

ORIGINAL ARTICLE

Patched-1 and Smoothed, a Hedgehog Receptor and Signal Transducer are Highly Expressed in Diffuse Large B-Cell Lymphoma

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ABSTRACT

Introduction: The Hedgehog (Hh) signalling pathway is a developmental signalling pathway involved in normal mammalian developmental and homeostasis of adult renewable tissues. In most adult tissues, this pathway remains silent and previous studies have shown that constitutive activation of Hedgehog signalling pathway leads to various types of malignancies including medulloblastomas, basal cell carcinoma, gastrointestinal, breast and prostate cancer. The purpose of this study was to investigate the immunohistochemical expression of Hedgehog pathway proteins in Diffuse Large B-cell Lymphoma and determine their association with overall survival (OS). **Methods:** Positive control using normal tonsils were included in each batch of immunohistochemical staining procedure. **Results:** PTCH1 proteins were highly expressed in DLBCL and showed strong staining intensity in 107 (100%) cases and SMO proteins were expressed in 105 (98.1%) cases. PTCH1 proteins were localised in the nucleus of tumour cells, whereas SMO proteins were mainly localised in the cytoplasm of tumour cells. Positive expression of PTCH1 and SMO proteins and overall survival of DLBCL patients were correlated with age, gender, race and tumour location. There was no significant correlation between the expression of these two proteins with any of the parameters. PTCH1 expression showed significant association with SMO expression ($P=0.03$). **Conclusions:** Our findings suggest that high expression of both PTCH1 and SMO may be important in the pathogenesis of DLBCL. However, additional mechanisms that may contribute to the activation of HH signalling in DLBCL needs to be further explored.

Keywords: Hedgehog signalling pathway, PTCH, SMO, Diffuse large B-cell lymphoma, Immunohistochemistry

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INTRODUCTION

DLBCL is the commonest subtype of Non Hodgkin Lymphoma. Despite its aggressive behaviour, it remains a treatable lymphoma with enhanced result combining the use of rituximab in standard regimens (1). According to the National Cancer Registry, 2007, lymphoma is the 6th commonest cancer among Malaysians. It is the sixth most common cancer in males and the eighth most common cancer in females (2). In 2010, the World Health Organization (WHO) recorded that lymphoma was the 21st top killer disease with 0.97% of the total death. Almost one thousand patients are diagnosed

every day (3). Studies have discovered useful prognostic markers with a subsequent deeper understanding of the various origins of DLBCL in order to provide potential therapeutic intervention (4). The hedgehog (HH) signalling pathway is a highly regulated signalling pathway that is important in the pathogenesis of several malignancies. The HH family of proteins comprises three ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)(5). Without the ligand, the 12-pass membrane receptor Patched (PTCH) will inhibit the action of another transmembrane G-protein-coupled receptor (GPCR); Smoothed (SMO)(6,7). This in return will switch off the pathway and the target gene transcription in the nucleus are inactivated. At the point when HH ligands binds to PTCH, inhibition of SMO is released and a complex signalling cascade is transduced to the cytoplasm by the serine/threonine protein kinase Fused (Fu), Suppressor of Fused (SuFu) and GLI (8).

A number of reports have shown that HH overexpression, that is accompanied by an increased in the expression of target genes play a role in gastric cancer, pancreatic cancer, glioma, breast, small cell lung carcinoma and prostate cancer. The initial link between HH signalling and human cancers was made from the findings that mutations in the HH pathway components PTCH1 was associated with a hereditary form of the basal cell nevus syndrome (BCNS) or also known as Gorlin syndrome (9). Patients with Gorlin syndrome are subject to developing advanced basal cell carcinoma (BCC) of the skin and are prone to rare tumours, medulloblastoma and rhabdomyosarcoma. Inappropriate activation of HH signalling pathway has also been shown in hematopoietic malignancies.

MATERIALS AND METHODS

Patient samples

This study was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia and Hospital Kuala Lumpur Research Committee. A total of 107 formalin fixed paraffin-embedded (FFPE) tissue samples of DLBCL were collected between year 2008 and 2014 for immunohistochemical assay. All tumours were histologically and clinically diagnosed by Hospital Kuala Lumpur according to the World Health Organization classification.

Immunohistochemistry Analysis

Immunohistochemistry was performed to examine PTCH1 and SMO expression in 107 DLBCL cases. The procedures were performed with classical protocols as previously described (10). Briefly, the FFPE tissue blocks received were serially cut and underwent heat-induced epitope retrieval in pH6.0 citrate buffer (Dako, Carpinteria, CA). Endogenous peroxidase was blocked using 3% H₂O₂ solution for 30 minutes. The following primary antibodies were applied: rabbit polyclonal anti-Ptch1 (1:150, AB53715; Abcam) and rabbit polyclonal anti-SMO (1:200, AB134906; Abcam). Incubation of appropriately diluted primary antibody for PTCH1 and SMO were at room temperature for 60 minutes. Secondary antibodies with DAKO Envision Kit, K5007, were applied for 30 minutes at 24°C. Detection of protein was visualized using chromogen diaminobenzine (DAB) (Dako, USA) for 5 minutes to develop the brown colour. Slides were counterstained lightly with hematoxylin. Cases were considered as positive if 10% or more (by estimation) of the tumour cells were expressing the proteins (11). Protein expression was scored as negative, low or high depending on the staining intensity. Three tonsil tissues were used as positive controls for both PTCH1 and SMO proteins.

Statistical Analysis

Statistical calculations were performed using the standard statistical software package, IBM SPSS Statistics for Windows, Version 17.0. Chi square (χ^2) test was used to evaluate the association between categorical variables. *Univariate analysis*: The Kaplan-Meier and the log-rank test were used to estimate overall survival distributions. Overall survival (OS) was calculated as the time from diagnosis to the date of death or last contact. Patients who were alive at last contact were treated as censored for OS analysis. In all of the statistical analyses, a p-value of <0.05 (95% confidence interval) was considered to be statistically significant.

RESULTS

Expression of PTCH1 and SMO proteins in DLBCL tissue

A total of 107 cases with DLBCL comprising 65 males and 42 females were retrieved. The mean age of male patients was 52 years (median: 55; range 3-87 years) and 56 years for female patients (median: 55; range 17-90 years). Most of the cases were elderly of over 55 years old, which approximately contribute to 53.3% of the total sample. Tumours were mostly located in the lymph nodes (n=57, 53.3%). Extranodal involvement includes CNS/brain, n=23, skin/soft tissue, n=7, gastrointestinal, n=7, head and neck; bone involvement, n=6, and genital, n=1 (Table I). As shown in Table 2, PTCH1 and SMO proteins are highly expressed in DLBCL. PTCH1 protein is overexpressed in all of 107 DLBCL cases. 19 cases (17.8%) had low expression, and 88 cases (82.2%) had high expression. In positive cases, the expression of PTCH1 was observed outlining the cell membrane and in the nucleus of malignant cells. SMO was positively expressed in 105/107 (98.1%) cases of DLBCL and was localized within the cytoplasm of the malignant cells. 82 cases (76.6%) showed high expression, 23 cases (21.5%) showed low expression and only two cases (1.9%) showed negative expression. Figure 1 shows a H&E stained photomicrograph of normal tonsils and DLBCL. Figure 2 shows the photomicrographs of immunohistochemical staining in normal tonsils and DLBCL tissues.

Correlation of immunohistochemical expression between Hh pathway proteins

The findings in this study showed that there was a significant correlation between the expression of PTCH1 with SMO (P=0.03). Tumour cells that are PTCH1 positive are more likely to express SMO.

Table 1

Characteristic	No. of cases (%)	PTCH1			SMO			P
		Low expression (%)	High expression (%)	P	Negative (%)	Low expression (%)	High expression (%)	
Age								
< 55	50 (46.7)	10 (20)	40 (80)	0.570	2 (4)	9 (18)	39 (78)	0.283
≥ 55	57 (53.3)	9 (15.8)	48 (84.2)		0 (0)	14 (24.6)	43 (75.4)	
Gender								
Male	65 (60.7)	10 (15.4)	55 (84.6)	0.424	2 (3.1)	10 (15.4)	53 (81.5)	0.082
Female	42 (39.3)	9 (21.4)	33 (78.6)		0 (0)	13 (31)	29 (69)	
Race								
Malay	76 (71)	16 (20.8)	61 (79.2)	0.376	2 (2.6)	16 (20.8)	59 (76.6)	0.468
Chinese	20 (18.7)	1 (5.3)	18 (94.7)		0 (0)	5 (26.3)	14 (73.3)	
India	7 (6.5)	1 (14.3)	6 (85.7)		0 (0)	0 (0)	7 (100)	
Others	4 (3.7)	1 (25)	3 (75)		0 (0)	2 (50)	2 (50)	
Tumour Location								
Nodal	57 (53.3)	10 (17.5)	47 (82.5)	0.951	2 (3.5)	11 (19.3)	44 (77.2)	0.518
Extranodal	50 (46.7)	9 (18)	41 (82)		0 (0)	12 (24)	38 (76)	

Table 2

Markers	P value
PTCH1-SMO	0.030*

*Significant P value <0.05 by chi-square test.

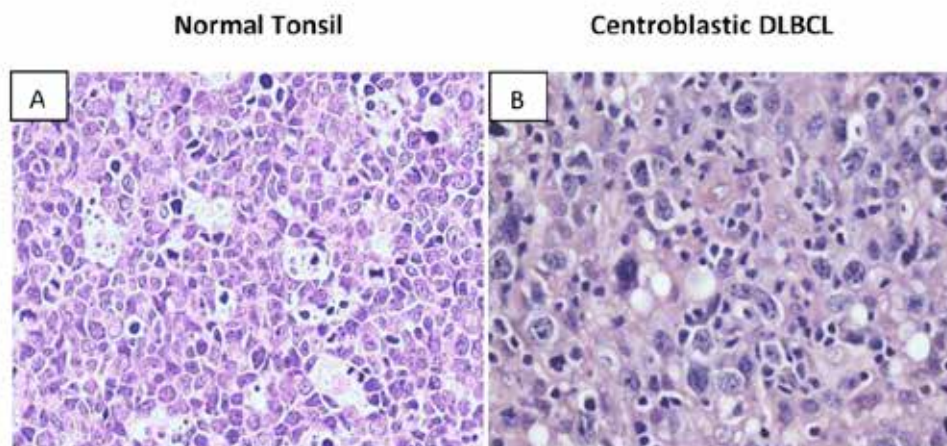


Fig. 1. H & E stained photomicrograph of normal tonsils (x200) and DLBCL (x200)

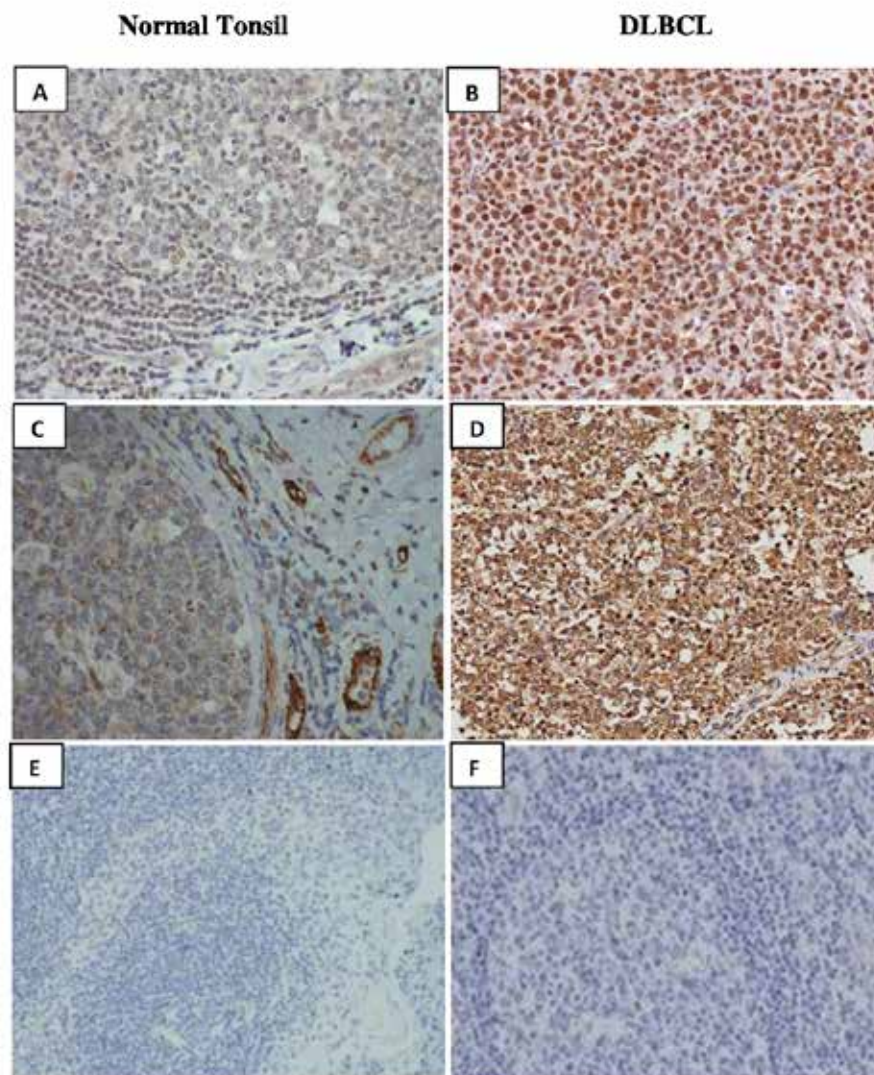


Fig. 2. Representative photomicrographs of immunohistochemical staining of (A) PTCH1 expressed in the germinal center of normal tonsil (x200) and (B) strong PTCH1 staining in the nucleus of DLBCL (x200). (C) SMO expressed in the germinal center of normal tonsil (x200) and (D) strong SMO staining in the cytoplasm of DLBCL (x200). (E) Negative PTCH1 staining in DLBCL (x200). (F) Negative SMO staining in DLBCL (x200)

Expression between HH proteins and their overall survival of DLBCL

The mean OS for DLBCL patients in this study was 65 months with 5-year overall survival of 74%. The overall survival curves were plotted against age, gender, race and tumour location, however no significant association with HH protein expression were shown.

DISCUSSION

Inappropriate activation of the HH signaling pathway has been shown in many cancers, including two well-recognized cancers; BCC and medulloblastoma (12–14). Modes of the HH signalling pathway in cancer development may vary according to tumour types. Aberrant HH pathway mechanism appears to be complex

and can either be in an autocrine and some may favour a paracrine model. Report has shown that haematological malignancies involved in a reverse paracrine mode and has only been observed in hematologic models of B-cell lymphoma, multiple myeloma and leukaemia (15). Our previous study showed that SHH and GLI1 were expressed at various levels in DLBCL and a large percentage of expression are at low levels (16). In this study, DLBCL commonly expressed PTCH1 and SMO at a very high levels. It has been shown that HH ligand, in a direct and/or indirect manner, upregulates the transcription of its own transcriptional effectors. Thus, suggesting the presence of positive autocrine loop with a positive feedback mechanism (17, 18). Because PTCH1 acts as HH receptor and SMO as the signal transducer that sends signals into the cytoplasm to activate transcription factor GLI, this might lead to cell proliferation and/or differentiation., thus, regulating the

transcription of target genes. In addition, the increased amount of PTCH1 may bind to SHH and alter ligand accessibility for different target cells by endocytosis.

Studies have found that genetically engineered mice with PTCH1 and SMO genes have generated more definite evidence for the critical role of HH signalling in cancer. Even in small cell lung cancer (SCLC) mouse model, the expression of oncogenic SmoM2 increases the tumour number, whereas SMO knockout reduces the tumour number. Therefore, high levels of SMO expression in this study might indicate the cause of an aberrant activation of HH signalling in DLBCL.

We reported that to the best of our knowledge, there is no study investigating the expression of SMO and PTCH1 and correlation with clinicopathological parameters and overall survival in DLBCL patients. However, Kim et al., 2009 showed significant correlation between expression levels of GLI1 with GLI2 and GLI3 in DLBCL (15). In summary, understanding the role of signalling pathways, particularly HH pathway in the development of cancer may provide new input and offer a possible target in therapeutic intervention. Among possible therapeutic interventions are inactivating mutations of *PTCH*, activating mutations of *SMO* and gene amplifications or gains involving *HH* ligands, GLI1 and GLI2.

CONCLUSIONS

In this study, PTCH1 and SMO were strongly expressed in DLBCL. These data provide a rationale for further investigation of the biological significance of PTCH1 and SMO in tumour biology particularly in the pathogenesis and progression of DLBCL

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