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# ORIGINAL ARTICLE

## Hedgehog signalling molecule, SMO is a poor prognostic marker in bladder cancer

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*Abstract*

*Introduction:* Hedgehog (HH) pathway is an important signalling cascade for growth and patterning during embryonic development. Constitutive activation of Hedgehog pathway can be found in various types of malignancies including medulloblastoma, basal cell carcinoma, gastrointestinal, breast, pancreatic, prostate cancer and leukaemia. Little is known about the expression and role of Hedgehog signalling in bladder cancer. *Materials and Methods:* The purpose of this study was to investigate the immunohistochemical expression of SMO in 112 bladder cancer cases and determine their association with demographic and clinicopathological parameters. Bladder cancer tissues were obtained from the Hospital Kuala Lumpur. *Results:* SMO was expressed in the cytoplasm of all cases of bladder cancer. 6 cases (5.4%) showed low expression, while 106 cases (94.6%) showed high expression. Positive expression of SMO protein was correlated with a few variables which include grade and stage of tumour, lymph node metastasis and distant metastasis. SMO expression showed statistically significant association with higher grade ($p$=0.001) and higher stage ($p$=0.042) of bladder cancer. SMO expression also showed borderline association with lymph node metastasis ($p$=0.056). *Conclusion:* These findings indicate that SMO expression may be a poor prognostic marker in bladder cancer.

*Keywords*: Hedgehog pathway, SMO, immunohistochemistry, bladder cancer

## INTRODUCTION

Bladder cancer is the most common malignancy of the urinary tract of both men and women. Based on the American Cancer Society, in 2015, approximately about 74,000 new cases of bladder cancer diagnosed with an estimated 16,000 bladder cancer-related deaths.[1] In Malaysia, it is the fourth most common cancer in men after lung, colorectal & nasopharyngeal cancers.[2] Men are affected 2 to 3 times more common than women[3] but women have a tendency to suffer from more advanced stage and have poor prognosis.[4] The 5-year survival rate of stage 4 bladder cancer is 15 %, while for stage 0 and 1 the survival rate is 98 and 88 %, respectively.[5] Older age group (>65 years) and Caucasians have a higher risk of bladder cancer compared to those of different ethnicities. Approximately eight in ten Caucasians are diagnosed with bladder cancer.[6]

Hedgehog signalling pathway was first discovered by Nusslein-Volhard and Wieschaus in 1990 during a genetic screening study to find mutations that affect larval body segment development in the fruit fly, Drosophila melanogaster (D. melanogaster).[7] The Hedgehog (HH) pathway is a crucial signalling pathway in many fundamental processes, including embryonic development and tissue homeostasis. Aberrant activation of HH is associated with birth defects.[8] At the molecular level, HH signalling pathway drives the progression of cancers by regulating cancer cell proliferation, metastasis, and expansion of cancer stem cells (CSCs). Therefore, it is a prospective target in

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cancer therapy. Among HH ligands are Sonic hedgehog (SHH), Indian hedgehog (IHH), Desert hedgehog (DHH), SMO and GLI. SMO is a seven-pass integral transmembrane protein, a member of the Frizzled (FzD) class of G-protein-coupled receptors (GPCRs) that functions as a positive regulator of the Hh signalling pathway. SMO binds to HH receptor, PCTH and the binding allows SMO to activate the intracellular signalling components, resulting in stabilisation of downstream transcriptional activator(s), resulting in activation of target genes.[9] Uncontrolled activation of HH signalling may lead to cancer progression and disease. Most colon cancer express high levels of HH mRNA or protein.[10] The expression levels of PTCH and SMO were found to be gradually increased as colon cancer progresses.[11] SHH signalling molecules are highly expressed in oral cancers.[12] Karhadkar *et al*. found that continuous Hedgehog signalling pathway activation transforms prostate progenitor cells and renders them tumourigenic. Metastatic disease shows elevated pathway activity.[13] In bladder cancer, SHH expression was found to be invariably lost during progression to invasive urothelial carcinoma. High levels of SHH protein were found in human benign bladder urothelium but none or little was detectable in the eight primary cancer cells of all invasive urothelial carcinomas.[14] Similarly, target genes GLI1 and PTCH1 also showed low expression in invasive urothelial carcinoma. SMO and GLI1 were genes were ablated to reduce the aggressiveness of tumours.[14] The expression of SMO however was not investigated in this study by Shin *et al*. Therefore, the aim of our study is to identify the expression of SMO in a larger sample size of 112 cases of urothelial carcinoma and correlate with clinicopathological parameters. The findings could be useful in the therapeutic intervention of urothelial carcinoma in future. Inhibitors of the HH signalling pathway have emerged in recent years as promising potential therapeutics for cancer therapy that target different members of this pathway, including SMO, SHH protein and GLI1.[15]

## MATERIALS & METHODS

### Tissue samples

This study was approved by the Medical Research and Ethics Committee and Hospital Kuala Lumpur Research Committee. A total of 112 formalin-fixed paraffin-embedded (FFPE) tissue samples of urothelial carcinoma were collected from the Pathology Department of Hospital Kuala Lumpur. All samples were from either cystoprostatectomy or cystectomy specimens.

### Immunohistochemical analysis

Immunohistochemistry was performed to examine SMO protein expression in 112 bladder cancer cases based on the classical immunohistochemistry protocol. Antigen retrieval was performed by microwaving the slides in 10 mM Citrate buffer at pH 6.0 for 20 minutes. Endogenous peroxidase activity was blocked using 3% $H_2O_2$ for 30 minutes at room temperature. The primary antibody used was rabbit polyclonal anti-SMO antibody (1:200; AB72130; Abcam). SMO antibody was incubated for 60 minutes at room temperature followed by secondary antibody. Visualisation was performed using chromogen diaminobenzidine and counterstained with hematoxylin. Gastric carcinoma tissue in which the Hedgehog pathway is known to be activated was used as positive control.

Scoring for bladder cancer was based on previous study[16] which considers [1] staining intensity and [2] percentage of positive tumour cells. The staining was evaluated by staining intensity: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining and number of positively stained cells: 0, no positive tumour cells; 1, <10% positive tumour cells; 2, 10-50% positive tumour cells; 3, >50% positive tumour cells The final score was calculated by multiplying [1] and [2]. Staining scores ≤4 and ≥6 was classified as tumours with low and high expression, respectively.

### Statistical analysis

Statistical Package for the Social Sciences (SPPS, Version 17.0 Network version, Inc., Chicago, Ill., USA) was used for all statistical analysis. Association between demographic and clinicopathological parameters with hedgehog pathway protein expression were assessed by chi-square tests. *P-value* < 0.05 was considered statistically significant.

## RESULTS

### Patient characteristics

Our cohort subjects included 112 bladder cancer

patients, 103 males (92%) and 9 females (8%). The age of our cohort ranged from 35 to 88 years with a mean value of 53 years. Tumour metastasis was detected in 56 patients (Table 1).

### Expression of SMO in bladder cancer

SMO showed moderate expression in normal bladder lining urothelium (Figure 1A) but was strongly expressed in all cases of bladder cancer. SMO was mainly located in the cytoplasm of malignant cells (Figure1B). Nuclear staining was not reported as significant.

### Association of SMO expression with demographic and clinicopathological parameters

Table 1 shows the statistical analysis of the association between SMO expression and demographic and clinicopathological parameters of bladder cancer. The expression of this protein was correlated with age, gender, ethnicity, grade, stage, lymph node metastasis and distant metastasis. The expression level of SMO protein was significantly associated with higher grade ($P$=0.001) and stage ($P$=0.042) of urothelial carcinoma. Statistical analysis indicated no correlation between the expression of SMO and other clinicopathological parameters such as age, gender, ethnicity, lymph node metastasis and distant metastasis.

## DISCUSSION

Aberrant HH signalling pathway was discovered in patients with Gorlin syndrome, a rare hereditary condition whereby patients developed basal cell carcinomas (BCC) and medulloblastomas (MB) during their lifetime.[17,18] Dysregulation of

**Table 1: Association of SMO protein expression with demographic and clinicopathological parameters**

| Characteristics | No of cases (%) | Low exp. (%) | High exp. (%) | *P* |
|---|---|---|---|---|
| Age | | | | |
| < 61 | 39 (34.8) | 3 (7.7) | 36 (92.3) | 0.418 |
| ≥ 61 | 73 (65.2) | 3 (4.1) | 70 (95.9) | |
| Gender | | | | |
| Female | 9 (8.0) | 1 (11.1) | 8 (88.9) | 0.402 |
| Male | 103 (92.0) | 5 (4.9) | 98 (95.1) | |
| Ethnicity | | | | |
| Malay | 65 (58.0) | 2 (3.1) | 63 (96.9) | |
| Chinese | 32 (28.6) | 3 (9.4) | 29 (90.6) | 0.459 |
| Indian | 11 (9.8) | 1 (9.1) | 10 (90.9) | |
| Others | 4 (3.6) | 0 (0) | 4 (100) | |
| Grade | | | | |
| G1 | 2 (1.8) | 1 (50.0) | 1 (50.0) | **0.001*** |
| G2 | 31 (27.7) | 4 (12.9) | 27 (87.1) | |
| G3 | 79 (70.5) | 1 (1.3) | 78 (98.7) | |
| Stage | | | | |
| Low | 46 (41.1) | 5 (10.9) | 41 (89.1) | **0.042*** |
| High | 66 (58.9) | 1 (1.5) | 65 (98.5) | |
| Lymph node metastasis | | | | |
| Yes | 41 (36.6) | 0 (0) | 41 (100.0) | 0.056 |
| No | 71 (63.4) | 6 (8.5) | 65 (91.5) | |
| Distant metastasis | | | | |
| Yes | 15 (13.4) | 0 (0) | 15 (100.0) | 1.000 |
| No | 97 (86.6) | 6 (6.2) | 91 (93.8) | |

*Significant p value ($p<0.05$)

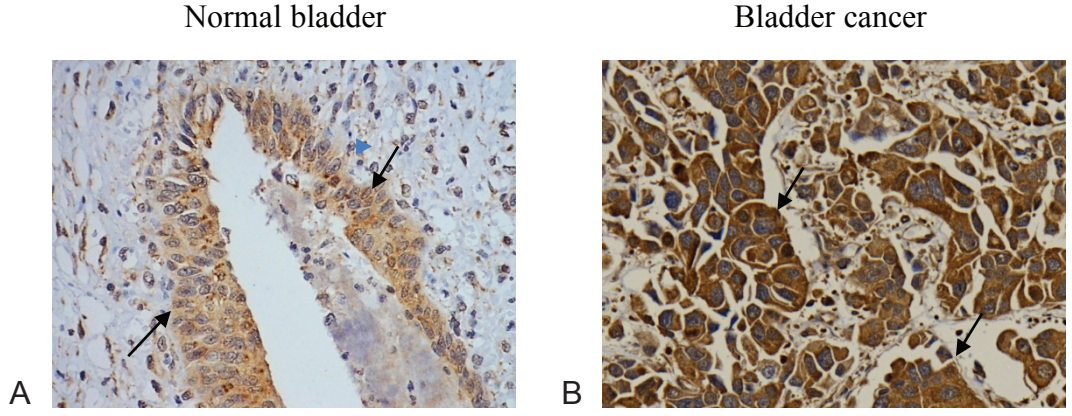


FIG. 1: Moderate expression of SMO proteins (arrowed) in normal bladder urothelium (A) and strong SMO expression in bladder carcinoma cells (×400) (B). SMO was mainly localised in the cytoplasm of benign urothelial cells and malignant cells (arrowed).

various signalling pathways has been implicated in bladder cancer tumourigenesis including the HH signalling pathway via mutations of PTCH and SMO genes.[19] Shin *et al*. found that SHH expression is invariably lost during progression to invasive urothelial carcinoma. Genetic blockade of stromal response to SHH dramatically accelerates progression and decreases survival time.[14] A few studies have shown that dysregulation of SHH ligand or one of its downstream mediators (PTCH1, SMO or GLI1) has been associated with urothelial carcinoma initiation and progression[20-22] and in regulating cancer stem cells activities.[18] Controlled HH pathway activity is linked to tissue repair and tissue homeostasis, whereas uncontrolled activation of the pathway promotes cancer.[23] Tumourigenesis can be ligand dependant or ligand independent. Ligand-independent activation is due to inactivating mutations in the negative regulators PTCH1 or SUFU or activating mutations in the positive regulator SMO, or amplification of GLI activators. Ligand-dependent activation occurs through paracrine or "reverse paracrine" mechanisms (24). To date, a number of small molecules have been discovered as HH pathway antagonists at different levels namely robotnikinin, purmorphamine and budesonide.[24] A wide variety of SMO agonists and antagonists have also been identified.

The "first-in-class" HH pathway regulator was vismodegib (also known as GDC-0449), a specific and potent SMO antagonist. It was used in 2012 for the treatment of metastatic or locally advanced BCC.[25] In 2015, another SMO inhibitor was approved by FDA, sonidegib (also known as LDE225), for treatment of locally advanced BCC.[26]

SHH signalling also mediates astrocyte response to injury. Following an acute, focal injury, reactive astrocytes exhibit a pronounced reduction in Shh activity in a spatiotemporally-defined manner.[27] Canonical SHH signalling occurs predominantly in astrocytes, which play key roles in both neuroprotective and neuroinflammatory actions in the injured CNS. SHH signalling in astrocytes is negatively regulated during acute reactive gliosis but is restored as inflammation and gliosis begin to subside and the injury response approaches resolution. Astrocytic SHH signalling plays a role in attenuating inflammation following an acute, focal injury.[27]

In our study, SMO showed statistically significant association with higher grade and stage of bladder cancer. These findings indicate that SMO expression may be a poor prognostic marker in bladder cancer. However, findings from Ha *et al*.[28] are different from ours where they found that SMO was significantly expressed in low-grade bladder tumours and tumours without progression. These findings suggest that SMO is closely correlated with the differentiation and progression of bladder cancer. These differences of findings may be due to their sample selection where majority (70.1%) were in the lower stage of disease compared to our sample where only 46% were in lower stage of disease. Besides that, scoring system used might contribute to the differences of our results.

Ha *et al*. and He *et al*. successfully proved that the positive expression of SHH, GLI1,

PTCH1 and SMO proteins in bladder cancer were significantly high.[28] Therefore, SHH, GLI1, PTCH1 and SMO may play important roles in the pathogenesis of bladder cancer.

Study limitations include small number of cases. Perhaps a multicentre study would give more samples and more significant results. Other clones of antibody should also be considered to confirm the validity of our study in future, as a polyclonal antibody used in this study may have produced large amount of non-specific binding.

## CONCLUSION

SMO protein was found to be significantly associated with higher grade and stage of bladder cancer. This suggests that SMO may be a poor prognostic marker for human bladder cancer. However further molecular studies are required to determine the role of SMO in the pathogenesis of bladder cancer.

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*Authors' contribution:*
KMA, HH, FAG and MAA designed the research and scored the immunohistochemical staining, KMA performed research and analysed data, SMS contributed to the statistical analysis, RY and AV contributed to clinical samples.

*Conflict of interest:* The authors declare no conflict of interest.

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