ORIGINAL ARTICLE

High CD14 Antigen Expression in Urothelial Bladder Carcinoma Establishes that the Inflammatory Microenvironment Promotes Tumour Cell Proliferation

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ABSTRACT

Introduction: CD-14 is an antigen found on the bladder cancer cells that mediated cancer development by providing a supportive inflammatory microenvironment. The study investigates the association of CD14 antigen expression in bladder cancer cells with demographic factors, clinicopathological parameters and recurrent cases by immunohistochemical (IHC) method. **Methods**: A retrospective study using 59 formalin-fixed paraffin-embedded tissue of urothelial bladder carcinoma cases were subjected to CD14 IHC staining. **Results:** Most patients were ≥ 65 years old (57.6%), Malay in ethnicity (55.9%) and male predominant (94.9%). CD14 was positively expressed in more than 50% of tumour cells in all grades and stages with the largest percentage seen in the highest grade (Grade III) and stage (Stage IV) of urothelial bladder carcinoma. There was a significant association between CD14 expression and tumour grade of urothelial bladder carcinoma (p = 0.043). However, there was no significant association between CD14 expression with demographic factors, tumour stage or recurrent cases. **Conclusion:** High CD14 antigen expression by cancer cells establishes that the inflammatory microenvironment promotes tumour cell growth and may suggest CD14 antigen as a poor prognostic marker in urothelial bladder carcinoma.
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INTRODUCTION

According to the Malaysian National Cancer Registry 2012-2016, bladder cancer is more common in males (1715 cases) than females (407 cases). This cancer is mainly seen in Malay (3.3%), followed by Chinese (2.8%) and Indian (2.5%) ethnicity (1). Approximately 70-80% of newly diagnosed bladder cancer patients present with non-invasive or early invasive disease (Stage Ta, Tis, or T1). Tumour recurrence is common, occurring in 50- 70% of cases, but cancer progression occurs in only 15-25% of cases (2).

Solid tumours are made up of a variety of cell types. Within tumour microenvironment (TME), cells produce a variety of soluble substances that form complex signaling networks. Tumor-promoting inflammation (TPI) is one of the results of this interaction. CD-14 antigen, a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein, plays a pivotal role in Toll-like receptor (TLR)-mediated signaling pathways. CD14 has been demonstrated to be critically imperative in the signaling pathways of TLR2, TLR3, TLR4, TLR7, and TLR9. The TLRs are well-known to function by recognising pathogen-associated molecular patterns (PAMPs). However, on top of that, TLRs also detect danger-associated molecular patterns. The latter is proven to be associated with some diseases, including cancer. Interestingly, recent evidence reveals that TLRs are also expressed in tumour cells. Over-expression of TLR2, TLR3, TLR4 and TLR5 are seen in ovarian cancer cells, while colonic cancers are associated with TLR2, TLR3 and TLR4 (3).

CD14-high bladder cancer cells mediate multiple cancer hallmarks. They produce signaling inflammatory mediators, including cytokines, chemokines, and small molecules that create the tumour's inflammatory

microenvironment. These inflammatory molecules released by CD14-high bladder cancer cells attract and polarise monocytes and macrophages to obtain immunosuppressive host properties. They are more capable of down-regulating major histocompatibility complex II (MHC II) on monocytes and polarising macrophages toward an M2-like phenotype (3).

A study has demonstrated a significant correlation between CD14 immunohistochemical expression with grades of urothelial carcinoma ($P<0.05$). CD14 expression was detected in 48.5% of bladder cancer cases with higher expression in lower grade urothelial carcinoma (4).

In the past decades, despite improvements in diagnostic fields and surgical and nonsurgical treatment advancements, bladder cancer recurrence remains high, ranging from 50% to 90%. As for non-muscle-invasive urothelial carcinoma, the recurrence rate is about 60% to 70% (5). Studies on CD14 antigen on bladder cancer recurrence are very limited. CD14+HLA-DR−/low cells were discovered in the peripheral blood mononuclear cells (PBMCs) of 64 bladder cancer cases and 14 non-diseased control cases in research. The fraction of CD14+HLA-DR-/low cells in bladder cancer patients was considerably greater than in control cases. The study showed no significant association between CD14+HLA-DR-/low cells and recurrent cases of bladder cancer. However, the level of CD14+HLA-DR−/low cells was correlated with gender, tumour size, tumour numbers, grade and stage (6).

The association of CD14 antigen with other types of cancer such as lung and gastric cancer, leukaemia and lymphoma have been studied. Positive CD14 tumour-associated macrophages were observed in the stroma of all six studied cases of non-small cell lung carcinoma (NSCLC) (7), and a study has demonstrated the role of CD14 in mediating the epithelial-mesenchymal transition (EMT) and invasion of gastric cancer cells in vitro (8). CD14 genes may also be implicated in the development of gastric MALT lymphoma (9). CD14 immunohistochemistry played a significant role as an alternative to flow cytometry for the classification of acute myeloid leukaemia especially AML-M4 to detect monocytic differentiation, in addition to MPO, CD34, CD68, and CD163 as diagnostic markers, to establish a diagnosis of monocytic leukaemia (10). A study on the association of intratumoral CD14 positive cells and clinical outcomes in follicular lymphoma showed that CD14+ follicular dendritic cells (FDC) as independent predictors of DLBCL transformation in follicular lymphoma (11).

MATERIALS AND METHODS

Tissue sampling

A total of 59 formalin-fixed paraffin-embedded (FFPE) tissue samples of urothelial bladder carcinoma were collected from the Histopathology Laboratory, Department of Pathology, Hospital Kuala Lumpur. The samples were selected from biopsy and cystectomy specimens.

Immunohistochemical analysis

FFPE tissue blocks were sectioned at 5 um thickness and mounted onto glass slides. Deparaffinisation followed by dehydration by xylene for 10 minutes was performed. The slides were then rinsed with 100% ethanol three times and 95% ethanol two times. The tissue slides were subjected to antigen retrieval by soaking in 10 µmol/L buffered citrate (pH 6.0) and heated in the microwave for 11 minutes. After cooling at room temperature, the slides were rinsed with distilled water followed by phosphate-buffered saline and were subsequently dipped in 0.03% hydrogen peroxide to block the endogenous peroxidase. Non-specific bindings were blocked by treating the tissue slides with normal goat serum for 40 minutes. The slides were then rinsed again with phosphate-buffered saline. Both positive and negative controls and the test slides were incubated with a specific primary antibody for CD14 (Rabbit monoclonal antibody, dilution1:100, Abcam EPR 3653) followed by secondary antibodies. The secondary antibody functioned as the binding site for chromogen, which gave brown colour, indicating a positive result. Lastly, the slides were counterstained with hematoxylin. The slides stained with CD14 antibody were evaluated using a light microscope. They were scored independently by a researcher and a pathologist according to the staining intensity and percentage of stained tumour cells displaying membranous positivity. Score 0 is when there is no staining, score 1 when there is weak staining in <25% of tumour cells, score 2 is moderate staining in 25%-75% of tumour cells, and score 3 is intense staining in >75% of tumour cells (Fig.1). Score 0 is considered as negative expression while score 1-3 is considered as positive expression.

Statistical analysis

The data was collected, sorted, and statistically analysed using Statistical Package for the Social Sciences (SPSS) version 23. The association between CD14 immunohistochemical expression score with demographic and clinicopathological parameters of urothelial bladder carcinoma was determined using coefficient correlation of spearmen system. The result of $p \leq 0.05$ was considered significant.

Ethics

The study was approved by the Medical Research and Ethics Committee Malaysia (NMRR-18-4003-44387).

RESULTS

Demographic and clinicopathological characteristics

From 59 studied cases, the majority age group was ≥65 years old (n=34, 57.6%), compared to those <65

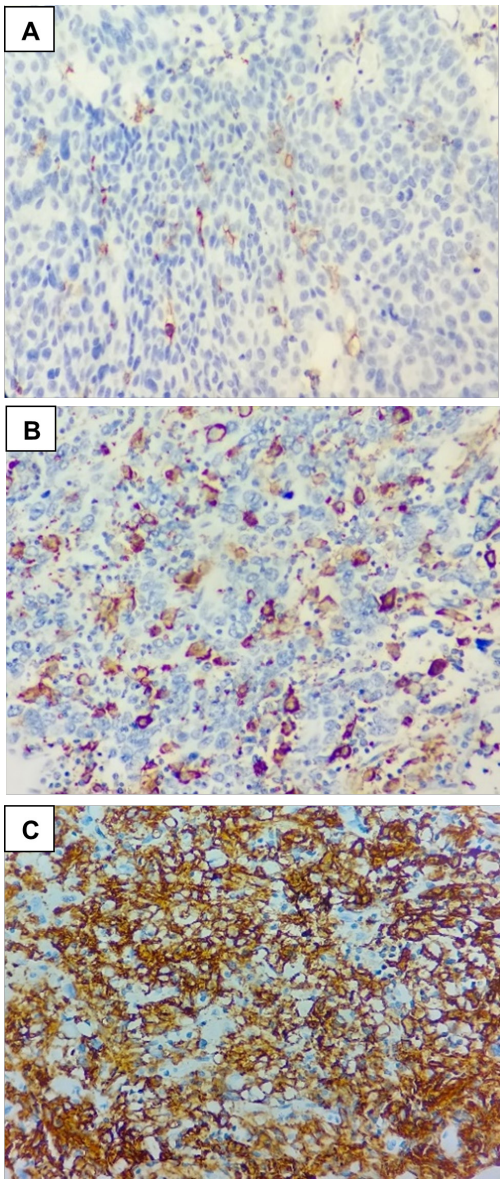

Figure 1: Scoring for CD14 immunohistochemical study exhibiting membranous staining of the tumour cells: A. Score 1 (400x magnification), B. Score 2 (400x magnification), C. Score 3 (400x magnification)

years old (n=25, 42.4%). The mean age was 66.8 years (SD: 9.46), with a range of 38 to 86 years. Male was much more common than female patients (94.9%, n=56). Malay ethnicity (55.9%, n=33) was the highest, followed by Chinese and Indian. Grade III was the most common (n=23, 39%), followed by Grade II (n=19, 32.2%) and Grade I (n=17, 28.8%). The number of cases was inversely proportionate with the tumour stage. More than half of the cases were Stage I (n=33, 55.9%), followed by Stage II (n=18, 30.5%), Stage III (n=6, 10.2%) and Stage IV (n=2, 3.4%) (Table I).

Immunohistochemical expression of CD14 across the grades and stages of urothelial bladder carcinoma

CD14 immunohistochemical expression was positively expressed in more than 50% of tumour cells in all grades and stages of urothelial bladder carcinoma. CD14 was largely expressed in Grade III (95.6%) and Stage IV (100%) tumours, where score 1 scored the highest percentage throughout the grades and stages. Among the grades and stages, intense CD14 expression (score 3) was largely found in Grade III (17.4%) and Stage II (22.2%) tumours (Table II).

Table I: Distribution of demographic and clinicopathological parameters in urothelial bladder carcinoma cases

Demographic and clinicopathological parameters		No. of cases (%) N= 59
Age		
	<65 years old	25 (42.4%)
	≥65 years old	34 (57.6%)
Gender		
	Male	56 (94.9%)
	Female	3 (5.1%)
Ethnicity		
	Malay	33 (55.9%)
	Chinese	21 (35.6%)
	Indian	5 (8.5%)
	Others	0 (0%)
Grade		
	I	17 (28.8%)
	II	19 (32.2%)
	III	23 (39.0%)
Stage		
	I	33 (55.9%)
	II	18 (30.5%)
	III	6 (10.2%)
	IV	2 (3.4%)

Association between CD14 immunohistochemical expression with demographic and clinicopathological characteristics and recurrent cases of urothelial bladder carcinoma

There was no significant association between CD14 immunohistochemical expression with demographic characteristics. A significant association between tumour grade and CD14 expression was detected (Rho=0.264, p-value = 0.043). However, there was no significant association between CD14 expression with tumour stage (Rho=0.159, p-value = 0.230) and recurrent cases of urothelial bladder carcinoma (Rho=0.162, p-value = 0.220) (Table III).

DISCUSSION

Urinary bladder carcinoma ranks as the tenth most common cancer across the globe. The annual incidence of this cancer is around 430,000 cases. In Malaysia, bladder cancer is the ninth most common cancer amounting to up to 1477 cases, equivalent to 3.2% of total registered cancer. The incidence increases with age, which peaks at more than 75 years old, with 322 recorded cases (1). The study showed a similar demographic pattern reported by the Malaysian National Cancer Registry 2012-2016 and the National Institute of Cancer United States 2019, where the majority of cases were elderly male patients (≥ 65 years old).

In this study, the majority of urothelial carcinoma of the bladder was grade III (39%), followed by Grade

Table II: Immunohistochemical expression of CD14 across different grades and stages of urothelial bladder carcinoma

Tumour		CD14 expression [No. of cases (%), N=59]			
		Negative expression	Positive expression		
		Score 0	Score 1	Score 2	Score 3
Grade	I	3 (17.6%)	11 (64.7%)	2 (11.8%)	1 (5.9%)
	II	5 (26.3%)	12 (63.2%)	2 (10.5%)	0 (0.0%)
	III	1 (4.3%)	13 (56.6%)	5 (21.7%)	4 (17.4%)
Stage	I	6 (18.2%)	22 (66.7%)	4 (12.1%)	1 (3.0%)
	II	2 (11.1%)	9 (50.0%)	3 (16.7%)	4 (22.2%)
	III	1 (16.6%)	4 (66.7%)	1 (16.7%)	0 (0.0%)
	IV	0 (0.0%)	1 (50.0%)	1 (50.0%)	0 (0.0%)

Table III: The association between CD14 immunohistochemical expression with demographic and clinicopathological characteristics and recurrent cases of urothelial bladder carcinoma

	CD14 expression	
	Rho	p-value
Demographic characteristics		
Age	0.054	0.684
Gender	0.048	0.716
Ethnicity	0.158	0.231
Clinicopathological characteristics		
Tumour Grade	0.264	0.043*
Tumour Stage	0.159	0.230
Recurrent cases	0.162	0.220

*Statistically significant ($p < 0.05$)

II (32.3%) and, lastly, Grade I (28.8%) (12,13). On the other hand, the tumour registry by the British Association of Urological Surgeons (BAUS) 2014 found that most of the bladder cancer was grade Grade I and II, which comprised 65% while Grade III tumours made up the remaining 35%. Histological grades showed a lack of reproducibility among the reporting pathologists. Inconsistencies occur as this is a subjective process depending on the template in mind (14). The histologic grade is dependent on tissue preservation, where the suboptimal tissue fixation will cause disruption and loss of visibility of mitotic figures, which is one of the components of the grading system.

The study also showed that more than half of the cases were at the early stage of the disease, in accordance with the Malaysian data where 29.8%, 26.5%, 17.6% and 26.2% of patients presented with Stage I, Stage II, Stage III and Stage IV respectively. These findings reflected that Malaysian health authorities had taken extraordinary measures for early bladder cancer detection such as health education on the common symptoms of bladder cancer which require further action. Recently, specialised haematuria clinics have made way for adequate radiological imaging and flexible cystoscopy in a single clinic visit, allowing for earlier detection and treatment of urological illnesses. (15).

There was no significant association between immunohistochemical expression of CD14 and demographic characteristics in this study. The findings concurred with a study where it showed no difference in CD14 positive cells compared to age, ethnicity, and gender (16). The result may suggest that CD14 expression was not affected by the demographic factors.

A significant correlation was detected between tumour grade and CD14 expression in this study, which concurred with two studies (4, 17). One of the studies also revealed that positive CD14 expression was largely seen in Grade III (92.3%), followed by Grade II (78.9%) and Grade I (60%). However, they found that intense CD14 expression (score 3) was largely found in Grade II, compared to Grade III, which contradicted our study findings. This might be due to the slight difference in their and our scoring system where in their study, the percentage of tumour cells within 50-80% was regarded as intense staining (score 3). However, our study used > 75% of tumour cells as intense staining. Their postulated explanation for this observation was due to the effect of down-regulation that occur during chronic inflammation. This inhibitory effect leads to a decrease in CD14 expression. The other possible reason was due to the consequence of shedding of dead urothelial cells that are frequently seen in high-grade urothelial carcinoma (4).

Tumour promoting inflammation is a regular feature of the TME (18). The role of CD14 in promoting inflammatory mediated tumour cell proliferation has been described using MB49 mouse bladder cancer cell line to study the microenvironment of bladder cancer that resembles the physiological environment of tumour formation (3). The study revealed that CD14-high tumour cells produce many signaling mediators including small molecules, cytokines, chemokines, growth factors, and angiogenic factors that act on host cells to promote tumour growth through different mechanisms. CD14-high bladder cancer cells also produce cytokines which have protumorigenic effects via CD14 knockouts. CD14-high bladder cancer cells produce bigger tumours with greater rates of vessel-forming endothelial cell invasion. Tumours generated by CD14-high cells have greater rates of myeloid cell recruitment, which includes monocytes, macrophages, neutrophils, and dendritic cells induced by immune mediators. Tumour-associated macrophages (TAMs) are an important part of TPI (19). TAMs enhance

tumour growth by generating soluble substances which promote tumour proliferation and induce new vessel formation. CD14-high tumour-factor–polarized monocytes and macrophages were shown to be more immune-suppressive and less capable of stimulating T-cell proliferation. Except for TLR3, several TLRs play a role in CD14 signaling in CD14-high bladder cancer cells. This redundancy guarantees that even when individual TLRs are inhibited, the important function of cytokine production is maintained. TLRs have long been linked to tumour formation through various methods. Our study findings support their findings when CD14 antigen was positively expressed in more than 50% of bladder cancer cells across the grades and stages. The expression in every scoring category (score 1-3) was relatively increasing as the grade and stage increased and score 3 was largely found in the highest grade (Grade III) tumours. However, for the tumour stage, score 3 was largely detected in Stage II tumours, not in Stage IV tumours. It might be because most samples are Stage I and Stage II tumours.

This study revealed no significant finding for the association of CD14 expression with different stages and recurrent cases of urothelial bladder carcinoma (p=0.230). To date, there was no similar study performed on the association between CD14 antigen and various tumour stages, as well as recurrent cases of urothelial bladder carcinoma by immunohistochemical assay. High levels of CD14+CD204+ cells by flow cytometry from the pulmonary vein in non-small cell lung carcinoma were found to have developed early recurrences. The study also suggests that tumour-associated macrophages from the primary tumour contribute to tumour metastasis (20). In another separate study performed in recurrent breast cancer cases, serum soluble CD14 (sCD14) could be used as a biomarker to predict the status of LN+ER/PR-Her2+ in recurrent breast cancer (21).

CONCLUSION

CD14 antigen expression in more than 50% of bladder cancer cells across the grades and stages establishes that an inflammatory microenvironment promotes tumour cell proliferation. The largest percentage of positively expressed CD14 antigen in the highest grade (Grade III) and stage (Stage IV) of urothelial bladder carcinoma may suggest that CD14 antigen is a poor prognostic marker. Future studies should include the association of CD14 antigen immunohistochemical expression with the survival rate of urothelial bladder carcinoma. Further molecular studies should be carried out to support the findings of this research.

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