UNIVERSITI PUTRA MALAYSIA

HEDGEHOG PATHWAY PROTEINS AND THEIR ASSOCIATION WITH CLINICOPATHOLOGICAL PARAMETERS OF BLADDER CANCER

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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August 2016

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Hedgehog pathway is important for growth and patterning during embryonic development. Previous studies have shown that constitutive activation of Hedgehog pathway can lead to various types of malignancies including medulloblastoma, basal cell carcinoma, gastrointestinal, breast and prostate cancer. The purpose of this study was to investigate the expression of Hedgehog pathway proteins in bladder cancer and determine their association with clinicopathological parameters. The expression of Sonic hedgehog (SHH), its receptor Patched (PTCH1), downstream transcription factor GLI1 and its signal transducer SMO in 112 bladder cancer tissues from Hospital Kuala Lumpur were determined by immunohistochemistry. SHH was expressed in 108 (96.4%) cases, GLI1 in 104 (92.9%) cases, PTCH1 in 111 (99.1%) cases and SMO in all cases. The relationship between the expression of these four proteins and its clinicopathological parameters were analysed statistically. Immunohistochemical staining results showed SHH, GLI1 and SMO proteins were mainly located in the cytoplasm of tumour cells, whereas PTCH1 was mainly located in the nucleus of tumour cells. Positive expression of SHH, PTCH1, GLI1 and SMO proteins were correlated with a few variables which include grade and stage of bladder cancer, lymph node metastasis and distant metastasis (Mx). Among all of the clinicopathological parameters, SMO was found to be the only protein that 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). These findings indicate that SMO expression may be a poor prognostic marker in bladder cancer.

Keywords: Hedgehog signalling pathway, Sonic hedgehog, Gli1, Patched, Smo, bladder cancer.

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LALUAN HEDGEHOG PROTEIN DAN HUBUNGKAITNYA DENGAN PARAMETER PATOLOGI KLINIKAL KANSER PUNDI KENCING

Oleh

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Laluan hedgehog penting untuk pertumbuhan dan corak semasa perkembangan embrio. Kajian sebelum ini menunjukkan bahawa pengaktifan berterusan laluan hedgehog boleh membawa kepada pelbagai jenis tumor termasuk medulloblastoma, karsinoma sel basal, kanser gastrousus, kanser payudara dan kanser prostat. Kajian ini bertujuan untuk menyiasat ekspresi protein laluan Hedgehog dalam kanser pundi kencing dan menentukan hubungkaitnya dengan parameter patologi klinikal. Ekspresi Sonic hedgehog (SHH), penerima (PTCH), faktor transkripsi, (GLI1) dan isyarat transduser, (SMO) bagi 112 tisu kanser pundi kencing daripada Hospital Kuala Lumpur telah ditentukan dengan teknik immunohistokima. SHH menunjukkan ekspresi dalam 108 (96.4%) kes, GLI1 dalam 104 (92.9%) kes, PTCH1 dalam 111 (99.1%) kes dan SMO dalam semua kes. Hubungan ekspresi untuk keempat-empat protein dan parameter patologiklinikal telah dianalisa secara statistik. Keputusan pewarnaan imunohistokimia menunjukkan protein SHH, GLI1 dan SMO kebanyakannya terletak di sitoplasma sel-sel tumor, manakala PTCH1 kebanyakannya terletak di dalam nukleus sel-sel tumor. Ekspresi positif daripada SHH, GLI1, PTCH1 dan SMO protein telah dikorelasi dengan beberapa pembolehubah yang merangkumi gred dan peringkat kanser pundi kencing, metastasis nodus limfa dan metastasis lebih jauh. Antara semua parameter patologi klinikal, SMO protein sahaja yang menunjukkan hubungkait yang signifikan secara statistik dengan gred tertinggi (p=0.001) dan peringkat tertinggi (p=0.042) kanser pundi kencing. Ekspresi SMO juga menunjukkan garis batas berkait dengan nodus limfa (p=0.056). Keputusan ini menunjukkan bahawa ekspresi SMO mungkin merupakan petanda prognostik yang lemah untuk kanser pundi kencing.

Kata kunci: Laluan hedgehog protein, Sonic hedgehog, GLI1, PTCH1, SMO, kanser pundi kencing.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

WHO	World Health Organization
TCC	Transitional cell carcinoma
HH	Hedgehog
Shh	Sonic hedgehog
PTCH1	Patched homologue 1
SMO	Smoothened homologue
GLI1	GLI family zinc finger 1
mL	mililitre
G1	well differentiated
G2	moderately differentiated
G3	poorly differentiated
WHO/ISUP	World Health Organization/International Society of Urological Pathology
PUNLMP	papillary urothelial neoplasms of low malignant
TNM	Tumour nodes metastasis
AJCC	American Joint Committee on Cancer
Ihh	Indian Hedgehog
Dhh	Desert Hedgehog
SUFU	Suppressor of fused
DNA	Deoxyribonucleic acid
Gli^R	GLI family zinc finger repressor
Gli^A	GLI family zinc finger activator
BCC	Basal cell carcinomas
MB	Medulloblastoma
CSCs	Cancer stem cells
FFPE	Formalin-fixed paraffin embedded
H&E	Hematoxylin & Eosin
DPX	Di-N-Butyle Phthalate in Xylene
Ag-Ab	Antigen antibody
ABC	Avidin-biotin complex
LSAB	Labelled streptavidin-biotin
PAP	Peroxidase-antiperoxidase
mM	mili molar
PBS	Phosphate buffered saline
TBST	Tris Buffered Saline with Tween 20
HRP	Horse radish peroxidase
µl	microlitre
DAB	Diaminobenzidine
SPSS	Statistical Package for the Social Sciences
Mx	Distant metastasis

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Cancer is one of the common health problems worldwide. In the United States, one in four deaths is due to cancer (Siegel, Naishadham, & Jemal, 2013). In year 2007, based on Ministry of Health Malaysia Hospitals after Heart Diseases & Diseases of Pulmonary Circulation and Septicaemia, cancer was the third common cause of death in Malaysia. A total of 18,219 new cancer cases were diagnosed in 2007 and registered at the National Cancer Registry. Among the cases reported, there were 8,123 (44.6%) males and 10,096 (55.4%) females (Zainal & Nor Saleha, 2011). Worldwide, bladder cancer ranks eleventh most common malignancy. It is the 7th most common cancer in men and the 19th most common cancer in women (Jacques Ferlay, Isabelle Soerjomataram, Rajesh Dikshit, 2012). The disease is more common in whites than blacks, and the average age at diagnosis is 65 years. In addition, it has been recommended that the stage survival of bladder cancer in female is worse than in male (Kirkali et al., 2005).

Approximately 90% of bladder tumours arise from the urothelial lining, and transitional cell carcinoma (TCC) is the most common histological type in cases arising in the United States. TCC or as known as urothelial carcinoma starts in the cells lining of the bladder and later will spread to the nearby organ or other body parts if it is not treated at early stage. Smoking habit, chemical exposure, age, sex and chronic bladder inflammation are risk factors that can contribute to the development of bladder cancer (Jemal et al., 2011). However, the pathogenesis of bladder cancer still largely remains unknown. Bladder cancer is treated based on the tumour stage and grade. Besides the treatment protocols, the incidence of therapeutic resistance and failure are still present.

Malignant cells aberrantly reactivate oncogenic developmental pathways such as Hedgehog, Wnt and Notch to provide growth and survival advantage to tumour cells (Ingham, Mcmahon, Ingham, & Mcmahon, 2001). Emerging evidence clearly suggesting the activation of Hedgehog pathway has been implicated in the tumour development of a large number of human cancers (Beachy, Karhadkar, & Berman, 2004).

The exact genetic events leading to urothelial transformation involve the activation and inactivation of oncogenes, inactivation or loss of tumour suppressor genes and alterations in the apoptotic gene products (Sandberg and Berger, 1994). Study shows that there are a lot of genetic abnormalities but the specific genes involved are still unidentified. This is because there are a lot of gene mutations detectable in bladder cancer (Cantile et al., 2003).

In 2010, Chen et al. have genotyped 177 single-nucleotide polymorphisms (SNP) in 11 SHH pathway genes in a study including 803 bladder cancer cases and 803 controls. They also analysed SNP associations with cancer risk and clinical outcomes in 318

cases of muscle-invasive and 419 cases of non-muscle-invasive bladder cancer and metastatic bladder cancer. As their findings, it has been confirmed that germ-line genetic variations in the SHH pathway could predict the clinical outcomes of non-muscle-invasive bladder cancer patients received transurethral resection and Bacillus Calmette-Guérin.

Hedgehog signalling pathway operates in organogenesis, control of proliferation, and the differentiation of embryonic adult stem cells, thus, not surprisingly it has been linked with several different tumours (Sverrisson et al., 2014) including gastrointestinal cancers and is frequently activated in esophageal (Ingham et al., 2001), pancreatic (Morton et al., 2007) and gastric cancer (Merchant, Saqui-Salces, & El-Zaatari, 2010) .On the other hand, the role of hedgehog signalling pathway has been less investigated in the development of bladder cancer and its involvement to the progression of transitional cell carcinoma of bladder is not clear (Haraguchi et al., 2007). However, He et al. successfully proved that the positive expression of SHH, GLI1 and PTCH1 proteins in bladder cancer were significantly higher than in adjacent normal bladder. Ha, Y. S. et al. (2007) has shown that SMO is closely related with the differentiation and progression of bladder cancer. Therefore, SHH, GLI1, PTCH1 and SMO may play important roles during development of bladder cancer (He et al., 2012). This study is necessary to contribute the knowledge in understanding the role of HH pathway proteins in bladder cancer.

1.2 Problem statement

Bladder cancer is generally diagnosed by cystoscopy and biopsy. It has a very high frequency of recurrence and therefore requires follow up cystoscopy as well as urine cytology as periodic surveillance to identify early recurrence. Failure to treat this cancer at an early stage will lead to advance stage which may further complicate the management of the patient. Currently, radical cystectomy is the treatment for urothelial carcinoma. As this cancer needs long-term follow up and surveillance procedure to monitor for tumour recurrence, many of the patients tend to default their follow up. The role of hedgehog signalling pathway in bladder development is well recognized but its role in human bladder cancer progression is uncertain and has been quite controversial. So, this study was done to determine the association of hedgehog pathway protein expression with the progression of bladder cancer so that it can be treated at an early stage with more effective treatments.

1.3 Significance of the study

The findings from this study will contribute knowledge in understanding the role of SHH, GLI1, PTCH1 and SMO in bladder cancer. This study will also offer overview on the expression of these proteins in bladder cancer to better evaluate the function of these proteins in tumour progression. It also provides fundamental background for further investigations on clinical use of related antibodies for personalised cancer management.

1.4 Research objectives

1.4.1 General objective

- To study the HH signalling pathway in bladder cancer.

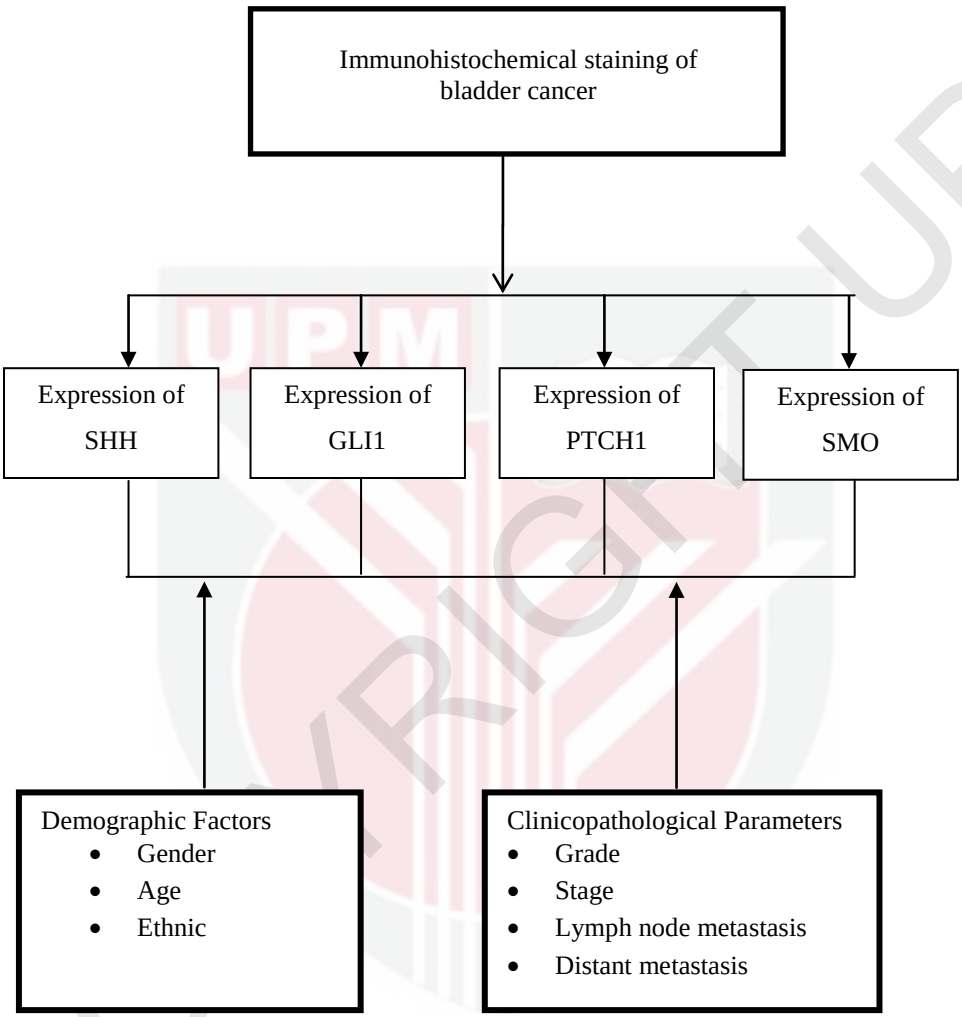
1.4.2 Specific objectives

1) To determine the demographic distribution of bladder cancer in Hospital Kuala Lumpur from 2008 to 2013.
2) To analyse the immunohistochemcal expression of SHH, GLI1, PTCH1 and SMO in bladder cancer.
3) To study the correlation in the immunohistochemical expression between Hedgehog pathway proteins (SHH, GLI1, PTCH1 and SMO) in bladder cancer.
4) To study the association of demographic factors (age, gender, ethnicity) and clinicopathological parameters (grade, stage, lymph node metastasis, distant metastasis) of bladder cancer with the expression of hedgehog pathway protein.

1.5 Research hypothesis

1) There is high expression of hedgehog pathway proteins (SHH, GLI1, PTCH1 and SMO) in bladder cancer.
2) There is significant correlation among the expression of hedgehog signalling proteins (SHH, GLI1, PTCH1 and SMO).
3) There is significant association between demographic factor and clinicopathological parameters of bladder cancer with the expression of hedgehog pathway proteins (SHH, GLI1, PTCH1 and SMO).

1.6 Conceptual Framework



REFERENCES

Al-Hajj, M., & Clarke, M. F. (2004). Self-renewal and solid tumor stem cells. *Oncogene*, *23*(43), 7274–7282.

Ara, J. A. R. A. (2005). Technical Aspects of Immunohistochemistry. *Veterinary Pathology*, *42*(4), 405–426.

Ayers, K. L., & Thérond, P. P. (2010). Evaluating Smoothened as a G-protein-coupled receptor for Hedgehog signalling. *Trends in Cell Biology*, 20(5), 287-298.

Baris, D., Karagas, M. R., & Verrill, C. et al. (2009). A case-control study of smoking and bladder cancer risk:emergent patterns over time. *Journal of the National Cancer Institute*, 101, 1553–1561.

Beachy, P. A., Karhadkar, S. S., & Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature*, 432(7015), 324–331.

Berman, D. M., Karhadkar, S. S., Hallahan, A. R., Pritchard, J. I., Eberhart, C. G., Watkins, D. N., Beachy, P. A. (2002). Medulloblastoma Growth Inhibition by Hedgehog Pathway Blockade. *Science*, 297(5586), 1559–1561.

Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Beachy, P. A. (2003). Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature*, 425(6960), 846–851.

Bitgood, M. J., Shen, L., & McMahon, A. P. (1996). Sertoli cell signaling by Desert hedgehog regulates the male germline. *Current Biology* , 6(3), 298–304.

Braud, F. De, Maffezzini, M., Vitale, V., Bruzzi, P., Gatta, G., Hendry, W. F., Tabor, M. (2002). Bladder cancer. *Crit Rev Oncol Hematol*, 41, 89–106.

Cantile, M., Cindolo, L., Napadono, G., Altieri, V., Cillo, C. (2003). Hyperexpression lof ocus C gene in the HOX network is strongly associated in vivo with human bladder transitional cell carinomas. *Oncogene*, 22, 6562–6468.

Chalasani, V., Chin, J. L., & J. I. Izawa. (2009). Histologic variants of urothelial bladder cancer and nonurothelial histology in bladder cancer. *Canadian Urological Association Journal*, 3, S193–198.

Chan, K. S., Espinosa, I., Chao, M., Wong, D., Ailes, L., & Diehn, M. et al. (2009). Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 14016-14021.

Chen, C. J., Chuang, Y. C., Lin, T. M., & Wu, H. Y. (1985). Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: high-arsenic artesian well water and cancers. *Cancer Research*, 45, 5895–5899.

Chen, M., Hildebrandt, M. A., Clague, J. et al. (2010). Genetic variation in the sonic hedgehog pathway affect clinical outcomes in non-muscle-invasive bladder cancer. *Cancer Prev Res (Phila), 3*, 1235–1245.

Cheng, W., Yeung, C. K., Ng, Y. K., Zhang, J. R., Hui, C. C., & Kim, P. C. W. (2008). Sonic Hedgehog Mediator Gli2 Regulates Bladder Mesenchymal Patterning.

Journal of Urology, 180(4), 1543–1550.

Clark, P. E., Agarwal, N., Biagioli, M. C., Eisenberger, M. A., Greenberg, R. E., Herr, H. W., Wilson, T. G. (2013). NCCN.org Bladder Cancer.

Coons, A. H., & Kaplan, M. H. (1950). Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. *The Journal of Experimental Medicine*, 91(1), 1–13.

Dahmane, N., Lee, J., Robins, P., Heller, P., & Ruiz i Altaba, A. (1997). Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature*, 389, 876–881.

DeSouza, K. R., Saha, M., Carpenter, A. R., Scott, M., & McHugh, K. M. (2013). Analysis of the Sonic Hedgehog Signaling Pathway in Normal and Abnormal Bladder Development. *PLoS ONE*, *8*(1), e53675.

Droller, M. J. (2001). *Bladder cancer: current diagnosis and treatment*. Retrieved from https://books.goggle.com.my/books?id=Pn31BwAAQBAJ

Echelard, Y., Epstein, D. J., St-Jacquess, B., Shen, L., Mohler, J., McMahon, J. A., & McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell*, 75(7), 1417–1430.

Edge, S., Byrd, D., & Compton, C. (2010). *AJCC cancer staging manual* (7th ed.). New york: Springer.

Edward, B. K., Ward, E., & Kohler, B. A. et al. (2010). Annual report to the nation on the status of cancer, 1975– 2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer*, 116, 544–573.

Elisabeth, H.-V., Davis, O.-W., & Philip, M.-I. (2000). The Sonic hedgehog-patched-Gli pathway in human development and disease. *American Journal of Human Genetics*, 67, 1047–1054.

Fajkovic, H., Halpern, J. A., & Cha, E. K. et al. (2011). Impact on gender on bladder cancer incidence, staging and prognosis. *World Journal of Urology*, 29, 457–463.

Fei, D. L., Sanchez-Mejias, A., Wang, Z., Flaveny, C., Long, J., Singh, S., Robbins, D. J. (2012). Hedgehog signaling regulates bladder cancer growth and tumorigenicity. *Cancer Research*, *72*(17), 4449–4458.

Freedman, N. D., Silverman, D. T., Hollenbeck, A. R., Schatzkin, A., & Abnet, C. C. (2011). Association between smoking and risk of blaader cancer among men and women. *Journal of the American Medical Association*, 306, 737–745.

Gonnissen, A., Isebaert, S., & Haustermans, K. (2013). Hedgehog signaling in prostate cancer and its therapeutic implication. *International Journal of Molecular Sciences*, 14(7), 13979–14007.

Goodrich, L. V, Milenković, L., Higgins, K. M., & Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science (New York, N.Y.)*, 277(5329), 1109–1113.

Gupta, P., Jain, M., Kapoor, R., et al (2009). Impact of age and gender on the clinicopathological characteristics of bladder cancer. *Indian J Urol*, 25, 207-10.

Gupta, S., Takebe, N., & Lorusso, P. (2010). Targeting the Hedgehog pathway in cancer. *Therapeutic Advances in Medical Oncology*, *2*(4), 237–250.

Ha, Y., Yun, S., Kim, Y., Lee, S., & Kim, W. (2007). 방광암의 예후 인자로서 Smo 의 가치 Utility of Smo as a Prognostic Marker for Human Bladder Tumors, 997–1003.

Hahn, H., Wicking, C., Zaphiropoulos, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Bale, A. E. (1996). Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell*, 85(6), 841-851.

Hamed, S., Larue, H., & Hovington, H. (2004). Accelerated Induction of Bladder Cancer in Patched Heterozygous Mutant Mice Advances in Brief Accelerated Induction of Bladder Cancer in Patched Heterozygous Mutant Mice, 1938–1942.

Haraguchi, R., Motoyama, J., Sasaki, H., Satoh, Y., Miyagawa, S., Nakagata, N., Yamada, G. (2007). Molecular analysis of coordinated bladder and urogenital organ formation by Hedgehog signaling. *Development*, *134*, 525–533.

Hartge, P., Harvey, E. B., Linehan, W.M., et al (1990). Unexplained excess risk of bladder cancer in men. *J Natl Cancer Inst*, 82, 1636-40.

He, H. C., Chen, J. H., Chen, X. Bin, Qin, G. Q., Cai, C., Liang, Y. X., Zhong, W. De. (2012). Expression of hedgehog pathway components is associated with bladder cancer progression and clinical outcome. *Pathology and Oncology Research*, 18(2), 349–355.

Horn, E. P., Tucker, Ma., Lambert, G., et al (1995). A study of genderbased cytochrome P450 1A2 variability: A possible mechanism for the male excess of bladder cancer. *Cancer Epidemiol Biomarkers Prev*, 4, 529-33

Ingham, P. W., & Mcmahon, A. P. (2001). Hedgehog signaling in animal development *Genes Dev*, 15(23), 3059-3087.

Islam, S. S., Mokhtari, R. B., Noman, A. S., Uddin, M., Rahman, M. Z., & Azadi, M. A. et al. (2015). Sonic hedgehog (Shh) signaling promotes tumorigenicity and stemness voa activation of epithelial-to-mesenchymal transition (EMT) in bladder cancer. *Molecular Carcinogenesis*, 55(5), 537-551.

Jacques Ferlay, Isabelle Soerjomataram, Rajesh Dikshit, S. E. (2012). Cancer incidence and mortality worldwide: sources, method and major patterns in Globocan 2012. *Interantional Journal of Cancer*, *Vol. 5*.

Jayendran, D., & Manoj, S. (2012). Bladder cancer. Retrieved from http://www.malaysiaoncology.org/article.php?aid=9

Jemal, A., Bray, F., & Ferlay, J. (2011). Global Cancer Statistics: 2011. *CA Cancer J Clin*, 61, 69–90.

Johnson, R. L., Rothman, a L., Xie, J., Goodrich, L. V, Bare, J. W., Bonifas, J. M., Scott, M. P. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science (New York, N.Y.)*, 272(5268), 1668–1671.

Kappler, R., Bauer, R., Calzada-Wack, J., Rosemann, M., Hemmerlein, B., & Hahn, H. (2004). Profiling the molecular difference between Patched- and p53-dependent rhabdomyosarcoma. *Oncogene*, 23, 8785–8795.

Karhadkar, S. S., Bova, G. S., Abdallah, N., Dhara, S., Gardner, D., Maitra, A., Beachy, P. A. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature*, 431(7009), 707–712.

Kim, J. E., Singh, R. R., Cho-Vega, J. H., Drakos, E., Davuluri, Y., Khokhar, F. A., Vega, F. (2009). Sonic hedgehog signaling proteins and ATP-binding cassette G2 are aberrantly expressed in diffuse large B-cell lymphoma. *Mod Pathol*, 22(10), 1312–1320.

Kinzler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Vogelstein, B. (1987). Identification of an amplified, highly expressed gene in a human glioma. *Science*, 236(70), 70–73.

Kirkali, Z., Chan, T., Manoharan, M., Algaba, F., Busch, C., Cheng, L., Weider, J. (2005). Bladder cancer: Epidemiology, staging and grading, and diagnosis. *Urology*, 66, 4–34.

Kogevinas, M., Mannetje, A., & Cordier, S. et al. (2003). Occupation and bladder cancer among men in Western Europe. *Cancer Cause Control*, 14, 907–914.

Kong, C. H. C., Singam, P., Hong, G. E., Cheok, L. B., Azrif, M., Tamil, A. M., et al. (2010). Clinicopathological features of bladder tumours in a single institution in Malaysia. *Asian Pacific J Cancer Prev*., 11(1), 149–52.

Kubo, M., Nakamura, M., Tasaki, A., Yamanaka, N., Nakashima, H., Nomura, M., Katano, M. (2004). Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. *Cancer Research*, 64(17), 6071–6074.

Lindemann, R. K. (2008). Stroma-initiated Hedgehog signaling takes center stage in B-cell lymphoma. *Cancer Research*, 68(4), 961-964.

Liu, C. Z., Yang, J. T., Yoon, J. W., Walterhouse, D., & Iannaccone, P. (1998). Characterization of the promoter region and genomic organization of GLI, a member of the Sonic hedgehog-Patched signaling pathway. *Gene*, 209(1-2), 1–11.

Luo, W., Li, S., Peng, B., Ye, Y., Deng, X., & Yao, K. (2013). Embryonic Stem Cells Markers SOX2 , OCT4 and Nanog Expression and Their Correlations with Epithelial- Mesenchymal Transition in Nasopharyngeal Carcinoma, *8*(2), e56324.

Malkowicz, S. B., van Poppel, H., Mickisch, G., Pansadoro, V., Thüroff, J., Soloway, M. S., Fukui, I. (2007). Muscle-Invasive Urothelial Carcinoma of the Bladder. *Urology*, 69, 3–16.

Marigo, V., Roberts, D. J., Lee, S. M., Tsukurov, O., Levi, T., Gastier, J. M., et al. (1995). Cloning, expression and chromosomal location of SHH and IHH: two human homologues of the Drosophilia segment polarity gene hedgehog. *Genomics*, 28(1), 44–51.

Merchant, J. L., Saqui-Salces, M., & El-Zaatari, M. (2010). *Hedgehog signaling in gastric physiology and cancer. Progress in Molecular Biology and Translational*

Science (Vol. 96). Elsevier Inc.

Montironi, R., & Lopez-Beltran, A. (2005). The 2004 WHO classification of bladder tumors: a summary and commentary. *Interantional Journal of Surgical Pathology*, 13, 143–153.

Morton, J. P., Mongeau, M. E., Klimstra, D. S., Morris, J. P., Lee, Y. C., Kawaguchi, Y., Lewis, B. C. (2007). Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5103–5108.

Ning, Z. F., Huang, Y. Z., Lin, T. X., Zhou, Y. X., Jiang, C., & Xu, K. W. et al. (2009). Subpopulation of stem-like cells in side population cells from the human bladder transitional cell cancer cell line T24. *Journal of International Medical Research*, 37, 621–630.

Nishimaki, H., Kasai, K., Kozaki, K., Takeo, T., Ikeda, H., Saga, S., Itoh, G. (2004). A role of activated Sonic hedgehog signaling for the cellular proliferation of oral squamous cell carcinoma cell line. *Biochemical and Biophysical Research Communications*, 314(2), 313–20.

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, 287(5785), 795–801.

Pasca di Magliano, M., & Hebrok, M. (2003). Hedgehog signalling in cancer formation and maintenance. *Nature Reviews. Cancer*, 3(12), 903–911.

Reuter, V. (1997). *Urinary bladder, ureter, and renal pelvis. In: Sternberg SS, ed. Histology for Pathologists* (2nd ed.). New york: Lippincott-Raven.

Ruiz I Altaba, A. (1999). Gli proteins encode context-dependent positive and negative functions: implications for development and disease. *Development (Cambridge, England)*, 126(14), 3205–3216.

Ruiz I Altaba, A., Mas, C., & Stecca, B. (2007). The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends in Cell Biology*, 17(9), 438-447.

Sanchez, P., Hernández, A. M., Stecca, B., Kahler, A. J., DeGueme, A. M., Barrett, A., Ruiz i Altaba, A. (2004). Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 101(34), 12561–12566.

Sandberg, A. A., and Berger, C. S. (1994). Review chromosomes studies in urologic tumors cytogenetics and molecular genetics of bladder. *Cancer Journals of Urology*, 151(3), 545–560.

Scales, S. J., & de Sauvage, F. J. (2009). Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends in Pharmacological Sciences*, 30(6), 303–312.

She, J. J., Zhang, P. G., Wang, Z. M., Gan, W. M., & Che, X. M. (2008). Identification of side population cells from bladder cancer cells by dyecycle violet staining. *Cancer Biology and Therapy*, 7(10), 1663–1668.

Shiroyanagi, Y., Liu, B., Cao, M., Agras, K., Li, J., Hsieh, M. H., … Baskin, L. S.

(2007). Urothelial sonic hedgehog signaling plays an important role in bladder smooth muscle formation. *Differentiation; Research in Biological Diversity*, 75(10), 968–77.

Siegel, R., Naishadham, D., & Jemal, A. (2013). Cancer statistics, 2013. *A Cancer Journal for Clinicians*, 63, 11–30.

Stanton, B. Z., Peng, L. F., Maloof, N., Nakai, K., Wang, X. et al. (2009). A small molecule that binds Hedgehog and blocks its signaling in human cells. *Nat. Chem. Biol.*, 5(3), 154–156.

Stenzl, A., Cowan, N. C., & Santis, M. D. et al. (2009). The updated EAU guidelines on muscle-invasive and metastatic bladder cancer. *European Urology*, 55(4), 815–825.

Sverrisson, E. F., Zens, M. S., Liang Fei, D., Andrews, A., Schned, A., Robbins, D., Seigne, J. D. (2014). Clinicopathological correlates of Gli1 expression in a population-based cohort of patients with newly diagnosed bladder cancer. *Urologic Oncology: Seminars and Original Investigations*, 32(5), 539–545.

Syed, I. S., Pedram, A., & Farhat, W. A. (2016). Role of Sonic Hedgehog (Shh) Signaling in Bladder Cancer Stemness and Tumorigenesis. *Current Urology Reports*, 17(2), 11.

Szkandera, J., Kiesslich, T., Haybaeck, J., Gerger, A., & Pichler, M. (2013). Hedgehog signaling pathway in ovarian cancer. *International Journal of Molecular Sciences*, 14(1), 1179-1196.

Taipale, J., Cooper, M. K., Maiti, T., & Beachy, P. A. (2002). Patched acts catalytically to suppress the activity of Smoothened. *Nature*, 418(6900), 892–7.

Taylor, M. D., Northcott, P. A., Korshunov, A., Remke, M., et al. (2011). Molecular subgroups of medulloblastoma: the current consensus. *Acta Neuropathologica*, 123(4), 465–472.

Teglund, S., & Toftgård, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochimica et Biophysica Acta*, 1805(2), 181-208.

Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Hebrok, M. (2003). Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature*, 425(6960), 851–6.

Theunissen, J. W., & de Sauvage, F. J. (2009). Paracrine Hedgehog signaling in cancer. *Cancer Research*, 69(15), 6007–6010.

Tian, H., Callahan, C. A., DuPree, K. J., Darbonne, W. C., Ahn, C. P., Scales, S. J., & de Sauvage, F. J. (2009). Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), 4254–9.

Tortora, G. J., & Derrickson, B. H. (2009). *Principle of anatomy and physiology* (12th ed.). Chichester: Wiley.

Tripathi, K., Mani, C., Barnett, R., Nalluri, S., Bachaboina, L., Rocconi, R. P., Palle, K. (2014). Gli1 protein regulates the S-phase checkpoint in tumor cells via bid protein, and its inhibition sensitizes to DNA Topoisomerase 1 inhibitors. *Journal*

of Biological Chemistry, 289(45), 31513–31525.

VanPutte, C. L., & Seeley, R. R. (2014). *Seeley's anatomy & physiology*. New York: McGraw-Hill.

Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A, & Baylin, S. B. (2003). Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature*, 422, 313–317.

Watkins, D. N., & Peacock, C. D. (2004). Hedgehog signalling in foregut malignancy. *Biochemical Pharmacology*, 68, 1055–1060.

Wayne W. Daniel. (2009). *Biostatistics: A foundation of analysis in the health sciences* (9th ed.). USA: Wiley.

Wicking, C., Smyth, I., & Bale, A. (1999). The hedgehog signalling pathway in tumorigenesis and development. *Oncogene*, 18(55), 7844–7851.

Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., de Sauvage, F. J. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature*, 391, 90–92.

Yauch, R. L., Gould, S. E., Scales, S. J., Tang, T., Tian, H., Ahn, C. P., de Sauvage, F. J. (2008). A paracrine requirement for hedgehog signalling in cancer. *Nature*, 455(7211), 406–410.

Yoon, J. W., Kita, Y., Frank, D. J., Majewski, R. R., Konicek, B. A., Nobrega, M. A., Iannaccone, P. (2002). Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *Journal of Biological Chemistry*, 277(7), 5548–5555.

Zainal, A. O., & Nor Saleha, I. T. (2011). *National Cancer Registry Report*. *Ministry of Health Malaysia*.

Zhu, G., Zhau, H. E., Wu, D., Zhang, L., Li, L., He, D., & Chung, L. W. K. (2013). Sonic hedgehog signaling in normal human bladder development. *Journal of Urology*, 189(4), e222.