Role of HER2 and Claudins in subtypes of urothelial carcinoma identified byGATA3 and Cytokeratin5\6

Immunohistochemical study

Abstract:

Background: Bladder cancer is globally the ninth, most common malignancy, and the thirteenth most common cause, of cancer death, associated with high morbidity and mortality, if not treated optimally. Bladder cancers can be divided into molecular subtypes, referred to luminal and basal with distinct clinical behaviors. HER2 is one of the established therapeutic targets in many cancers. Claudins are tight junction proteins, known to modulate therapy resistance in cancer cells.

Aim: This study aimed to assess Claudins and HER2 status in the context of tumor molecular subtypes, identified by GATA3 and CK5/6 expression, that may help to select urothelial carcinoma patients, most likely to respond to immunotherapy.

Materials and Methods: This retrospective study was done upon 50 cases of conventional urothelial carcinoma. GATA3, CK5/6, HER2, Claudins1&4 and P53immunostaining were done and correlated with clinico-immuno-pathological parameters.

Results: Bladder cancers could be assigned to main intrinsic molecular subtypes, referred to luminal, basal and double negative. Basal & double negative bladder cancers were more aggressive, when compared to luminal cancers. Positive significant statistical correlation was found between HER2, claudin1 and P53 and clinic-immuno-pathological parameters as tumor size, grade, TNM stage, LVI, tumor budding and aggressive molecular subtypes (P-value< ***0.05).*** Negative significant statistical correlation was found between claudin4 and fore mentioned clinico-immuno-pathological parameters (P-value< ***0.05***).

Conclusions: The molecular subtypes of bladder cancers, HER2, claudin1&4 and P53 can be used for prognostic and therapeutic stratification of bladder cancers patients, and may affect patient outcome.

Keywords: Molecular subtypes, HER2, Claudins1&4

Abbreviations: Cytokeratin (CK) lympho-vascular invasion (LVI)

Introduction:

Bladder cancer is one of common globally, increasing cancer. Worldwide, it is the 7th most common cancer in men, and the 17th most common cancer in women, and the 9th most common in both sexes **(1).** Bladder cancer is the most common malignancy among Egyptian males, and previously has been attributed to *Schistosoma* infection, a major risk factor for squamous cell carcinoma **(2).**

Bladder cancers could be assigned to main intrinsic molecular subtypes, referred to luminal and basal. This may provide prognostic information, and may help to identify a subgroup of patients, with increased sensitivity to chemotherapy **(3).** KRT5/6 and GATA3 immunohistochemical markers, may have a great role in classifying the urothelial bladder tumors into different molecular subtypes. **(4)**

The HER2 is one of the epidermal growth factor receptors, which contribute to physiological mechanisms of cell proliferation, by intrinsic tyrosine-kinase activity. The overexpression of HER2 was shown in several malignancies, and it is known to affect proliferation, angiogenesis and metastasis of malignant cells. **(5)**

Claudins are tight junction proteins (TJs), responsible for maintaining cellular polarity, and cell-cell communication**.** A disruption of TJs leads to invasiveness, loss of cohesion, and lack of differentiation in cancer cells. **(6)**

The aim of this study, is to evaluate the IHC expression of HER2, claudins1&4, P53 in molecular subtypes of bladder cancer, identified by GATA3 and CK5/6 expression, and correlate these results with clinico-pathological data, to clarify its diagnostic, prognostic and predictive roles.

Material and Methods:

This is a retrospective study, on 50 archival formalin fixed, paraffin-embedded, tissue specimens, of Egyptian patients of conventional urothelial carcinoma, collected from Benha Pathology Department, Faculty of Medicine, Benha University and International Medical Center, during the period from 2015 to 2019, with available demographic, and clinico-pathological data. This research plan, was approved by ethical committee, of Benha Faculty of Medicine and International Medical Center.

A- Histopathological Examination:

Hematoxylin and eosin-stained slides, on all cases, were revised by two observers simultaneously, to confirm the diagnosis. Conventional urothelial carcinoma cases, were graded, according 2016 WHO, into low grade and high grade **(7).** Cases were staged by, TNM staging, pT stage classified into pT0, pT1, pT2, pT3, pT4, and stage grouping into, 0, I, II, III, IV as stages0/1, were considered low stage, and stages I/III/IV were considered high stage **(8).**

B-Immunohistochemical Procedure:

For immunohistochemical analysis, 4-micron thick sections were obtained, from formalin-fixed, paraffin-embedded tissue, blocks, on coated slides. According to manufacture instructions, antigen retrieval for Claudin1, claudin4, P53 and CK5/6 was done, by using 10 mmol/Lcitrate monohydrate buffer (pH 6.0), and for HER2 and GATA3, was done by using 10ml solution of EDTA buffer (pH 9.0). The slides, were immunostained for HER2 polyclonal antibody Cat.#A0485 (**DAKO Agilent Pathology Solution**), Claudin1 polyclonal antibody Cat.#RB-9209-R7 (T**hermo Fisher scientific anatomical pathology, USA**), Claudin4 polyclonal antibody Cat.#RB-9043-R7 **(Thermo Fisher scientific anatomical pathology, USA**), P53 monoclonal antibody Cat.#CBL-422 (**DAKO Agilent Pathology Solution**), GATA3 polyclonal antibody Cat.#YPA1589 (**Chongqing Biospes Co.,Ltd**) and CK5/6 monoclonal antibody Cat.#MA5-12429 (**DAKO Agilent Pathology Solution**), at a dilution of 1:100, at room temperature for 30 minutes. Immunodetection was executed, using a standard labeled streptavidin-biotin system, (***Dako Cytomation, Denmark, A/S).***

Negative & positive controls

- The epidermis of apparently normal skin was used as positive control for claudin1 & epithelium of apparently normal colon was used as positive control for claudin4**(9)**
- Epithelium lining the renal collecting ducts, was used as positive control, for GATA3 & apparently normal breast tissue, was used as positive control for CK5/6 **(4**).
- Apparently normal tonsil germinal centers B cells, was used as positive control for P53 & HER2 positive breast cancer were used as positive control for HER2 **(5)**

For negative controls, omitting the primary antibody and replacing it with normal rabbit serum IgG.

Immunostaining evaluation:

Claudin1 & Claudin4 expression, was detected as, homogeneous, brown, cytoplasmic, or membranous coloration. Immunoreactivity was assessed, based on a combined score of the extent, and intensity of staining. Scores 0–3 were assigned, according to the percentage of positive tumor cells (0=0%; 1=<25%; 2=25–50%; 3=>51%), and the intensity of staining in tumo,r (0=negative; 1=weak; 2=moderate; 3=strong). The two scores were multiplied, to give an overall score (H-score) of 0–9, of which 0 was considered negative, 1–2 was considered weak, 3–6 moderate, and 9 strong staining. Negative and weak expression was considered as low, whereas moderate and strong as high. **(9)**

HER2 expression was scored, using the latest ASCO/CAP guidelines published in 2016,

: negative (0/1+), equivocal (2+) and positive (3+), with a cut-off for score 3+, if more than 10% strongly positive, complete, membrane, staining of cells. (10)

Immunoreactivity of GATA3, was assessed, as any intensity of nuclear staining, with greater than 5% of cells, was considered positive. (**11)** Immunoreactivity of CK5/6 was assessed, as a score of 0 corresponded to 0%; 1, <1%; 2, 1% to <10%; 3, 10% to <33 %; 4, 33% to <66%; and 5, ⩾66 % of diffuse, homogenous, cytoplasmic, and/ or membranous staining of any intensity of tumor cells. It was determined that, scores 0, 1, and 2 indicate negativity and scores 3, 4, and 5 to indicate positivity. ***(12)*** Immunoreactivity of P53 was considered positive, if any cancer cell showed strong nuclear staining. **(13)**

Statistical analysis: Results were analyzed by SPSS (version 16) statistical package, for Microsoft windows. The Pearson correlation coefficient, was used for statistical analysis. P value <0.05, was considered statistically significant, and highly statistically significant when it was <0.01. ROC curve was also used to determine AUC, Sensitivity & Specificity of all markers, as AUC > 0.7, considered good.

Results:

Clinico-histo-pathological results:

There was, a significant, statistical, correlation, between pathological T stage, and other clinico-histo-pathological parameters, as tumor size, grade, nodal metastasis, distant metastasis, LVI, Associated CIS, Tumor budding, and focality of tumors, as P-value< ***0.05***. ***Table*** **(1)**

Immunohistochemical results:

- **Regarding** molecular subtypes, the studied cases were classified according to the study of **(4**), **as 56%** were luminal **subtype (**(GATA3+/CK5/6+), 32% were basal, and 12% were double negative. Then luminal cases reclassified, regarding morphology**,** according to the study of **(14) into,** 57% of luminal cases were papillary-morphology and 43% were infiltrated non papillary morphology. **Table (2)**
- There was a statistically, significant correlation, between molecular subtypes, and tumor size, tumor grade, pT stage, Stage grouping in radical cystectomy cases, lympho-vascular invasion, tumor budding, and Tumor focality (P-value< ***0.05***) **Table (3),**
- Regarding HER2 expression, there was a statistically significant correlation, between HER2 expression, and tumor size, tumor grade, pT stage, lymph node metastasis, distant metastasis in radical cystectomy, lympho-vascular invasion, tumor budding, and molecular subtypes P-value< ***0.05*** **Table (4), Figure (1)**
- Regarding p53 expression, Positive significant statistical correlation was found between **P53** and clinico-immuno-pathological parameters, as tumor size, grade, stage, nodal metastasis, distant metastasis, LVI, Tumor budding, focality of tumors, molecular subtypes, with aggressive behavior. as P-value < ***0.05.*** **Table (4), Figure (1)**
- Regarding claudins expression, Positive significant statistical correlation, was found between claudin1 and clinico-immuno-pathological parameters, as tumor size, grade, stage, nodal metastasis, distant metastasis, LVI, Tumor budding, focality of tumors, molecular subtypes with aggressive behavior, HER2 expression, and p53 expression, as P-value< ***0.05***, and negative significant statistical correlation was found between claudin4, and fore mentioned clinic-immuno-pathological parameters P-value< ***0.05***, **Table (5), Figure (2).**

Table* (1) *Correlation between different clinic-pathological parameters and pathologic T stage (pT):

Clinico -pathological variants		Total	*pT stage*		P Value
			NMI(pTa,T1)	**MI(pT2,pT3,pT4)**	
			NO	**NO**	
Histopathological variant	**Papillary**	**17**	**15 (88%)**	**2 (12%)**	**P= 0.000**
	non papillary	**33**	**3 (9%)**	**30 (91%)**	**HS**
Tumor size	**Up to 3cm**	**21**	**15 (71%)**	**6 (29%)**	**P= 0.000**
	More than 3cm	**29**	**3 (9%)**	**26 (91%)**	**HS**
Grade	**Low**	**18**	**15 (83%)**	**3 (17%)**	**P= 0.000**
	High	**32**	**3 (9%)**	**29 (91%)**	**HS**
Nodal Metastasis in radical cystectomy cases	**N0**	**7**	**4 (57%)**	**3 (43%)**	**P= 0.01**
	N+	**19**	**0 (0%)**	**19 (100%)**	**HS**
Lymph vascular invasion	**Absent**	**20**	**16 (80%)**	**4 (20%)**	**P= 0.000**
	Present	**30**	**2 (7%)**	**28 (93%)**	**HS**
Associated CIS	**Absent**	**28**	**17 (60%)**	**11 (40%)**	**P= 0.000**
	Present	**22**	**1 (5%)**	**21 (95%)**	**HS**
Tumor focality	**Unifocal**	**32**	**18 (56%)**	**14 (44%)**	**P= 0.000**
	Multifocal	**18**	**0 (0%)**	**18 (100%)**	**HS**
Tumor Budding	**Present**	**25**	**17 (68%)**	**8 (32%)**	**P= 0.000**
	Absent	**25**	**1 (4%)**	**24 (96%)**	**HS**

Table (2): Molecular subtypes of the studied cases identified by GATA3 & CK5/6 status:

Molecular subtypes	*NO.*		
Luminal (GATA3+/CK5/6+)	28 (56%)	Luminal with papillary morphology	16 (57% of luminal cases)
		Luminal with infiltrated non papillary morphology	12 (43% of luminal cases)
Basal (GATA3- /CK5/6+)	16 (32%)		
Double Negative (GATA3 - and CK5/6 -).	6 (12%)		
Total	50(100%)		

Table* (3) *Correlation between different clinic-pathological parameters and Molecular subtypes:

			Total	Molecular subtypes				P Value
				Luminal – papillary morphology	**Luminal – infiltrated non papillary morphologyy**	**Basal**	**Double Negative**	
				NO	**NO**	**NO**	**No**	
Histopathological variant	**Papillary**		**17**	**16 (94%)**	**0 (0%)**	**1 (6%)**	**0 (0%)**	**0.001**
	non papillary		**33**	**0 (0%)**	**12 (36%)**	**15 (45%)**	**6 (18%)**	**HS**
Grade	**Low grade**		**18**	**15 (83%)**	**3 (17%)**	**0 (0%)**	**0 (0%)**	**0.001**
	High grade		**32**	**1 (3%)**	**9 (28%)**	**16 (50%)**	**6 (19%_)**	**HS**
pT stage	**NMI**	**pTa**	**8**	**8 (100%)**	**0(0%)**	**0 (0%)**	**0 (0%)**	**0.000**
		pT1	**11**	**7 (64%)**	**3 (27%)**	**1(9%)**	**0 (0%)**	**HS**
	MI	**pT2**	**21**	**1 (5%)**	**6 (29%)**	**10 (48%)**	**4 (19%)**	
		pT3	**9**	**0 (0%)**	**3 (33%)**	**4 (44%)**	**2 (22%)**	
		pT4	**1**	**0 (0%)**	**0 (0%)**	**1 (100%)**	**0 (0%)**	
Stage grouping in radical cystectomy cases	**Low stage (0&I)**		**7**	**3 (43%)**	**3 (43%)**	**1 (14%)**	**0 (0%)**	**0.004** **HS**
	High stage (II,III&IV)		**19**	**1 (5%)**	**5 (26%)**	**8 (42%)**	**5 (26%)**	
Associated CIS	**Present**		**22**	**0 (0%)**	**2 (9%)**	**14 (64%)**	**6 (27%)**	**0.002**
	Absent		**28**	**16 (57%)**	**10(36%)**	**2(7%)**	**0 (0%)**	**HS**
Tumor budding	Present		**25**	**1 (4%)**	**4 (16%)**	**14 (56%)**	**6 (24%)**	0.000
	Absent		**25**	**15 (60%)**	**8 (32%)**	**2 (8%)**	**0 (0%)**	HS
Tumor focality	**Unifocal**		**32**	**16 (50%)**	**10(31%)**	**6 (19%)**	**0 (0%)**	**0.000**
	Multifocal		**18**	**0 (0%)**	**2 (11%)**	**10(56%)**	**6 (33%)**	**HS**

Table (4) Correlation between clinic-immuno-pathological Variants and HER2 and P53 Expression:

Clinicoimmunopathological variants		Total		HER2 expression		P Value	P53 expression		P Value
				Negative	**Positive**		**Negative**	**Positive**	
Histopathological variant	**Papillary**	**17**		**15 *(88%)***	**2 (12%)**	**P= 0.108**	**13 (76%)**	**4 (24%)**	**P=0.000**
	non papillary	**33**		**16 (48%)**	**17 (52%)**		**8 (24%)**	**25 (76%)**	**HS**
Grade	**Low**	**18**		**18 (100%)**	**0 (0%)**	**P= 0.001**	**14 (78%)**	**4 (22%)**	**P=0.005**
	High	**32**		**13 (41%)**	**19 (59%)**	**HS**	**7 (22%)**	**25 (78%)**	**HS**
pT stage	**NMI**	**pTa**	**8**	**8 (100%)**	**0 (0%)**	**P= 0.01**	**7 (88%)**	**1 (12%)**	**P=0.001**
		pT1	**11**	**11 (100%)**	**0 (0%)**	**S**	**6 (55%)**	**5(45%)**	**HS**
	MI	**pT2**	**21**	**9 (42%)**	**12 (58%)**		**6 (29%)**	**15 (71%)**	
		pT3	**9**	**3 (33%)**	**6 (67%)**		**2 (22%)**	**7 (78%)**	
		pT4	**1**	**0 (0%)**	**1(100%)**		**0 (0%)**	**1(100%)**	
Staging group in radical cystectomy cases	**Low-stage(0/I)**	**7**		**7(100%)**	**0 (0%)**	**P=0.006**	**5(71%)**	**2 (29%)**	**P=0.006**
	High stage(II/III/IV)	**19**		**5 (26%)**	**14 (74%)**	**HS**	**8 (42%)**	**11 (58%)**	**HS**
Associated CIS	**Absent**	**28**		**18 (65%)**	**10 (35%)**	**P= 0.371**	**19 (68%)**	**9 (32%)**	**P=0.000**
	Present	**22**		**13 (59%)**	**9 (41%)**		**2 (9%)**	**20 (91%)**	**HS**
Tumor budding	**Absent**	**25**		**24 (96%)**	**1 (4%)**	**P= 0.01**	**18 (72%)**	**7 (28%)**	**P=0.001**
	Present	**25**		**7 (28%)**	**18 (72%)**	**S**	**3 (12%)**	**22 (88%)**	**HS**
Tumor focality	**Unifocal**	**32**		**22 (70%)**	**10 (30%)**	**P= 0.09**	**19 (59%)**	**13 (41%)**	**P=0.001**
	Multifocal	**18**		**9 (50%)**	**9 (50%)**		**2 (11%)**	**16 (86%)**	**HS**
molecular subtypes	**Luminal -papillary**	**16**		**15 (94%)**	**1 (6%)**	**P= 0.022**	**12 (75%)**	**4 (25%)**	**P=0.000**
	Luminal-infiltrated	**12**		**1 (8%)**	**11 (92%)**	**S**	**6 (50%)**	**6 (50%)**	**HS**
	Basal	**16**		**5 (94%)**	**1 (6%)**		**3 (13%)**	**13 (87%)**	
	Double-Negative	**6**		**0 (0%)**	**6 (100%)**		**0 (0%)**	**6 (100%)**	

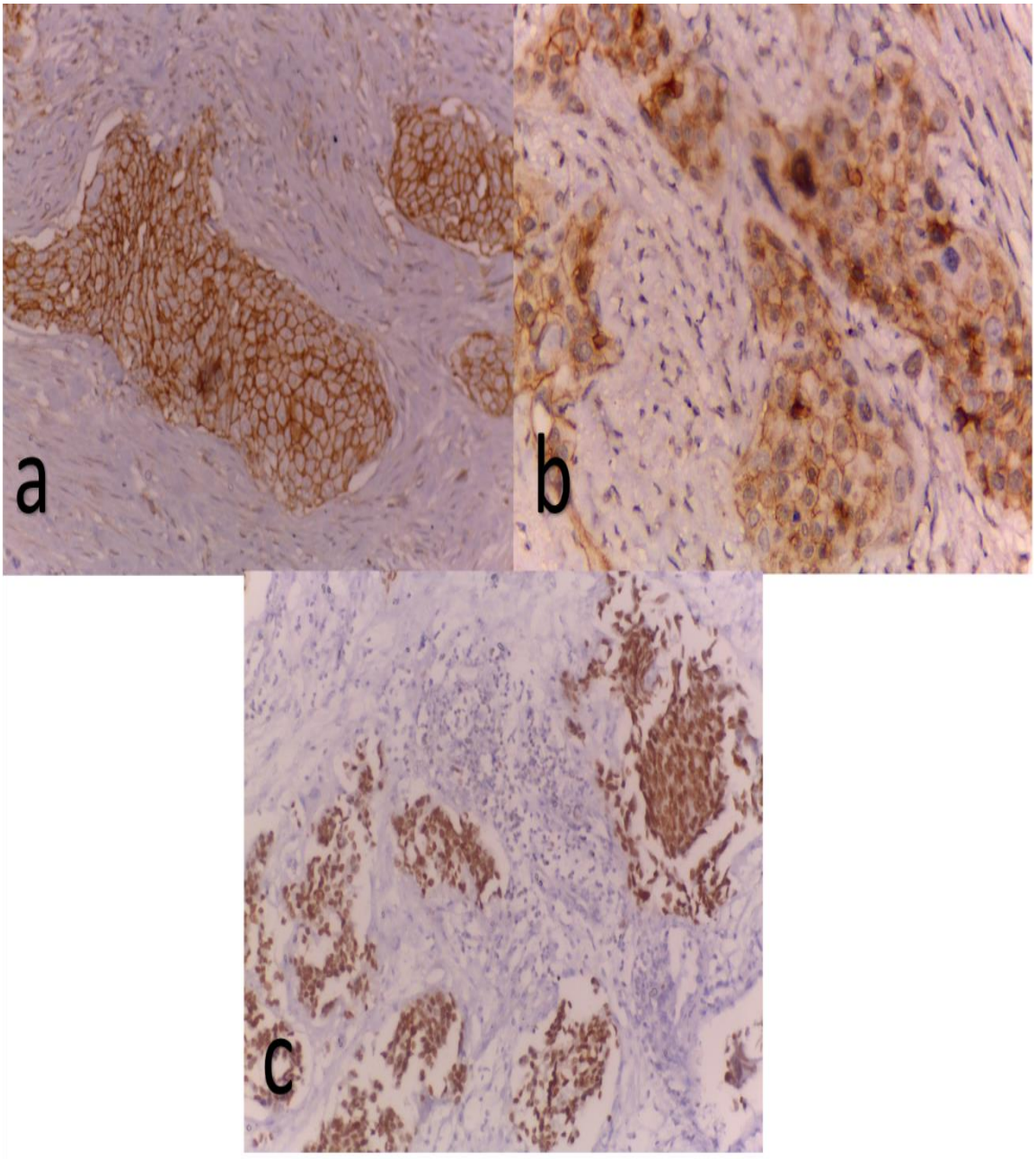

Figure (1): a) *high grade invasive non papillary urothelial carcinoma showing strong complete membrane staining forHER2 in more than 10% of tumor cells score 3*(ABCX200). *b) high grade invasive non papillary urothelial carcinoma showing incomplete membrane staining for HER2 expression in more than 10% of tumor cells score2***(ABCX400).** c) high grade invasive urothelial carcinoma with evident LVI showing strong positive nuclear P53 staining in tumor cells and tumor emboli (ABCX200***)***

***Table* (5) *Correlation between* clinic-immuno-pathological Variants and *Claudin1&claudin4* expression**:

			Total	*claudins expression*				P Value	
				Claudin1-low	Claudin1-high	Claudin4-low	Claudin4-high		
				NO	NO	NO	NO	Claudin1	Claudin4
Histo-pathological variant	Papillary		17	14(82%)	3 (18%)	2 (12%)	15 (88%)	0.009	0.003
	non papillary		33	6 (18%)	27 (82%)	26 (79%)	7 (21%)		
Grade	Low grade		18	14 (78%)	4 (22%)	2 (11%	16 (89%)	0.005	0.003
	High grade		32	6 (19%)	26 (81%)	26 (75%)	6(25%)		
pT stage	NMI	pTa	8	6 (75%)	2 (25%)	1 (13%)	7 (87%)	0.03	0.022
		pT1	11	7 (64%)	4 (36%)	3 (27%)	8 (73%)		
	MI	pT2	21	6 (29%)	15 (71%)	17 (80%)	4 (20%)		
		pT3	9	1 (11%)	8 (89%)	7 (78%)	2 (22%)		
		pT4	1	0 (0%)	1 (100%)	0 (0%)	1 (100%)		
Stage grouping in radical cystectomy cases	Low stage(0/I)		7	7 (100%)	0 (0%)	2 (29%)	5 (71%)	0.04	0.03
	High-stage (II/III/IV)		19	8 (40%)	11 (60%)	12 (63%)	7 (37%)		
Associated CIS	**Negative**		28	9 (32%)	19 (68%)	13 (46%)	15 (54%)	**0.145**	**0.098**
	Positive		22	11 (50%)	11 (50%)	15 (68%)	7 (32%)		
Tumor budding	**Absent**		25	17 (**68%**)	8 (**32%**)	7 (**28%**)	18 (**72%**)	**0.001**	**0.001**
	Present		25	3 (**12%**)	22 (**88%**)	21 (**84%**)	4 (**16%**)		
Tumor focality	**Unifocal**		32	17 (**53%**)	15 (**47%**)	13 (**40%**)	19 (**60%**)	**0.011**	**0.003**
	Multifocal		18	3 (**16%**)	15 (**84%**)	15 (**83%**)	3 (**17%**)		

Immuno-pathological parameters		Total	*claudins expression*				P value	
			Claudin1-low	Claudin1-high	Claudin4-low	Claudin4-high	Claudin1	Claudin4
Molecular subtypes	Luminal-papillary	16	13 (80%)	3 (20%)	2 (12%)	14 (88%)	0.002	0.000

	Luminal-infiltrated non papillary	**12**	**2 (17%)**	**10 (83%)**	**9 (75%)**	**3 (25%)**		
	Basal	**16**	**3 (19%)**	**13 (81%)**	**13 (81%)**	**3 (19%)**		
	Double negative	**6**	**2 (33%)**	**4 (67%)**	**4 (67%)**	**2 (33%)**		
HER2 expression	**Negative**	**31**	**17 (54%)**	**14 (48%)**	**13 (46%)**	**18 (55%)**	***0.02***	**0.03**
	Positive	**19**	**3 (15%)**	**16 (85%)**	**15 (78%)**	**4 (22%)**		
P53 expression	**Negative**	**21**	**12 (57%)**	**9 (43%)**	**7 (33%)**	**14 (67%)**	**0.036**	**0.005**
	Positive	**29**	**8 (27%)**	**21 (73%)**	**21(73%)**	**8 (27%)**		

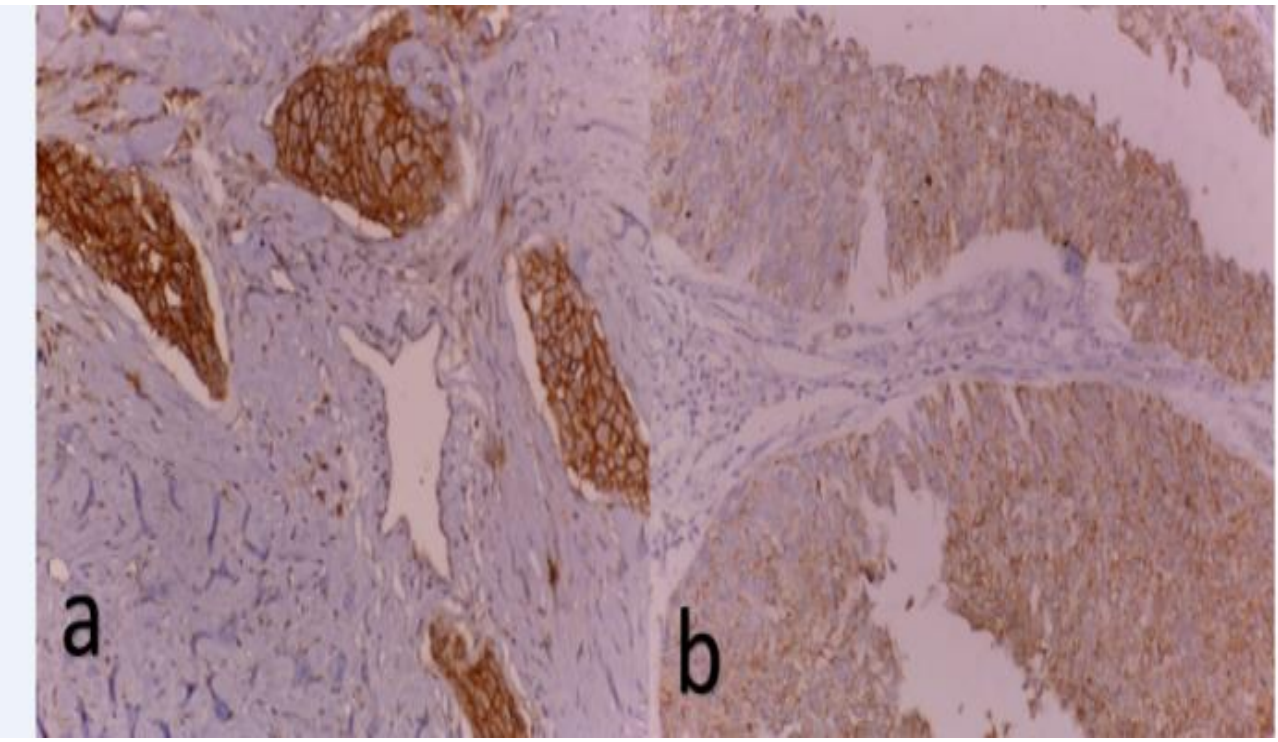

Figure (2): a) High grade, urothelial carcinoma, showing strong membranous expression in more than 50% of tumor cells, claudin1 expression, in tumor cells (claudin1 high, score9), (ABCX200). b) low grade, papillary urothelial carcinoma, showing moderate membranous, &cytoplasmic claudin4, expression in more than 25% of tumor cells, score 4(ABCX200).

***Table (6) Statistical determination of sensitivity and specificity of HER2, Claudin1, Claudin4 and P53S biomarkers results and Molecular subtypes by using ROC curve** analysis:*

Molecular type	Marker	AUC	Sensitivity (%)	Specificity (%)
Luminal – papillary	HER2	0.296	6.2	53
	Claudin1	0.197	18.8	20.6
	Claudin4	0.820	90.91	67.44
	P53	0.257	25.0	26.5
Luminal –infiltrated non papillary morphology	HER2	0.770	81.82	67.44
	Claudin1	0.645	73.7	57.1
	Claudin4	0.375	25.0	50.0
	P53	0.447	50.0	39.5
Basal	HER2	0.297	6.2	52.9
	Claudin1	0.656	81.2	50.0
	Claudin4	0.314	18.8	43.1
	P53	0.717	85.7	63.64
Double negative	HER2	0.875	95.6	70.9
	P53	0.738	86.7	67.8
	Claudin4	0.439	33.3	45.6
	Claudin1	0.644	83.6	64.6

AUC for claudin4 in luminal papillary subtype is0.820. AUC for HER2 in luminal non-papillary subtype is 0.770. AUC for p53 in Basal subtype is 0.717. AUC for HER2 &p53 in double negative subtype are 0.875&0.738respectively. Table (6)& figure (3)

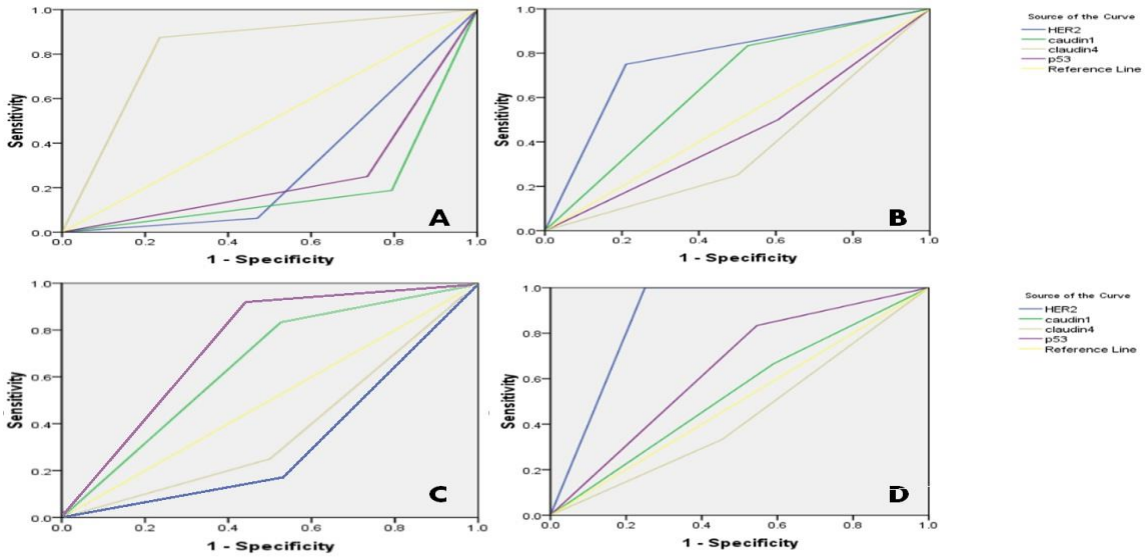

Figure (3): Diagnostic performance, using ROC curve analysis for, A) luminal papillary subtype B) luminal infiltrated subtype, C) Basal subtype, D) Double negative subtype

Discussion:

Bladder cancer, is one of common increasing cancer worldwide. In Egypt, it is the third, most common cancer, in both sexes **(15).**

This retrospective study, was carried on 50 cases, of conventional transitional cell carcinoma (TCC) of urinary bladder. The mean age of the studied cases, was 61.34 years. This agrees with the study of **(16),** the male to female ratio of the studied cases, was 3.5:1. This agrees with the study of **(17).**

Conventional TCC cases, in this study were 34% papillary transitional cell carcinoma, and 66% infiltrating-non papillary transitional cell carcinoma, this in line with previous study **(18)**. In disagreement with previous study **(19), who** had majority of patients with superficial tumors. According to **2016 WHO grading system,** the studied cases, were (36%) low grade and (64%) high grade. This finding is in agreement, with previous study on bladder urothelial cancer **(7),** which found that, (32%) of the cases were low grade, and (68%) were high grade. While previous study **(20),** found, (57%) of the cases, were low grade, and (43%) were high grade.

Regarding muscle invasion, (38%) of the cases were non-muscle invasive and (62%)were muscle invasive, this is in agreement with previous study **(8),** which stated that, most of urinary bladder TCC cases, present in an advanced stage. While**,** previous study **(19),** showed that, majority of cases were non muscle invasive**.** The differences in results of the present study, and other studies regarding grade and muscle invasion, may be contributed to differences in patient awareness, and late diagnosis in Egyptian patients. **(21**)

On basis of IHC analysis, using GATA3 and CK5/6 markers, the studied cases were classified into, three molecular subtypes, 56% were Luminal, 32% were basal subtype and 12% were Double Negative subtype. This distribution of molecular subtypes, is nearly similar to the study of **(22).** Then luminal cases reclassified, regarding morphology**,** according to the study of **(14) into,** 57% were luminal papillary and 43% were luminal infiltrated morphology. There was a statistically, significant, correlation, between molecular subtypes, and tumor grade, pT stage, TNM stage in radical cystectomy cases, LVI, associated CIS, tumor budding, and tumor focality as (P-value< ***0.05***) revealing that, Luminal-papillary subtype was associated with the best prognosis, while Basal and Double Negative subtypes had a more aggressive behavior, with a tendency to early invasion and metastases. **Th**ese results were in line with previous study **(4**) which showed that the molecular subtypes were significantly associated with tumor grade &stage and luminal subtype was associated with more favorable outcome when compared to basal subtype.

Immuno-histochemical evaluation of HER2 expression, revealed a statistically significant correlation between HER2 expression, and tumor size, tumor grade, pT stage, TNM stage in radical cystectomy cases, LVI, tumor budding, and molecular subtypes as (P-value< ***0.05***). These results

were in parallel with study of **(23), (24), (25**). The poor prognostic impact of Her2/neu in the studied cases, may be contributed to the role of HER2, as one of receptor tyrosine kinase proto-oncogenes, that enhances cancer cell proliferation, motility, invasion and metastasis **(26).** However, **(27)** on his study on gastric carcinoma, found that, HER2 expression was not related to gastric cancer patient prognosis. This differences may be contributed to different tissue on which this study done.

This study also revealed a statistically significant correlation, between HER2 expression and molecular subtypes, (P value= ***0.022)*** showing that, tumors with a luminal molecular subtype, had a significantly higher rate of Her2 alterations, than those of the basal subtype This result was in line with previous study **(10),** ***which*** suggested that, HER2 activity was also associated with subtype status.

The p53 gene, is the most frequently altered gene, in human cancers, and P53 mutations have been associated with genomic instability, and hence progressive development, of further mutations. ***(28).***

Immuno-histochemical evaluation of P53 expression, revealed a significant statistical correlation between P53 and clinico-immuno-pathological parameters, as tumor size, grade, stage, LVI, Tumor budding, focality of tumors, and molecular subtypes with aggressive behavior as P-value< ***0.05***. This result was in parallel with study of **(29), (30)**, **(13)**. However, previous study ***(31***) suggested that p53 expression was not significantly associated with the stage or grade of bladder cancer.

This study also revealed, a statistically, significant, correlation, between p53 expression, and molecular subtypes (P value= ***0.000),*** as all cases with Double Negative molecular subtypes, showed positive nuclear expression of p53. This was in line with previous studies ***(4***) and (**32).** That study revealed, there was no a significant, statistical, correlation between p53 expression, and HER2 expression, (P value=0.08).

Immuno-histochemical evaluation of Claudin1&4 expression, revealed that a positive significant statistical correlation was found, between claudin1 and clinico-immuno-pathological parameters as tumor size, grade, stage, LVI, Tumor budding, focality of tumors, as p value< ***0.05,*** and a negative significant statistical correlation was found, between claudin4 and fore mentioned clinic-immuno-pathological parameters, as P-value< ***0.05***. These previous results were in line with ***the study of*** **(9**). These results could be explained by, the role of Claudin1 in directly promoting epithelial mesenchymal transition (EMT), through its interaction with, defined EMT-related transcription factors, and signaling pathways. while Claudin-4 expression increased the barrier function of tight junctions, and inhibited the migration, and invasion of cancer cells **(33)**

In contrast with ***previous studies (34***), ***(35) and*** **(36)** ***which*** reported that, increased expression of Claudin-4, together with claudin3 and KI67, was correlated to advanced stage and poor prognosis,

and loss of Claudin1 expression, together with claudin7, was associated with high grade, and stage tumors. This different results may have contributed to, claudins action was influenced and modulated by interaction with other claudins members as claudins 3&7 and other proliferating genes as KI67**.**

This study also revealed a statistically significant correlation between claudins (1&4) expression and molecular subtypes (P value= ***0.002 &0.000 for claudin1and claudin4 respectively)*** as 81% of cases with basal molecular subtypes, showed high expression of claudin1, and low expression of claudin4, and 67% of double negative cases, showed high expression of claudin1, and low expression of claudin4. These results were in line with, ***previous studies (37),*** and **(4**), which stated that, major subset of basal-like, and double-negative tumors, showed downregulation of claudin target genes (claudins 3, 4, and 7). And in line with the study of **(38),** on ductal breast carcinoma, who demonstrated that majority of luminal subtype showed low claudin-1 expression, and triple negative cases, showed high claudin1 expression.

This study also revealed a statistically significant correlation between claudins (1&4) expression and HER2 expression (P value= ***0.02 &0.037 for claudin1and claudin4 respectively)***. This results were in line with study done by **(39)** on breast carcinoma, who demonstrated that, absence HER2 expression, was associated with low expression claudin4. And in line with previous study **(38),** that demonstrated that, claudin-1 overexpression, was associated with HER2 enriched, breast carcinoma. This study also revealed, a statistically, significant, correlation, between p53 expression, and claudins (1&4)***, as P*** value= ***0.036 &0.005 for claudin1and claudin4 respectively***. This result was in line with **previous report (40)** which stated that expression of p53 and claudin1 was significantly increased in SCC of vulva.

Regarding, results of ROC analysis, Claudin4 may be considered, better biomarker, in prediction of Luminal – papillary subtype, HER2 may be considered, better biomarker, in prediction of Luminal infiltrated subtype, p53 may be considered, better biomarker in prediction of basal subtype. HER2, P53 and, claudin1 may be considered, better biomarkers in prediction of double negative subtype.

Conclusion: Molecular subtypes of bladder cancers, and expressions of HER2, claudin1&4 and P53 can be used for, prognostic, and therapeutic purpose of BC patients, that may affect patient outcome.

Conflicts of interest: No conflicts of interest**.**

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