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## **Abstract**

SLIT2 has been classified as a major tumour suppressor gene due to its frequent inactivation in different cancer types. However, alterations of SLIT2 expression and relation to patient outcomes in diffuse large B-cell lymphoma (DLBCL) remain undefined. The aim of this study was to investigate the expression and the methylation status of SLIT2 gene as well as its relation to patient outcomes in DLBCL. Immunohistochemical (IHC) staining was carried out to detect the expression of SLIT2 in a series of 108 DLBCL cases. Re-analysis of previously published dataset (GSE10846) that measured gene expression in DLBCL patients who had received CHOP or R-CHOP therapy was performed to identify associations between SLIT2 and patients survival. Laser capture microdissection was performed to isolate GC B cells and DLBCL primary tumor cells. Bisulfite treatment and methylation-specific PCR (MSP) analysis were done to assess SLIT2 promotor methylation status. The expression of SLIT2 protein was reduced in a subset of DLBCL cases and this was significantly correlated with advanced clinical stage ( $p= 0.041$ ) and was an independent predictor of worse overall survival (OS) ( $p= 0.012$ ). Re-analysis of published gene expression data showed that reduced SLIT2 mRNA expression was significantly correlated with worse OS in R-CHOP-treated ABC DLBCL patients ( $p= <0.01$ ). Hypermethylation of the SLIT2 promotor was significantly correlated with low SLIT2 expression ( $p=0.009$ ). Our results provide a novel evidence of reduced expression of SLIT2 that is associated with promoter hypermethylation and adverse outcomes in patients with DLBCL.

**Key words:** SLIT2, DLBCL, hypermethylation, patient survival

## Introduction

Diffuse large B cell lymphoma (DLBCL) is a heterogeneous disease with distinct molecular features and clinical behavior [1]. It is the most common of the non-Hodgkin lymphomas [2]. About 30-40 percent of the non-Hodgkin lymphomas are DLBCL. Based on gene expression profiling, two main classes of DLBCL can be identified, these are the germinal center B-cell (GCB) and the activated B-cell like subtypes (ABC) with a small percentage (10-15%) being un-classifiable [3, 4].

DLBCL is usually an aggressive cancer, with a median survival of less than 12 months in untreated patients. Since the 1970s, the standard treatment has been the CHOP regimen consisting of adriamycin, vincristine, and cyclophosphamide [5]. Use of this regimen is associated with around 40% progression-free survival and 50% long-term survival [6]. There has been a considerable improvement in the prognosis of DLBCL within the last few years, mainly due to the inclusion of rituximab, an anti-CD20 monoclonal antibody [7]. Notwithstanding these notable achievements, a sizable percentage of patients have refractory disease or will relapse reducing their chances of survival [8].

The pathogenesis of DLBCL is complex involving interactions between genomic and epigenomic changes [9,10]. *SLIT2* has been classified as a major tumour suppressor gene due to its frequent inactivation in different cancer types, including deletion or hyper-methylation of its promoter [11,12]. *SLIT2* plays a role in the suppression of tumour growth [13,14], via the regulation of the  $\beta$ -catenin and PI3-K signaling pathways leading to enhancement of  $\beta$ -catenin and E-cadherin mediated cell-cell adhesion [15].

In hematolymphoid malignancies, only in leukemias and mantle cell lymphoma, few reports are available for the role of *SLIT2* and its main receptor *ROBO1* [16–18]. These reports showed frequent *SLIT2* methylation in leukemia cell lines, also in acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL) primary samples [17]. Moreover, the expression of *SLIT2* was restored after treating ALL lines with the demethylating agent (5-aza-2'-deoxycytidine) [17]. In Mantle cell lymphoma, *ROBO1* displayed a high level of methylation, which correlated well with its low mRNA expression [16, 18], also correlated with aggressive cliniopathological features and shorter survival [18]. Here, we have studied the expression and the methylation status of *SLIT2* gene as well as its relation to patient outcome in DLBCL.

## Materials and Methods

### Tissue specimens

This is a retrospective cohort study in which Formalin-fixed paraffin-embedded tissues were used from 108 DLBCL patients diagnosed between 1997 and 2012 at the University Hospital, Birmingham. Diagnosis was confirmed by histological review. Detailed clinical and follow-up data was available for all patients. Follow up ranged from 1- 180 months with a mean of 45 months. Based on the Hans algorithm, DLBCL were grouped by IHC into either GCB or ABC-DLBCL types [19]. The baseline pathologic and clinical characteristics of the patients are listed in Table 1. Different normal/reactive lymphoid tissues (tonsils and lymph nodes) obtained through

tonsillectomy or excision biopsy, fixed in formalin and embedded with paraffin, were used for both positive and negative control as well as for GC B cells microdissection for the methylation analysis (see below).

### **Immunohistochemistry**

Paraffin-embedded tissues from both DLBCL samples and normal lymphoid tissues were sectioned at a thickness of 4µm and placed on X-tra Adhesive micro slides. The expression of SLIT2 protein was detected by IHC using Leica BOND-MAX™ autostainer (Leica Biosystems) according to the protocol instruction. In brief, the tissue sections were deparaffinized and then rehydrated through graded alcohols. Sections were immersed into preheated EDTA (PH 8.0) for 20 minutes. Endogenous peroxidase activity inhibited in 0.3% hydrogen peroxide, and non-specific binding sites blocked with normal goat serum at room temperature. Primary mouse monoclonal anti-SLIT2 [Clone EPR2771; Abcam Ltd. 134166 USA; diluted at 1:800] was used for 1 hr. A cold PBS was used in place of the primary antibody for the negative control staining.

### **Microdissection of GC B cells and DLBCL cells**

DLBCL cases were selected with variable SLIT2 staining intensity as well as GC B-cells from normal/reactive lymphoid tissues expressing strong SLIT2 staining. A total of 6 unstained sections per each case were cut at 8µm thickness and stained with Haematoxylin with available matched SLIT2 immunostained sections for each case. Sections were placed onto membrane-covered slides for microdissection (PEN membrane slide, Carl Zeiss Ltd. Germany). Using a PALM Microbeam microdissection (Carl Zeiss Ltd, Germany), laser capture microdissection was performed to capture GC B cells and DLBCL tumor cells from areas of which matched to the SLIT2 stained sections. Microdissected tissues were put into PALM adhesive cap (Carl Zeiss Ltd.) tubes for further methylation analysis.

### **Analysis of SLIT2 expression**

The entire tumour section was scanned at low power to survey overall staining intensity in DLBCL tumour cells. SLIT2 protein was found to be expressed in both tumour cell cytoplasm and the membrane. The intensity was judged by the comparing the intensity between tumour and GCs of normal lymphoid tissue (tonsillar/lymph nodal tissue as an external positive control or residual GCs in the affected lymph node as an internal positive control).

The expression intensity was scored as follows: 0 =negative, 1 = weak, 2 = moderate, comparable to normal germinal centers and 3 = strong, presence of cells with staining obviously stronger than in normal GCs. Staining percentage was scored 4 (if greater than 75% of tumour cells were positive), 3 (50-75%), 2 (25-50%), or 1 (< 25%). The staining was graded as “low expression” if staining intensity multiplied by percentage was less than or equal to 4 [20, 21].

### **Re-analysis of gene expression data**

A previously published dataset (GSE10846) that had measured gene expression in DLBCL patients who received CHOP or R-CHOP therapy [22] was downloaded from GEO

(<https://www.ncbi.nlm.nih.gov/geo/>). The Bioconductor project's affy package was used to conduct a probe level quantile normalization [23] and robust multi-array analysis [24] on the raw CEL files. The R survival package was used to perform survival analysis (<http://www.r-project.org/>), including log-rank test with samples split by median SLIT2 expression.

### **Bisulfite treatment and methylation-specific PCR (MSP) analysis**

DNA was extracted from the microdissected samples using QIAamp DNA FFPE Tissue Kit (Catalogue no. 56404). Microdissected cells were placed in proteinase K digestion solution, then incubated at 90°C. Residual contaminants were washed away. DNA was then eluted in Buffer ATE. Purified DNA was checked on Nanodrop spectrophotometer. DNA was stored at -20°C.

Bisulfite modification of genomic DNA was carried out as described previously [25, 26]. 50 ng of bisulfited DNA for each sample was used for methylation-specific PCR (MSP) analysis. Multiple methylated primer sets targeting SLIT2 promoter were designed and tested. The best pair (m5/m7) with clear methylation band, inversely correlated with expression, and not amplifying any not-bisulfited DNA, was chosen. MSP primer set used in the study was: SLIT2m5: 5'-GATCGGTTTAGGTTGCGG C; SLIT2m7: 5'-AACAACTAAACATAACGCGCG. MSP was performed for 38 cycles using AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific) [25-27].

### **Statistical Methods**

Data were analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 23 (SPSS Inc., Chicago, IL). Numerical data was described as median and interquartile range or range or mean and standard deviation as appropriate, while qualitative data were described as number and percentage. Chi-square (Fisher's exact) test was used to examine the relation between qualitative variables as appropriate. Survival analysis was done using the Kaplan-Meier method. Comparison between two survival curves was done using the log rank test. Multivariate analysis was done by Cox regression model to test for independent prognostic effects of statistically significant variables on univariate level with calculating hazard ratio and its 95% confidence interval. A p-value less than 0.05 was considered statistically significant. All tests were two tailed. Overall survival (OS) was calculated from date of diagnosis till date of death or last follows up. Event free survival (EFS) was calculated from date of treatment till date of relapse, death or last known follow up.

### **Results**

#### **Pattern of SLIT2 protein expression in normal lymphoid tissue and DLBCL**

Immunohistochemical analysis of SLIT2 was performed for both normal lymphoid tissue (tonsil/lymph nodes) and primary DLBCL. In normal lymphoid tissue, high SLIT2 expression was restricted to the germinal centers with only few inter-follicular lymphocytes showing low SLIT2 expression [Figure 1a&b]. SLIT2 was

located in both the cytoplasm and membrane of DLBCL tumour cells. Different intensities of SLIT2 protein expression were observed among different cases; 41/108 cases (38%) showed high expression (SLIT2 intensity multiplied by the percentage  $>4$ ), 66/108 (61.1%) had low expression (SLIT2 intensity multiplied by the percentage  $\leq 4$ ). Only one case (1/108, 0.9%) was negative (Low expression  $\leq 4$ ). Figure 1c&d show representative DLBCL cases with different levels of SLIT2 protein expression. Of the 108 cases, 49 (45.4%) were of GCB type, while 58 (53.7%) cases were of ABC-DLBCL type. One case was unclassifiable due to inconclusive IHC results. 55.1% (27 cases) of GCB DLBCL and 65.5% (38 cases) of the ABC-DLBCL showed low SLIT2 expression. There was no significant correlation between subtype and SLIT2 protein expression ( $p= 0.27$ ).

### **Association between SLIT2 protein expression and clinicopathological features**

In our study, a significant correlation between low SLIT2 intensity and advanced clinical stage was found (stages 3&4 vs 1&2;  $p=0.041$ ). There was a tendency for cases with low SLIT2 expression to be associated with the poor prognostic group as defined by the Revised International Prognostic Index (R-IPI) ( $p=0.099$ ). No significant relationships between SLIT2 protein expression and other parameters were found [Table 2].

### **Impact of SLIT2 protein expression on the survival of DLBCL patients**

SLIT2 protein expression was then assessed for its impact on patient outcome. A univariate Kaplan-Meier (K-M) analysis revealed a significant reduction in overall survival (OS) for DLBCL patients with low SLIT2 protein, with a median of 28 months, while the median survival was not reached in patients with strong SLIT2 expression ( $p= 0.044$ ) [Figure 2A]. In a multivariate analysis, SLIT2 was an independent significant predictor of OS ( $p= 0.012$ ). Although the median EFS was only 12.6 months for patients with low tumour expression of SLIT2, compared with 88.9 months for those with strong SLIT2 expression, though this difference was not significant ( $p= 0.108$ ), [Figure 2B]. We also evaluated the impact of SLIT2 protein expression on outcome separately for patients with each of the DLBCL subtypes. We found no correlation between SLIT2 protein expression and OS or EFS in patients with either GC- ( $p= 0.26$  and  $p= 0.35$ , respectively) or ABC-DLBCL ( $p= 0.26$  and  $p= 0.31$ , respectively).

### **SLIT2 gene expression predicts survival in DLBCL (ABC subtype) patients treated with R-CHOP**

To further explore the potential effects of SLIT2 expression on outcome we took advantage of the dataset previously published by Lenz et al (2008) (GSE10846) that had measured gene expression in the tumour tissues of 181 DLBCL patients treated with either CHOP or R-CHOP and which had used microarray gene expression to define subtype (Lenz et al. 2008). We found that low SLIT2 mRNA expression was significantly correlated with worse OS in R-CHOP-treated ABC DLBCL patients ( $p= <0.01$ ) [Figure 3]. SLIT2 expression was not significantly correlated with OS or EFS in patients with the GC subtype.

## **Methylation of the SLIT2 promoter in DLBCL**

We measured SLIT2 promoter methylation in 26 DLBCL cases with different SLIT2 protein expression as well as microdissected GC B cells with high SLIT2 expression, 14/26 (54%) DLBCL had low SLIT2 protein expression and 12/26 (46%) strong expression.

Higher SLIT2 methylation (strong/moderate) was found in cases with reduced SLIT2 protein expression, and that was statistically significant (Fisher's exact test,  $p=0.009$ ). In samples with low/negative SLIT2 expression, 13/14 (92.9%) had a strong/moderate-methylation status. On the other hand, among samples with higher SLIT2 expression, 7/12 (58.3%) had weak/un-methylation status, while 5/12 (41.7%) had strong/moderate-methylation status [Figure 4A]. In contrast the microdissected GC B cells with strong SLIT2 protein expression, showed unmethylated SLIT2 gene [Figure 4B]. In this small series there was no statistically significant correlation between SLIT2 methylation and DLBCL subtypes (7/18 ABC-DLBCL and 4/8 GCB DLBCL had weak/un-methylation status;  $p=0.65$ ).

## **DISCUSSION**

The SLIT family of genes is large extracellular matrix-secreted and membrane-associated glycoproteins. SLIT2 is known to function through its two main receptors Robo1 and Robo4. Robo1 is mainly expressed in cancer cells [28].

The present study aimed to identify if SLIT2 might be a potential tumour suppressor also in DLBCL. After careful search of existing literatures, no such study has yet been reported in DLBCL. Our results show that a subset of DLBCL is characterized by reduced SLIT2 expression correlated significantly with promotor hypermethylation. Furthermore, reduced SLIT2 expression was also associated with poor prognosis, suggesting that SLIT2 might serve as a prognostic biomarker in DLBCL patients.

In our study, reduced expression of SLIT2 protein was correlated significantly with advanced clinical stage ( $p=0.041$ ). Moreover, a significant reduction in OS of DLBCL patients was correlated with low expression of SLIT2 protein ( $P=0.044$ ). This has been supported by multivariate analysis where poor survival was associated with lower SLIT2 expression ( $p=0.012$ ).

Consistent with our data, several studies indicate that inactivation of the SLIT/ROBO1 pathway is important for cancer development and that its low expression is associated with a poor prognosis [13, 20]. Thus, Tseng et al. (2015), found that low SLIT2 expression is correlated with late stage and with poor OS and DFS in oesophageal cancer patients [21], effects which might be explained by their observation that cancer cell motility is reduced when SLIT2 is stably overexpressed. These observations were confirmed by Kim et al. (2008), who found that SLIT2 expression was decreased or abolished in human oesophageal squamous cell carcinomas (SCCs) compared to normal tissues as detected by in situ hybridization [29]. Low expression of SLIT2 also increased cell migration in lung cancer and invasive cervical cancer [30,31]. The underlying mechanism is that inactivation of the SLIT2-ROBO1 pathway enhances cell migration through accumulation of activated Cdc42 (a member of the Rho-

family of GTPases ), resulting in loss of the interaction between E-cadherin and B-catenin and the inactivation of EGFR degradation [15, 21, 32, 33, 34].

SLIT2 protein can also inhibit the migration of endothelial cells which might explain the promotion of angiogenesis following SLIT2 loss [35]. Apoptosis was also shown to be increased and proliferation decreased in SLIT2-transfected tumours, suggesting another mechanism for SLIT2-mediated tumour suppression [29]. Interestingly, Chang et al reported a correlation between low SLIT2 expression in the stromal fibroblasts with lymph node metastasis in breast cancer, indicating an important role for the expression of SLIT2 in the tumour microenvironment on the progression of tumours [36].

In our study, re-analysis of the published dataset that had measured mRNA expression among DLBCL patients treated with either CHOP or R-CHOP [22], revealed that downregulation of SLIT2 mRNA was significantly correlated with reduced overall survival of R-CHOP-treated ABC DLBCL patients. We did not find such an association in our IHC analysis possibly because of the smaller numbers of FFPE samples studied or because of the reported deficiencies in the correct delineation of gene expression sub-entities by IHC.

It was evident that SLIT2 gene methylation was significantly correlated with its low protein expression in DLBCL, whereas normal GC B cells strongly expressed SLIT2 and lacked methylation of the promoter. Our results suggest that promoter methylation could explain the reduced expression of SLIT2 in DLBCL. In keeping with our findings, previous studies have reported that promoter hypermethylation is responsible for the down-regulation of SLIT2/ROBO1 signaling in various solid tumours, including breast, head and neck squamous cell carcinoma (HNSCC), cervical, oesophageal, hepatocellular carcinomas and gliomas, where it is also associated with poor prognosis [20,21,37,38,39]. In hematolymphoid malignancies, Dunwell et al (2009) found that SLIT2 was methylated in all ten leukemia cell lines analyzed, whilst DNA from peripheral blood and bone marrow from healthy control samples showed no SLIT2 methylation [17]. The expression of SLIT2 was restored after treating ALL lines with 5-aza-2'-deoxycytidine [17]. These findings suggest that reactivating the SLIT2 function by reversing epigenetic inactivation could represent a novel therapeutic opportunity to attenuate human cancer.

In conclusion, the results of the present study indicate for the first time that reduced expression of SLIT2 gene is associated with poorer patient outcomes in DLBCL. Furthermore, we have shown that SLIT2 protein down-regulation correlates with promoter hypermethylation. The functional impact of reduced SLIT2 inhibition in DLBCL remains to be established.

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## **Compliance with Ethical Standards**

### **Conflict of interest**

The authors declare no conflict of interest

### **Ethical approval**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the UK Health Research Ethics Committee (North West-Haydock; reference 15/NW/0079).

### **Author contributions**

Ghada Mohamed undertook pathologic review of the cases, interpreted and analyzed the IHC results, performed laboratory work and co-wrote the manuscript; Soha Talima undertook clinical data analysis and co-wrote the manuscript; Lili Li performed laboratory work and co-wrote the manuscript; Wenbin Wei undertook bioinformatic and statistical analyses, interpreted the data and co-wrote the manuscript; Zbigniew Rudzki Undertook pathologic review of the cases ; Rasha Allam undertook statistical analysis and interpreted the data; William Simmons undertook cases' data collection; Qian Tao supervised laboratory work and co-wrote the manuscript; Paul G Murray designed and oversaw the research and co-wrote the manuscript.

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## Figure legends

**Fig. 1: SLIT2 protein expression in normal lymphoid tissue and DLBCL primary tumours** (a) High SLIT2 protein expression in the reactive GCs, also in normal squamous epithelium of the tonsil, with low expression in few scattered interfollicular cells (Mag. 10x). (b) Higher magnification of one of the GCs expressing high SLIT2 protein. (c) Example of high SLIT2 expression (score 3) in DLBCL, sparing the residual normal/reactive lymphocytes. (d) A representative case of DLBCL with low SLIT2 expression (score 1).

**Fig. 2: Kaplan-Meier survival analysis for DLBCL cases expressing low/high SLIT2 protein.** (A) Low SLIT2 protein expression correlates with poor OS in DLBCL patients ( $p=0.044$ ). (B) Patients with low SLIT2 protein or negative expression show difference in Event-free survival with a median of only 12.6 months as compared to 88.9 median survival of patients experienced high SLIT2 protein, however that was not statistically significant ( $p=0.108$ ).

**Fig. 3: Re-analysis of a previously published gene expression dataset GSE10846.** High SLIT2 gene expression correlates with better overall survival in R-CHOP treated ABC DLBCL patients ( $p < 0.01$ ).

**Fig. 4: Methylation-specific PCR (MSP) analysis of the SLIT2 promotor in microdissected GC B cells and DLBCL samples.** (A) DLBCL cases with variable SLIT2 protein intensities as detected by IHC. Samples

highlighted in red express a low/negative SLIT2 protein. Reduced SLIT2 protein expression correlates with promoter methylation status (strong/moderate) ( $p=0.009$ ). **(B)** GC B cells expressing high SLIT2 protein show unmethylated status Methylated (OCI-LY3) and unmethylated (HEK293) cell lines were used as positive controls. M: methylated; U: unmethylated.