Expression and Alteration of *p16* in Diffuse Large B Cell Lymphoma

Ee-Soo Lee^a Lian-Hua Kim^c Wan Ariffin Abdullah^b Suat-Cheng Peh^a

Departments of ^aPathology and ^bPaediatrics, Faculty of Medicine, University of Malaya, and ^cDepartment of Mechatronic and BioMedical Engineering, Faculty of Engineering and Science, University of Tunku Abdul Rahman, Kuala Lumpur, Malaysia

Key Words

p16 • Diffuse large B cell lymphoma • Immunohistochemistry • Fluorescence in situ hybridization

Abstract

Objectives: This study aimed to examine (1) the expression of P16 protein relative to sites of presentation, immunophenotypic subgroups and proliferative indices of tumour cells, and (2) the relationship between *p16* gene alterations and P16 protein overexpression in 70 cases of diffuse large B cell lymphoma (DLBCL). **Methods:** Expression of P16, CD10, BCL-6, MUM-1 and proliferation marker (Ki-67) was demonstrated by immunohistochemistry. Fluorescence in situ hybridization (FISH) was employed to detect *p16* alterations. **Results:** P16 overexpression was shown in 45.7% (32/70) of the DLBCL cases, and was significantly correlated with CD10 ($p = 0.022$) and germinal centre B-cell-like (GCB) phenotype ($p = 0.022$). High expression of P16 was inversely associated with high proliferative activity (Ki-67 index greater than 75%) ($p = 0.020$). Of the 47 cases that yielded interpretable FISH results, 57.4% (27/47) showed deletions of *p16* and 27.7% (13/47) showed gains of *p16*. P16 overexpression and *p16* deletions were mutually exclusive ($p = 0.019$). There was no correlation between P16 overexpression and *p16* gains ($p = 0.621$). **Conclusions:** The GCB and non-GCB subgroups of

DLBCLs show different patterns of P16 expression. High levels of P16 may mitigate tumour cell proliferation. Gains of *p16* do not necessarily increase P16 protein expression.

Copyright © 2010 S. Karger AG, Basel

Introduction

Progression of cell cycle is tightly controlled at multiple checkpoints to ensure normal cell proliferation. Passage through each checkpoint is regulated by a series of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors [1]. *p16* (also known as *CDKN2A*, *MTS1* or *INK4A*) is a tumour suppressor gene that resides in the 9p21 chromosomal region [1, 2]. Expression of *p16* has first been discovered as a 16-kDa protein that binds exclusively to CDK4 and CDK6 [3]. In the proliferative state, CDK4 and CDK6 are activated by cyclin D to phosphorylate the PRb. Phosphorylated PRb releases E2F, a transcription factor responsible for cell cycle transition from G₁ to S phase. Binding of P16 to CDK4 and CDK6 may inhibit phosphorylation of PRb and induce cell growth arrest at the G₁ checkpoint [1, 2]. There is controversy concerning the expression of P16 in carcinogenesis. Loss of P16 as a result of genetic inactivation has been a common event in tumour cell lines and primary tumours [2, 4]. However, varying results have

Fig. 1. Stratification of DLBCL into GCB and non-GCB phenotypic subgroups based on the expression of three immunomarkers.

been reported in the past decade, suggesting a prevalence of P16 overexpression in human neoplasms [5–8].

Diffuse large B cell lymphoma (DLBCL) is a highly heterogeneous lymphoid malignancy comprising several disease subentities [9]. Based on cell origin, gene expression profiling using cDNA microarrays has identified three distinct subgroups of DLBCL: (1) the germinal centre B-cell-like (GCB) subgroup, with features of GCB cells, (2) the activated B-cell-like and type 3 subgroups, with features of post-GCB cells [10, 11], and (3) primary mediastinal B cell lymphoma (PMBL), with features of thymic B cells [12]. It was claimed that it is possible to identify DLBCL subgroups similar to those of cDNA microarrays by immunohistochemical analysis using a panel of antibodies [13]. In immunohistochemical analysis, expression of CD10 and BCL-6 is used to define the GCB phenotype whereas expression of MUM-1 is related to the non-GCB phenotype [13]. The GCB phenotypic subgroup is known as a predictor of a favourable outcome, while the non-GCB phenotypic subgroup appears to predict an unfavourable outcome [10, 13].

GCB and non-GCB DLBCLs may develop through different oncogenic pathways [14] and express differential levels of cell cycle regulators [15]. Loss of P16 in DLBCL has been studied by different research groups [15, 16] and has been shown to associate with the non-GCB phenotype [15]. In order to further elucidate the role of *p16* in lymphomagenesis, we investigated P16 overexpression in de novo DLBCL by immunohistochemistry in order to clarify the sites of presentation, immunophenotypic subgroups and proliferation profiles as denoted by the expression of Ki-67. FISH was employed to analyse genetic alterations of *p16* and their associations with high levels of P16 protein in DLBCL.

Materials and Methods

Tissue Samples

A total of 70 formalin-fixed, paraffin-embedded specimens from DLBCL cases (34 nodal and 36 extranodal) obtained between the years 1996 and 2006 were retrieved from the archives of a single institution. All cases were reconfirmed as DLBCL based on the criteria of the WHO classification of haematopoietic and lymphoid tissue tumours [9]. One patient had DLBCL with features of classical Hodgkin lymphoma. Colon carcinoma and reactive hyperplastic lymphoid tissues, including lymph nodes and tonsils, were used as controls.

Immunohistochemistry

The Envision system was employed for the staining of CD10, BCL-6 and MUM-1. The avidin-biotin complex system was employed for the staining of P16 and Ki-67. Three-micrometer-thick sections were cut from paraffin-embedded tissue blocks, deparaffinised in xylene and dehydrated through a graded ethanol series. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Heat-induced antigen retrieval was achieved by pressure cooking. After cooling to room temperature, antigen-retrieved sections were incubated with primary antibodies. Reactions against CD10 (1:50; clone 56C6, Novocastra Laboratories, UK), BCL-6 (1:10; clone PG-B6p, DakoCytomation, Denmark), MUM-1 (1:25; clone MUM1p, DakoCytomation) and Ki-67 (1:50; clone MIB-1, DakoCytomation) were run for 2 h at room temperature; overnight incubation at 4°C was performed for P16 (1:200; clone 16P07, Neomarkers, USA). Chromogenic development of all staining was achieved using diaminobenzidine (DakoCytomation). Haematoxylin was used as nuclear counterstain. External positive controls, i.e. reactive lymphoid tissues for staining of CD10, BCL-6, MUM-1 and Ki-67, and colon carcinoma for staining of P16 were included in every run of the assay.

All stained slides were evaluated by a pathologist (S.C.P.) blinded to the clinicopathological data and FISH results. The percentages of tumour cells showing protein immunoreactivity were determined visually. CD10 was considered to be expressed when $\geq 30\%$ of tumour cells showed membrane staining. BCL-6 and MUM-1 were considered to be expressed when $\geq 30\%$ of tumour cells showed nuclear staining. The Ki-67 indices (percentages of cells expressing Ki-67) were stratified into five groups based on the extent of protein immunoreactivity: (1) $<10\%$ of positive tumour cells, (2) 10–25% of positive tumour cells, (3) 26–50% of positive tumour cells, (4) 51–75% of positive tumour cells, and (5) $>75\%$ of positive tumour cells. Staining was regarded as inconclusive if immunoreactivity was not observed in the internal positive controls, i.e. fibroblasts and neutrophils for CD10, and reactive lymphocytes for BCL-6, MUM-1 and Ki-67. The algorithm proposed by Hans et al. [13], based on CD10, BCL-6 and MUM-1 expression, was employed to stratify all the DLBCL cases into GCB and non-GCB phenotypic subgroups (fig. 1).

P16 expression was separately classified into three categories: (1) simultaneous nuclear-cytoplasmic staining, (2) purely cytoplasmic staining, and (3) purely nuclear staining. Both the intensity and extent of staining were considered during the evaluation. Staining intensity was scored as follows: 1+ as weak, 2+ as moderate, and 3+ as strong. The extent of staining was graded as the percentage of positively stained tumour cells. Cut-off points for increased P16 expression were derived from seven reactive hyper-

Table 1. Cut-off values for *p16* alterations derived from means and standard deviations of normal cells with altered signals

	Losses of <i>p16</i>			Gains of <i>p16</i>
	monosomy 9	hemizygous deletion	homozygous deletion	polysomies 9
Mean $\pm$ SD	38.84 $\pm$ 8.67	8.85 $\pm$ 2.74	0.86 $\pm$ 1.02	0.07 $\pm$ 0.18
Cut-off values	56.19 [mean + 2 SD]	17.07 [mean + 3 SD]	4.96 [mean + 4 SD]	0.61 [mean + 3 SD]
Specificity, %	100	100	100	100
Sensitivity, %	2	42	38	30

plastic lymphoid tissues. P16 overexpression was defined as $\geq 5\%$ of tumour cells showing simultaneous nuclear-cytoplasmic staining, and/or $>75\%$ of tumour cells showing purely cytoplasmic staining, and/or $\geq 25\%$ of tumour cells showing P16 staining with 3+ intensity score. Staining of normal lymphocytes and endothelial cells was used as internal positive controls.

Fluorescence in situ Hybridization

Interphase FISH analysis was performed using a commercially available dual-colour probe consisting of locus-specific identifier (SpectrumOrange) and centromere enumeration probe 9 (SpectrumGreen) (Abbott Molecular Inc., USA). Locus-specific identifier *p16* spans approximately 190 kb and hybridizes to the 9p21 chromosomal region, containing the following genetic loci D9S1749, D9S1747, *p16*, *p14*, D9S1748, *p15* and D9S1752. Centromere enumeration probe 9 is a chromosome 9 control probe designed to detect the α -satellite sequences 9p11–q11. The FISH assay was performed as previously described [17]. A reactive lymphoid tissue section was incorporated in every run of the assay as experimental control.

All FISH specimens were evaluated using a fluorescence microscope equipped with appropriate filter cubes. Specimens were scanned using a 40 $\times$ objective to identify the tumour areas. Digital images of the tumour areas were acquired using an integrating monochrome CCD camera and a 100 $\times$ objective. At least 190–210 intact and non-overlapping nuclei were scored for the copy numbers of *p16* (visualized as red signal) and chromosome 9 (visualized as green signal). Only nuclei with at least 1 green signal were enumerated. The scoring system was based on the percentage of nuclei showing different copy numbers of *p16* and chromosome 9. An additional slide was prepared for specimens with less than 190 evaluated nuclei. Cases were considered non-informative if the supplemental slide failed to yield sufficient nuclei for analysis. Cases with poor signal intensity and/or excessive background autofluorescence were also regarded as non-informative.

Cut-off values for *p16* alterations were determined from 15 normal controls (reactive hyperplastic lymphoid tissues) based on the mean percentages of normal cells exhibiting altered signals and dispersions (as defined by standard deviations) of the incidence values (table 1). All established cut-off values were verified in terms of specificity and sensitivity using receiver operating characteristic curve analysis. Thresholds with 100% specificity were considered. Cases with altered signals exceeding the corresponding cut-off values were interpreted as alterations of *p16*.

Statistical Analysis

Pearson's χ^2 and Fisher's exact probability tests were used to examine the relationships between the different investigated parameters. The Mann-Whitney U test was employed to determine the differences in the frequencies of *p16* alterations between normal controls and DLBCL. The results were considered statistically significant when $p < 0.05$. SPSS for Windows, version 13 (SPSS Inc., USA) was used to perform all statistical analysis.

Results

Patient Data

The DLBCL series included 38 (54.3%) male and 32 (45.7%) female patients. The mean age of these patients at the time of diagnosis was 52.4 ± 16.5 years (median, 53.5 years; range, 3–86 years). 40.0% (28/70) of patients were aged 50 years or less. The studied subjects were comprised of three ethnic groups: Chinese (33/70, 47.1%), Malay (28/70, 40.0%) and Indian (9/70, 12.9%). The clinico-pathological features of each patient are presented in table 2.

Immunophenotypic Profiles

Of all the DLBCL cases, 32.9% (23/70) exhibited positive CD10 expression, 62.9% (44/70) expressed BCL-6 and 71.4% (50/70) demonstrated positive MUM-1 expression. 38.6% (27/70) of the cases were assigned to the GCB subgroup, and 61.4% (43/70) to the non-GCB subgroup (fig. 1).

Proliferation Profiles

The cell proliferation marker Ki-67 was expressed in all (100%) DLBCL cases; positivity ranged from 10 to $>75\%$. No cases had Ki-67 indices $<10\%$. Ki-67 indices of 10–25, 26–50 and 51–75% were noted in 11.4% (8/70), 21.4% (15/70) and 22.9% (16/70) of the cases, respectively.

Fig. 2. Overexpression and alterations of *p16* gene in DLBCL. **a** Overexpression of P16 protein is shown in both nucleus and cytoplasm of the tumour cells. $\times 25$. **b** Hemizygous deletion of *p16* gene is in the tumour cells (arrowheads). $\times 100$. **c** Homozygous deletion of *p16* gene shown in the tumour cells. $\times 100$. **d** Tumour cells revealed gains of *p16* gene with trisomy and tetrasomy of chromosome 9 (arrows). $\times 100$.

The majority (31/70; 44.3%) of the cases showed strong Ki-67 expression with indices higher than 75%.

Expression of P16

P16 expression was interpretable in all (100%) DLBCL cases. Overall, 45.7% (32/70) of the cases showed P16 overexpression. High levels of P16 were observed predominantly as simultaneous nuclear-cytoplasmic staining (29/32; 90.6%) as shown in figure 2a or purely cytoplasmic staining (3/32; 9.4%). P16 overexpression and CD10 positivity ($p = 0.022$) were significantly associated. Increased P16 expression was significantly associated with the GCB subgroup ($p = 0.022$). An inverse correlation was found between P16 overexpression and a high proliferative index (Ki-67 index $>75\%$) of tumour cells ($p = 0.020$). No statistically significant correlations were found between P16 overexpression and sites of presentation ($p = 0.826$) and expression of BCL-6 ($p = 0.152$) and MUM-1 ($p = 0.129$) (table 3).

Alterations of *p16*

FISH signals were interpretable in 67.1% (47/70) of the DLBCL cases. The Mann-Whitney U test demonstrated that the frequencies of monosomy 9 ($p = 0.019$), hemizygous *p16* deletion ($p = 0.002$), homozygous *p16* deletion ($p < 0.001$) and polysomies 9 ($p = 0.032$) were significantly different between DLBCL cases and normal controls.

There was no significant difference between tumour cases and normal controls in the frequencies of *p16* gene amplification ($p = 0.771$). Monosomy 9 and hemizygous and homozygous deletions were referred to as losses of *p16*. Gains of *p16* in the present DLBCL series were observed as polysomies 9.

Of the 47 interpretable hybridizations, hemizygous and/or homozygous deletions were evident in 27 (57.4%) cases. The concurrent occurrence of hemizygous and homozygous deletions in individual cases was statistically significant ($p = 0.043$). Simultaneous hemizygous and homozygous deletions were present in 11 of 27 (40.7%) cases, purely hemizygous *p16* deletion was shown in 9 of 27 (33.3%) cases, and purely homozygous deletion was shown in 7 of 27 (25.9%) cases. None of the examined specimens showed monosomy 9. Gains of *p16* due to polysomies 9 were noted in 27.7% (13/47) of the cases. Representative examples of DLBCL harbouring cytogenetic alterations are illustrated in figure 2b–d.

p16 deletions were significantly associated with extranodal presentation ($p = 0.047$). No statistically significant associations or differences were observed in different subgroups of DLBCL relative to *p16* deletions ($p = 0.137$) and *p16* gains ($p = 0.726$). P16 protein overexpression was not significantly associated with gains of *p16* gene ($p = 0.621$), but was inversely related to *p16* gene deletions ($p = 0.019$) (table 4).

Table 2. Patient data, sites of presentation, phenotypic subgroups, P16 overexpression and genetic alterations in DLBCL

Case No.	Age years	Gender	Ethnic group	Site of presentation	Phenotypic subgroup	P16 over-expression	<i>p16</i> alteration
1	54	M	C	LN	GCB	+	NI
3	38	M	C	LN	non-GCB	+	G
4	61	F	M	LN	non-GCB	–	NI
7	62	M	M	LN	non-GCB	+	NI
8	32	F	M	LN	non-GCB	–	NA
10	32	M	C	LN	GCB	–	D
11	12	M	C	LN	GCB	+	NI
12	25	M	C	LN	GCB	–	G
14	53	F	M	LN	non-GCB	–	G
15	52	M	C	LN	GCB	+	NA
18	65	M	I	LN	GCB	+	NA
19	58	F	C	LN	non-GCB	–	G
23	61	F	C	LN	non-GCB	+	NA
26	38	F	C	LN	non-GCB	–	D
27	60	F	I	LN	GCB	–	D
29	64	F	C	LN	GCB	+	D
31	49	M	M	LN	non-GCB	–	NA
32	34	M	C	LN	GCB	+	D
34	69	M	C	LN	non-GCB	–	G
36	74	M	C	LN	non-GCB	+	D
37	54	M	C	LN	non-GCB	–	D
38	43	F	M	LN	non-GCB	–	D
40	63	M	C	LN	GCB	–	D/G
41	46	F	M	LN	GCB	+	G
43	43	F	M	LN	non-GCB	–	D
46	29	M	M	LN	non-GCB	+	NI
47	63	F	M	LN	non-GCB	–	NI
51	84	F	I	LN	GCB	+	NI
53	49	M	C	LN	GCB	+	G
54	3	F	C	LN	GCB	+	NA
56	39	M	C	LN	GCB	–	NA
57	74	M	I	LN	non-GCB	–	D
58	51	M	C	LN	GCB	+	NI
60	42	M	M	LN	non-GCB	–	NI
2	63	F	C	tonsil	non-GCB	+	NI
6	58	F	C	tonsil	GCB	+	NA
9	31	M	M	tonsil	non-GCB	+	NI
28	57	F	C	tonsil	non-GCB	–	D
33	63	F	M	tonsil	non-GCB	+	NA
52	52	M	C	tonsil	GCB	–	NI
65	60	M	M	tonsil	non-GCB	–	D
68	56	F	M	tonsil	non-GCB	–	D/G
70	70	F	M	tonsil	non-GCB	+	D
5	53	F	I	GI tract	GCB	+	NA
13	51	F	C	GI tract	non-GCB	+	NI
25	81	M	C	GI tract	non-GCB	–	D
44	40	F	C	GI tract	non-GCB	+	G
48	59	M	M	GI tract	non-GCB	–	NI
50	64	M	I	GI tract	non-GCB	–	NI
55	26	M	C	GI tract	GCB	+	D
17	86	F	M	nasal region	non-GCB	–	D
64	65	F	M	nasal region	GCB	–	NI
66	45	F	C	nasal region	non-GCB	–	NI

Table 2 (continued)

Case No.	Age years	Gender	Ethnic group	Site of presentation	Phenotypic subgroup	P16 over-expression	<i>p16</i> alteration
67	58	F	I	nasal region	GCB	–	D
69	48	M	M	nasal region	GCB	+	D
24	40	F	M	bone	non-GCB	+	D/G
30	77	M	M	bone	non-GCB	–	D
20	56	M	C	skin	non-GCB	+	NI
42	83	M	C	skin	non-GCB	–	NI
16	61	M	M	testis	non-GCB	–	D
21	74	M	M	thyroid	GCB	–	D
22	29	M	I	lung	non-GCB	+	NI
35	36	M	I	mediastinum	non-GCB	+	NI
39	54	M	C	brain	GCB	–	NA
45	36	F	M	oropharynx	GCB	+	NI
49	71	M	M	spinal-cauda equina	non-GCB	–	D
59	37	F	C	submandibular gland	non-GCB	–	D
61	51	F	M	neck region	non-GCB	–	D/G
62	49	M	M	soft tissue	GCB	+	G
63	49	F	C	breast	non-GCB	–	NI

M = Male; F = female; C = Chinese; M = Malay; I = Indian; LN = lymph node; GI = gastrointestinal; GCB = germinal centre B-cell-like; G = gain; D = deletion; NA = no alteration; NI = non-informative.

Table 3. P16 overexpression in DLBCL in relation to sites of presentation, expression of CD10, BCL-6 and MUM-1, phenotypic subgroups and proliferation profiles (Ki-67 indices)

	Overexpression of P16		P
	positive (n = 32)	negative (n = 38)	
Site of presentation, n			
Nodal	16 (47.0)	18 (52.9)	0.826
Extranodal	16 (44.4)	20 (55.6)	
CD10, n			
Positive (≥30%)	15 (65.2)	8 (34.8)	0.022
Negative (<30%)	17 (36.2)	30 (63.8)	
BCL-6, n			
Positive (≥30%)	23 (52.3)	21 (47.7)	0.152
Negative (<30%)	9 (34.6)	17 (65.4)	
MUM-1, n			
Positive (≥30%)	20 (40.0)	30 (60.0)	0.129
Negative (<30%)	12 (60.0)	8 (40.0)	
Phenotypic subgroup, n			
GCB	17 (63.0)	10 (37.0)	0.022
Non-GCB	15 (34.9)	28 (65.1)	
Ki-67 index, n			
10–25%	6 (75.0)	2 (25.0)	0.020
26–50%	9 (60.0)	6 (40.0)	
51–75%	9 (56.3)	7 (43.8)	
>75%	8 (25.8)	23 (74.2)	

Figures in parentheses are percentages.

Discussion

There are but few studies on the expression profile and alterations of *p16* in DLBCL. In the present study, we found that a large proportion of the DLBCL cases (45.7%) showed overexpression of P16. The frequency is higher than that reported by Møller et al. [18] (17.1%), but is lower than that reported by Jehan et al. [19], i.e. 72.0%. This discrepancy is most likely due to the differences in sample size and/or variations in the scoring system. In agreement with the findings of Finegan et al. [6] and Melck et al. [7], overexpression of P16 in the present series was predominantly the result of increased simultaneous nuclear-cytoplasmic expression (90.6%). Prior surveys indicated that P16 expression is low or undetectable in the normal physiological state, but may be markedly increased during tumourigenesis [5, 20]. Hence, it is conceivable that DLBCLs without P16 overexpression may be found among those having basal physiological levels of P16. Alternatively, it could be attributed to inactivation of *p16* by gene deletion or methylation of the gene promoter region [15, 16, 18].

We noted in both reactive lymphoid tissues and DLBCL that highly proliferative regions, particularly germinal centres, displayed relatively higher expression of P16 than others. Similar observations were also report-

Table 4. *p16* alterations in DLBCL in relation to sites of presentation, phenotypic subgroups and P16 overexpression

	Deletions of <i>p16</i>			Gains of <i>p16</i>		
	positive (n = 27)	negative (n = 20)	p	positive (n = 13)	negative (n = 34)	p
Site of presentation, n						
Nodal	11 (44.0)	14 (56.0)	0.047	8 (32.0)	17 (68.0)	0.478
Extranodal	16 (72.7)	6 (27.3)		5 (22.7)	17 (77.3)	
Phenotypic subgroup, n						
GCB	9 (45.0)	11 (55.0)	0.137	5 (25.0)	15 (75.0)	0.726
Non-GCB	18 (66.7)	9 (33.3)		8 (29.6)	19 (70.4)	
Overexpression of P16, n						
Positive	7 (36.8)	12 (63.2)	0.019	6 (31.6)	13 (68.4)	0.621
Negative	20 (71.4)	8 (28.6)		7 (25.0)	21 (75.0)	

Figures in parentheses are percentages.

ed by Villuendas et al. [16]. Our findings revealed P16 overexpression and CD10 expression were correlated ($p = 0.022$). Given that CD10 is a marker of GCB cells [21], we speculate that increased expression of P16 is related to antigen-dependent germinal centre development, where follicular centre cells are actively proliferating [21]. Overexpression of P16 could be one of the regulatory mechanisms controlling B cell generation and proliferation.

In this study, overexpression of P16 was significantly more common in GCB (63.0%) than in non-GCB DLBCLs (34.9%) ($p = 0.022$). These results further strengthen the notion that GCB and non-GCB subgroups exhibit distinct molecular features and oncogenic events [9, 14]. The prognostic significance of P16 overexpression in non-Hodgkin lymphoma is not apparent, but entities with loss of P16 expression have always been related to an aggressive clinical course [15, 16, 22]. By extrapolation, it is conceivable that high-level P16 may lead to a favourable outcome in DLBCL. Current findings indicating an association of P16 overexpression with GCB DLBCL may support this hypothesis. It was reported that DNA replication stress and oncogene-induced senescence could potentially upregulate P16 expression, leading to a halt in lymphomagenesis [23]. Sandig et al. [24] also showed that ectopic overexpression of P16 may lead to apoptosis as well as cell growth arrest both in vivo and in vitro. On this basis, we suggest the favourable prognosis in the GCB subgroup as reported previously [10, 13] may be related to the role of P16 in inhibiting cell proliferation and inducing apoptosis.

The Ki-67 index has always been considered to reflect the rate of cell proliferation [21]. It was observed in the present study that the majority (75.0%) of the DLBCL cases with high levels of P16 showed Ki-67 indices $\leq 75\%$. The inverse relationship between P16 overexpression and high Ki-67 index ($>75\%$) ($p = 0.020$) is consistent with the role of P16 as a cell cycle inhibitor [1, 2]. In contrast to our results, an investigation in breast cancer showed a parallel relationship ($p = 0.005$) between strong expression of P16 and a high Ki-67 index ($>50\%$) [8]. The reasons for these discordant results await elucidation. Expression of P16 [reviewed in 1] and Ki-67 [25] may fluctuate at different levels along the cell cycle. P16 appears to be more accentuated at the G₁/S transition [reviewed in 1], whereas Ki-67 expression seems to peak at the G₂/M transition [25]. In support of these findings, it is speculated that increased expression of P16 may be an early event in the cell cycle and may mitigate proliferation of tumour cells.

There was no significant association between overexpression of P16 and site of presentation, although it was previously shown that P16 upregulation was more prevalent in extranodal DLBCLs [19]. In agreement with our data, Paik et al. [15] showed no significant difference in the expression of P16 between nodal and extranodal DLBCLs. The reasons for these discordant results are not clear.

In our DLBCL series, losses of *p16* were typically detected as deletions of one allele (hemizygous deletion) and/or deletion of both alleles (homozygous deletion). Chromosomal loss (monosomy 9) was not observed in any cases even though it has been reported as an underly-

ing mechanism of P16 loss in DLBCLs [26]. Overall, deletions of *p16* were noted in 57.4% (27/47) of the DLBCL cases. Variable *p16* deletion rates have been reported in de novo DLBCL (0–52.2%) [18, 22, 27–29]. The variability of the data could be linked to differences in techniques of analysis, hence variations in the sensitivity and specificity of detection [1, 4]. Current publications agree that FISH is a more sensitive and precise method in detecting gene deletions than multiplex PCR and Southern blot analysis [4, 30]. Since FISH allows cell-by-cell assessment, we are able to perform analyses without contaminating normal cells [30]. This might also explain the higher rate of deletions in our series than in studies performed using comparative genomic hybridization: 15.3% in Lenz et al. [28] and 52.2% in Tagawa et al. [29].

In the subset showing *p16* deletions, 40.7% (11/27) of the cases were found to harbour simultaneous hemizygous and homozygous deletions, suggesting that partial loss and complete loss of *p16* alleles are significantly interrelated ($p = 0.043$). Our observation confirms that of Cairns et al. [4], who reported high frequencies of homozygous *p16* deletion in various primary tumours showing loss of heterozygosity at 9p21. It is conceivable that tumour cells with partial loss of *p16* may have an increased life span and genomic instability, thus predisposing the remaining allele to undergo deletion; tumour cells with biallelic deletion of *p16* are most probably derived from entities harbouring loss of heterozygosity [1]. On the other hand, Konemann et al. [31] revealed that human neoplasms may comprise different populations of tumour cells, and each population may display distinct clonality and genetic features. The simultaneous occurrence of hemizygous and homozygous *p16* deletions may explain the clonal and molecular heterogeneity in DLBCL [9, 31, 32]. The presence of neoplastic cells with a distinct heterogeneous molecular background in a single sample may reflect a process of tumour progression [32].

In the present study, the frequency of *p16* deletions was higher in extranodal DLBCLs (16/22; 72.7%) than in nodal DLBCLs (11/25; 44.0%) ($p = 0.047$). This observation reinforces the notion that DLBCLs arising in extranodal sites harbour genetic alterations that differ from those in the lymph nodes [33].

Gains of *p16* were detected in 27.7% (13/47) of the DLBCL cases. Notably, *p16* gains in our series were associated with chromosomal duplication (polysomies 9) rather than *p16* gene amplification. This finding is in concordance with earlier reports that gene amplification is rarely detected in lymphoma [34, 35]. Previous studies reported that gains in the 9p21 chromosomal region in-

volving *p16* loci are a characteristic of PMBL [36, 37]. In the absence of gene expression profiles, we were not able to decide whether the DLBCL cases with extra copies of *p16* were cases of PMBL.

It appeared in this study that P16 overexpression was not attributed to gains of *p16* gene ($p = 0.621$). Indeed, gains of a gene do not necessarily lead to protein overexpression since the overrepresented genes can potentially be silenced by genetic alterations such as point mutations and promoter methylation [38]. Our data revealed an inverse relationship between P16 overexpression and gene deletions ($p = 0.019$), compatible with the notion that protein expression and gene inactivation are mutually exclusive [16, 18]. Finegan et al. [6] suggested the high level of P16 could be attributed to accumulation of mutant protein with extended half-life. It has been revealed in prostate cancer that P16 overexpression should be attributed to upregulation of *p16* [5] by different molecular mechanisms, including lack of functional PRb [reviewed in 1], overexpression of cyclin D and/or CDK4 [5] and cellular senescence [23]. Further studies are needed to clarify the involvement of these mechanisms in P16 overexpression in DLBCL.

In conclusion, our study provides evidence that P16 overexpression is common in DLBCL. The level of P16 expression may differ between GCB and non-GCB subgroups. Increased expression of P16 may mitigate cell proliferation and potentially lead to cell growth arrest. Losses of *p16* are attributed to *p16* gene deletions rather than monosomy 9, and gains of *p16* are associated with polysomies 9 rather than *p16* gene amplification. Deletions of *p16*, including hemizygous and homozygous deletions, are more common than gains of *p16*, and overexpression of P16 protein is not associated with gains of *p16* gene. Further investigations on cellular signalling and molecular mechanisms responsible for P16 overexpression may provide further insight into the importance of *p16* in the development of DLBCL.

Acknowledgements

This study was supported by grant No. FS172/2007C from the University of Malaya. Special thanks to Alice K.H. Shia, Kah Keng Wong and Lin Kiat Lee for their kind assistance in the staining of CD10, BCL-6 and MUM-1.

References

- Ruas M, Peters G: The p16^{INK4a}/CDKN2a tumor suppressor and its relatives. *Biochim Biophys Acta* 1998;1378:115–177.
- Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavitian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH: A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994;264:436–440.
- Xiong Y, Zhang H, Beach D: Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev* 1993;7:1572–1583.
- Cairns P, Polascik TJ, Eby Y, Tokino K, Califano J, Merlo A, Mao L, Herath J, Jenkins R, Westra W, Rutter JL, Buckler A, Gabrielson E, Tockman M, Cho KR, Hedrick L, Bova GS, Isaacs W, Koch W, Schwab D, Sidransky D: Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. *Nat Genet* 1995;11:210–212.
- Lee CT, Capodiceci P, Osman I, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C: Overexpression of the cyclin-dependent kinase inhibitor p16 is associated with tumor recurrence in human prostate cancer. *Clin Cancer Res* 1999;5:977–983.
- Finegan MM, Han AC, Edelson MI, Rosenblum NG: p16 expression in squamous lesions of the female genital tract. *J Mol Histol* 2004;35:111–114.
- Melck A, Masoudi H, Griffith OL, Rajput A, Wilkins G, Bugis S, Jones SJ, Wiseman SM: Cell cycle regulators show diagnostic and prognostic utility for differentiated thyroid cancer. *Ann Surg Oncol* 2007;14:3403–3411.
- Milde-Langosch K, Bamberger AM, Rieck G, Kelp B, Löning T: Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. *Breast Cancer Res Treat* 2001;67:61–70.
- Stein H, Warnke RA, Chan WC, Jaffe ES, Chan JK, Gatter KC, Campo E: Diffuse large B-cell lymphoma, not otherwise specified; in Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman VJ (eds): WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, IARC, 2008, pp 233–237.
- Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000;403:503–511.
- Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Gilt-nane JM, Hurt EM, Zhao H, Averett L, Yang L, Wilson WH, Jaffe ES, Simon R, Klausner RD, Powell J, Duffey PL, Longo DL, Greiner TC, Weisenburger DD, Sanger WG, Dave BJ, Lynch JC, Vose J, Armitage JO, Montserrat E, López-Guillermo A, Grogan TM, Miller TP, LeBlanc M, Ott G, Kvaloy S, Delabie J, Holte H, Krajci P, Stokke T, Staudt LM: The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med* 2002;346:1937–1947.
- Rosenwald A, Wright G, Leroy K, Yu X, Gaulard P, Gascoyne RD, Chan WC, Zhao T, Haioun C, Greiner TC, Weisenburger DD, Lynch JC, Vose J, Armitage JO, Smeland EB, Kvaloy S, Holte H, Delabie J, Campo E, Montserrat E, Lopez-Guillermo A, Ott G, Muller-Hermelink HK, Connors JM, Braziel R, Grogan TM, Fisher RI, Miller TP, LeBlanc M, Chiorazzi M, Zhao H, Yang L, Powell J, Wilson WH, Jaffe ES, Simon R, Klausner RD, Staudt LM: Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favourable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med* 2003;198:851–862.
- Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Müller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO, Chan WC: Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004;103:275–282.
- Lossos IS: Molecular pathogenesis of diffuse large B-cell lymphoma. *J Clin Oncol* 2005;23:6351–6357.
- Paik JH, Jeon YK, Park SS, Kim YA, Kim JE, Huh J, Lee S-S, Kim WH, Kim CW: Expression and prognostic implications of cell cycle regulatory molecules, p16, p21, p27, p14 and p53 in germinal centre and non-germinal centre B-like diffuse large B-cell lymphomas. *Histopathology* 2005;47:281–291.
- Villuendas R, Sánchez-Beato M, Martínez JC, Saez AI, Martínez-Delgado B, García JF, Mateo MS, Sánchez-Verde L, Benítez J, Martínez P, Piris MA: Loss of p16/INK4A protein expression in non-Hodgkin's lymphomas is a frequent finding associated with tumor progression. *Am J Pathol* 1998;153:887–897.
- Kim LH, Peh SC, Poppema S: Expression of retinoblastoma protein and P16 proteins in classic Hodgkin lymphoma: Relationship with expression of p53 and presence of Epstein-Barr virus in the regulation of cell growth and death. *Hum Pathol* 2006;37:92–100.
- Møller MB, Ino Y, Gerdes AM, Skjødtt K, Louis DN, Pedersen NT: Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma. *Leukemia* 1999;13:453–459.
- Jehan Z, Siraj AK, Abubaker J, Ruiz C, Simon R, Sultana M, Uddin S, Bavi P, Hussain A, Razack S, Ezzat A, Al-Dayel F, Sauter G, Al-Kuraya KS: Distinct gene expression profiles: nodal versus extranodal diffuse large B-cell lymphoma. *Oncology* 2008;75:71–80.
- Lukas J, Aagaard L, Strauss M, Bartek J: Oncogenic aberrations of p16INK4/CDKN2 and cyclin D1 cooperate to deregulate G1 control. *Cancer Res* 1995;55:4818–4823.
- Lossos IS, Morgensztern D: Prognostic biomarkers in diffuse large B-cell lymphoma. *J Clin Oncol* 2006;24:995–1007.
- Pinyol M, Cobo F, Bea S, Jares P, Nayach I, Fernandez PL, Montserrat E, Cardesa A, Campo E: P16^{INK4a} gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. *Blood* 1998;91:2977–2984.
- Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA: Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 2005;436:660–665.
- Sandig V, Brand K, Herwig S, Lukas J, Bartek J, Strauss M: Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. *Nat Med* 1997;3:313–319.
- Sasaki K, Murakami T, Kawasaki M, Takahashi M: The cell cycle associated change of the Ki-67 reactive nuclear antigen expression. *J Cell Physiol* 1987;133:579–584.
- Hunt KE, Reichard KK: Diffuse large B-cell lymphoma. *Arch Pathol Lab Med* 2008;132:118–124.
- Stranks G, Height SE, Mitchell P, Jadavay D, Yuille MA, De Lord C, Clutterbuck RD, Treleaven JG, Powles RL, Nacheva E, Oscier DG, Karpas A, Lenoir GM, Smith SD, Millar JL, Catovsky D, Dyer MJS: Deletions and rearrangement of CDKN2 in lymphoid malignancy. *Blood* 1995;85:893–901.
- Lenz G, Wright GW, Emre NC, Kohlhammer H, Dave SS, Davis RE, Carty S, Lam LT, Shaffer AL, Xiao W, Powell J, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Campo E, Jaffe ES, Delabie J, Smeland EB, Rimsza LM, Fisher RI, Weisenburger DD, Chan WC, Staudt LM: Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. *Proc Natl Acad Sci USA* 2008;105:13520–13525.

- 29 Tagawa H, Suguro M, Tsuzuki S, Matsuo K, Karnan S, Ohshima K, Okamoto M, Morishima Y, Nakamura S, Seto M: Comparison of genome profiles for identification of distinct subgroups of diffuse large B-cell lymphoma. *Blood* 2005;106:1770–1777.
- 30 Ventura RA, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason DY, Siebert R: FISH analysis for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded tissue. *J Mol Diagn* 2006;8:141–151.
- 31 Konemann S, Schuck A, Malath J, Rupek T, Horn K, Baumann M, Vormoor J, Rube C, Willich N: Cell heterogeneity and subpopulations in solid tumors characterized by simultaneous immunophenotyping and DNA content analysis. *Cytometry* 2000;41:172–177.
- 32 Heppner GH: Tumor heterogeneity. *Cancer Res* 1984;44:2259–2265.
- 33 López-Guillermo A, Colomo L, Jiménez M, Bosch F, Villamor N, Arenillas L, Muntanola A, Montoto S, Giné E, Colomer D, Beà S, Campo E, Montserrat E: Diffuse large B-cell lymphoma: clinical and biological characterization and outcome according to the nodal or extranodal primary origin. *J Clin Oncol* 2005;23:2797–2804.
- 34 Magrath I: Molecular basis of lymphomagenesis. *Cancer Res* 1992;52:5529s–5540s.
- 35 Ben-Yehuda D, Houldsworth J, Parsa NZ, Chaganti RS: Gene amplification in non-Hodgkin's lymphoma. *Br J Haematol* 1994;86:792–797.
- 36 Bentz M, Barth TF, Bruderlein S, Bock D, Schwerer MJ, Baudis M, Joos S, Viardot A, Feller AC, Müller-Hermelink HK, Lichter P, Dohner H, Moller P: Gain of chromosome arm 9p is characteristic of primary mediastinal B-cell lymphoma (MBL): comprehensive molecular cytogenetic analysis and presentation of a novel MBL cell line. *Genes Chromosomes Cancer* 2001;30:393–401.
- 37 Bea S, Zettl A, Wright G, Salaverria I, Jehn P, Moreno V, Burek C, Ott G, Puig X, Yang L, Lopez-Guillermo A, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Gascoyne RD, Connors JM, Grogan TM, Brazier R, Fisher RI, Smeland EB, Kvaloy S, Holte H, Delabie J, Simon R, Powell J, Wilson WH, Jaffe ES, Montserrat E, Müller-Hermelink HK, Staudt LM, Campo E, Rosenwald A: Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction. *Blood* 2005;106:3183–3190.
- 38 Bird A: DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16:6–21.