Reelin expression in human prostate cancer: a marker of tumor aggressiveness based on correlation with grade

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Reelin is a glycoprotein that plays a critical role in the regulation of neuronal migration during brain development and, since reelin has a role in the control of cell migration, it might represents an important factor in cancer pathology. In this study, 66 surgical specimens of prostate cancer were analyzed for reelin expression by immunohistochemical method. The reelin expression was correlated with Gleason score and individual Gleason patterns. Reelin expression was found in 39% prostate cancers. Stromal tissues, normal epithelial cells and prostate intraepithelial neoplasia (PIN) of any grade around and distant from cancer were always negative for reelin. Reelin was found in malignant prostatic epithelial glands of 50% cases Gleason score 10, 52% Gleason score 9, 56% Gleason score 8, 18% Gleason score 7, while no sample of prostate cancers with Gleason score 6 showed reelin expression ($P = 0,005$). As reelin staining is frequently found in high Gleason score prostate cancers, we explored whether reelin expression is influenced by single Gleason patterns. While Gleason 3 pattern did not show reelin immunoreactivity, reelin expression was found in 35% Gleason 4 patterns and 45% Gleason 5 patterns ($P < 0.001$). Our results demonstrated for the first time that reelin is expressed in prostate cancer and not in benign prostate tissue and its expression occurs in higher Gleason score and correlates significantly with increasing of single Gleason patterns. This suggests reelin may behave as a specific histological marker and may represent a useful biomarker to predict aggressive phenotypic behavior of prostatic cancer cells.
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Reelin is a 420 kDa secreted extracellular glycoprotein that plays a critical role in the regulation of neuronal migration during brain development.[1] Reelin is thought to guide migrating neurons by interacting with two cell surface receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), and by activating a tyrosine kinase signalling cascade that instructs

neurons to reach their correct laminar position in the cortex;[2] reelin modulates the cytoskeletal organization and behavior of migrating neurons by regulating the phosphorylation state of the microtubule-stabilizing protein *tau*; mice that are mutant for reelin show high levels of tau phosphorylation suggesting that defective reelin signalling leads to tau hyperphosphorylation.[3] Although most studies have focused on the role of reelin in central neural system (CNS) development, recent data have shown that reelin is also expressed in adult peripheral tissues, including the peripheral neural system,[4] liver, kidney, testis, ovary,[5] odontoblasts,[6] plasma cells,[7] serum,[8] acinar and ductal pancreatic epithe-

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lial cells, Langherhans islet cells and autonomic peripheral nerves,[9] thereby proposing a wide spectrum of biological functions.

Although reelin dysfunction has been implicated in the pathogenesis of brain malformation and several psychiatric disorders,[10] its biological role in the development and function of other organs remains obscure. As reelin has a role in the control of cell migration, it might represents an important factor in cancer invasion and metastasis.

Wetmore et al[11] demonstrated for the first time reelin expression in cancer pathology reporting the presence of reelin mRNA in mice medulloblastoma tissue.

Reelin expression in non-cerebral tumor pathology has also been reported by immunohistochemical methods in esophageal[12] and pancreatic[9] cancer.

In order to further understand the role of reelin in neoplastic pathology, we initially investigated reelin expression by immunohistochemistry in a large series of most frequent human cancers (skin, colorectal, gastric, neuroendocrine, breast, prostate). In this initial study, reelin expression was found only in two prostate cancers. This finding led us to further investigate reelin expression in a larger series of prostate cancers.

Materials and methods

Case Selection

Reelin expression was evaluated in ten skin, colorectal, gastric, breast, neuroendocrine tumors and prostate cancer resection specimens (total of 60 cases), retrieved from the files of the Surgical Pathology Unit of the University Campus Bio-Medico. Tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Routine hematoxylin- and eosin-staining was performed on the sections for histopathologic evaluation. Tumor histology and grade were evaluated at primary diagnosis and extracted from the pathology reports. They were re-evaluated according to the International Union Against Cancer.[13] Consecutive $3 \mu m$ sections were cut from samples used for routine diagnostic purposes. Reelin expression was evaluated by immunohistochemical methods.

According to the initial immunohistochemical results, another 56 prostate cancer cases were further selected from the files of the Surgical Pathology Unit of the University Campus Bio-Medico and the Institute of Pathology of the University of Palermo. No patient had received chemotherapy, hormonotherapy or radiation therapy before surgery.

One of the pathologists (MZ) re-examined the hematoxylin- and eosin-stained slides, classified the tumors according to commonly used criteria[13] and representative blocks were chosen for reelin immunohistochemical evaluation. Within the 66 prostate cancer cases studied, 31 were radical prostatectomies, 19 enucleations and 16 TURPs. The Gleason score findings are reported in Table 2. High-grade prostatic intraepithelial neoplasia (PIN), the direct precursor of prostate cancer, was present in 31 of the 66 prostate cancers included in this study. For reelin immunohistochemical evaluation, $3 \mu m$ sections were cut from the paraffin blocks corresponding to the slides selected.

Antibodies and Reagents

Unless otherwise stated, all reagents were from DakoCytomation Inc. (Carpinteria, CA, USA). In the present study, two primary antibodies against reelin protein were used: the mouse monoclonal antibody (Calbiochem: 142 clone; 1/500) that recognizes an epitope localized in the N-terminal region of reelin[14] and, to confirm the specificity of reelin staining, 10 randomly select cases of prostate cancer were immunostained with the rabbit polyclonal antibody (Santa Cruz: H-221 clone; 1/200) that recognizes an epitope localized in the C-terminal region of reelin.[15]

Immunohistochemical Staining

Immunohistochemical staining was performed by the streptavidin–biotin method. In brief, sections were deparaffinized and endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide. The slides were washed with TBS, treated with horse serum for 30 min at room temperature to block non-specific binding, incubated with the anti-reelin mouse monoclonal (142 clone) and rabbit polyclonal (H-221 clone) antibodies for 2 h at room temperature. After washing three times with TBS (5 min each wash), sections were incubated with a biotinylated anti-mouse/anti-rabbit (LSAB2) antibody for 10 min. They were then washed three times with in TBS, treated with streptavidin–biotin–peroxidase complex for 10 min and then washed again with TBS for three times. Finally, specimens were incubated with 3,3′-diaminobenzidine (DAB) for 3 min, followed by hematoxylin counterstaining. All the sections were examined by light microscopy by two investigators (GP and DL) blind to the corresponding clinicopathological data to assess the presence or absence of reelin immunostaining and its distribution. Neural structures (fibres and ganglion) present in the tissue sections were used as positive internal control.[9] As negative control some slides were processed without primary antibody and were included in each staining run.

Reelin immunostaining was evaluated in terms of the percentage of reelin immunoreactive cells over a total of at least 1000 tumor cells. Only prostate cancers with a percentage of immunostained cells $\geq 10\%$ were considered as positive.

We evaluated reelin expression by assessing immunostaining in the various Gleason patterns in addition to Gleason score.

Statistic Methods

Reelin expression between different Gleason score and different Gleason patterns were compared using logistic regression analysis. Spearman's rank correlation test was used to assess relationships between ordinal data. $P < 0.05$ were regarded as statistically significant in tailed tests. SPSS software (version 13.00, SPSS, Chicago) was used for statistical analysis.

Results

Reelin Expression in Different Cancer Types

Among the different cancers studied in the initial analysis, only two prostate cancers resulted positive for reelin. Table 1 summarizes the principal pathological characteristics of these neoplastic lesions. Stromal tissue, vascular structures, normal epithelial cells did not show reelin expression. On the contrary, neural structures were positively stained for reelin. In particular, ganglia showed cytoplasmic staining for reelin.

On the basis of these preliminary results, reelin expression was further evaluated in a larger series of prostate cancers.

Reelin Expression in Prostate Cancer

Another 56 prostate cancers were selected on the basis of the corresponding Gleason score and were processed by immunohistochemistry for reelin expression.

Stromal tissues and normal glandular epithelial cells around and distant from the cancer were always negative for reelin. All prostate intraepithelial neoplasia (PIN) lesions of any grades were also negative. The neural structures present in the sections examined were positively stained for reelin, thus were used as internal positive control (Figure 1).

Of a total of 66 cases, 26 (39%) prostate cancers showed reelin expression. Table 2 reports the Gleason score and the immunohistochemical findings obtained with anti-reelin clone 142 antibody of all prostate cancers studied. Reelin expression was evenly distributed throughout the cancer in those cases with heterogeneous staining. Any difference in staining relating to the different types of tissue examined was found (data not shown). Furthermore, no difference in terms of reelin expression was found among cytoarchitectural patterns.

Similar immunohistochemical results were obtained with the two different antibodies (142 clone and H-221 clone) showing a clear cytoplasmic positivity for reelin in malignant neoplastic cells (data not shown).

Table 1 Preliminary study on reelin expression in cancer pathology

Cancer lesions	No.	Histotype	No.	Grade	No.	No. of reelin positive tumors
Skin	10	Basal cell	5		5	0
		Squamous cell	5	Low	3	0
				High	2	0
Colorectal	10	Adenocarcinoma	10	Moderate	5	0
				High	5	0
Gastric	10	Intestinal type	5	Low	3	0
				High	2	0
		Diffuse type	5	High	5	0
Breast	10	Ductal	5	Low	3	0
				High	2	0
		Lobular	5	High	5	0
Neuroendocrine	10	Gastric	4		4	0
		Duodenum	2		2	0
		Appendix	4		4	0
Prostate	10	Adenocarcinoma	10	Gleason 6	2	0
				Gleason 7	2	0
				Gleason 8	2	0
				Gleason 9	2	1
				Gleason 10	2	1

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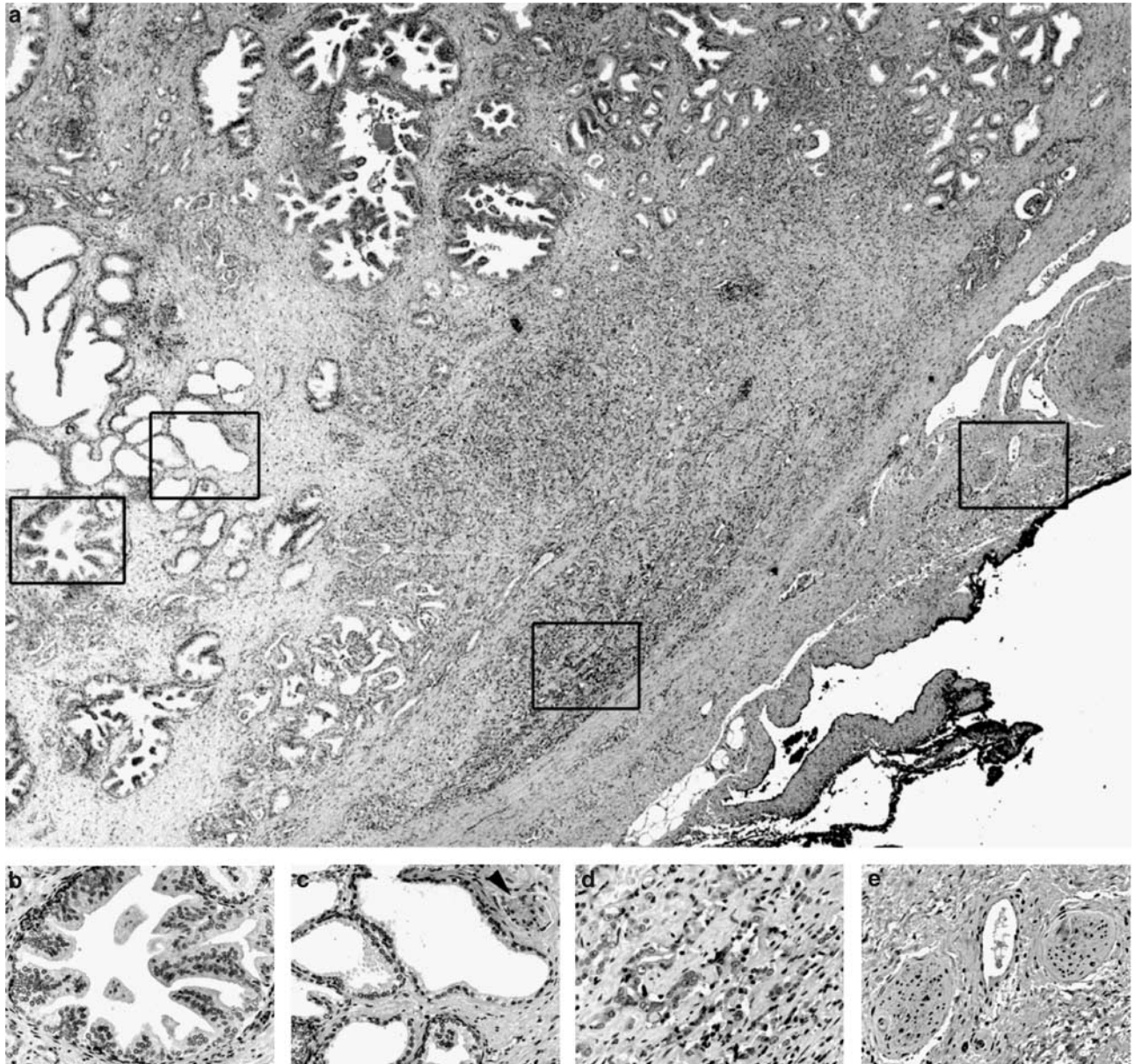
Figure 1 (**a**) Immunohistochemical analysis of reelin expression (Mab 142) in a prostate specimen with a Gleason 8(4 + 4) cancer. (**b–e**) Details of (**a**). (**b**) PIN lesions, (**c**) nonneoplastic prostate epithelial cells and stromal tissue resulted negative for reelin staining in contrast to positive labelling in a peripheral nerve tissue (arrow). Immunolabelling for reelin is identified diffusely in prostate cancer. (**d**) Clearly shows the cytoplasmic staining pattern of reelin in prostate cancer at higher magnification. (**e**) The neural structures present in the section were positively stained for reelin, thus were used as internal positive control. Original magnification: (**a**) $\times 20$; (**b–e**) $\times 200$.

Relationship between Reelin Expression and Gleason Score

Of 66 prostate cancers, 10 (15%) specimens were categorized as Gleason score 6, 11 (17%) as Gleason score 7, 18 (27%) as Gleason score 8, 23 (35%) as Gleason score 9 and 4 (6%) as Gleason score 10.

Reelin expression was found in malignant prostatic epithelial glands of 2/4 (50%) cases Gleason score 10, 12/23 (52%) Gleason score 9, 10/18 (56%) Gleason score 8, 2/11 (18%) Gleason score 7, while no sample of prostate cancers with Gleason score 6 showed reelin expression.

With the logistic regression analysis, a significant statistical difference was detected among Gleason scores as it concerns reelin expression ($P = 0.005$), suggesting that reelin expression is modified by differentiation grade (Table 3).

The Gleason score is the sum of the two most frequently occurring Gleason patterns within the same prostate specimen.[16]

As reelin staining is frequently found in high Gleason score prostate cancers, we explored whether reelin expression is influenced by single Gleason patterns.

Table 2 Gleason grade and Gleason patterns of all prostate cancers studied and immunohistochemical findings obtained with anti-reelin clone 142 antibody

No.	Age	Primary Gleason pattern	% of reelin positive cells on primary Gleason	Secondary Gleason pattern	% of reelin positive cells on secondary Gleason	Gleason score
1	71	5	43	—	—	10
2	58	5	71	—	—	10
3	75	5	0	—	—	10
4	68	5	0	—	—	10
5	85	5	59	4	51	9
6	81	5	21	4	0	9
7	59	5	46	4	0	9
8	74	5	0	4	0	9
9	62	5	0	4	0	9
10	56	5	5	4	0	9
11	66	5	85	4	0	9
12	77	5	89	4	10	9
13	61	5	72	4	0	9
14	66	5	0	4	0	9
15	73	4	0	5	0	9
16	76	4	0	5	77	9
17	65	4	0	5	0	9
18	71	4	0	5	0	9
19	65	4	0	5	0	9
20	73	4	0	5	75	9
21	78	4	0	5	0	9
22	54	4	71	5	73	9
23	74	4	0	5	0	9
24	78	4	41	5	0	9
25	65	4	0	5	0	9
26	81	4	52	5	55	9
27	70	4	37	5	29	9
28	70	5	0	3	0	8
29	75	5	0	3	0	8
30	73	5	49	3	0	8
31	74	3	0	5	0	8
32	62	4	0	—	—	8
33	66	4	58	—	—	8
34	76	4	0	—	—	8
35	77	4	92	—	—	8
36	80	4	89	—	—	8
37	68	4	0	—	—	8
38	72	4	49	—	—	8
39	64	4	53	—	—	8
40	68	4	0	—	—	8
41	80	4	20	—	—	8
42	80	4	43	—	—	8
43	77	4	62	—	—	8
44	75	4	0	—	—	8
45	59	4	34	—	—	8
46	63	4	57	3	0	7
47	70	4	0	3	0	7
48	54	4	0	3	0	7
49	64	4	0	3	0	7
50	68	4	0	3	0	7
51	81	4	5	3	0	7
52	62	3	0	4	3	7
53	67	3	0	4	0	7
54	66	3	0	4	0	7
55	68	3	0	4	0	7
56	68	3	0	4	54	7
57	57	3	0	—	—	6
58	75	3	0	—	—	6
59	64	3	0	—	—	6
60	75	3	0	—	—	6
61	66	3	0	—	—	6
62	68	3	0	—	—	6
63	68	3	0	—	—	6

Table 2 Continued

No.	Age	Primary Gleason pattern	% of reelin positive cells on primary Gleason	Secondary Gleason pattern	% of reelin positive cells on secondary Gleason	Gleason score
64	71	3	0	—	—	6
65	61	3	0	—	—	6
66	72	3	0	—	—	6

Table 3 Regression analysis between Gleason score and reelin expression

|	Reelin expression	Total (%)
Gleason grade		
6	0 (0)	10
7	2 (18)	11
8	10 (56)	18
9	12 (52)	23
10	2 (50)	4
Total	26 (40)	66

Logistic regression: odds ratio = 2.136; $P = 0.005$.

As expected, while Gleason 3 pattern did not show reelin immunoreactivity, reelin expression was found in 17/48 (35%) Gleason 4 patterns and 14/31 (45%) Gleason 5 patterns (Figure 2). Also the difference in terms of reelin expression within different Gleason patterns resulted statistical significant ($P < 0,001$) (Table 4). Furthermore, in this context, a highly positive statistical correlation was found between the percentage of reelin positive cells and the Gleason pattern (rho: 0.372; $P < 0.0001$). This finding suggests that reelin expression in higher Gleason scores is a result of the increased expression of reelin associated with an increased Gleason pattern.

Discussion

Reelin is a large protein of the extracellular matrix that plays an important role in the lamination of several brain structures during the cortical development.[17,18] Recently, it was demonstrated that reelin is also expressed in organs and compartments outside of the CNS[4–7,9] where it might regulate cellular differentiation and cellular migration. As these processes participate in neoplastic transformation and progression, we investigated reelin expression in a large series of neoplastic lesions and report for the first time reelin expression in prostate cancers.

In our study, we observed 39% prostate cancers showing reelin expression. Stromal tissues and normal glandular epithelial cells around and distant
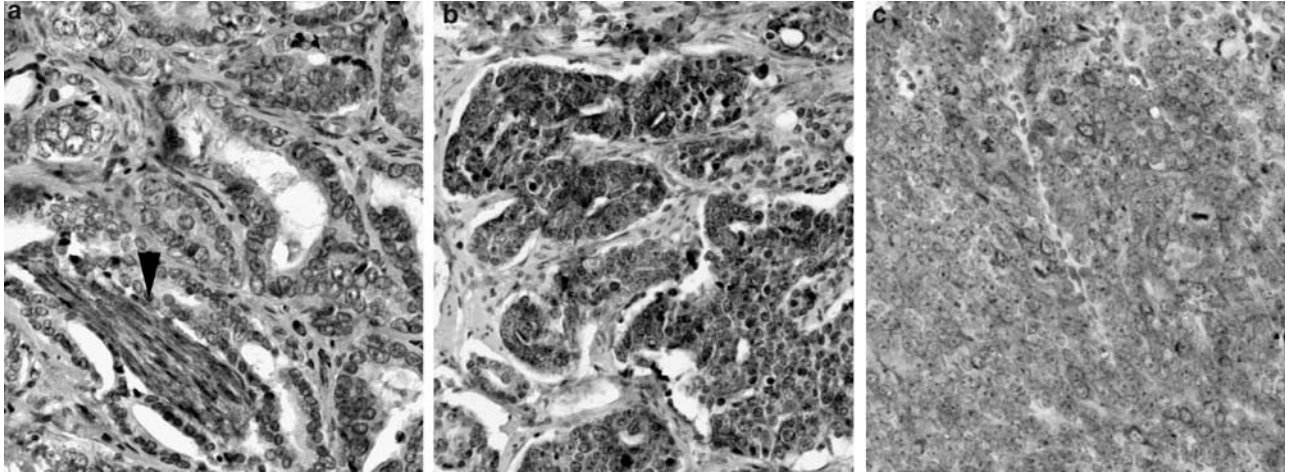
Figure 2 Immunohistochemical analysis of reelin in prostate cancer. (**a**) Gleason 3 pattern did not show reelin immunoreactivity. The neural structures present in the section were positively stained (arrow), which serve as an internal control. Reelin immunoreactivity was found in 35% Gleason 4 (**b**) and in 45% Gleason 5 pattern (**c**). Original magnification: (**a–c**) ×200.

Table 4 Regression analysis between differ ent Gleason patterns and reelin expression

	Reelin expression	Total (%)
Gleason pattern		
3	0 (0)	25
4	17 (35)	48
5	14 (45)	31
Total	31 (30)	104

Logistic regression: odds ratio $= 3.266$; $P = 0.001$.

from cancer were always negative for reelin. PIN lesions of any grade were also reelin negative. The neural structures present within the tissue examined sections showed a positive immunoreactivity for reelin, thus were used as an internal positive control.[9] According to our results reelin is expressed specifically in cancer lesions while it does not show up in normal and hypertrophic tissue. Reelin is not also expressed in PIN lesions, thus suggesting it behaves as a specific histological marker for prostate cancer.

Our results are consistent with another recent report about reelin expression in cancer pathology. Wang *et al*[12] found reelin expression in esophageal cancer through RT-PCR and immunohistochemical analysis on normal and tumor esophageal tissue. Low expression of reelin was detected in all normal esophageal tissues, while increased expression and strong immunoreactivity for reelin was detected in 87,5% of esophageal carcinoma tissues studied.

Furthermore, Sato *et al*[9] have recently demonstrated the expression of reelin protein in primary pancreatic cancers by immunohistochemical experiments performed using tissue microarrays on a large series of primary pancreatic adenocarcinomas and corresponding nonneoplastic pancreatic tissues. Of 294 primary pancreatic cancers, 28% showed diffuse immunolabeling for reelin. In nonneoplastic pancreas, immunolabeling for reelin was identified in the cytoplasm of various cell types, including normal ductal epithelial cells, acinar cells, islet cells, and autonomic peripheral nerves. Moreover they demonstrated that reelin is a frequent target for epigenetic silencing in pancreatic neoplasms, and loss of reelin function can confer a more aggressive phenotype to pancreatic cancer cells, thus showing an association between reelin expression and the progression of cancer.

In the present study, we show that an increase of reelin expression is more frequently associated with higher Gleason scores of prostate cancers. The results of our study, in agreement with the other reports, are consistent with the hypothesis that reelin may play a key role in cancer pathology.

Moreover, prostate cancer is the leading cause of death due to malignancy, accounting for 40% of all cancers diagnosed in men, and the frequency of all forms of the disease increases with age.[19] The early detection of molecular changes which are predictive of invasiveness and metastatic behavior is essential to clinical decision-making. Interestingly, in our study, a positive statistical correlation was found between reelin expression and Gleason score.

Gleason grading is the most widely used pathological grading system in patients with prostate cancer.[16] Although Gleason grading is commonly used as a predictor of disease progression, it is limited by lack of total accuracy in distinguishing patients at risk of disease progression from patients with stable disease. The 20-year prostate cancer mortality rate for patients with Gleason 5, 6, 7 and 8–10 is 14, 27, 45 and 66% respectively, suggesting that each Gleason score consists of an heterogeneous population of patients with both progressive and stable forms of disease.[20] Therefore, there is a clear

need to identify novel biomarkers that can further improve or predict clinical progression in addition to the Gleason scoring system. Our predictive capability of prostate cancer incidence, recurrence following treatment and disease progression has improved since the introduction of validated nomograms, which use multiple continuous variables for prediction.[21] In addition to clinical parameters such as tumor grade and stage, serum PSA and other serum and tissue molecular markers, such as interleukin receptor 6 and transforming growth factor β1, are now being included in experimental nomograms to improve the predictive accuracy.[22] Since the present study demonstrates that reelin expression correlates significantly with increasing Gleason score, it is conceivable that reelin may represent a useful biomarker to predict aggressive phenotypic behavior of prostatic cancer cells and thus may be potentially incorporated in future nomograms.

Besides demonstrating reelin expression in high-grade prostate cancers, we have also shown that reelin occurs more frequently in $\geq$Gleason 4 pattern compared to Gleason 3 pattern prostate cancer. There is a debate in the urologic community with regard to the clinical significance of individual Gleason patterns within the total Gleason scoring system.[23] For example, patients with a Gleason score 7 which show a Gleason score $4+3$ tend to have higher biochemical recurrence rate than patients with a Gleason score $3+4$, suggesting that an individual Gleason pattern can also represent an independent predictor of disease progression.[24] Our study confirms that reelin expression occurs at significantly higher rate in Gleason patterns 4 or 5 compared to Gleason patterns 3, which suggests that reelin could be a novel biomarker for high Gleason patterns. The finding that reelin expression differs significantly between Gleason patterns 3 and 4 suggests an association of reelin with a more aggressive malignant phenotype. Characteristic gene expression patterns characteristically associated with specific Gleason scores have already been shown for prostate cancers.[25]

As reelin is involved in the control of cell migration and the latter represents an important factor in cancer invasion and metastasis, further research on the role of reelin in prostate cancer may be helpful to understand the molecular mechanisms of prostate cancer aggressiveness and may provide a new target for diagnosis, prognosis and therapy of prostate carcinoma.

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