

ORIGINAL ARTICLE

The correlation of EMT and p53 immunohistochemical markers with cisplatin resistance in muscle invasive bladder cancer patients: A single-centred study

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Abstract

Introduction: Although epithelial-mesenchymal transition (EMT) and p53 have been established to play a pivotal role in the aggressiveness of muscle-invasive bladder cancer (MIBC), its pathological correlation to cisplatin treatment in the Malaysian patient cohort is lacking. This study aimed to evaluate the association of EMT markers, e-cadherin, vimentin and actin, as well as tumour suppressor gene, p53, in cisplatin-receiving MIBC patients. **Materials and Methods:** Formalin-fixed paraffin-embedded (FFPE) blocks of muscle-invasive bladder cancer patients receiving cisplatin-based chemotherapy between January 2010 to December 2020 were traced. Immunohistochemistry staining was performed on traced blocks using antibodies to e-cadherin, vimentin and actin, and p53. **Results:** p53 and e-cadherin were stained positive in most cases ($p=0.515$ and 0.242 respectively), although e-cadherin showed stronger positive expression in pre-cisplatin receiving MIBC cases. All the cases stained negative for actin and vimentin except for faint staining observed in one pre-cisplatin case. **Conclusion:** Although this study does not show a significant correlation between EMT markers and p53 with cisplatin-responsiveness in MIBC patients, the results serve as preliminary findings on the heterogeneous outcomes of molecular staining in the Malaysian MIBC patient cohort.

Keywords: Muscle-invasive bladder cancer, epithelial-mesenchymal transition, cisplatin resistance

INTRODUCTION

Bladder cancer is among the most common urological malignancies in the world. In 2020, it was reported to be the sixth most common cancer in males worldwide with 573,000 new cases reported that year, which is an alarming increase of 20% compared to the new cases reported in 2018.¹⁻³ In Malaysia, bladder cancer is the ninth most common malignancy affecting males with the most common stage at presentation being muscle-invasive bladder cancer (MIBC).⁴ Cisplatin is a platinum-based chemotherapy drug which is the first-line treatment for MIBC. Although this treatment has high initial response rates, its clinical efficacy is hampered by the development of intrinsic or extrinsic resistance which contributes to a high rate of disease recurrence, rendering a poor

prognosis for MIBC patients in Malaysia with a mean overall survival of only 8-33 months.^{4,9}

The mechanisms underpinning cisplatin resistance in bladder cancer have been widely studied over the years^{5,10-12}, with epithelial-mesenchymal transition (EMT) and mutations of cell cycle regulatory genes prominently implicated in chemoresistance and the aggressive phenotype in MIBC. EMT is a biological process that leads to a phenotypic switch of epithelial cells to become more mesenchymal-like. The phenotype plasticity signified by the loss of epithelial markers such as e-cadherin and gain of mesenchymal markers such as vimentin and actin alter tumour cell behaviour and induces chemoresistance in various cancers including pancreatic, lung and bladder cancers.¹³⁻¹⁸

The mutations of cell-cycle regulatory genes,

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mainly p53 which is a tumour suppressor gene that predominantly regulates programmed cell death, are involved in bladder cancer progression and survival.¹⁹⁻²³ Studies have implicated that the nuclear expression of p53 correlates to higher muscle invasion and disease progression in bladder cancer.^{22,23} As molecular changes instigate phenotypic characteristics that are precursors to chemoresistance, understanding the immunohistochemical expression of p53 in cisplatin-receiving MIBC patients and its correlation to EMT may predict the therapeutic outcome of cisplatin treatment in MIBC. This observation prompted us to investigate the pathological expression of EMT markers e-cadherin, vimentin and actin, and tumour suppressor gene, p53, in a Malaysian subpopulation of MIBC patients receiving cisplatin-based chemotherapy.

MATERIALS AND METHODS

Patient cohort

A single-centre retrospective study was conducted at the University Malaya Medical Center (UMMC) in Kuala Lumpur, Malaysia. It was approved and carried out with the oversight of the institutional research ethics board, under the identification code 2019618-7527, and was conducted in compliance with the International Conference on Harmonisation – Guidelines for Good Clinical Practice (ICHGCP) and the Declaration of Helsinki. All muscle-invasive bladder cancer (confirmed via imaging, clinical and/or histologically) patients above 18 years of age who received cisplatin-based chemotherapy from January 2012 till December 2020 at the University Malaya Medical Centre were included in the study. Patients who had undergone chemotherapy for other types of cancers prior to bladder cancer diagnoses or possess incomplete chemotherapy records or who are unable to provide tissue samples were excluded from this study. Retrospective and clinical information were retrieved from the patient's medical records and were tallied with the chemotherapy records of the Inpatient Pharmacy Department.

The reports and histopathological slides of these cases were traced and examined. 17 cases with a definite diagnosis of urothelial carcinoma and a cisplatin-based chemotherapy regimen were selected for analysis, yielding 33 formalin-fixed paraffin-embedded (FFPE) blocks that contain an adequate amount of tissue for immunohistochemical (IHC) staining, including

blocks from follow-up biopsies with or without recurrent tumours.

Patient tissue samples

Paraffin-embedded tissue blocks of muscle-invasive bladder cancer were archived in the Department of Pathology, University Malaya Medical Centre (UMMC). All surgical specimens were collected from patients undergoing Transurethral Resection of Bladder Tumour (TURBT) or cystectomy from the operation theatre and were fixed in 10% buffered formalin until the samples were processed and embedded into FFPE blocks. To further ensure the confidentiality of the patients in this study, tissue blocks and clinicopathological data were assigned with specific coded numbers.

Immunohistochemical analysis

Immunohistochemistry was performed to elucidate the expression of e-cadherin, vimentin, actin and p53. FFPE sections were stained and scored by double-blinded pathologists at UMMC. The paraffin blocks were sectioned at 3-5 μm of thickness before IHC staining. The staining was performed by the standard protocol using the BondMax autostainer and antibodies e-cadherin (DAKO, NCH-38, 1:50 dilution), vimentin (DAKO, V9, 1:200 dilution), α -SMA or actin (DAKO, 1A4, 1:1000 dilution) and p53 (DAKO, DO-7, 1:300 dilution). The sections were visualised using the Bond polymer refine detection kit (Leica) using diaminobenzidine chromogen as substrate. Each slide was optimised with suitable control tissues. Each case was assessed for e-cadherin, vimentin, actin and p53 cytoplasmic localisation immunopositivity (Figure 1). The IHC score for e-cadherin, vimentin and actin was based on any expression of >1% of tumour cells to be considered positive and ranged from the strength of 0 (negative/nil), 1+ (weak), and 2-3+ (strong). As for p53, IHC scoring used the percentage of tumour stained that were positively stained and were stratified as <1% (negative/nil), 1-25% (weak), 25-50% (moderate) and 50-100 % (strong).

Statistical analysis

The qualitative variables of the association between histoscores and clinical parameters were described as frequencies with percentages and were analysed using Pearson's chi-square tests with Cramer's V as the strength of association (SPSS, version 26.0), or Fisher's exact test. P-values < 0.05 were considered statistically significant.

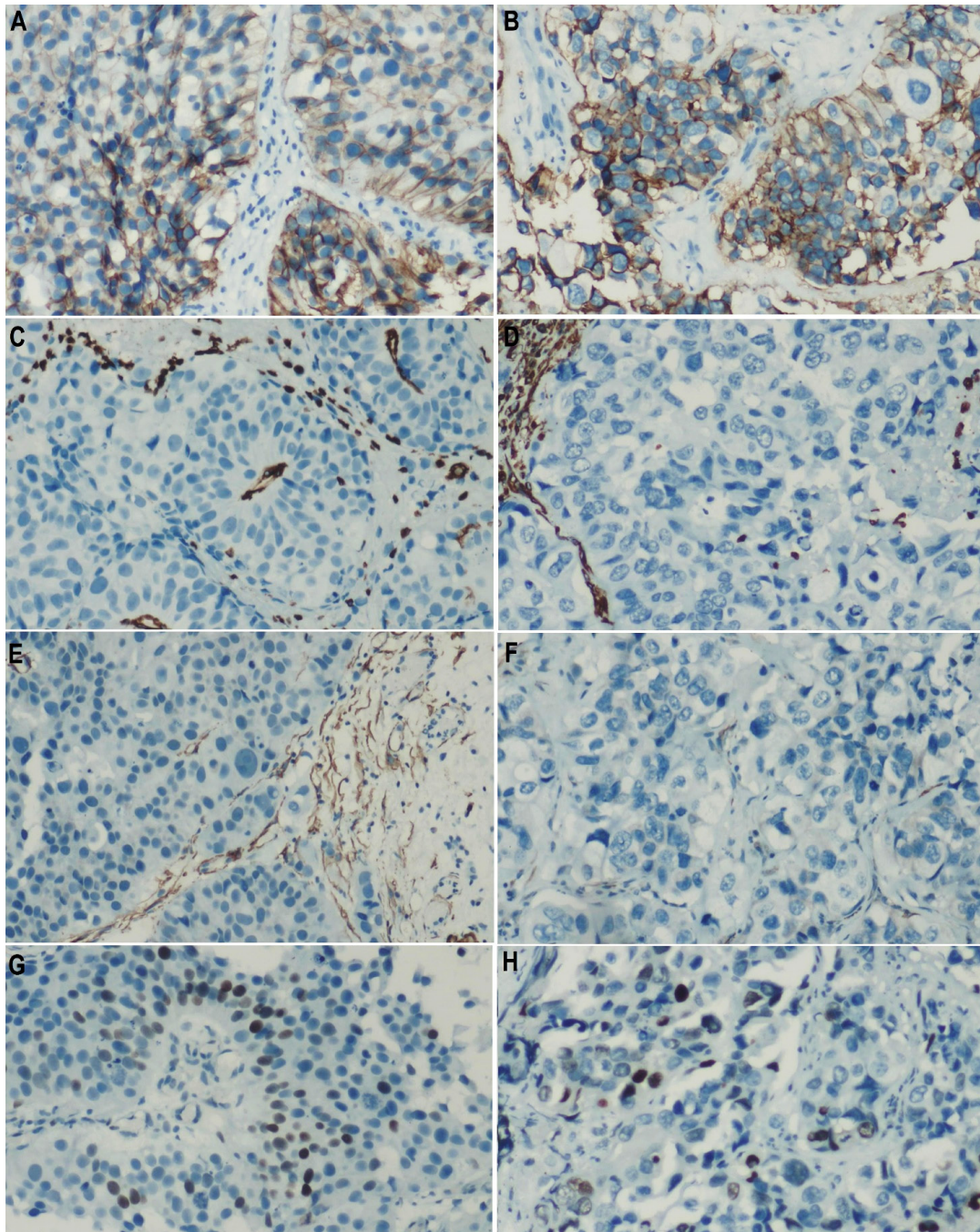


FIG. 1: Panel showing the immunohistochemistry staining at 10x magnification pre- and post-cisplatin from the same patient. The pre-cisplatin case (A, C, E, G) was a diagnosis of invasive papillary urothelial carcinoma and the post-cisplatin case (B, D, F, H) was a diagnosis of invasive urothelial carcinoma with brain metastasis. (A and B) Expression of e-cadherin pre-and post- cisplatin (pre, 3+; post, 1+). (C and D) Expression of vimentin pre- and post-cisplatin (pre and post, negative). (E and F) Expression of actin pre-and post- cisplatin (pre and post, negative). (G and H) Expression of p53 pre-and post-cisplatin (pre, 10%; post 5%).

RESULTS

A total of 243 urothelial carcinoma cases were admitted to the University Malaya Medical Center (UMMC) between January 2010 to December 2020. However, as this study was conducted on retrospective cases, only 17 cisplatin-treated muscle-invasive bladder cancer patients and 33 FFPE blocks were available for the study. This was due to incomplete patient medical records or insufficient pathological samples. The demographics of the cases admitted in this study are illustrated in Table 1. The majority of the muscle-invasive urothelial carcinoma cases

in UMMC were characterised as transitional cell carcinoma (n=25 cases; 75.7%) and males were the dominant gender (n=15 patients; 87.9%). All 33 cases were stratified according to the chemotherapy status at the time of sampling. While most cases had a definite chemotherapy status of pre-and post- cisplatin treatment, 27.3 % of them were termed 'unsure' due to incomplete medical records of chemotherapy regimens.

The most common pathological diagnosis was urothelial carcinoma, which included invasive urothelial carcinoma (IUC, n=17), followed by low-grade urothelial carcinoma and high-grade

TABLE 1: The demographic and clinicopathological parameters of the sample cohort

Demographics and clinical characteristics	
Variables	n (%)
Demographics	
Patients	17 (100)
Cases	33 (100)
Gender	
Male, n (%)	15 (88)
Female, n (%)	2 (12)
Chemotherapy status	
Pre	15 (45.4)
Post	9 (27.3)
Unsure	9 (27.3)
Clinical characteristics	
Diagnosis	
IUC	17 (51.52)
LGUC	2 (6.06)
HGUC	2 (6.06)
IPUC	2 (6.06)
HG-IPUC	1 (3.03)
Bladder NAD	2 (6.06)
Cystitis	2 (6.06)
Post-radiation atypia	1 (3.03)
Bladder adenocarcinoma	2 (6.06)
SCC	1 (3.03)
Post-BCG changes	1 (3.03)
Cellular changes	
Transitional cell	25 (75.7)
Squamous	5 (15.2)
Papillary	3 (9.1)
Metastasis	
Yes	4 (12.1)
No	29 (87.9)

urothelial carcinoma (LGUC and HGUC, n=2 respectively). The other malignant cases were different grades of papillary urothelial carcinoma, including invasive papillary urothelial carcinoma and high-grade invasive papillary urothelial carcinoma (IPUC and HG-IPUC, n=3), adenocarcinoma (n=2) and squamous cell carcinoma (n=1). Tumour recurrence was observed in two cases (no abnormality detected or bladder NAD, n=2), while non-malignant changes were observed in several cases for cystitis (n=2), post-radiation atypia (n=1) and post-BCG changes (n=1). Metastatic lesions including colon and brain metastases were also

observed in 4 cases (n=4; 12.12%) (Table 1).

A significant statistical correlation wasn't observed between the difference in gender and pathological diagnosis (p=0.721). Similarly, the diagnosis and cellular changes in FFPE cases were not significantly correlated to the chemotherapy status of the patient at the point of sampling (p=0.289 and p=0.153). However, the pathological diagnosis and cellular changes observed in the FFPE samples were correlated (p=0.003).

Histoscores are illustrated as e-cadherin (Table 2), vimentin and actin (Table 3) and p53 (Table 4). E-cadherin expression was stronger

TABLE 2: The association between immunohistochemical expressions of e-cadherin with clinicopathological parameters

Clinical characteristics	Immunoreactivity of E-cadherin			df	p-value
	Expression				
	Negative	Weak	Strong		
Chemotherapy status					
Unsure	2	0	7	4	0.154
Pre	0	4	11		
Post	2	3	4		
Diagnosis					
IUC	0	5	12	20	0.242
LGUC	0	0	2		
HGUC	0	0	2		
IPUC	0	1	1		
HG-IPUC	0	0	1		
Cystitis	1	0	1		
Bladder NAD	1	0	1		
Post-radiation atypia	0	1	0		
Dysplastic colonic epithelium	0	0	2		
Bladder adenocarcinoma	1	0	0		
SCC	1	0	0		
Post-BCG changes	0	5	12		
Cellular changes					
Transitional cell	3	4	18	4	0.741
Squamous	1	2	2		
Papillary	0	1	2		
Metastasis					
Yes	1	1	2	2	0.806
No	3	6	20		
Muscle invasion					
Yes	0	0	2	2	0.724
No	4	7	20		

TABLE 3: The association between immunohistochemical expressions of vimentin and actin with clinicopathological parameters

Clinical Characteristics	Immunoreactivity of Vimentin				Immunoreactivity of Actin			
	Expression		df	p-value	Expression		df	p-value
	Negative	Weak			Negative	Weak		
Chemotherapy status								
Unsure	9	0	2	0.539	9	0	2	0.539
Pre	14	1			14	1		
Post	9	0			9	0		
Diagnosis								
IUC	16	1	10	1.0	17	0	11	0.1
LGUC	2	0			2	0		
HGUC	2	0			1	1		
IPUC	2	0			2	0		
HG-IPUC	1	0			1	0		
Cystitis	2	0			2	0		
Bladder NAD	2	0			2	0		
Post-radiation atypia	1	0			1	0		
Bladder adenocarcinoma	2	0			2	0		
SCC	1	0			1	0		
Post-BCG changes	1	0			1	0		
Cellular changes								
Transitional cell	25	0			25	1		
Squamous	4	1	2	0.056	5	0	2	0.848
Papillary	3	0			3	0		
Metastasis								
Yes	4	0			3	0		
No	28	1	1	0.879	30	1	1	0.879
Muscle invasion								
Yes	2	0			1	1		
No	30	1	1	0.939	32	0	1	0.061

in pre-cisplatin cases compared to post-cisplatin cases. Pre-cisplatin cases observed strong e-cadherin expression in 11 cases compared to the weak (n=4) and negative expressions (n=0). In post-cisplatin cases, the majority of the expression of e-cadherin was strong (n=4), compared to the weak (n=3) and negative expressions (n=2). Three out of four cases with metastatic lesions were stained positive for e-cadherin, including the two cases with histologically proven muscle invasion. Although there was no significant correlation observed between the strength of e-cadherin expression and the variables studied in this patient cohort, including cisplatin treatment status (p=0.154), e-cadherin was positively expressed in all

urothelial and papillary urothelial carcinoma cases regardless of the grade.

Vimentin was not expressed in any of the cases except in one pre-cisplatin case which was diagnosed as IUC with squamous cell differentiation (p=0.05). Similarly, the expression of actin was negative in all cases except for one weak expression in a pre-cisplatin case with a diagnosis of high-grade urothelial carcinoma and transitional cell differentiation, therefore not showing any significant correlation between the expression of actin with different pathological diagnoses (p=0.539) and cellular changes (p=0.1). While all four FFPE cases with metastatic lesions stained negatively for actin (p=0.879), one of the two cases with a

TABLE 4: The association between immunohistochemical expressions of p53 with clinico-pathological parameters

Clinical Characteristics	Immunoreactivity of p53				df	p-value
	Expression					
	Negative	Weak	Moderate	Strong		
Chemotherapy status						
Unsure	7	1	1	0	6	0.093
Pre	4	4	1	6		
Post	4	4	0	1		
Diagnosis						
IUC	8	3	1	5	30	0.515
LGUC	1	1	0	0		
HGUC	0	0	1	1		
IPUC	0	2	0	0		
HG-IPUC	0	0	0	1		
Cystitis	1	1	0	0		
Bladder NAD	1	1	0	0		
Post-radiation atypia	0	1	0	0		
Bladder adenocarcinoma	2	0	0	0		
SCC	1	0	0	0		
Post-BCG changes	1	0	0	0		
Cellular changes						
Transitional cell	11	7	3	5	6	0.349
Squamous	3	0	0	2		
Papillary	0	2	0	1		
Metastasis						
Yes	2	1	0	0	3	0.953
No	12	8	3	8		
Muscle invasion						
Yes	0	0	0	2	3	0.048
No	16	9	2	5		

histological expression of muscle invasion had a weak expression of actin ($p=0.061$).

The expression of p53 was positive in most of the cases in this cohort. While there wasn't a statistically significant correlation between cisplatin treatment status and p53 expression ($p=0.093$), pre-cisplatin cases saw higher numbers of stronger expression of p53 ($n=6$) compared to the post-cisplatin cases ($n=1$). The expression of p53 varied across the pathological diagnosis ($p=0.515$) and was positively expressed in 13 out of the 25 cases with transitional cell differentiation, and 2 out of 5 cases with squamous cell differentiation. Interestingly, p53 was strongly stained in both cases that had histological characteristics of muscle invasion ($p=0.048$).

DISCUSSION

This study showed a predominance of males compared to females at a ratio of 7.5: 1, which was almost similar to the 9.4:1 ratio reported by Kong *et al.* (2010).⁹ The higher incidence in males could be attributed to the higher exposure to industrial or outdoor carcinogens and smoking compared to females, all of which are major predisposing factors for bladder neoplasms.²⁴

Although 243 patients were diagnosed with bladder cancer between January 2010 and December 2020, the low number of muscle-invasive bladder cancer patients admitted in this study ($n=17$), cases ($n=33$) and lack of pre or post-cisplatin treatment status (unsure, 27.3%) being traced in this study was due to the

incomplete chemotherapy records of patients and insufficient pathology samples. Similarly, this was also observed in a study conducted on the malignant bladder cancer patient records at the Malaysian National Cancer Registry, whereby more than half (51.4%) of the cases lacked records of the stage at diagnosis and receipt of chemotherapy or radiotherapy.⁴ UMMC is a teaching hospital which primarily attends to patients subsidised by the government pension scheme. However, due to varied preferences in treatment, patients often seek treatment from different centres. As routine follow-up is a cornerstone of oncology treatment, insufficient patient medical records due to the lack of a nationwide integrated medical record system often hinder follow-up and overall survival monitoring, leading to discrepancies in treatment outcomes.^{25,26}

Urothelial carcinoma, which is also known as transitional cell carcinoma, was the most common pathological diagnosis in this study (75.7%), similar to that in previously reported Malaysian studies which were 90.4% and 78.2%.^{4,9} However, due to the inclusion criteria in this study, only 6.06% of the cases were tumour sections with muscle invasion and 12.1% of the cases were samples with metastatic lesions. While the low numbers of cases are unrepresentative of the patient cohort, the skewed distribution provides inconclusive correlative outcomes on the molecular staining patterns in parts of the tumour that has muscle invasion or metastatic lesions. Furthermore, the tumour sampling process in bladder cancer involves the cutting and burning of tissues during cauterisation performed in Transurethral Bladder Resection (TURBT). Inadequate tissue or tissue cauterised during this process hampers the analysis of the excisional specimen and is therefore among the limitations in this study.²⁷

The loss of e-cadherin which leads to the loss of cell-cell adhesion, and the gain of mesenchymal markers such as vimentin and actin, is known to be common in the process of epithelial to mesenchymal transition (EMT) in various cancers including bladder cancer.^{21,28} Several studies have shown the loss of e-cadherin in the more invasive, higher grade, and drug-resistant bladder cancer.^{21,29-31} In this study, e-cadherin was positively expressed in 29 cases while only 4 cases saw a negative expression. The expression of e-cadherin was heterogeneous pre- and post-cisplatin treatment; however, pre-cisplatin cases observed a higher ratio of

positive versus negative expressions (15:0) compared to the post-cisplatin cases (7:2). However, there weren't significant correlations observed between the e-cadherin expression and chemotherapy status, pathological diagnosis and cellular changes.

The expression of mesenchymal intermediate filament proteins vimentin and actin are common markers in myofibroblasts and smooth muscle cells of the bladder and have been associated with increased tumour grade and stage in bladder cancer.^{18,32} In a study by Council *et al.* (2009)³³, bladder cancer tumour samples with predominant muscularis mucosa were reported to stain homogeneously for vimentin while bladder cancer tumour samples with myofibroblast infiltration were positively stained for actin. However, in this study, vimentin and actin were both absent in all the cases except for one pre-cisplatin receiving case. While vimentin and actin show the possible recruitment of mesenchymal cells from the muscle, invasion of the tumour into the muscle layer, or the heterogeneous population of mesenchymal cells in the host tumour, it does not serve as a marker for the epithelial to mesenchymal switch in the urothelial carcinoma cases of this cohort.

The lack of epithelial-mesenchymal switch observed in this sample cohort is inconsistent with the correlation found in the acquisition of mesenchymal-like phenotype in muscle-invasive bladder cancer^{16,32} and cisplatin-resistance in bladder cancer.³¹ Increasing evidence reports the shift of e-cadherin from membrane localisation to cytoplasmic and nuclear localisation plays a role in the generation of cancer stem cells (CSCs), the acquisition of cisplatin resistance, and invasive bladder cancer.^{29,31} Initially termed a tumour suppressor due to its implications in various invasive and metastatic cancers³⁴⁻³⁸, recently e-cadherin has been reported to play other tumour-promoting roles than just inhibiting a mesenchymal switch. For example, e-cadherin mediates the intracellular adhesion and aggregation among adjacent cells which promotes the formation and maintenance of multicellular spheroids/ multicellular tumour spheroids (MCS/MCTs) which are vital in the metastasis and resistance of cancer lesions.³⁹⁻⁴¹ The shift from membrane to cytoplasmic and nuclear localisation of e-cadherin shows the possibility of another mechanism interplay in this patient cohort.

The prognostic significance of the tumour suppressor gene p53 has been extensively

reported in bladder cancer. p53 is located on chromosome 17p13.1, and mutations on this locus are commonly reported in bladder cancer.⁴² A study by Wu *et al.* (2019) reported that bladder cancer patients with mutant p53 were especially sensitive to chemotherapeutic agents mitomycin-C, doxorubicin and gemcitabine.⁴³ Compared to its wild-type counterpart, mutant p53 is stable and has a longer half-life, thus allowing its accumulation in the nucleus and detectable by immunohistochemistry.⁴⁴ In this study, p53 was positively expressed in most cases and saw a higher number of cases with strong expression in the pre-cisplatin receiving samples ($p=0.231$) compared to post-cisplatin receiving cases. As the overexpression of p53 has been coherently associated with bladder cancer of higher grades and stages⁴⁴, the lower expressions in the post-cisplatin receiving cohort may be parallel to the reduced tumour burden. However, the p53 staining observed in this study is based on the cytoplasmic expression and therefore doesn't allow the correlation between wild-type p53 and mutant p53 to treatment outcomes.

In summary, the heterogeneous distribution of pathological diagnoses and cellular subtypes in this patient cohort allowed us to explore molecular markers commonly used to study cisplatin resistance pathways in muscle-invasive bladder cancer in our subpopulation. This study did not find a significant correlation between e-cadherin, vimentin and actin to be molecular markers denoting the epithelial to mesenchymal switch which has been shown in the interplay of cisplatin resistance in other subpopulations of muscle-invasive bladder cancer patients. Furthermore, while the cytoplasmic expression of p53 was established in this patient cohort, its correlation to cisplatin responsiveness wasn't significant. The outcomes observed in this study shows the differences in the molecular characteristics expressed in this patient cohort and probable avenues to explore the localisation changes in molecular markers between different grades of bladder cancer and chemotherapy status, as well as the possibility of alternative mechanisms employed by patients with local genetic predispositions. Furthermore, the correlation between molecular markers and cisplatin-responsiveness in small patient cohorts may be achieved in a methodological approach which requires observational follow-up with patients and adequate sampling. This may be possible with the implementation of complete and comprehensive medical history and data

collection and sharing process which should be enforced to ensure optimised oncology care and future research possibilities.

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