



Research article

IMMUNOHISTOCHEMICAL ANALYSIS ON THE ROLE OF GP130 IN EARLY-STAGE UROTHELIAL CARCINOMA OF THE URINARY BLADDER

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Introduction

Urinary bladder urothelial carcinoma is a common malignant disease with a high rate of local recurrence when diagnosed in its early stages. Multiple factors in the disease morphology and cell biology have been studied with little prognostic significance toward recurrence prediction. In the present study, we set out to study the role of glycoprotein with mol. weight of 130 kD (gp130) in disease recurrence by means of immunohistochemistry.

Materials and Methods

The study design was a non-clinical and non-interventional, retrospective, observational one. Patients with biopsy-proven early-stage urothelial carcinoma of the urinary bladder, diagnosed in a single tertiary healthcare institution in the period 2007-2011, with a five-year follow-up until 2011-2016 at the same institution, were included in the study. Demographic and biopsy data were collected. Gp130 immunohistochemistry was performed via routine methods, and the results were analyzed for statistical significance.

Results

The demographic results showed a typical cohort spread of patients with early-stage urothelial carcinoma – male dominant, high rate of recurrence, and shorter progression-free survival for high-grade and pT1 tumors. Gp130 h-score showed a cut-off value of 140; however, no significance was found with tumor differentiation, stage, occurrence, and time to local recurrence.

Conclusion

While the place of gp130 in cellular biology and especially its proven role in other malignant diseases are promising for research, our result did not show any correlation in relation to early-stage urothelial bladder carcinoma, beckoning a more in-depth study into the topic out of the scope of the currently presented one.

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INTRODUCTION

Urothelial, also referred to as transitional-cell carcinoma of the urinary bladder, is an extremely insidious malignant condition that progresses for an extended period of time without producing clinical symptoms (1). According to international data, it is the ninth most common malignancy in the world, with over 90-95% of all bladder tumors being of urothelial origin. In contrast, other histological types, such as squamous cell and glandular malignancies, are exceedingly rare (1,2).

With a diagnostic purpose and based on the defined mandatory morphological prognostic and predictive parameters, attention is increasingly focused on the search for new markers involved in the process of angiogenesis, cell proliferation, and differentiation, which could help unravel tumor biology (3). These markers are of interest in different types of carcinomas, including urothelial carcinoma, which, although in the early stages of its development, has a favorable prognosis and carries a risk of recurrence and progression. As such, the identification and application of markers to predict the biological behavior of the tumor in the early stages of its development, when no recurrences have yet occurred, combined with applied adequate treatment, can increase the chances of a favorable outcome for patients.

The identification and use in practice of more precise molecular markers in urothelial carcinoma, in combination with the morphological characteristics of the tumor and the staging of the cancer process, makes it possible to refine the diagnosis and, if possible, determine the prognosis of the patients (4,5). In some cases, it is necessary to apply markers that are generally used in tumors with a different histogenesis than that of the examined tumor. They could serve as predictors of the disease. Such a marker could be glycoprotein 130 (gp130) (6).

Gp130 is a signal-inducing receptor protein that forms part of the receptor complexes of some cytokines, including interleukin-6, interleukin-11, interleukin-27, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotropin-1, and cardiotropin-like cytokines (7). The interaction between gp130 and interleukin-6 is similar to what gp130 has with the other interleukins listed. When interleukin-6 binds to the corresponding receptor interleukin-6 receptor alpha, a heterodimeric association with gp130 follows, which forms a signaling complex composed of a gp130 homodimer and two interleukin-6 alpha receptors (8,9). Bound in this way to the two cytokine-specific alpha-chains, it activates the JAK-STAT pathways. Under normal circumstances, the Janus kinases JAK 1 and JAK 2, as well as tyrosine kinase 2, associate with gp130,

but the activation of alpha receptors results in phosphorylation and further activation of the kinase pathways (10,11). The specified enzyme activation is expressed in the translocation of these molecules toward the nucleus while simultaneously initiating gene transcription. The promoted action of gp 130 leads to the additional engagement of the cytoplasmic protein tyrosine phosphatase SHP2, which activates RAS/ERK, MAPK, and PI3K/AKT oncogenic cascades (12).

Further stimulated, these pathways are exclusively associated with oncogenesis (13), the aggressiveness of the respective tumor, invasiveness, early metastasis, resistance to apoptosis, and enhanced uncontrolled cell proliferation (14–16). This, in turn, makes gp130 an attractive therapeutic target. It is now known that increased activity of gp 130 is often found in colorectal cancer (17), breast carcinoma (18), as well as in invasive and as a rule aggressive carcinoma of the bladder (19,20). In urothelial carcinoma, the frequent recurrences and limitations of conservative treatment require the introduction of more effective and new diagnostic immunohistochemical markers for the disease in order to predict the progression of the disease and achieve a more complete therapeutic approach (21–23).

MATERIALS AND METHODS

Study design

The present study is a non-clinical and non-interventional, retrospective, observational one. Patients with biopsy-proven early-stage urothelial carcinoma of the urinary bladder (pTa and pT1 as per the 8th revision of AJCC tumor staging manual) from a single tertiary healthcare center were included. The inclusion period was 2007-2011, five full calendar years. A total of 163 patients with the specified selection criteria were included, separated into two groups based on disease progression:

1. The first group consisted of 95 patients (n=95). In these patients, no histological or endoscopic evidence of recurrence was detected in a five-year follow-up period (2011-2016).
2. The second group consists of 68 patients (n=68). This group comprises patients with verified local disease recurrence in the five-year follow-up period (2011-2016).

For both groups, data such as patient demographics – age and gender, grade of differentiation, disease stage at presentation, and time to local recurrence were collected for statistical analysis.

Immunohistochemical protocol for gp130 detection

An indirect immunoperoxidase method was applied for im-

munohistochemical analysis using mini KIT high Ph DAKO K8024 and antibody gp130 (E-8) with cat. No. sc-376280, a murine monoclonal primary antibody, at a dilution of 1:250. Positive antibody control was performed as per antibody specification with adult adrenal gland. Antibody expression visualization was carried out using an anti-polyvalent HRP/DAB detection system, and the reaction was visualized using an appropriate substrate chromogen (DAB/Diaminobenzidine) reagent.

Preparation of biopsy materials for immunohistochemical examination

- Biopsy materials from the initial biopsy diagnosis (fixed in neutral formalin and embedded in paraffin blocks) were cut into 4 µm thick sections and mounted on silanized glass slides.
- Deparaffinization was carried out in descending order of alcohols as follows: Ethanol 100% 3 min., Ethanol 90% 3 min., Ethanol 80% 3 min., Ethanol 70% 3 min., Xylene 3 x 10 min. Then, sections were washed with running water and placed in distilled water.
- Antigen retrieval was performed with EnVision FLEX Target Retrieval Solution62 preheated to 65°C (working solution) in a PT Link container, and the sections were incubated for 20 min at a temperature of 97°C and pH of 9. After cooling, samples were washed at room temperature with FLEX Wash Buffer (20x) for 1-5 minutes.
- All sections were stained with automatic stainer AUTO-STAINER LINK 48 SYSTEM – DAKO.

Immunohistochemical protocol

Sections were stained according to the FLEX protocol, using a wet chamber for all steps, followed by incubation with peroxidase blocking solution (3% H₂O₂) for 5 min at room temperature to block endogenous peroxidase activity and rinsing with wash buffer for 5 min.

- Incubation with the primary antibody, with dilution for 20 min at room temperature.
- Wash with washing buffer for 2 x 5 min. at room temperature
- Incubation with labeled HRP polymer for 20 min at room temperature.
- Wash with washing buffer for 3 x 5 min. at room temperature
- Incubation of the sections with chromogen DAB peroxidase solution for 2 x 5 min., with continuous microscopy.
- Wash with buffer for 2 min.

- Rinse with distilled water for 2 min.
- Counterstaining with Mayer's hematoxylin for 5 min.
- Rinse the samples with distilled water for 5 min.
- Dehydration in reverse ascending order ethanol 70%, ethanol 80%, ethanol 90%, ethanol 100% for the same duration as for deparaffinization.
- Placement in a mounting environment.

Expression analysis

Immunohistochemical evaluation was done by examining 10 fields at the highest magnification (x400) for each case. Scanned images of the cases were made using the Leica Aperio Scan Scope AT2 device (Aperio 50 Technologies, Vista, CA), with subsequent analysis of the scanned images with ImageScope V12.1.0.5029 (Aperio).

We evaluated the immunohistochemical expression using the H-score (histo-score) on the tissue samples. First, the intensity (I) of the reaction (0, 1+, 2+, or 3+) was determined for each cell in different fields. The number of positive cells (P) was scored as 0 (less than 10% positive cells), 1 (10–49% positive cells), 2 (50–74% positive cells), or 3 (more than 75% positive cells); finally, the H-score was calculated using the following formula:

$(1 \times (\% \text{ cells with } 1+) + 2 \times (\% \text{ cells with } 2+) + 3 \times (\% \text{ cells with } 3+))$, ranging from 0 to 300

The final score was dichotomously divided according to the median (determining a cut-off value). All cases above the median were reflected as high expression levels and those below the median as low.

Statistical analysis

The statistical analysis was performed using MS Excel 2016 software and IBM SPSS Version 25 statistical program for Windows and included the following methods:

- Descriptive analysis to determine statistical values: mean value ($\mu(X)$), standard deviation (SD), minimum (min) and maximum value (max).
- Crosstabulation and Chi-square (χ^2) when looking for significant differences in the frequency representation of categorical values. Statistical significance in Chi-square tests was accepted at $p \leq 0.05$.
- Correlation analysis to assess the dependence between the studied indicators and establish the strength of their interaction. The assessment of the strength of the dependency between the variables is based on the Pearson coefficient (Pearson r) results. The degree of association between variables was defined as significant at $r > 0.5 < r = 0.7$; large at

$0.7 < r = 0.9$, and extremely large at $r > 0.9$ at $p \leq 0.05$.

- Analysis of survival until the occurrence of recurrence - Kaplan-Meier test to study the survival of patients with urothelial carcinoma according to the influence of low and high expression of antibodies and the examined clinical-morphological indicators.
- Comparative analysis (hypothesis evaluation) - Student's t-test for comparing quantitative and qualitative indicators and studying their differences. The obtained data are presented as an arithmetic mean value and standard deviation for the individual studied groups. 95% confidence intervals (CI) were used.
- ROC graph analysis – to determine the role of accuracy and specificity in the predictability of specific metrics. The value of the area under the curve ranges between 0.5 and 1.0. A complete separation of values by a given indicator should be done at a result above 0.75 or 75%. Differences between groups were statistically significant at $p \leq 0.05$.
- In all conducted analyses, an acceptable significance level of $p < 0.05$ with a confidence interval of 95% CI is accepted.
- Graphical and tabular presentation of statistical data was carried out using Microsoft Office 2016.

RESULTS

Distribution of patients with urothelial carcinoma of the bladder by age

A total of 163 patients with stage pTa and pT1 urothelial carcinoma of the bladder were included in the present study; the average age of the patients was 65.92 ± 11.06 years, with the youngest patient being 31 and the oldest being 89 years old (Table 1). The patients were divided into six age interval groups, with the highest percentage of patients being in the 61-70 years age group (63%) and the lowest in the 31-40 years age group (1.8%).

Table 1. Distribution of patients with urothelial carcinoma by age group ($n=163$)

Age group	n	%
31-40	3	1.8
41-50	11	6.7
51-60	28	17.2
61-70	63	38.7
71-80	46	28.2
81-90	12	7.4
Total	163	100

Distribution of patients with urothelial carcinoma of the bladder by gender

In the studied cohort, male patients dominate - 119 of the cases (73%), while females are almost three times fewer - 44 (27%) (Tab. 2).

Table 2. Distribution of patients with urothelial carcinoma by gender ($n=163$)

	n	%
Female	44	27.0
Male	119	73.0
total	163	100

pT stage of urothelial carcinoma – wall invasion

Depending on the degree of invasion into the bladder wall, patients in stage pTa and pT1 were selected in the study group, who are subject to intravesical local therapy and follow-up by cystoscopies. It is impressive that the group is strongly dominated by urothelial carcinomas in stage pTa - 109 of the cases (66.9%), while patients with urothelial carcinoma in stage pT1 were nearly twice fewer - 54 (33.1%) (Table 3).

Table 3. Distribution of patients with urothelial carcinoma according to stage ($n=163$)

Stage	n	%
pTa	109	66.9
pT1	54	33.1
Total	163	100

Degree of differentiation of urothelial carcinoma – grade (G)

We determined the degree of differentiation of urothelial carcinoma of the urinary bladder according to the WHO and ISUP classification from 2004. According to it, urothelial carcinomas in the studied group are divided into low-grade and high-grade carcinomas. The majority of patients were assessed as low-grade – 107 (65.6%), while patients with high-grade tumors were approximately twice as many – 56 (34.4%) (Table 4).

Table 4. Distribution of patients with urothelial carcinoma of the bladder according to differentiation ($n=163$)

Tumor grade	n	%
low	107	65.6
high	56	34.4
Total	163	100

The biological behavior of bladder urothelial carcinoma in stages pTa and pT1 is expressed in the fact that they often produce local recurrence and, less often, invasion in the detrusor of the bladder wall. The examined materials are from transurethral resections, and in a large percentage of cases, it cannot be definitively determined whether it is a tumor in stage pTa or pT1. Therefore, in the present study, we divided the patients into two groups with invasive and non-invasive tumors according to their biological behavior – recurrent and non-recurrent tumors.

In the studied group of 163 patients with urothelial carcinoma of the urinary bladder, the male gender clearly dominates - 53 cases (77.9%) have relapsed, and 66 (69.5%) have not relapsed.

pT (stage) in patients with recurrent and non-recurrent urothelial carcinoma

Depending on the invasion of the bladder wall, in the group of patients with developed tumor recurrence, it was found that those with a tumor defined as pTa predominated - 43 cases (63.2%), and approximately two times less were those in stage pT1 – 25 patients (36.8%). In the group of patients in whom the tumor did not relapse, it was found that most were those with a tumor classified as pTa - 66 patients (69.5%), and nearly twice as few of them were those in pT stage T1 – 29 patients (30.5%), (Tab. 5).

Table 5. Distribution of urothelial carcinoma patients by stage - pT

Stage	Recurrent disease		Non-recurrent disease	
	n=	%	n=	%
pTa	43	63.2	66	69.5
pT1	25	36.8	29	30.5
total	68	100	95	100

Concerning the occurrence of recurrence in the study group using statistical progression free survival analysis (Kaplan-Meier test) with a view to assessing the prognostic significance of the tumor stage (pT-stage), it was proved that invasive urothelial carcinomas (pT1) had a definite prognostic significance regarding the occurrence of local recurrence (Fig 1). There was a significant difference in overall local recurrence-free survival among different stages. The occurrence of local recurrence in the group of patients with stage pT1 urothelial carcinoma (8.12 months, (95%CI, 5.16-11.07)) was much earlier when compared to the group

of patients with stage pTa tumors (11.53 months, (95%CI CI, 8.03-15.03) (Tab. 6).

Table 6. Average time (months) until recurrence of urothelial carcinomas in stage pTa and pT1; 95%CI, $p < 0.01$

Stage	mean	Standard deviation
pTa	11.535	1.787
pT1	8.120	1.509
total	10.279	1.268

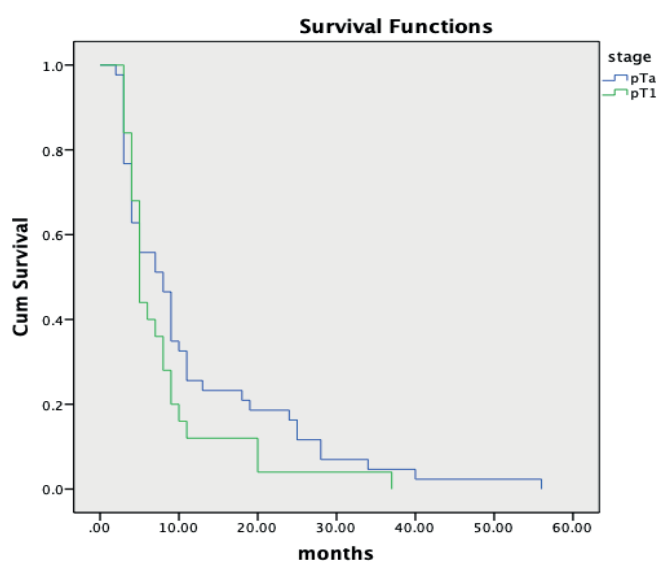


Figure 1. Kaplan-Meier curves of months to relapse according to stage (T-stage), ($p=0.01$). The conducted statistical correlation analysis, according to Spearman (ρ), proved that there is no relationship between the stage of the tumor and the occurrence of local recurrence ($\rho=0.065$, $p=0.407$). The probability of local recurrence cannot be predicted according to the pT stage category.

Degree of differentiation (grade) in patients with recurrent and non-recurrent urothelial carcinoma

In the studied group of patients with urothelial carcinoma with local recurrence, we determined the degree of differentiation according to the classification of WHO and ISUP (2004), with the majority of patients defined as low-grade 38 (55.9%) and the smaller are high-grade – 30 (44.1%). In the group of carcinomas without local recurrence, most of the patients were defined as low-grade 69 (72.6%), and approximately three times less were those with high-grade carcinoma – 26 (27.4%) (Table 7).

Table 7. Distribution of patients with urothelial carcinoma depending on the degree of differentiation

Tumor differentiation	Recurrent disease		Non-recurrent disease	
	n=	%	n=	%
low	38	55.9	69	72.6
high	30	44.1	26	27.4
total	68	100	95	100

Regarding the occurrence of recurrence in the study group with the Kaplan-Meier test (Fig. 2), it is clear that the group of urothelial carcinomas with a low grade of differentiation has a longer survival period without local recurrence (11.44 months, 95%CI, 7.66-15.22), while patients from the group of urothelial carcinomas with a high-grade of differentiation have a shorter period prior to the occurrence of local recurrence (8.8 months, 95%CI, 5.84-11.75), (Table 8).

Table 8. The average value of the time (months) until recurrence in low-grade and high grade differentiation of urothelial carcinomas; 95%CI, $p < 0.01$

Tumor differentiation	mean	Standard deviation
low	11.447	1.928
high	8.800	1.507
total	10.279	1.268

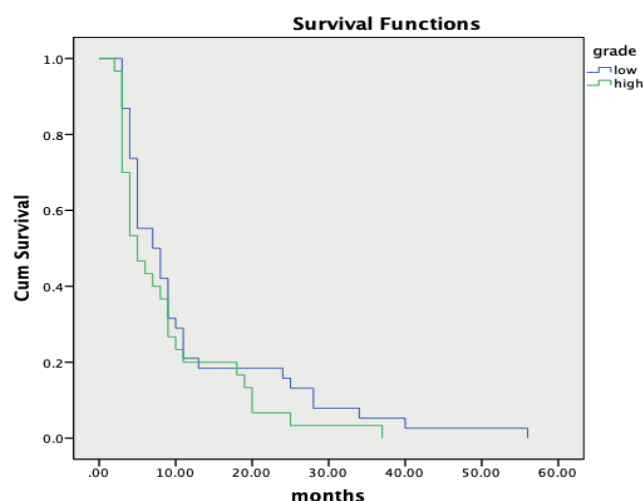


Figure 2. Kaplan-Meier curves of months to relapse according to tumor differentiation (Grade), ($p = 0.01$)

The conducted statistical correlation analysis, according to Spearman (ρ), proves that there is a relationship between the

differentiation (Grade) and the occurrence of local recurrence ($\rho = 0.174$, $p = 0.026$). The probability of local recurrence is greater in high-grade urothelial carcinomas than in low-grade tumors.

Gp130

The immunohistochemical examination revealed expression of gp130 in the cytoplasm of the tumor cells. We evaluated the expression levels with the h-score scale, ranging from 0 to 300. Figure 3 shows the expression of gp130 in low and high-grade urothelial carcinomas.

The expression of gp130 differs in the two groups of urothelial carcinomas with different degrees of differentiation - low and high-grade, stage (pT), and the occurrence of local recurrence. Using ROC Curve analysis, we determined the cut-off value that helped us divide the expression levels of gp130 into low and high expression levels. For the cut-off value of gp130 expression, we found 140. The analysis showed that at optimal cut-off values of cytoplasmic gp130 expression from tumor cells, no relationship was established between the expression levels and the stage of urothelial carcinomas (AUC = 0.465, 95% CI: 0.319- 0.611, $p = 0.634$), (Fig. 4).

Comparing gp130 expression levels and tumor differentiation, no statistically significant difference was also found (AUC = 0.514, 95% CI: 0.383-0.645, $p = 0.827$) (Fig. 5).

DISCUSSION

One of the most characteristic features in the biological behavior of urothelial carcinomas of the bladder is the extremely high recurrence rate compared to malignant epithelial tumors of other localization. With this in mind, we set out to conduct a study of patients with endoscopically proven urothelial carcinoma of the bladder with a follow-up over five years for the eventual occurrence of local recurrence. Possible mechanisms for the occurrence of recurrences are due to incomplete resection or secondary implantation. More significant are those recurrences, which represent the growth of microscopic tumors not visible at the initial examination or the de novo appearance of the tumor. Biopsied from our study did not provide sufficiently informative clinical data to allow us to stratify recurrent carcinomas into subgroups by this metric. We assume that the cases in our study are mostly from the first and third groups (incomplete resection and undetected at biopsy) because, with endoscopic biopsy, the possibility of implantation is small, and de novo tumors appear significantly later (24). The role of gp130 in urothelial carcinoma of the bladder is not

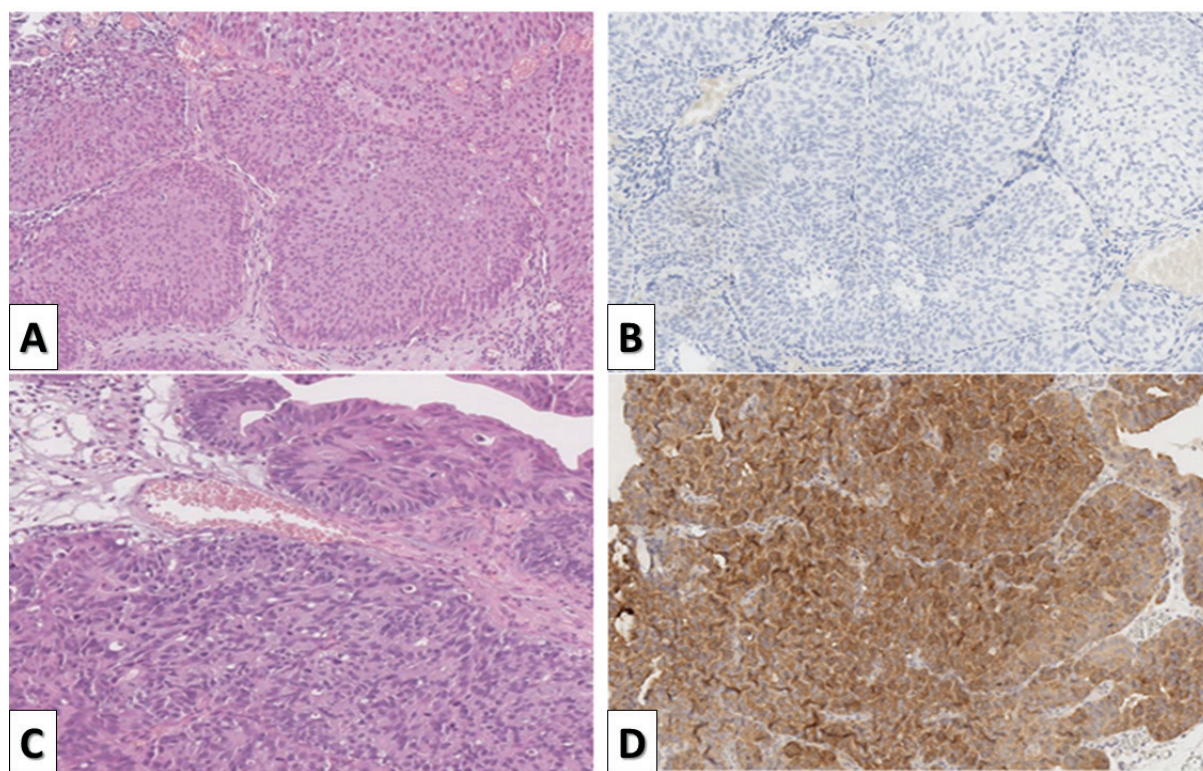


Figure 3. Expression of gp130 in low and high-grade urothelial carcinomas. **A.** Low-grade urothelial carcinoma, H&E staining, magnification x100; **B.** Lack of gp130 expression in low-grade urothelial carcinoma, magnification x100; **C.** High-grade urothelial carcinoma, H&E staining, magnification x100; **D.** Intense cytoplasmic expression of gp130 in high-grade urothelial carcinoma, magnification x100

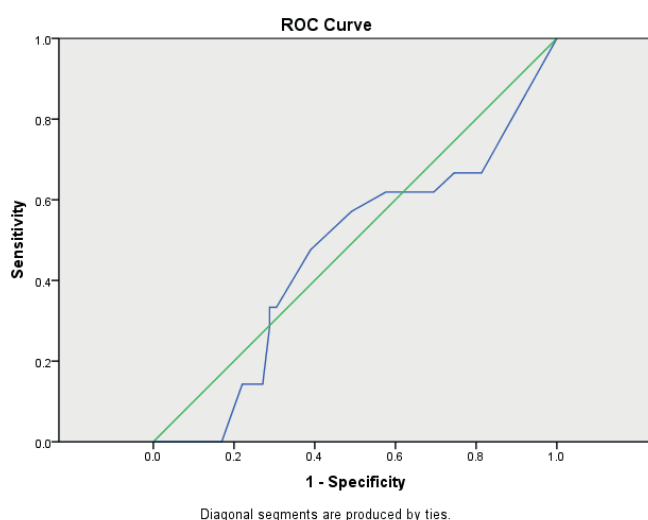


Figure 4. ROC Curve analysis and determination of cut-off value for gp130 (AUC = 0.465, 95% CI: 0.319-0.611, $p = 0.634$)

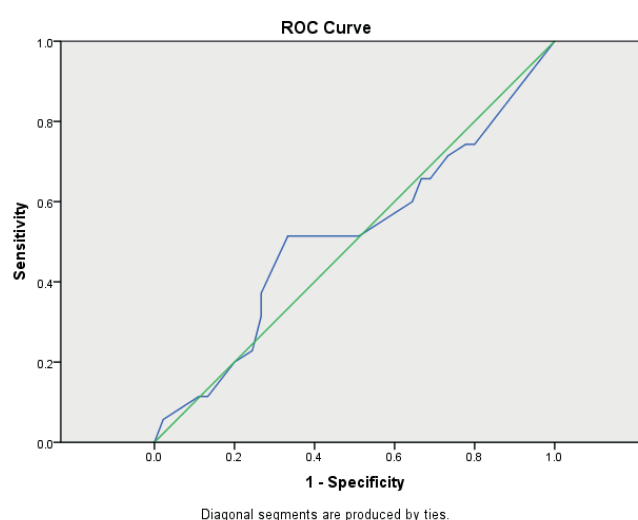


Figure 5. ROC Curve analysis and determination of the cut-off value for gp130 (AUC = 0.514, 95% CI: 0.383-0.645, $p = 0.827$). From the performed statistical analyses, no relationship was established between the expression of gp130 and the clinico-morphological parameters (gender, age, and occurrence of local recurrence) at a statistically significant $p < 0.05$.

sufficiently well understood. According to Omokehinde T *et al* (18). gp130 regulates a wide range of processes, including tumor pathogenesis and metastasis. Authors such as Xu S *et al* (15) suggested that gp130 may have a role in the initiation of urothelial carcinoma through coexpression with genes promoting tumor cell proliferation.

Martin D *et al* (20), evaluating the expression of gp130, Bcl-xL, and VEGFR2 on 11 urothelial carcinomas, demonstrate that it is associated with tumor aggressiveness. In particular, gp130 expression significantly correlated with tumor differentiation grade, nodal status, and patient survival. The same authors noted low expression of gp130 mainly in non-aggressive papillary tumors of the bladder, while high levels correlated with malignant tumors with a poor prognosis. In the present study, we did not find such a relationship, which can be explained by the fact that the number of patients we studied is greater, and the significance of the study is accordingly greater. The established positive expression of gp130 in the cytoplasm of the tumor cells of the carcinomas did not show a relationship with the clinicopathological indicators we examined (gender, age, differentiation, stage, and occurrence of local recurrence) at a statistically significant $p < 0.05$.

Despite the data from the literature and the research carried out so far, there are still uncertainties regarding the mechanisms and stages of action in tumorigenesis.

A better understanding of the signaling pathways and behavior of tumor cells, their surrounding microenvironment, and their interaction would provide knowledge of the mechanisms responsible for the stimulation and/or inhibition of tumorigenesis. There is great hope that elucidation of these mechanisms may lead to the development of new therapeutic targets to prevent both tumor onset and progression, as well as tumor recurrence.

STUDY LIMITATIONS

The present study's findings indicate no association between gp130 expression and the clinical, demographic, morphological, and prognostic parameters in urinary bladder urothelial carcinoma. These findings have several indications for further research into the field and validation. First, as gp130 has been shown to play a role in multiple malignancies and different cellular pathways, molecular variation of these results. Second, the expression pattern in recurrent and non-recurrent UCs should be looked at with different clones of poly and monoclonal antibodies, as the lack of correlation may be due to the lack of sensitivity of the antibody used by us or the detection of mutant phenotype

protein by it. Sadly, our study falls short in these perspectives due to its initial design. Furthermore, a larger cohort with more extensive follow-up, more advanced stages of the disease, and possible urothelial carcinomas in other locations than the urinary bladder will significantly enrich the medical literature on the topic far beyond the scope of the present study.

CONCLUSION

Urothelial carcinomas of the urinary bladder are one of the most commonly diagnosed malignancies worldwide, more often affecting males and with a high tendency for recurrence in their initial presentation stages (pTa and pT1). In the present study, we have studied the link between gp130 and its established role in cellular processes in the occurrence of local recurrence of bladder urothelial carcinomas by means of immunohistochemistry. Despite gp130 being a promising marker to study and the variability in our cohort's clinical, demographic, and progression characteristics, the result yielded no correlation with expression levels whatsoever.

CONFLICT OF INTEREST

The authors declare no conflicting interests

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