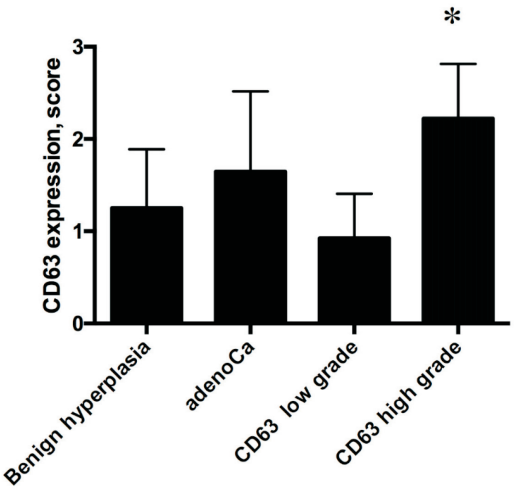

Fig. 1. CD63 expression in prostate benign hyperplasia, acinar adenocarcinoma and low-risk (Grade groups I–II) and high-risk (Grade groups III–V) prostate acinar adenocarcinoma, p = 0.03, compared high-risk to low-risk adenocarcinoma. The Chi-square test was used.

The median age of the study subjects with prostate cancer and benign hyperplasia was 64.5 (43–85). Low-grade prostate cancer (Grade groups I and II) was observed in 32 patients, and high-grade cancer (Grade groups III–V) was observed in 30 patients with acinar adenocarcinoma.

CD63 expression in prostate tissue. The obtained results showed that there was no difference in CD63 expression between benign prostate hyperplasia and prostate carcinoma (1.25 (0–2) vs. 1.64 (0–3), score).

However, when the low and high risk patients were independently analysed, CD63 expression was significantly higher in patients with Grade III–V prostate cancer compared to Grade I–II prostate cancer, respectively, 2.23 (1–3) vs 0.92 (0–2) score, p = 0.001 (Fig. 1).

In addition, there was a significant positive correlation between CD63 expression and grade group (Rho = +0.54; $p <$ 0.0001).

MMR expression was absent in ten patients (two patients with Grade group III, five patients with Grade group IV and three patients with Grade group V.

The Kaplan–Meier method using the log-rank test demonstrated that the progression-free survival was significantly higher in patients low CD63 expression (score 0 and 1), compared to high CD63 expression (scores 2 and 3) (Fig. 2).

A representative photomicrograph of CD63 expression in patients with benign prostate hyperplasia and prostate acinar adenocarcinoma is shown in Figure 3.

MSH2, MSH6, MLH1, and PMS2 expression in prostate tissue. MMR was present in all cases of benign prostate hyperplasia (mild to moderate staining).

Immunohistochemically staining for 4 MMR proteins was conducted.

Overall, MLH1, MSH2, MSH6, and PMS2 loss occurred in 10 (16%), 12 (19%), 8 (13%), and 14 (22%) prostate cancers, respectively. All cases with MLH1 loss concurrently occurred with loss of three other proteins, while all cases with MSH2 loss showed concurrent MSH6 loss.

Thus, the loss of at least 1 MMR protein was identified in 14 (22%) cases; from these patients nine patients had Grade III–V cancer and five patients had Grade I–II cancer.

The study revealed negative correlation between loss of MMR proteins and grade group, Rho = –0.43; $p = 0.0005$).

DISCUSSION

Prostate cancer is one of the most common malignancies in developed countries and the second cause of cancer death for men (Siegel *et al.,* 2019). A large proportion of prostate cancer (PCa) cases are latent, which never destined to progress to metastatic disease. It is of utmost importance to identify which PCa are destined to progress and which would benefit from an early radical treatment. The prostate-specific antigen (PSA) remains the most used test to detect PCa. Its limited specificity and an elevated rate of overdiagnosis are the main problems associated with PSA testing.

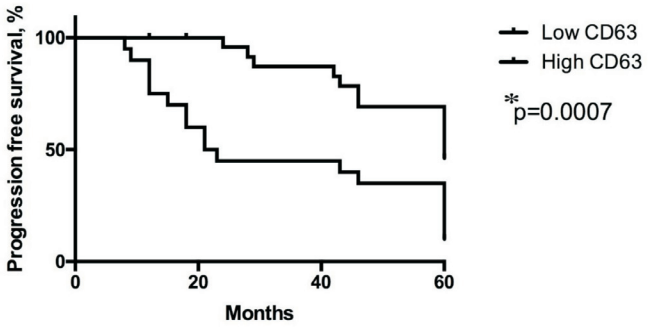

Fig. 2. Progression-free survival of prostate cancer with low CD63 and high CD63 expression. The Kaplan–Meier method using the log-rank test was used.

The classic prostate cancer predictive criteria, which stratify the risk of disease progression, are histopathological criteria like cancer histopathological subtype, Gleason grading system, and Grade groups, which are assessed in prostate biopsy samples by a pathologist.

In the past decade, advances in precision oncology have resulted in an increased demand for predictive assays that enable risk stratification for prognosis and risk stratification for disease progression (Tarhan *et al*., 2005; Thompson, 2006; Azmi *et al*., 2013; Soekmadji *et al*., 2013; Kharaziha *et al*., 2015; Del Re *et al*., 2017).

Approximately 10% of advanced/metastatic prostate tumours have a markedly elevated rate of single nucleotide mutations, almost always due to underlying somatic and/or germline inactivation of genes in the mismatch repair (MMR) family (MSH2, MSH6, MLH1 or PMS2) and often accompanied by microsatellite instability (MSI) (Guedes *et al*., 2017).

Our study demonstrated loss of MMR expression in 18% of low-risk prostate cancer and 25% of high risk prostate cancer cases. In addition, correlation of loss of MMR expression and Grade groups was revealed in our study.

Only a few immunohistochemical studies have reported the incidence of MMR deficiency in prostate cancer, with ranges from 1.2 to to 22.7% (Guedes *et al*., 2017; Albero-González *et al*., 2019; Sharma *et al*., 2020).

MSH2 loss was significantly more often seen in tumours with Gleason score 9–10/Grade Group V than in those with Gleason score ≤ 8/Grade Group ≤ IV (Miki *et al*., 2018). In contrast, other studies did not demonstrate significant associations between MLH1/MSH2/PMS2 loss and Gleason and grade groups (Albero-González *et al*., 2019; Sharma *et al*., 2020).

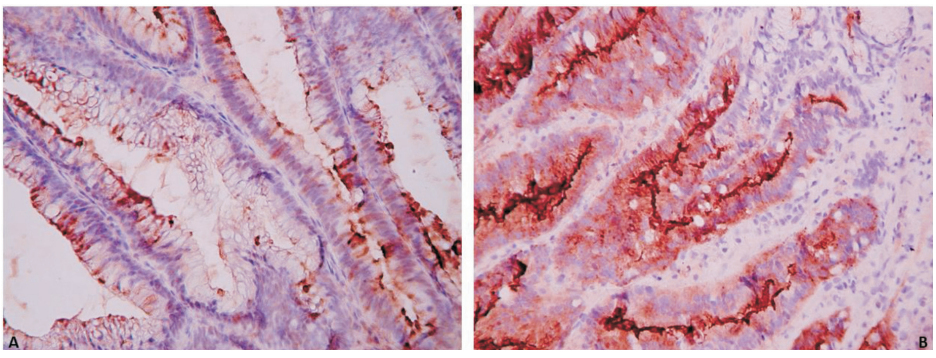

Fig. 3. Representative photomicrograph of CD63 expression in patients with benign prostate hyperplasia (A) and prostate acinar adenocarcinoma (B). Immunohistochemical staining method, magnification ×400.

Recently, it has been reported that exosomes from cancer cells might be associated in intracellular communication involved in the development of the tumour microenvironment, such as metastatic niche formation and angiogenesis, resulting in the progression of carcinoma (Boucheix *et al*., 2001).

In this study, we found that CD63 expression in prostate cancer was an independent significant prognostic factor. As CD63 is a surface marker of exosomes, our data might suggest that exosomes derived from prostate cancer cells play an important role in cancer progression. To the best of our knowledge, this is the first study to investigate the clinicopathological significance of exosome marker CD63 expression in prostate cancer tissue assessed by immunohistochemistry.

It has been demonstrated that positive areas for prostate-specific membrane antigen (PSMA) were significantly different in patients with benign prostate hyperplasia (BPH), and low-risk, intermediate-risk, and high-risk PCa, shown using transmission electron microscope (TEM) with immunoperoxidase/diaminobenzidine (DAB) methods and immunogold enhancement and immunofluorescence imaging (Park *et al*., 2016). In addition, analysis of prostatic tissue by confocal microscopy showed a punctate pattern of co-localised CD63 (green) and PSMA (red), confirming the TEM results (Park *et al*., 2016). However, plasma CD63-positive EV concentration did not significantly differ among patients with different disease status.

Previous studies showed that urinary exosomes markers can aid in the decision-making process regarding whether to carry out a prostate biopsy and in the design of a therapeutic strategy (Fujita and Nonomura, 2018). Urinary exosomes and their cargo, especially miR-21 and miR-375, have become an emerging source of biomarkers in the detection and prognosis of PCa (Foj *et al*., 2017). Moreover, the expression of serum exosomal miRNAs induced by radiotherapy may have potential value as prognostic and predictive biomarkers PCa (Soekmadji, 2013).

It has been observed that after DRE and correction for urinary PSA, CD9 and CD63 were significantly higher in men with prostate cancer (Duijbesz *et al*., 2015).

One of potential surrogate methods to detect exosomes in the tissue is the immunohistochemical method. There are only a few studies that used immunohistochemistry to investigate the role of exosomales biomarkers in prostate tissue.

It has been previously shown that CD9 expression is significantly reduced and even lost during prostate cancer progression. Moreover, deletions and mutations of CD9 mRNA may be associated with loss of protein expression observed in tumour cells. Our data suggest that CD9 inactivation may play an important role in prostate cancer progression.

Furthermore, CD9 was observed to be well expressed in nonmetastatic disease but less expressed or absent in metastatic prostate (Thompson, 2006). However, there are no data in the literature about using immunohistochemistry to examine CD63 biomarker expression in prostate cancer tissue. However, CD63 expression by immunohistochemistry has been demonstrated in other human cancer types.

In pancreatic cancer, the expression of CD63 has been reported to be higher in cancerous tissue than in normal tissue (Khushman *et al*., 2017). In addition, it was shown that CD63 expression in gastric cancer cells was a significant independent prognostic factor in patients with gastric cancer (Miki *et al*., 2018).

Our study demonstrated by immunohistochemical examination of prostate tissue that CD63 expression was significantly higher in patients with Grade III–V prostate cancer compared to Grades I–II. In addition, the significant positive correlation between CD63 expression and grade group was revealed. Furthermore, the progression-free survival was significantly higher in patients with low CD63 expression, compared to high CD63 expression.

In conclusion, CD63 tissue immunohistochemical detection might be a prognostic marker for patients with prostate cancer. CD63-positive exosomes are associated with the malignant potential of cancer cells through the interaction between stromal cells and cancer cells.

Routine immunohistochemical staining for CD63 in primary prostate cancer tissue in clinical practice could be beneficial and cost-effective for the risk stratification of disease progression.

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CD63 UN DNS NESAKRITĪBAS LABOJOŠO PROTEĪNU EKSPRESIJA PACIENTIEM AR PROSTATAS VĒZI

Tiek ziņots, ka ar olbaltumvielu ekspresijas līmeņu noteikšanu imūnhistoķīmijā un molekulārajos biomarķieros ir iespējams prognozēt slimības recidīvu, progresēšanu, metastāzēšanos un pacienta dzīvildzi. Zemas un augstas pakāpes prostatas vēža molekulārās īpatnības varētu atšķirties ārstēšanas lēmuma pieņemšanā un prognozē. Pētījuma mērķis bija salīdzināt eksosomu biomarķieru CD63 un nesakritības labojošo proteīnu (MSH2, MSH6, MLH1 un PMS2) ekspresiju ar imūnhistoķīmijas metodi pacientu, kam ir prostatas vēzis un labdabīga prostatas hiperplāzija, audos. Kopumā šajā retrospektīvajā pētījumā tika iekļauti 62 pacienti ar prostatas acināru adenokarcinomu un 20 pacienti ar prostatas labdabīgu hiperplāziju. CD63, MSH2, MSH6, MLH1 un PMS2 ekspresija tika analizēta ar imūnhistoķīmijas metodi. Iegūtie rezultāti parādīja, ka, salīdzinot ar 1.–2. pakāpes prostatas vēzi, CD63 ekspresija bija ievērojami augstāka pacientiem ar 3.–5. pakāpes prostatas vēzi – attiecīgi 2,23 (1–3) pret 0,92 (0–2), p = 0,001. Turklāt tika atklāta nozīmīga pozitīva korelācija starp CD63 ekspresiju un vēža pakāpes grupām (Rho = + 0,54; p < 0,0001). Arī dzīvildze bez slimības progresēšanas bija ievērojami augstāka pacientiem ar zemu CD63 ekspresiju, salīdzinot ar augstu CD63 ekspresiju (p = 0,0007). MMR ekspresija nebija vērojama 14 pacientiem (četriem pacientiem ar 1.–2. pakāpes vēzi un desmit pacientiem ar 3. pakāpes vēzi). MMR ekspresija bija visos labdabīgas prostatas hiperplāzijas gadījumos (viegla vai mērena krāsošanās). Secinājums: augstas pakāpes prostatas vēzis (3.–5. pakāpes grupas), kam raksturīga paaugstināta CD63 ekspresija, korelēja ar dzīvildzi bez slimības progresēšanas.