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Immunoexpression of Claudins -3, -4 and -7 in prostate adenocarcinomas

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Abstract

Claudins are a family of essential tight junction proteins, abnormally expressed in human carcinomas. The studies that indicated the involvement of claudins in tumor biology and progression suggest the possibility of their utility as markers for diagnosis or prognosis, but also as possible targets for therapy. We investigated 50 prostate adenocarcinomas (PAs) for which we followed the expression of Claudins -3, -4 and -7 in relation to *International Society of Urological Pathology* (ISUP) grades. We observed the positivity for Claudin-3, Claudin-4, and Claudin-7 in 76%, 74% and 46% of cases. Analysis of the immunoexpression pattern revealed the cytoplasmic and nuclear translocation for Claudins -3 and -4, and only cytoplasmic for Claudin-7. For all claudins investigated, we noted a final staining score with significantly higher values or at the limit of statistical significance for PA belonging to ISUP groups 1–4. The internalization of Claudins -3, -4 and -7 expression, regardless of the degree of PA, indicates their involvement in prostate carcinogenesis. In addition, the similar immunoexpression patterns of the three investigated claudins and their positive linear correlation suggest a coordinated regulation and indicate the possibility of a targeted treatment strategy.

Keywords: prostate carcinoma, ISUP grade, claudins.

Introduction

The dysfunctionality of cell adhesion molecules is essential in the process of tumor progression. During carcinogenesis in the prostate tissue, biomolecular changes occur, which are regulated by intercellular adhesion molecules, and lead to the alteration of the relationship between the glandular structures and the stroma [1]. In this context, changes in key components of adherens and tight junctions have been studied as potential biomarkers for PA progression, but most studies have only included single molecules.

The molecular structure of tight junctions comprises the following groups: integral (intrinsic) membrane proteins (IMPs) including occludin, claudins, junctional adhesion molecules (JAMs), and peripheral (extrinsic) membrane proteins including *zonulae occludentes*, pilt, symplekin, cingulin, membrane-associated guanylate kinase (MAGI-1) [2]. Claudins represent a family of essential tight junction proteins, abnormally expressed in human carcinomas, the barrier function of this protein family being fundamental for maintaining the tissue integrity.

The studies that indicated the involvement of claudins in tumor biology and progression suggest the possibility of their utility as markers for diagnosis, prognosis, and therapy. This point of view was supported by studies that suggests the involvement of claudins in the transduction of regulatory signals and that they may play an important role in carcinogenesis, their expression being frequently dysregulated in the context of neoplastic transformation [3, 4]. As a result, the expression pattern of claudins could represent a new diagnostic marker and an additional predictive factor, sensitive for unfavorable prognosis [5].

Aim

The present study aims to evaluate the expression of Claudins -3, -4 and -7 in PA, depending on the *International Society of Urological Pathology* (ISUP) grade of the tumors, evaluated on the fragments of transurethral resection of the prostate (TURp) tumor.

Materials and Methods

We investigated 50 cases of PA from the Urology Clinic of the Emergency County Clinical Hospital, Craiova, Romania, obtained through TURp. Surgical excision samples were treated in 10% neutral buffered formalin, later being paraffin-embedded and routine stained (Hematoxylin–Eosin). The classification of the selected PAs was performed according to ISUP recommendations [6].

Serial sections obtained from selected paraffin blocks were processed immunohistochemically, using the EnVision™ FLEX+ (code K8002, Dako) amplification system, which detected the investigated targets through heat-induced epitope retrieval (HIER), and the reactions were visualized with 3,3'-Diaminobenzidine (DAB) chromogen (code 3467, Dako). The immunohistochemical (IHC) reactions have been validated by controls: staining protocol without primary antibody (negative control) or using positive tissues to the marker (positive controls) (Table 1).

Table 1 – ***IHC data for the used markers***

Biomarker	Clone / Producer	Dilution	Antigen retrieval (HIER)	Positive controls
Claudin-3	Polyclonal / Invitrogen	1:200	Citrate buffer, pH 6	Tegument
Claudin-4	Polyclonal / Invitrogen	1:150	Citrate buffer, pH 6	Ovarian carcinoma
Claudin-7	Polyclonal / Invitrogen	1:150	Citrate buffer, pH 6	Colon carcinoma

HIER: Heat-induced epitope retrieval; IHC: Immunohistochemical.

The semi-quantitative assessment of the investigated markers was done by evaluation of the intensity of reactions and the percentage (%) of labeled cells, using models adapted from the literature [7]. The score of intensity was evaluated as: 1 (mild), 2 (moderate) and 3 (increased). The number (%) of positive cells was scored: 6–25% (score 1), 26–50% (score 2), 51–75% (score 3) and >75% (score 4), with a cutoff value of 5%. By multiplying the two scores, we calculated a final staining score (FSS); we considered that values of 1–4 designated low FSSs and values of 6–12 high FSSs. Further, we also calculated their average values (FSSm).

For the statistical investigation, we used the average values, standard deviations, and comparative tests [χ^2 (*chi-squared*) and Pearson] included in Statistical Package for the Social Sciences (SPSS) 10 software, the values $p<0.05$ being considered significant.

In this investigation, the rules of ethics in research were respected, the study being approved by the Local Ethics Commission.

Results

For the 50 PAs which were investigated, the histopathological assessment revealed ISUP-grade 1 tumors in 16 cases, ISUP-grade 2 tumors in seven cases, ISUP-grade 3 tumors in four cases, ISUP-grade 4 tumors in 10 cases, and ISUP-grade 5 tumors in 13 cases.

The reactions for Claudin-3 were present in 76% of the considered PAs. The analysis of Claudin-3 expression indicated different FSSm values, depending on the ISUP groups, with values between 7.9–2.7 (Table 2). The staining intensity was moderate or increased in tumors from ISUP groups 1–4, and moderate or mild in tumors from ISUP group 5. The staining pattern was membranous and cytoplasmic in the case of PA ISUP group 1, and membranous, cytoplasmic, and nuclear in tumors from ISUP groups 2–5 (Figure 1, A–D).

Table 2 – ***FSSm distribution according to ISUP groups***

ISUP group		1	2	3	4	5
Claudin-3	Positive cases (No.)	13	5	3	7	10
	Positive cells (%)	66.9±9.9	58±14.4	35±8.6	37.1±13.1	32.5±12.9
	Intensity	2, 3	2, 3	2, 3	2, 3	1, 2
	FSSm	7.9	6.4	6.4	4.8	2.7
Claudin-4	Positive cases (No.)	12	5	2	7	11
	Positive cells (%)	68.7±7.1	72±4.4	70	70±10	59±11.3
	Intensity	2, 3	2, 3	2, 3	2, 3	2, 3
	FSSm	8.5	9	6	8.8	6.4
Claudin-7	Positive cases (No.)	8	3	1	6	5
	Positive cells (%)	66.2±9.5	70±8.6	60	57.5±6.8	54±10.2
	Intensity	2, 3	2, 3	2	2, 3	2
	FSSm	8.2	7	6	6.1	4.8

FSSm: Final staining score mean values; ISUP: *International Society of Urological Pathology*.

For ISUP-grade 1 carcinomas, the mean value of marked cells was 66.9±9.9, with variation of 40–75%. In PA ISUP-grade 2 tumors, we found a mean value of marked cells of 58±14.4, with variation of 40–70% for the tumoral cells. ISUP-grade 3 carcinomas presented a mean value of marked cells of 35±8.6, with variation of 30–45%. ISUP-grade 4 tumors indicated a mean value of marked cells of 37.1±13.1, with variation of 20–55% of tumor cells. In ISUP-grade 5 tumors, we found a mean value of marked cells of 32.5± 12.9, with variation of 20–55% for tumor cells.

Claudin-4 immunoreaction was identified in 74% of the PAs investigated. The analysis of Claudin-4 expression indicated different values of FSSm depending on the ISUP groups, the values being between 8.5–6 (Table 2). The staining intensity was moderate or increased in all tumor groups, with a membrane and cytoplasmic staining pattern in the case of ISUP groups 1–3, and membrane, cytoplasmic and nuclear for ISUP groups 4 and 5 (Figure 2, A–D).

For ISUP-grade 1 carcinomas, we found a mean value of labeled cells of 68.7±7.1, with variation of 60–80% for positive cells. In PA corresponding to ISUP group 2, the average percentage of positive cells was 72±4.4, with variations between 70–80%. ISUP-grade 3 tumors presented a percentage of positive cells of 70%. ISUP-grade 4 carcinomas presented a mean value of labeled cells of 70±10, with variation of 60–85% for positive cells. For the ISUP-grade 5 carcinomas, we observed a mean value of labeled cells of 59±11.3, with variation of 45–75% for neoplastic cells.

The immunoreaction for Claudin-7 was identified in 46% of the studied cases (Table 2). The analysis of Claudin-7 expression indicated different FSSm values depending on the ISUP groups, respectively between 8.2–4.8. The staining intensity was moderate or increased in PA from ISUP groups 1, 2 and 4, or only moderate in tumors from ISUP groups 3 and 5. The staining pattern was membranous for ISUP 1–3 tumors, and membranous and cytoplasmic in the case of PA from ISUP groups 4 and 5 (Figure 3, A–D).

For ISUP-grade 1 tumors, the average percentage of positive cells was 66.2±9.5, with variations between 55–75%. In PA corresponding to ISUP-grade 2 carcinomas,

we found a mean value of labeled cells of 70±8.6, with variation of 60–75%. The ISUP-grade 3 positive tumors had a mean value of 60% marked cells. For ISUP-grade 4 carcinomas, we observed a mean percentage of 57.5±6.8 positive cells, with variation of 50–70%. In the case of ISUP-grade 5 carcinomas, there was a mean value of positive cells of 54±10.2, with variation of 45–65% for neoplastic cells.

The statistical analysis indicated the association of high FSS Claudin-3 values with the ISUP group 1, these also prevailing in the intermediate ISUP groups 2–5, in contrast to the low FSS values associated with the ISUP group 5, aspects that were statistically significant (p=0.002, χ^2 test) (Figure 4A). For Claudin-4, the immunoexpression indicated the association of high FSS values with all ISUP grading groups, but also the association of low FSS values with the ISUP-grade 5 group, differences that were statistically significant (p=0.008, χ^2 test) (Figure 4B). Also, exclusive high FSS were present for ISUP groups 1–4. The statistical analysis of the obtained results related to Claudin-7 immunoexpression indicated the predominance of high FSS in ISUP groups 1–4, the exclusive association of high FSS with ISUP groups 1–3, the presence of low FSS only in ISUP groups 4 and 5, respectively the predominance of high FSS in ISUP 5 group, differences that were at the limit of statistical significance (p=0.065, χ^2 test) (Figure 4C).

The analysis of percentage values for Claudins -3, -4 and -7 immunoreactions (Pearson's test) indicated a positive linear correlation both for Claudins -3/-4 (p<0.001) and for Claudins -3/-7 (p<0.001) and Claudins -4/-7 (p<0.001) (Figure 4D).

Discussions

Tight junctions are made up of three proteins located in membrane, represented by claudins, occludin, and JAMs, with roles that are incompletely understood, but claudins are thought to be the most important components of tight junctions [8]. Numerous studies indicate that their expression is increased in many human cancers, including PA. Of the 27 members of this family [9], Claudins -3, -4 and -7 are most frequently dysregulated in epithelial cancers, their functional importance in their progression being well established [10, 11].

Li *et al.* indicated an abnormal Claudin-3 expression which is closely linked with the initiation and evolution of tumors [12]. In prostate tissue, Claudin-3 expression was identified both in normal glandular epithelium and in PA [8, 13], with overexpression in the latter [13]. The aberrant expression of Claudin-3 usually accompanies the damage of cellular polarity, and the reduction of intercellular adhesion affects epithelial permeability and thus promotes the appearance and development of tumors [14]. Liu *et al.* reported the Claudins -3 and -4 overexpression in aggressive high-grade PA specimens [15]. In the conducted study, the analysis of Claudin-3 expression in relation to the ISUP groups, indicated statistically significant aspects, the low FSS values being associated with the ISUP group 5, while the overexpression was associated with the ISUP groups 1–4. Although it is a tight junction protein, it is worth noting that in addition to membrane and cytoplasmic staining, similar to our study, cytoplasmic and nuclear staining was also reported [16].

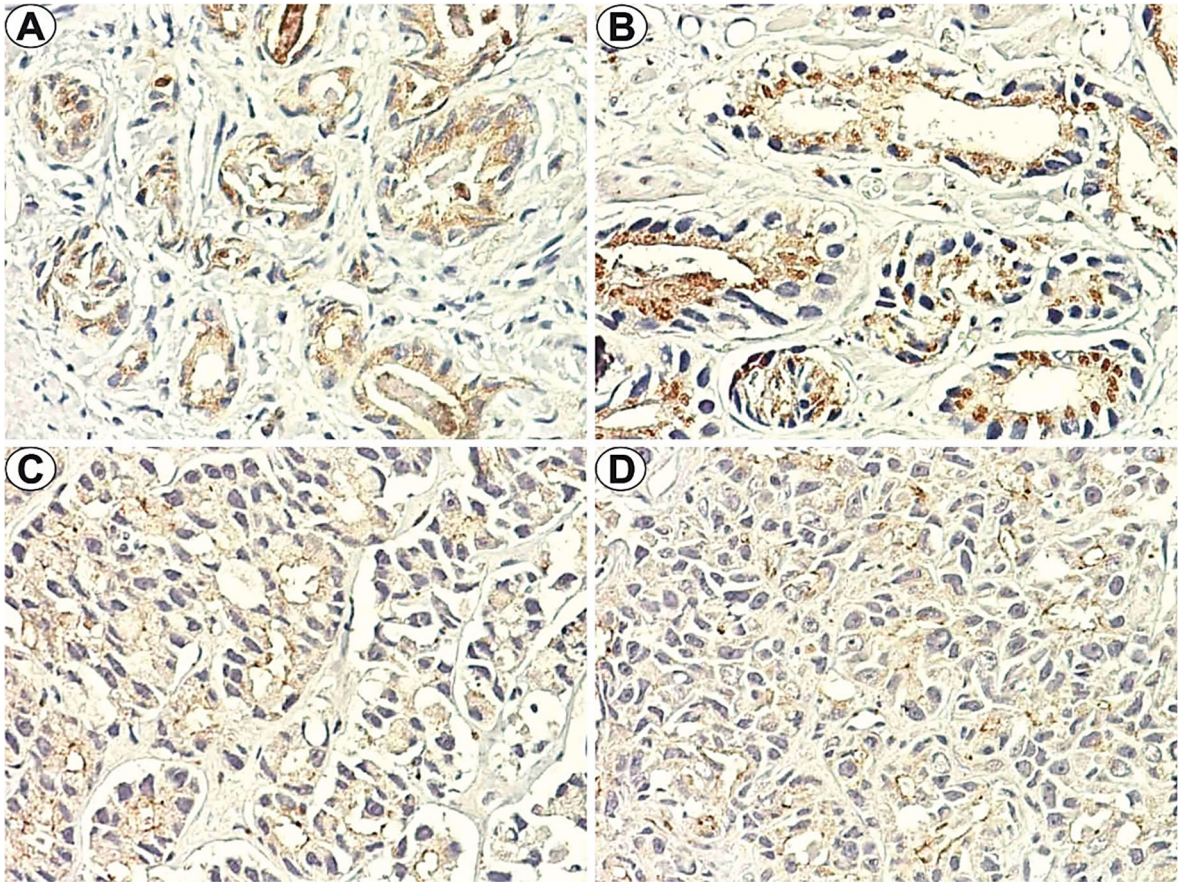

Figure 1 – ***Claudin-3 immunoexpression, ×400: (A) ISUP-1 (3+3) group; (B) ISUP-3 (4+3); (C) ISUP-4 (4+4) group; (D) ISUP-5 (5+5) group. ISUP: International Society of Urological Pathology.***

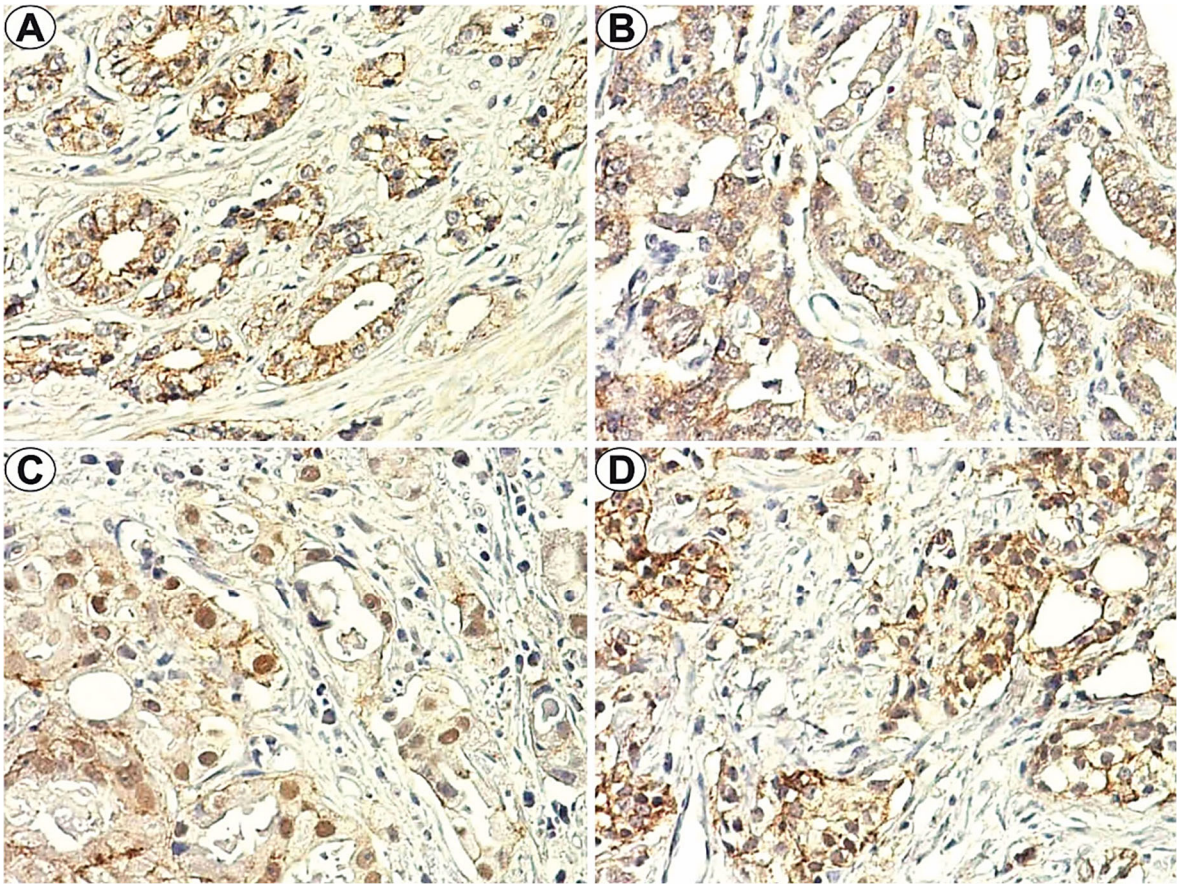

Figure 2 – ***Claudin-4 immunoexpression, ×400: (A) ISUP-1 (3+3) group; (B) ISUP-3 (4+3) group; (C) ISUP-4 (4+4) group; (D) ISUP-5 (4+5) group. ISUP: International Society of Urological Pathology.***

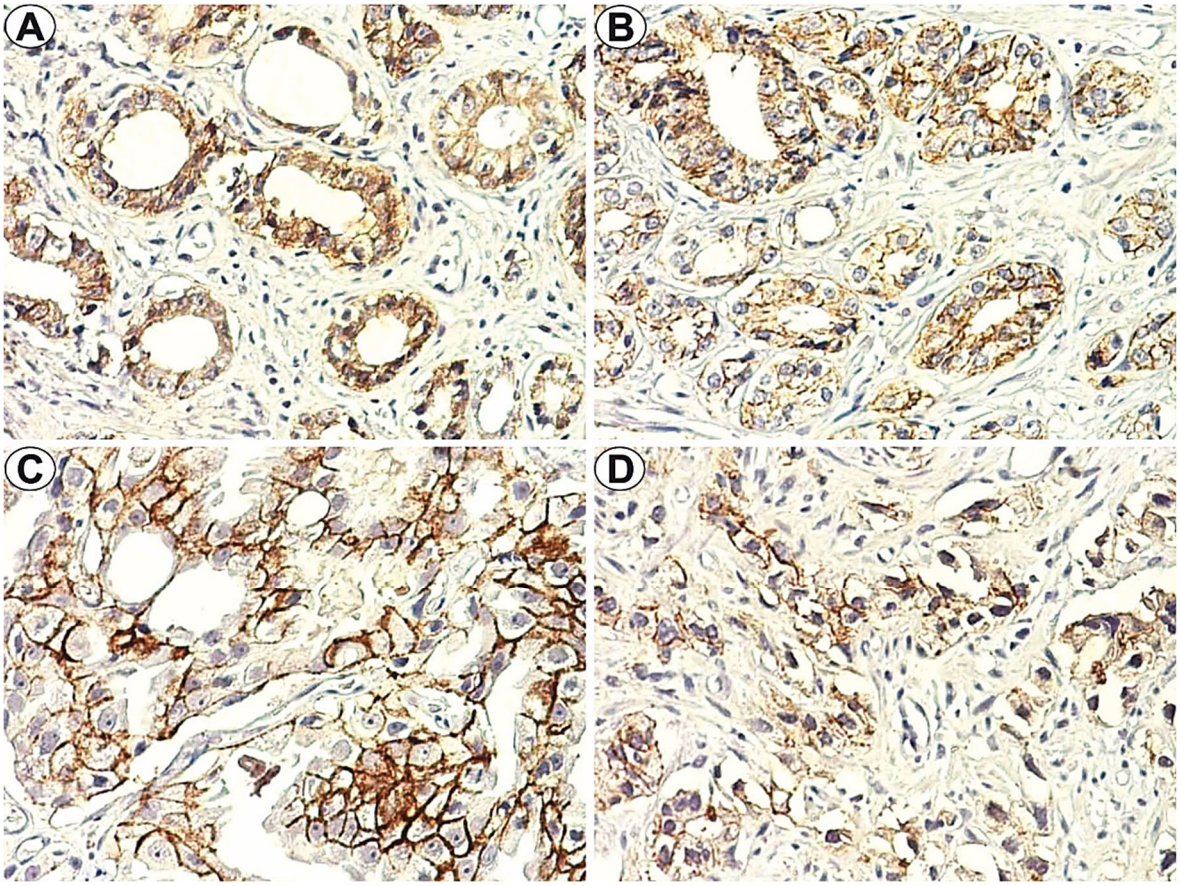

Figure 3 – ***Claudin-7 immunoexpression, ×400: (A) ISUP-1 (3+3) group; (B) ISUP-2 (3+4) group; (C) ISUP-4 (4+4) group; (D) ISUP-5 (4+5) group. ISUP: International Society of Urological Pathology.***

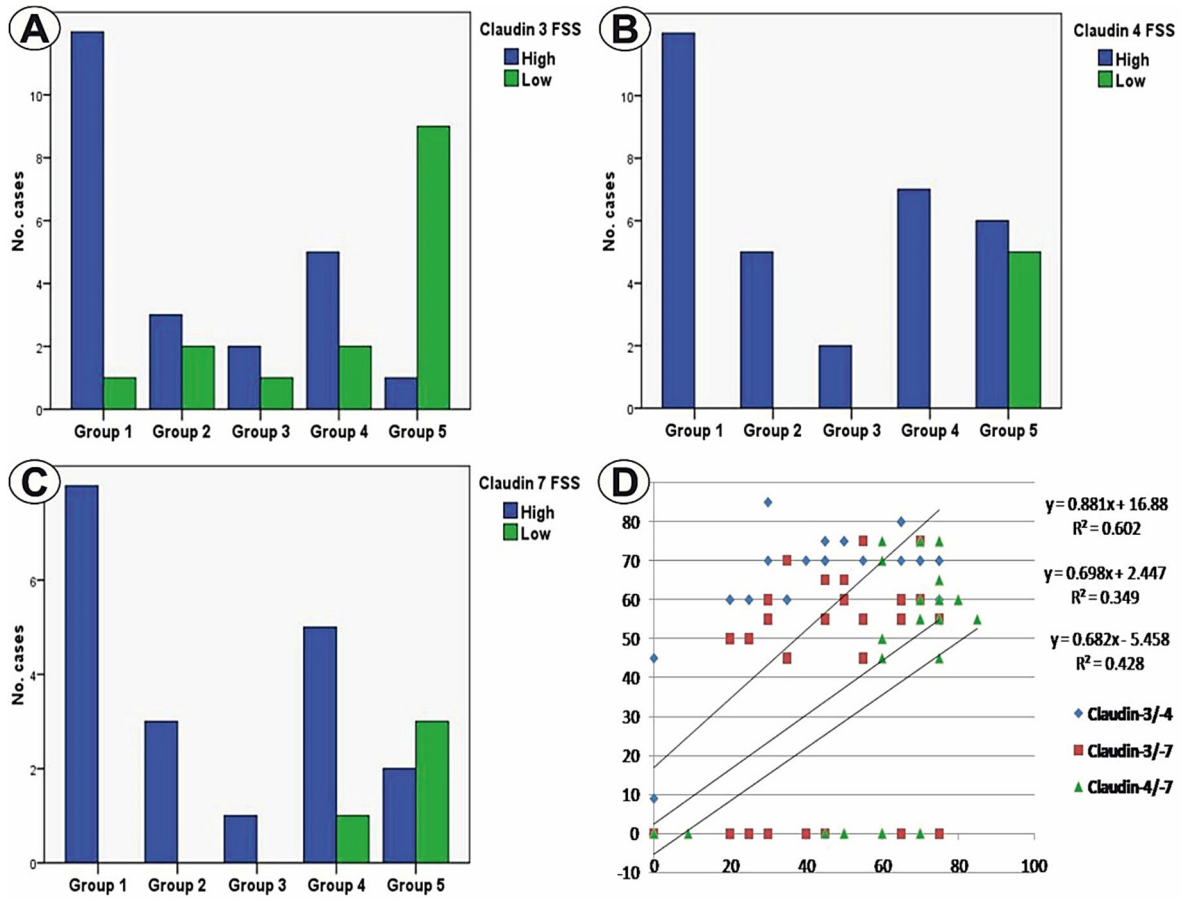

Figure 4 – ***Distribution of cases in relation to ISUP grading groups and Claudin-3 FSS (A), Claudin-4 FSS (B), and Claudin-7 FSS (C). The percentage values distribution for Claudins -3, -4 and -7 (D). ISUP: International Society of Urological Pathology; FSS: Final staining score.***

The basic mechanisms which contribute to the translocation of expression are still unexplained but are thought to be based on protein kinases that lead to their phosphorylation [17]. Among the potential mechanisms that may explain the abnormal localization is the expression downregulation of other molecules involved in adhesion, such as E-cadherin [4]. On the other hand, nuclear claudins are found mainly in malignant tissues, always in protein-associated complexes [*β*-catenin, yes-associated protein (YAP), *zonula occludens*-1 (ZO-1), nucleic acid binding protein ZO-1 and cyclin D1], inducing their nuclear accumulation to enhance cell proliferation and tumor progression [17–19].

Claudin-4 encodes a protein containing four transmembrane regions, which shows close homology to Claudin-3. In normal prostate glands, Claudin-4 expression is membranous [20], but its expression was observed in premalignant and malignant lesions [21], in most cases with increased and very increased intensity [13]. In primary tumors, Claudin-4 is more intensely expressed in low-grade lesions (Gleason score 6) compared to high-grade ones (Gleason score ≥7) [13, 21]. On the contrary, in other studies no significant differences were observed depending on the Gleason score [22]. Moreover, Radi & Abd-Elazeem indicated that increased Claudin-4 immunoexpression of is associated with high grade of tumor, lymphovascular invasion, lymph node metastases, suggesting that the protein may be related to poor prognosis [23]. In our study, the analysis of Claudin-4 immunoexpression indicated the association of high FSS values with all ISUP grading groups, but also the association of low FSS values only with ISUP group 5. In addition, we found, as for Claudin-3, that besides membrane expression, Claudin-4 had cytoplasmic and nuclear expression. Recently, similar aspects have been reported for clear cell renal carcinoma, the authors suggesting that the release of Claudin-4 from the affected tight junction could be a mechanism underlying the malignant properties of these tumors [17].

The exact biological role of Claudin-7 remains only partially known, but in PA it is appreciated that it has a potential role in cell differentiation, and it has been shown to regulate the expression of prostate-specific antigen (PSA) [24]. Cases with lower expression of Claudin-7 were associated with significantly higher values of PSA and, similarly, cases with high Gleason score had higher values [13].

Sheehan *et al.* reported a decrease in Claudin-7 expression correlated with high degree of PA [20]. Similarly, in our study, the statistical analysis of the results related to Claudin-7 immunoexpression revealed the predominance for ISUP groups 1–4 of high FSS in the and also the presence of low FSS only in the ISUP groups 4 and 5, differences which were at the limit of statistical significance. Also, in addition to membrane expression, we also observed cytoplasmic expression. Similarly, Väre *et al.* reported both membranous and aberrant cytoplasmic localization of Claudin-7, its intensity being increased and very increased [13]. The redistribution of claudins has been attributed to

several mechanisms, including down-regulation of the protein, which results in its cytoplasmic internalization [25]. Based on these results, it can be assumed that Claudin-7 could be a candidate for differentiating indolent from aggressive cancers [26].

Due to their location on the cell surface, claudins are ideal molecules for targeted therapies. They are known to be targets for various viruses and bacteria that hijack claudins and infect cells, a phenomenon that has been exploited in therapeutic strategies. *Clostridium perfringens* enterotoxin binds strongly to Claudins -3 and -4, and with lower affinity to Claudins -5, -6, -7, -8, -9, -14 and -19 [27, 28]. Claudin binding has a cytotoxic effect on carcinomatous cells with several locations, including PA, positive for Claudin-3 and/or Claudin-4 [29]. These studies support the use of *C. perfringens* enterotoxin-based therapies targeting those tumors that have an appropriate claudin expression profile.

Conclusions

New molecular targets are needed to identify and prevent the progression of advanced PA. The similar expression patterns of the three investigated claudins suggest a coordinated regulation and indicate the possibility of new targeted therapeutic strategies. In addition, the internalization of Claudins -3, -4 and -7 expression, regardless of the degree of PA, indicates their involvement in prostate carcinogenesis.

Conflict of interests

The authors declare that they have no conflict of interests.

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