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The Expression of the RUVBL1 Component of the R2TP Complex Correlates with Poor Prognosis in DLBCL

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ORIGINAL ARTICLE

Metallothionein levels in gingival crevicular fluid, saliva, and serum of smokers and non-smokers with chronic periodontitis

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Keywords
Diffuse large B-cell lymphoma · DNA damage · Double-strand break · Gamma H2AX · Immunohistochemistry · R2TP

Abstract

Introduction: Diffuse large B-cell lymphoma (DLBCL) is the most prevalent subtype of non-Hodgkin's lymphoma (NHL) accounting for 30% of adult NHL worldwide and 50% in developing countries like India. DNA damage and Myc-induced transformation are well-known contributing factors towards development of DLBCL. A recently identified HSP90 co-chaperone complex R2TP has been shown to contribute towards DNA damage and Myc-induced transformation. This study aimed to analyse the immunohistochemical (IHC) expression of R2TP complex components RUVBL1, PIH1D1, and RPAP3 in DLBCL patients and correlate with prognosis. ***Methods:*** DLBCL (n = 54) histological slides were retrieved from archives, and detailed histomorphological and clinical features were noted. IHC staining of R2TP complex components RUVBL1, PIH1D1, and RPAP3 was performed on 54 cases (FFPE) of DLBCL. Expression data were correlated with survival and clinical features. ***Results:*** Out of the 54 DLBCL cases, 59.26% (n = 32) stained positive for RUVBL1. The RUVBL1 expression was associated with poor prognosis in both progression-free survival (PFS) ($p = 0.0146$) and overall survival (OS) ($p = 0.0328$). The expression was positively correlated with bone marrow involvement ($p = 0.0525$). The expression of PIH1D1 was observed in 68.51% (n = 32) of DLBCL cases, and positive correlation was observed with international prognostic index score ($p = 0.0246$); however, no correlation was observed with PFS or OS. Finally, RPAP3 was found immunopositive in only 1 case of DLBCL. ***Conclusions:*** Immunopositivity for RUVBL1 is associated with poor prognosis along with a higher relapse rate amongst the DLBCL patients. PIH1D1 immunopositivity correlated with a higher IPI score.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive neoplasm of B cells with varied clinical features, morphology, molecular profile, and immunophenotype. It is the most prevalent subtype of non-Hodgkin lymphoma

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(NHL) accounting for 30% of NHL worldwide [1]. The frequency of DLBCL, not otherwise specified (NOS), is much higher in developing countries like India, constituting for ~50% of total DLBCL cases [2]. With a clinically heterogenous spectrum and no consistent definition, it is of utmost importance to identify prognostically and biologically distinct subgroups [3–5]. Although morphological, clinical, and biological studies have subdivided DLBCL into different subtypes, a vast majority of cases are still classified as DLBCL, NOS, which can be further subcategorized on the basis of morphology, cell of origin, and genetics. Morphologically, immunoblastic, centroblastic, and anaplastic types can be distinguished [6, 7], and genetically they can be categorized into the germinal centre B-cell (GCB) and the activated B-cell subtype [7] by their genetic signatures; however, 10–15% of cases still remain unclassified [8–10]. The R-CHOP regimen has been established as the therapy of choice even for aggressive cases. However, relapse or a refractory disease to R-CHOP confers a dismal prognosis. Genomic instability is considered a cancer hallmark, suggesting a propensity to acquire DNA damage with continuous gain of mutation and genomic abnormalities, favouring the growth of cancer, metastatic phenotypes, and chemoresistance [11, 12]. Recent studies have reported a subset of DLBCLs displaying mutations of genes involved in DNA repair [13]. And hence, the DNA repair mechanism has been seen to be altered in DLBCL [14].

DNA damage culminates into γ2AX phosphorylation at serine 139 by members of the P13K family, and once phosphorylated, it binds to the damaged DNA. The kinases involved in the overhaul of DNA damage are serine protein kinases (ATM), phosphatidylinositol 3-kinase-related kinase (DNA-PK), and the serine/threonine-protein kinase (ATR) [15, 16]. ATM defects upsurge the occurrence of cancer in patients and can contribute to the condition of ataxia telangiectasia syndrome associated with increased radiation exposure [17, 18]. The ATM phosphorylates the downstream target like p53, checkpoint kinase 1 and 2, Mdm2, BRCA1, and nibrin alongside H2AX upon DSB induction, and thus has a number of effects on cell cycle, DNA repair, and apoptosis [19–21]. However, in cells that lack DNA-PK and have decreased ATM levels, the formation of γH2AX foci is strongly reduced [22]. So far, molecular understanding of the mechanism involved in etiopathogenesis of DLBCL has not been elucidated to an extent that they can serve as diagnostic and prognostic biomarkers.

A novel mechanism has been proposed for the regulation of PI3Ks like ATM, ATR, DNA-PK, and Myc-induced transformation [23, 24]. PI3Ks have been shown to be stabilized by a unique Hsp90 co-chaperone complex called PAQosome/also known as the R2TP complex. The components of this complex have also been shown to interact with Myc and help in transformation [24]. In mammals, the R2TP complex consists of 4 core subunits (RUVBL1, RUVBL2, PIH1D1, and RPAP3). RUVBL1/RUVBL2 is part of several cellular complexes like the TIP60 complex which forms a complex with c-Myc and helps in Myc-driven oncogenesis [24]. In addition to helping in Myc-induced transformation, the R2TP complex has a prominent role in phosphatidylinositol 3-kinase-related protein kinase (PI3K) stabilization [25]. The c-Myc translocation has been reported as a hallmark of Burkitt's lymphoma where c-Myc rearrangement occurs in the immunoglobulin heavy chain (IGH) on chromosome 14 [4]. Recent studies have reported that 5–10% of the DLBCL cases show Myc rearrangement [26, 27]. Unlike Burkitt lymphoma, the c-Myc rearrangements typically involve a non-IGH partner like PAX5 t {8:9} and light chain [28].

Keeping in view the role of the R2TP complex in stabilizing the PIKK and Myc-driven oncogenesis, the expression of core R2TP complex components may be altered in DLBCL which forms the aim of our study. In this pilot study, expression levels of R2TP complex components RUVBL1, PIH1D1, and RPAP3 were analysed in 54 DLBCL patients. Their expression levels were correlated with clinicopathological parameters and patient survival.

Materials and Methods

Patients and Specimens

This is a retrospective study wherein a total of 54 cases of histopathologically diagnosed of DLBCL NOS and treated at our institute between 2014 and 2016 were included. Using the WHO classification of haematopoietic and lymphoid malignancies (2016), diagnosis was made and cases were reviewed. The histopathological material was retrieved from the archives of the Department of Pathology, and the follow-up and clinical case details were obtained from the archives of the treating unit. Twenty-seven biopsies of reactive lymph nodes were taken as control. Ethical clearance was obtained from the institutional review board before the start of the study (IEC-485/September 1, 2017).

Immunohistochemical Staining and Quantification

Immunohistochemistry was done on 5-μm-thick sections from the corresponding formalin-fixed, paraffin-embedded tissue samples which were collected on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in graded series of alcohol. Microwave-mediated antigen retrieval was done using low-pH and high-pH buffers (800 W, 15 min; 480 W, 5 min) as per manufacturer's instruction for the antibodies. The primary antibodies anti-RUVBL1 (10210-2-AP), anti-PIH1D1 (19427-1-AP),

Table 1. Immunohistochemistry performed as indicated

Antibodies	Manufacturer	Source	Buffer	Dilution	Tissue (controls)	Localization
RUVBL1	Proteintech (10210-2-AP)	Rabbit	Tris-EDTA, pH 9.0	1:3,500	Human ovarian cancer	Nuclear+cytoplasmic
PIH1D1	Proteintech (19427-1-AP)	Rabbit	Citrate, pH 6.0	1:200	Human lung tissue	Cytoplasmic
RPAP3	Proteintech (23741-1-AP)	Rabbit	Citrate, pH 6.0	1:800	Human tonsil	Cytoplasmic

and anti-RPAP3 (23741-1-AP) incubation was standardized to overnight (shown in Table 1). The secondary detection was performed using CRF™ Anti-Polyvalent HRP Polymer (DAB) Lab Pack (Ref. CPP050; Scytech Laboratories) according to the manufacturer protocol. Each batch was run with an appropriate positive and negative control.

Immunohistochemical scoring was done using semiquantitative H-score (stained intensity and positive cell percentage). The rule for stained cell percentage was described as 0: no stained cells, 1: 1–25%, 2: 26–50%, 3: 51–75%, and 4: 76–100%. Staining intensity was graded as 0: no staining, 1: weak staining, 2: moderate staining, and 3: intense staining. High expression (significant positive population) was documented if the total score was more than or equal to 6 points; otherwise, the result was regarded as low expression or immunonegative (Table 2).

Fluorescence in situ Hybridization

The fluorescence in situ hybridization (FISH) was performed using break apart standard probes from Sure FISH (8q24.21 MYC 5′ Cat No. G101109G-8, MYC 3′ Cat. No. G101110R-8; 3q27.3 BCL2 5′ Cat. No. G110931G-8, and BCL2 3′ Cat. No. G110926R-8). The DAKO implementation kit was used for visualization. A total of 100 non-overlapping nuclei were counted.

Statistical Analysis

All statistical analyses were performed using STATA version 12 and the EZR plugin of R console. A χ^2 test or Fischer's exact test was used to investigate associations. One-way ANOVA, Student's *t* tests, and rank sum tests were used as applicable. Survival analyses were used to investigate time-to-event outcomes including progression-free survival (PFS) and overall survival (OS). To estimate the proportion surviving at a point in time and to compare the curves from different groups, the log-rank test and Kaplan-Meier curve were used. The Cox proportional hazard regression model was used to assess the impact of RUVBL1 and PIH1D1 on the OS and PFS. A p value of < 0.05 was considered significant throughout.

Results

This is a retrospective cohort study that included a total of 54 patients of DLBCL NOS. A slight female predominance with a female to male ratio of 1.3:1 ($F = 57.4\%$, $M = 42.6\%$) was noted with a mean age of presentation of 54 years (youngest participant being 26 and the eldest 77 years old). All the cases were classified into GCB and non-GCB type by the HANs algorithm. As per the classification based on cell of origin, 72.22% ($n = 39$) of patients were GCB type and 27.78% ($n = 15$) were non-GCB type.

RUVBL1 Expression Correlates with Overall Survival, Progression-Free Survival, and Bone Marrow Involvement

RUVBL1 immunopositivity was noted as either only cytoplasmic or nuclear + cytoplasmic (Fig. 1b). For all the 54 cases evaluated, 59.26% ($n = 32$) of cases showed strong positivity for RUVBL1 while immunonegativity was seen in 40.74% ($n = 22$). All the reactive lymph nodes (controls, $n = 27$) were negative for RUVBL1. The expression of RUVBL1 was correlated with clinicopathological features and was found statistically significant with PFS, OS, and BM involvement (Table 3). Among all the 54 patients, 48.15% of cases ($n = 26$) had no relapse in which 57.69% ($n = 15$) showed negative expression for RUVBL1 and 42.30% ($n = 11$) were positive for RUVBL1. Among the patients with relapse, i.e., 51.85% ($n = 28$), 25% ($n = 7$) were immunonegative for RUVBL1 and 75% ($n = 21$) showed immunopositivity for RUVBL1, with a significant p value of *0.0146*. Similarly, for the OS, 70.37% of cases ($n = 38$) were alive in which 50% of cases ($n = 19$) were negative for RUVBL1, and the other 50% of cases ($n = 19$) were immunopositive for RUVBL1. However, among the lost lives of 29.63% of cases ($n = 16$), 81.25% of cases ($n = 13$) were immunopositive for RUVBL1 and only 18.75% of cases ($n = 3$) were negative for RUVBL1, and hence a significant p value of *0.0328* was observed. Furthermore, 87.04% of cases ($n = 47$) did not have bone marrow involvement among which 46.80% ($n = 22$) had no expression for RUVBL1 and 53.19% ($n = 25$) had RUVBL1 expression. However, 100% ($n = 7$) of patients that had bone marrow involvement stained for RUVBL1 with a significant p value of *0.0525* (Table 3). The other clinicopathological factors like stage, LDH levels, lymph node status, and presence of bulky disease did not show any statistical correlation with the RUVBL1 expression.

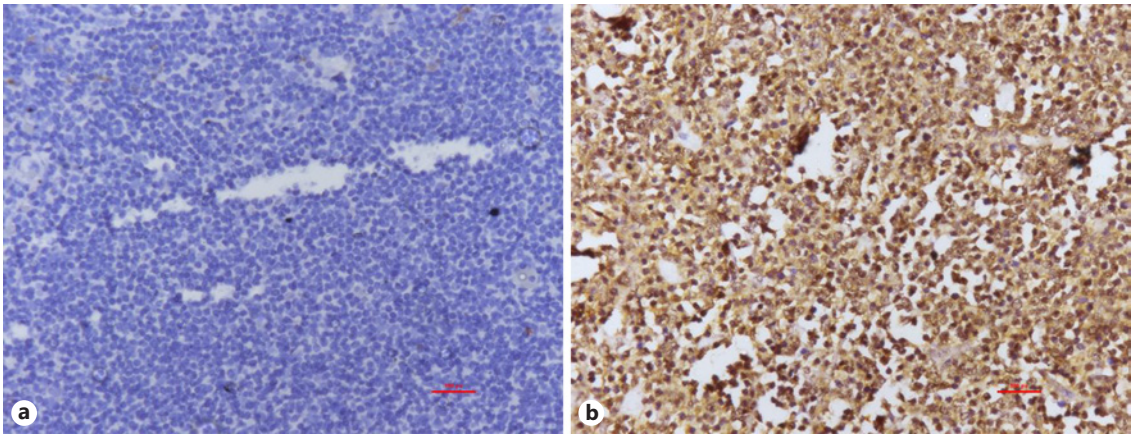

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Fig. 1. Representative photomicrograph of immunohistochemical expression for RUVBL1. Reactive lymph node showing no reactivity (×100) (**a**); intermediate-to-large-sized atypical lymphoid cells showing strong nuclear and cytoplasmic positivity for RUVBL1 in a DLBCL case (×100) (**b**). DLBCL, diffuse large B-cell lymphoma.

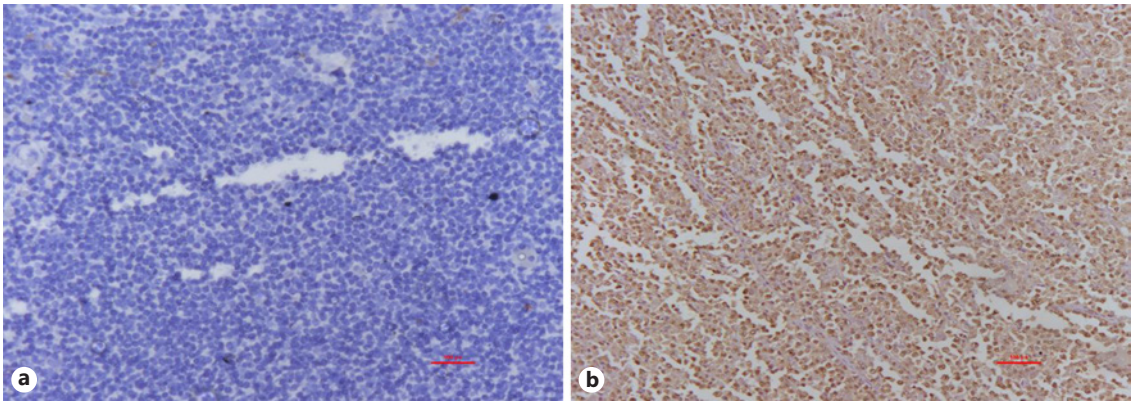

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Fig. 2. Photomicrograph of immunohistochemical expression of PIH1D1. Reactive lymph node negative for PIH1D1 (×100) (**a**); atypical lymphoid cells showing cytoplasmic immunopositivity for PIH1D1 in a DLBCL case (×200) (**b**). DLBCL, diffuse large B-cell lymphoma.

Expression of PIH1D1

The PIH1D1 staining was observed as cytoplasmic (Fig. 2b). Strong positivity was observed in 68.51% of cases ($n = 37$) while PIH1D1 was negative in 31.48% of cases ($n = 17$). Although not completely understood, 40.7% ($n = 11$) of reactive lymph nodes (controls) stained positive for PIH1D1, and on evaluation of their clinical history, no features of any lymphoproliferative or infectious disease were revealed. The PIH1D1 correlation was observed only with the International Prognostic Index (IPI) score with a p value of *0.0246* (Table 3).

Expression of RPAP3

The immunopositivity of RPAP3 was observed in the cytoplasm of the cells (Fig. 3b). Out of all the 54 cases of DLBCL, only one turned out to be positive. However, RPAP3 did not show any statistical significance with any of the clinicopathological features.

Association of RUVBL1 and PIH1D1 with c-Myc and BCL6

The immunopositivity of c-Myc and BCL6 was observed in the nucleus (Fig. 4a, b). Among the 40.74% ($n = 22$) of cases which were RUVBL1 negative, 90.91% ($n = 20$) were BCL6 positive. A significant inverse relationship was noted between BCL6 and RUVBL1, as expected indicating that BCL6 might be suppressing the RUVBL1 expression ($p = 0.027$) [29]. c-Myc was not significantly related to RUVBL1 ($p = 0.582$) with equal distribution of positives and negatives out of the 22 RUVBL1 negatives.

Table 2. Association of the protein expression with the age, gender, and lactate dehydrogenase levels

Characteristics	Patient frequency (n = 54)	RUVBL1		p value	PIH1D1		p value	RPAP3		p value
		negative	positive		negative	positive		negative	positive	
Sex										
Male	23 (42.59)	9 (39.13)	14 (60.86)		7 (30.43)	16 (69.56)		23 (100)	0 (0)	
Female	31 (57.41)	13 (41.93)	18 (58.06)	0.836	10 (32.25)	21 (67.74)	0.887	30 (96.77)	1 (3.22)	1.000
Age, years										
<60	31 (57.41)	13 (41.93)	18 (58.06)		9 (29.03)	22 (70.96)		30 (96.77)	1 (3.22)	
>60	23 (42.59)	9 (39.13)	14 (60.86)	0.836	8 (34.78)	15 (65.21)	0.653	23 (100)	0 (0)	1.000
LDH										
≤290	22 (40.74)	10 (45.45)	12 (54.54)		6 (27.27)	16 (72.72)		22 (100)	0 (0)	
>290	32 (59.26)	12 (37.5)	20 (62.5)	0.559	11 (34.37)	21 (65.62)	0.581	31 (96.87)	1 (3.12)	1.000

Table 3. Correlation of the expression of the proteins with the clinicopathological parameters

Characteristics	Patient frequency (n = 54)	RUVBL1		p value	PIH1D1		p value	RPAP3		p value
		negative	positive		negative	positive		negative	positive	
Stage										
1–2	26 (48.15)	13 (50)	13 (50)	0.182	5 (19.23)	21 (80.76)		25 (96.15)	1 (3.84)	0.97
3–4	28 (51.85)	9 (32.14)	19 (67.85)		12 (42.85)	16 (57.14)		28 (100)	0 (0)	
Lymph node status										
Nodal	25 (46.30)	11 (44)	14 (56)	0.651	8 (32)	17 (68)	0.939	25 (100)	0 (0)	1.0
Extranodal	29 (53.70)	11 (37.93)	18 (62.06)		9 (31.03)	20 (68.96)		28 (96.55)	1 (3.44)	
Bulky disease										
Absence	44 (81.48)	17 (38.63)	27 (61.36)	0.509	14 (31.81)	30 (68.18)	1.0	43 (97.27)	1 (2.27)	1.0
Presence	10 (18.52)	5 (50)	5 (50)		3 (30)	7 (70)		10 (100)	0 (0)	
BM involvement										
Not involved	47 (87.04)	22 (46.80)	25 (53.19)	**0.0525***	13 (27.65)	34 (72.34)	0.189	46 (97.87)	1 (2.12)	1.0
Involved	7 (12.96)	0 (0)	7 (100)		4 (57.14)	3 (42.85)		7 (100)	0 (0)	
IPI score										
>2	34 (62.96)	16 (47.05)	18 (52.94)	0.218	7 (20.58)	27 (79.41)	**0.0246***	33 (97.05)	1 (2.94)	1.0
>2	20 (37.04)	6 (30)	14 (70)		10 (50)	10 (50)		20 (100)	0 (0)	
PFS events										
No relapse	26 (48.15)	15 (57.69)	11 (42.30)	**0.0146***	7 (26.92)	19 (73.07)	0.487	26 (100)	0 (0)	1.0
Relapse	28 (51.85)	7 (25)	21 (75)		10 (35.71)	18 (64.28)		27 (96.42)	1 (3.57)	
OS events										
No death	38 (70.37)	19 (50)	19 (50)	**0.0328***	10 (26.31)	28 (73.68)	0.208	37 (97.36)	1 (2.63)	1.0
Death	16 (29.63)	3 (18.75)	13 (81.25)		7 (43.75)	9 (56.25)		16 (100)	0 (0)	

BM, bone marrow; IPI, International Prognostic Index; PFS, progression-free survival; OS, overall survival. * Bold numbers in the table indicate significance.

No significant difference (p = 0.849) was noted between the average percentage of c-MYC among RUVBL1 negative (34.09 ± 21.75, n = 22) and that among RUVBL1 positive (36.88 ± 24.16, n = 32). Moreover, no significant difference (p = 0.849) was noted between the average percentage of c-MYC among PIH1D1 negative (37.06 ± 25.44, n = 17) and that among PIH1D1 positive (35.14 ± 22.19, n = 37). RPAP3 has only 1 positive observation, and hence the comparison of c-MYC percentage was not possible. Among the 54 patients, only 22.22% (n = 12) were double expressor lymphoma, whereas all the other cases are non-double expressor lymphoma constituting about 77.77% (n = 42). Among them, only 1 double hit case was observed with c-Myc break apart and BCL2 amplification (Fig. 5a, b).

All the 54 cases were processed for EBV LMP and only one turned immunopositive for EBV. However, the EBER ISH was processed only in 15 cases as a part of a different project, and none of them turned out to be positive for EBER.

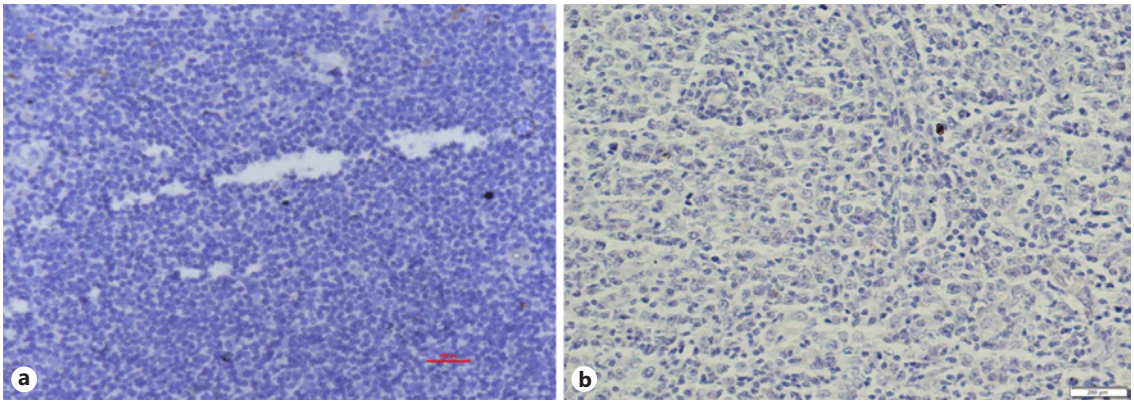

Fig. 3. Representative photomicrograph of immunohistochemical expression of RPAP3. Reactive lymph node showing no reactivity for RPAP3 (×100) (**a**); DLBCL case negative for RPAP3 (×100) (**b**). DLBCL, diffuse large B-cell lymphoma.

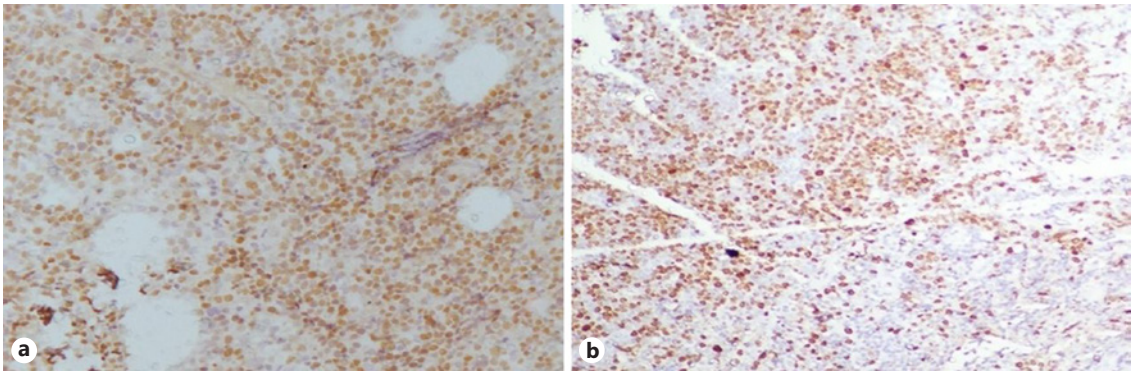

Fig. 4. Representative photomicrograph of immunohistochemical expression of c-Myc and BCL6. c-Myc showing strong nuclear immunopositivity in DLBCL (×100) (**a**); Bcl6 showing nuclear immunopositivity in DLBCL (×100) (**b**). DLBCL, diffuse large B-cell lymphoma.

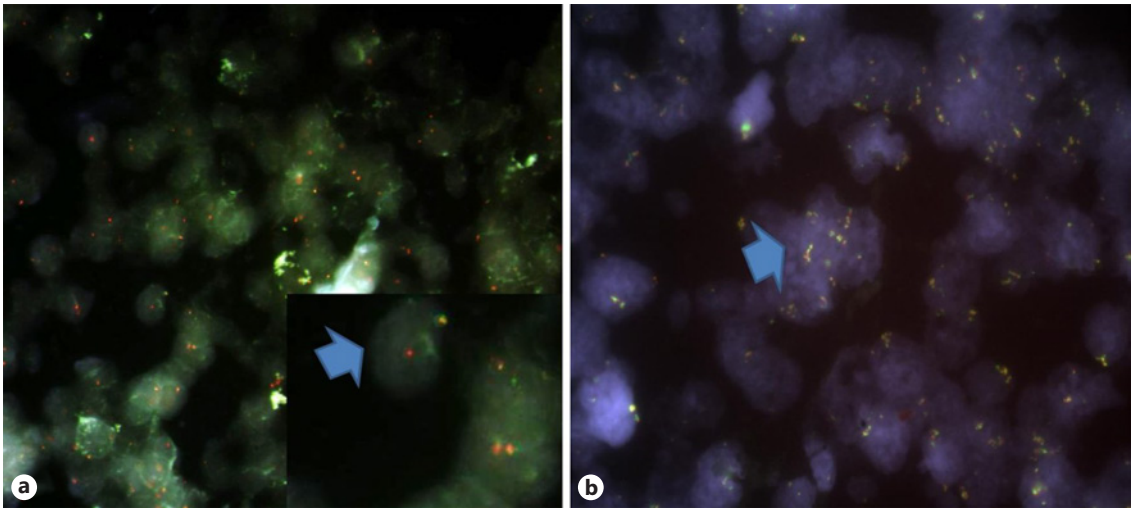

Fig. 5. Representative FISH image of c-Myc and BCL2. c-Myc showing break apart (**a**); BCL2 showing amplification in DLBCL (**b**). FISH, fluorescence in situ hybridization; DLBCL, diffuse large B-cell lymphoma.

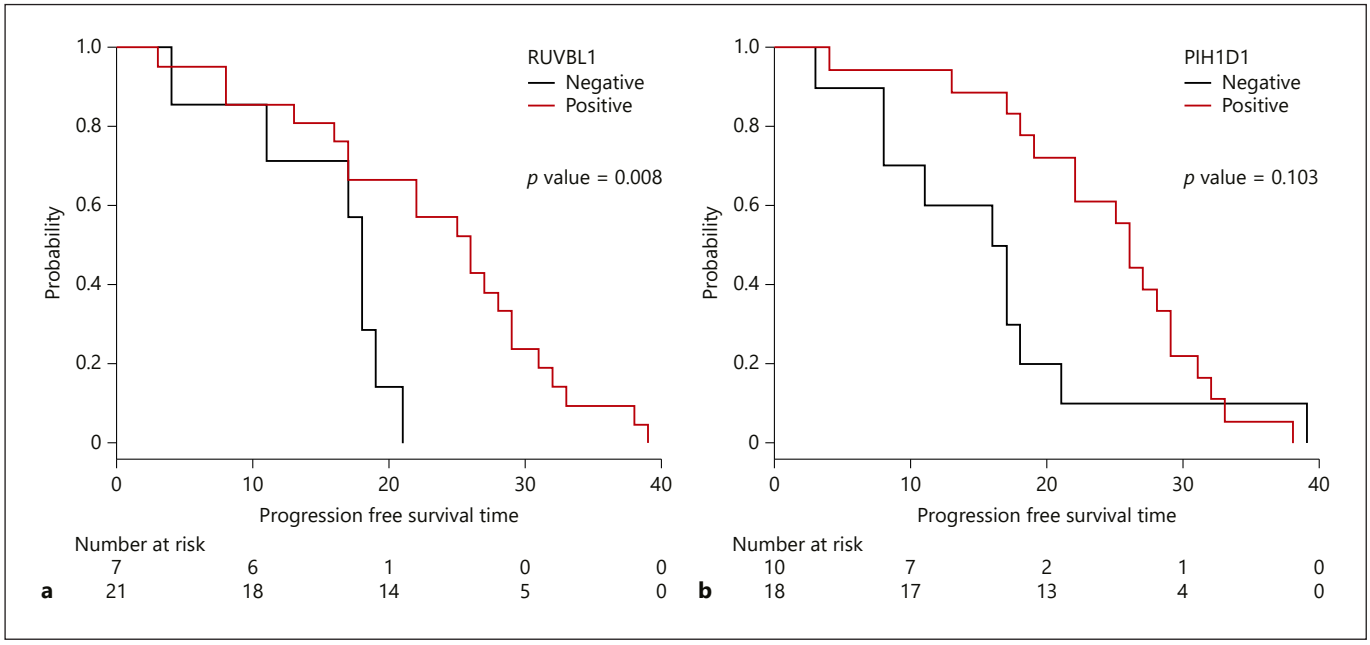

Fig. 6. a, **b** Kaplan-Meier plots of RUVBL1 and PIH1D1 for PFS events. PFS, progression-free survival.

Table 4. The time-to-event analysis results on progression-free survival and the overall survival across the 2 core subunits of the human R2TP complex ($n = 54$)

Protein coding gene	Progression-free survival			Overall survival		
	no relapse, *n* (%)	relapse, *n* (%)	hazard ratio (95% CI)	no death, *n* (%)	death, *n* (%)	hazard ratio (95% CI)
RUVBL1 positive	11 (34.38)	21 (65.63)	2.56 **(1.07, 6.13)**	19 (59.38)	13 (40.63)	3.75 **(1.04, 13.47)**
RUVBL1 negative	15 (68.18)	7 (31.82)		19 (86.36)	3 (13.64)	
PIH1D1 positive	19 (51.35)	18 (48.65)	0.51 (0.23, 1.12)	28 (75.6)	9 (24.3)	0.72 (0.26, 2.01)
PIH1D1 negative	7 (41.18)	10 (58.82)		10 (58.8)	7 (41.1)	

Bold numbers indicate significance.

Survival Analysis

The findings presented in Table 4 reveal that at any time *t*, RUVBL1-positive cases had 2.56 times chances of a PFS event (relapse) proportionally to those RUVBL1 negative. At any time, *t*, 3.75 times as many with RUVBL1 positive experience an OS event (death) proportionally to those with RUVBL1 negative. However, the wide confidence interval of the hazard ratio, 3.75 (1.04, 13.47), indicates greater dubiety involved, and this could be attributed to the lesser number of RUVBL1-negative cases experiencing death.

The Kaplan-Meier survival plot showed a significant difference in the PFS observed across RUVBL1-positive and -negative cases ($p = 0.008$). Those with RUVBL1 immunonegativity were expected to experience a relapse in around 21 months, whereas those with RUVBL1 immunopositive in around 39 months (Fig. 6a).

No significant difference in the PFS was observed among those with PIH1D1 positive and those with PIH1D1 negative ($p = 0.103$). However, it was observed that those with PIH1D1 negativity are expected to survive without progression till 39 months if they manage to survive event free till the 21 months (Fig. 6b). Patients who experienced relapse at around 39 months did not show any correlation with the PIH1D1 immunostaining.

No significant difference in the OS was observed among the participants with respect to the core subunits

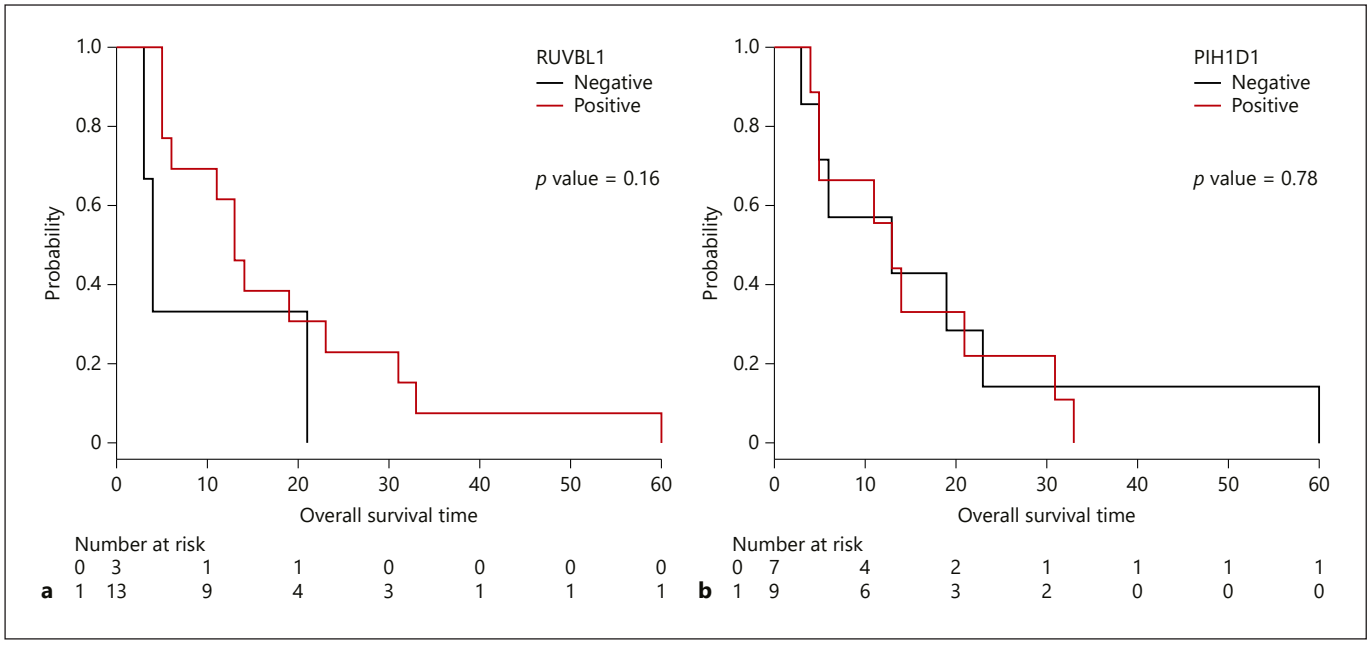

Fig. 7. a, **b** Kaplan-Meier plots of RUVBL1 and PIH1D1 for OS events. OS, overall survival.

including RUVBL1 ($p = 0.160$) and PIH1D1 ($p = 0.78$). However, it was observed that those with RUVBL1 positivity, if survived till 32 months, were expected to survive till 60 months. Those in the RUVBL1-negative cohort were expected to survive till 21 months if they survive till 4 months. Among those with PIH1D1 negative, those who survive till the 23 months will survive till 60 months (Fig. 7a, b).

The median PFS time for RUVBL1 positive and negative was 26 (17, 29) and 18 (4, 19), respectively. The median OS time for RUVBL1 positive and negative was 13 (5, 23) and 4 (3, na), respectively. Three individuals with RUVBL1 negativity died, and hence due to the smaller number, the upper limit of the confidence interval was not computed and presented as "na (not applicable)" (Table 4).

The median PFS time for PIH1D1 positive and negative was 26 (19, 29) and 16.5 (3, 18), respectively. The median OS time for PIH1D1 positive and negative was 13 (4, 31) and 13 (3, 23) months, respectively (Table 4).

Discussion

This is the first study to evaluate prognostic implications of R2TP co-chaperone complex components in DLBCL. We evaluated the immunohistochemical expression of the 3 components of the R2TP complex. Notably, the individual components show association with the prognosis of DLBCL. However, taken together, all the 3 components of this complex did not show a significant association with prognosis of DLBCL. The component RUVBL1 of this complex showed a strong correlation with the poor prognosis of DLBCL. Among the patients which had overexpressed RUVBL1, bone marrow involvement was seen, and this expression correlated well with PFS event ($p = 0.0146$) and OS events ($p = 0.0328$). This is in line with previous studies showing an association of RUVBL1 expression with the development of hepatocellular carcinoma [30–33]. Moreover, overexpression of RUVBL1 has been shown to be associated with poor prognosis of HCC [33, 34]. Besides being the major component of the R2TP complex, RUVBL1 is known to be part of several other cellular complexes and is involved in numerous physiological and pathological processes ranging from the cell cycle to telomere biogenesis [35, 36]. Nonetheless, most of these cellular processes are deregulated in DLBCL [37]. Another interesting function of RUVBL1 along with its partner RUVBL2 is in causing aberrant expression of c-Myc. Myc is known to be deregulated in DLBCL, and its overexpression has a worse prognosis in DLBCL [38, 39]. Thus, the overexpression of RUVBL1 may help Myc-induced transformation in DLBCL, but we did not find a significant correlation by immunohisto-

chemistry between RUVBL1 and Myc. FISH localized the RUVBL1 gene to 3q21, a region often rearranged in leukaemia and deleted in solid tumours [40]. RUVBL1/2 proteins have been described as novel co-factors in c-Myc-driven oncogenic transformation [24]. A study also showed that the RUVBL1 gene is upregulated in DLBCL in advanced scenarios compared to the early stages of the malignancy [41]. RUVBL1 interacts with mutant p53 protein, promoting tumorigenesis by regulating the transcriptional activity of mutant p53, thereby increasing migration, invasion, metastasis, and anchorage-independent cell growth of tumour cells. High RUVBL1 expression levels have been found to be associated with poor prognosis, especially for tumours carrying mutant p53 [42]. RUVBL1 has also been identified as a potential target of BCL6 repression. Interestingly, we found that BCL6 is repressing RUVBL1 expression in DLBCL patients as well. In a knockdown study of BCL6, a significant increase in the expression of endogenous RUVBL1 was observed [29]. Studies on renal cell carcinoma have revealed that cytoplasmic occurrence of RUVBL1 has been associated with poor survival of patients [43]. In a leukaemic model, it was found that RUVBL1 is needed not for cell survival but for the proliferation of the cells [44]. RUVBL1 and RUVBL2 have also been stated as novel interactors of the ebola virus (EBOV) nucleoprotein. This study suggested a possible role of the R2TP complex in EBOV capsid formation [45]. Keeping in view the above-allotted roles to RUVBL1, it was interesting to demystify the role of RUVBL1 expression in DLBCL as we attempted in our study.

Although we did not find an association of PIH1D1 with OS and PFS, its role cannot be ruled out completely. PIH1D1 is a phosphoreader component of the R2TP complex, and it interacts with casein kinase 2 phosphorylated motif DS/DDE of several proteins like ECD, Tel2, UBR5, etc [46, 47]. It is possible that the expression of these bridging partners of PIH1D1 might be either altered or mutated in DLBCL and will be explored in future studies. Notably, PIH1D1 expression correlates with IPI score and was found to be significant, thus hinting that PIH1D1 might be contributing towards relapse of DLBCL patients, and this can be tested in different groups of DLBCL patients. In future studies, greater sample size may be taken to account for the uncertainty as revealed in our study with wide 95% confidence intervals.

Another component of the R2TP complex RPAP3 was also evaluated immunohistochemically in our DLBCL cohort. Out of all the 54 cases of DLBCL, only 1 case was found to be positive. No study has looked for the role and expression of this protein in human malignancies. Our study is the first to evaluate the expression of this protein. It is possible that this gene might be silenced or lost in DLBCL. Our future studies will address the molecular mechanism responsible for the loss of RPAP3 expression in DLBCL. It is of note that PIH1D1 and RPAP3 may show some interesting characteristics during therapy when most of the tumour is under stress conditions. Our future studies will look at that aspect.

Conclusion

High immunohistochemical expression of RUVBL1 in DLBCL is associated with relapse and poor survival.

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Statement of Ethics

This study was approved by the Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi, India (IEC-485/September 1, 2017). Patient Informed Consent Form (PICF) was not needed as this is a retrospective study, and the formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from the archives of the Department of Pathology.

Conflict of Interest Statement

The authors declare that they do not have any conflicts of interest.

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Author Contributions

Moien Lone and Mahaiwon Shadang contributed to arrangement, conduction of the work, collection and analysis of all the data, and writing and editing of the manuscript including all pictures. Qulsum Akhter contributed to coordination of the research and editing. Mithilesh Kumar contributed to helping in editing of the manuscript. Saumyaranjan Mallick contributed to helping with the specimen collection and H-scoring. Ajay Gogia provided all the clinical case details and follow-up data. Nilima Nilima helped with statistical analysis. Shyam S. Chauhan contributed to coordination of research and design. Riyaz A. Mir contributed to design, writing, editing, and revision of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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ORIGINAL ARTICLE

Metallothionein levels in gingival crevicular fluid, saliva, and serum of smokers and non-smokers with chronic periodontitis

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Abstract

Background: Metallothionein (MT), a cysteine rich protein is involved as a radical scavenger in several pathological conditions associated with oxidative stress; however, its role in periodontal disease still remains elusive. The aim of this cross-sectional study is to determine the serum, saliva and gingival crevicular fluid (GCF) levels of MT in smokers (S) and non-smokers (NS) with chronic periodontitis (CP), and compare them with those of periodontally healthy (PH) individuals.

Methods: A total of 85 participants were enrolled: 45 patients with CP (23 S [CP+S] and 22 NS [CP+NS]) and 40 PH individuals (20 S [PH+S] and 20 NS [PH+NS]). In all the study participants, clinical periodontal parameters (plaque index, gingival index, sulcus bleeding index, probing depth, and clinical attachment level) were recorded and samples of serum, saliva and GCF were collected. Enzyme-linked immunosorbent assay was used to determine the levels of MT in the samples.

Results: All periodontal clinical parameters were significantly higher in the CP groups as compared to PH groups ($P < 0.05$). MT levels in CP+S group were significantly raised in comparison to other three groups. There was no statistically significant difference in MT levels among CP+NS and PH+S groups ($P > 0.05$); however, relatively higher levels were observed in GCF and saliva in CP+NS group. When all the study groups were observed together, MT levels were positively correlated with clinical parameters.

Conclusions: Results of present study suggest that smoking and CP can induce the synthesis of MT owing to increased oxidative stress and heavy metals intoxication. Further longitudinal studies with large sample size and an interventional arm are needed to substantiate the role of MT as a potential biomarker in periodontitis.

KEYWORDS
antioxidants, chronic periodontitis, gingival crevicular fluid, metallothionein, serum, smoking

1 | INTRODUCTION

Reactive oxygen species (ROS) are produced as a result of normal cellular metabolism by the cells of innate immune system (polymorphonuclear leukocytes and monocytes) through oxygen-dependent mechanisms.[1] These include oxygen-derived free radicals (superoxide [O_2^-], hydroxyl [OH^-], and nitric oxide [NO]) and non-radical derivatives of oxygen (hydrogen peroxide [H_2O_2] and hypochlorous acid [HOCL]). ROS are highly toxic to the microbes internalized through phagocytosis and therefore play an important role in host defense against the pathogens.[2] However, extracellular release of ROS because of their increased production at the sites of chronic inflammation results in damage to surrounding tissues.[1]

Periodontitis is a chronic inflammatory disease associated with release of inflammatory cytokines from host cells and subsequent overproduction of ROS resulting in breakdown of periodontal tissues in a susceptible host.[3] In physiological conditions, there is a dynamic equilibrium between ROS activity and antioxidant defense mechanisms that may be disturbed by different factors such as smoking.[4,5] Smoking is a major environmental risk factor for the development and progression of periodontitis.[5] Smokers demonstrate an increased prevalence and more rapid destruction of alveolar bone than non-smokers.[6]

Cigarette smoke consists of a mixture of about 7,000 chemical compounds in tar and gas phase.[7] Cadmium is also present in substantial amounts (1 to 3 μg per cigarette) in tobacco smoke.[8] Smoking exerts its deleterious effects by impairing the host response and through direct effect of cigarette constituents on periodontal tissues.[9–11] Cigarette smoke also results in the release of potentially damaging ROS (O_2^-, H_2O_2, and OH^- radicals) via stimulation of neutrophil oxidative bursts.[11]

Antioxidant enzymatic and non-enzymatic systems prevent tissue damage from free radicals. The non-enzymatic radical scavenger systems include reduced glutathione, ceruloplasmin, albumin, bilirubin and trasferrin. Glutathione peroxidase (G-Px), superoxide dismutase (SOD) and catalase (CAT) constitute antioxidant enzyme system. SOD catalyzes O_2^- into H_2O_2 by reduction which is then transformed into water (H_2O) and oxygen (O_2) by G-Px and CAT. G-Px also reduces organic peroxides into their corresponding alcohols while oxidizing glutathione.[12] However; there is no specific antioxidant mechanism against the hydroxyl radicals which are the most potent oxygen free radicals. In last few years, role of metallothionein as radical scavenger has captured wide attention because the rate constant for its reaction with hydroxyl radicals in vitro is very high and it scavenges these radicals through a mechanism that could involve metal loss and sulfhydryl oxidation.[13]

Metallothionein (MT) is a non-enzymatic, intracellular low molecular weight (6.8 kDa), heat stable protein of 61 to 68 amino acids characterized by high cysteine (30%) and metal (7 to 12 metal atoms per molecule) content.[14] Other characteristic features are absence of disulfide bond and aromatic amino acids. It is induced in response to various stimuli such as metals, cytokines, free radicals, glucocorticoids, catecholamines, and interferons.[14] Broadly, four mammalian MT isoforms have been identified. MT-1 and MT-2 are major isoforms and are present in almost all types of tissue. These isoforms are mainly involved in much diverse functions of essential metal (Zn, Cu) homeostasis, protection against heavy metals (Cd, Hg), and oxidative stress.[14] MT-3 and MT-4 are expressed as minor isoforms in neurons and differentiating stratified squamous epithelial cells, respectively.

MT has been implicated in protection against the damaging effects of ROS in some inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and atherosclerosis, which share a pathogenesis and cytokine profile similar to periodontitis. Elevated levels of metallothionein have been found to be associated with certain pathogenic stages of RA.[15] MT overexpression has been observed in intestinal epithelial cells of patients with IBD (ulcerative coloitis and Crohn's disease) and correlated significantly with the grade of inflammation.[16] MT immunoreactivity within smooth muscle cells in atherosclerotic plaque further suggests the role of MT in protecting these active matrix-producing cells.[17]

Levels of different local and systemic oxidative stress-related biomarkers in chronic periodontitis (CP) have been widely studied[18,19] but the role of MT as an antioxidant is still obscure. In this context, Katsuragi et al. investigated the expression and distribution of MT in gingival tissues of smokers and non-smokers with advanced periodontitis using monoclonal antibody and immunohistochemical staining.[20] Results of this study demonstrated that smokers had a higher MT-positive cell ratio in the prickle cell layer of gingival epithelium compared with non-smokers, suggesting the possible role of MT in defense against free radicals in smoker's gingiva.

Previous studies have demonstrated the raised levels of MT both in smokers[21,22] and inflammatory diseases.[15–17,20] Although smoking is a well-established risk factor for periodontitis, correlation of periodontitis and MT levels in blood and oral fluids in smokers is still unknown. We hypothesized that tobacco smoking can increase the MT levels in patients with periodontitis and controls as compared to the non-smoker counterparts. Hence, the present study was planned to assess the levels of MT in serum, saliva, and gingival crevicular fluid (GCF) of periodontally healthy and CP patients with respect to their smoking behavior (smokers and non-smokers).

2 | MATERIALS AND METHODS

2.1 | Study population and experimental design

The present study was conducted as a comparative cross-sectional study. A total of 146 participants were screened from the patients referred for the diagnosis and treatment of periodontal disease to the Division of Periodontics, Centre for Dental Education and Research, All India Institute of Medical Sciences, New Delhi, India from September 2018 to December 2019. The study protocol was reviewed by Institute Project Review Committee and ethical clearance was obtained from Institutional Ethics Committee (IEC-271; Project code: A-511) for Human Subjects, AIIMS, New Delhi in July 2017. Each participant provided a signed informed consent before participating in the study. Principles outlined in the Declaration of Helsinki, as revised in 2013, were followed.

After reviewing the medical and previous dental history, patients with any of the following conditions were excluded: (1) presence of any systemic disease; (2) use of antibiotics, antioxidants, anti-inflammatory drugs or any other medication that can impact the periodontal status in previous 6 months; (3) alcoholics; (4) history of any periodontal therapy within the preceding 3 months; (5) regular use of antiseptic mouthrinses; (6) pregnancy and/or lactation.

It was a prerequisite for the participants to have ≥ 20 teeth in their mouths to be included in the study. For inclusion of patients in CP groups, they should have teeth with 30% periodontal bone loss, presence of at least two non-adjacent sites per quadrant with probing depth (PD) ≥ 5 mm and bleeding on probing.[23] According to 2017 World Workshop Classification of Periodontal and Peri-implant Diseases and Conditions, these patients could be classified into Stage II or Stage III, Grade B or Grade C, and generalized periodontitis.[24] Individuals were diagnosed as periodontally healthy (PH) if they had no attachment loss and periodontal disease history, full-mouth bleeding scores < 10% and a probing depth ≤ 3 mm in all teeth. Patients who were currently smoking for > 5 years were included in smoker group. Smoking status was determined based on the number of cigarettes smoked per day as reported by the study participants and were categorized as: light smokers (≤ 10 cigarettes/day), moderate smokers (> 10 cigarettes/day but less than or equal to 20 cigarettes/day) and heavy smokers (> 20 cigarettes/day).[25] Those who claimed to have never smoked were assigned to the non-smoker groups. Former smokers and individuals using tobacco products in any other form along with cigarettes were not included in the study.

In 2017 classification, as the risk stratification is established on well-validated risk factors such as smoking, thus, taking this grade modifier into consideration, the participants were categorized in following groups: smokers with CP (CP+S), non-smokers with CP (CP+NS), PH smokers (PH+S) and PH non-smokers (PH+NS).

After the first screening, 61 subjects were excluded from final sample as they did not meet the inclusion criteria ($n = 52$), refused to participate ($n = 4$), or did not attend the first appointment ($n = 5$). Thus, a total of 85 individuals i.e., 23 CP+S, 22 CP+NS, 20 PH+S and 20 PH+NS were finally included in this study.

2.2 | Periodontal clinical examination

Plaque index (PI),[26] gingival index (GI),[27] sulcus bleeding index (SBI),[28] probing depth (PD), and clinical attachment level (CAL) were recorded as clinical periodontal parameters for all the teeth present except third molars. Digital pantomographs were taken to determine the level of alveolar bone destruction in patients with chronic periodontitis. PI and GI were recorded at four sites whereas PD and CAL were measured at six sites (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual) of the teeth with a periodontal probe* and values were rounded up to nearest millimeter. Average scores of all the clinical parameters were calculated separately for each participant. All the clinical parameters were recorded by a single blinded calibrated investigator (VSY). Intra-examiner reproducibility of probing measurements was assessed with calibration exercises on ten patients before commencement of study. Intra-examiner agreement exhibited kappa values of 0.87 and 0.84 for PD and CAL measurements, respectively.

2.3 | Collection and preparation of samples

All of the samples were collected in the morning following an overnight fast and after 48 hours of recording the clinical parameters. Participants were instructed not to brush their teeth, eat or drink anything except water at that morning.

2.4 | Saliva sampling

Participants were asked to rinse their mouth with water, and unstimulated whole saliva was collected after

* UNC-15, Hu-Friedy Mfg. Inc, Chicago, IL, USA

10 minutes by passive drooling method. Patients were asked to sit upright and accumulate the saliva in the floor of mouth for 5 minutes, and then expectorate into a polypropylene tube. Three milliliters of the collected saliva was transferred into the Eppendorf tubes to standardize the volume collected for each patient. The samples were then centrifuged[†] to remove the cell debris at 4000 × g at 4°C for 10 minutes. Supernatants were stored at -80^0 C until analysis.

2.5 | Venous blood sampling

Five milliliters of blood sample was collected in plain tube without clot activator[‡] from the antecubital vein using a standard venipuncture method. The blood sample was allowed to clot at room temperature for 2 hours and then serum was separated by centrifugation at 1000 × g for 15 minutes. The serum was transferred to sterile Eppendorf tubes and stored at -80°C until the day of laboratory analysis.

2.6 | GCF sampling

GCF samples were collected from four different interproximal sites of teeth in periodontally healthy and CP patients. Periodontal sites for GCF collection exhibited PD ≥ 5 mm, CAL ≥ 4 mm, GI score > 1 and presence of BOP in periodontitis subjects and PD ≤ 3 mm, GI score 0 and absence of BOP in PH individuals. At the sites of sample collection, supragingival plaque was removed by sterile curets, isolated with cotton rolls and slightly air-dried to avoid contamination. A sterile standardized paper strip[§] was gently inserted into the periodontal pocket/gingival sulcus until mild resistance was felt in an attempt to minimize the mechanical trauma of gingival tissues. Strips were kept in place for 1 minute to collect sufficient quantity of GCF for MT estimation.[29] Paper strips contaminated with blood were discarded and collection was repeated after 30 minutes. All the four strips were placed in an Eppendorf tube containing phosphate buffered saline (400 μL, pH 7.2). The tubes were then vortexed[**] and homogenized for 1 minute, and centrifuged[††] at 3,000 × g at 4°C for 15 minutes. Supernatants were collected and stored at -80°C for MT analysis.

2.7 | MT assay

Saliva, GCF, and serum metallothionein levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using commercial kit[‡‡] according to the manufacturer's instructions. The kit is intended for measuring MT in serum, plasma, cell culture supernates, urine, saliva, and tissue homogenates. The detection range was 6.25 to 400 pg/mL. The concentration of MT in each of the samples was estimated by comparing the average sample optical density readings with the concentrations from the assay standard curve as per the given protocol. However, if the sample generated results higher than the highest standard value, standardization for the optimal sample dilution was used and results were reported considering multiplication with the intended dilution factor. Thus, a sample dilution of 1:10 was optimally standardized for serum and GCF and by convention; results were expressed in ng/mL for serum and GCF and as pg/mL for saliva. A single blinded biochemist evaluated all the biochemical analysis (RY*).

2.8 | Statistical analysis

Sample size was calculated using power calculations with statistical software[§§]. Assuming Cohen's fixed effect size to be 0.5 with α (alpha) error 0.05 and power of 0.80 with allocation ratio of 1, sample size calculated was 19 per group. Normality of data distribution was assessed using Shapiro-Wilk test. As the data showed vigorous non-normal distribution, non parametric statistics were applied. Descriptive analysis was done and all the continuous periodontal clinical and biochemical parameters in serum, saliva and GCF were expressed as median values and categorical variables were presented as frequencies.

Chi- square statistics were applied to assess the significance of difference of gender distribution among groups. Differences among various groups were determined using Kruskal-Wallis test followed by multiple pair-wise comparisons with post-hoc Dunn's test for determining the groups leading to differences. Each individual was considered as the unit for statistical analysis. Correlation between various clinical parameters and serum, saliva and GCF MT levels was determined using Spearman's correlation coefficient. All statistical analyses were two-tailed, with a P value of $< .05$ as a threshold for significance. A statistical software[***†††] was used for data analysis.

[†] Refrigerated Sigma 2-16KL Centrifuge, Germany
[‡] BD Vacutainer Tube, Becton-Dickinson and Company; Franklin Lakes, NJ
[§] PerioPaper, Oraflow, Smithtown, NY
[**] Fisherbrand, Digital Vortex Mixer, Germany
[††] Sigma 1-14K Refrigerated Microcentrifuge, Germany

[‡‡] Cusabio Human Metallothionein ELISA Kit, Wuhan, China
[§§] G* Power v.3.0.10, Heinrich-Heine-Universität Düsseldorf Düsseldorf, Germany
[***] SPSS, v.19.0 for Windows, IBM, Chicago, IL
[†††] Excel, Microsoft, Redmond, WA

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TABLE 1 Demographic characteristics for all groups

Characteristics	CP+S	CP+NS	PH+S	PH+NS	*P* value
Age (years)	45.18 ± 5.16[a]	42.51 ± 6.50[a]	37.4 ± 5.07	34.65 ± 6.87	.001
Sex (M/F)[b]	18/5	13/9	15/5	13/7	.488
Smoking (cigarettes/day)	22.37 ± 8.05	–	15.7 ± 6.24	–	–
Light smokers	3	–	2	–	–
Moderate smokers	11	–	15	–	–
Severe smokers	9	–	3	–	–
Smoking duration (years)	14.92 ± 5.09	–	12.5 ± 5.19	–	–

[a]Significant difference compared with PH+S and PH+NS.
[b]Chi-square test.

TABLE 2 Comparison of clinical periodontal parameters among four groups (Median [Min-Max])

Clinical Parameter	CP+S	CP+NS	PH+S	PH+NS	*P* value
PI	1.93 (1.00-2.80)[a]	1.81 (0.40-2.75)[a]	0.98 (0.24-2.11)	0.49 (0.11-1.67)	<.001
GI	1.68 (0.83-2.78)[a]	1.87 (0.49-2.72)[a]	0.61 (0.08-1.86)	0.89 (0.33-2.02)	<.001
SBI	2.06 (0.97-2.85)[a,b]	2.68 (1.01-3.66)[a]	0.78 (0.21-1.87)	0.96 (0.52-1.97)	<.001
PD	3.86 (1.87-7.10)[a]	3.81 (2.67-6.80)[a]	1.78 (0.78-2.65)	1.55 (0.46-2.08)	<.001
% sites with PD 1-3 mm	45.79	52.67			
% sites with PD 4-5 mm	33.63	26.11			
% sites with PD ≥6 mm	20.58	21.22			
CAL	4.96 (1.27-7.90) [a]	4.33 (2.33-6.56) [a]	1.76 (0.07-3.20)	1.34 (0.53-2.12)	<.001
% sites with CAL 1-2 mm	13.29	29.92			
% sites with CAL ≥3 mm	86.71	70.08			

[a]Significant difference compared with PH+S and PH+NS.
[b]Significant difference from CP+NS.

3 | RESULTS

A total of 85 individuals (59 males and 26 females; age range: 30 to 60 years; mean age: 40.67 ± 6.98 years) were subjected to the estimation of MT levels in serum, saliva and GCF.

3.1 | Participant characteristics and clinical parameters

The demographic data of age, gender and smoking status of the study participants are summarized in Table 1. Patients with CP were significantly older than PH individuals. Regarding gender distribution, number of female participants in smoker groups (CP+S and PH+S) was less in comparison to males. CP groups demonstrated significantly higher values of periodontal clinical parameters than those of PH groups ($P < .001$). All the clinical parameters except SBI did not differ significantly among both the CP groups, though the values of clinical variables (PI, PD, CAL) were higher in CP+S group (Table 2).

3.2 | Laboratory findings

The median values of MT levels in serum, saliva and GCF among all the four groups are shown in Figure 1.

Serum MT levels: PH+NS demonstrated significantly lower levels than in CP groups and PH+S ($P < .001$). No marked difference was found between CP+NS and PH+S groups ($P > .05$). Among CP groups, significantly higher levels were observed in smokers (CP+S).

Salivary MT levels: A trend similar to the serum MT levels was observed. Significantly raised levels were found in CP+S compared to CP+NS and PH individuals ($P < .001$). Levels in CP non-smokers and PH smokers did not differ markedly. Among PH groups, smokers (PH+S) exhibited significantly increased levels ($P < .05$)

GCF MT levels: CP smokers showed significantly increased levels than other three groups ($P < .001$). Levels in CP+NS were higher than PH individuals; however, the difference was statistically significant only with PH+NS. Comparison between PH individuals yielded no significant differences, though the levels were higher in smokers.

A comparative evaluation of MT levels in serum, saliva and GCF between participants of CP+S and other groups

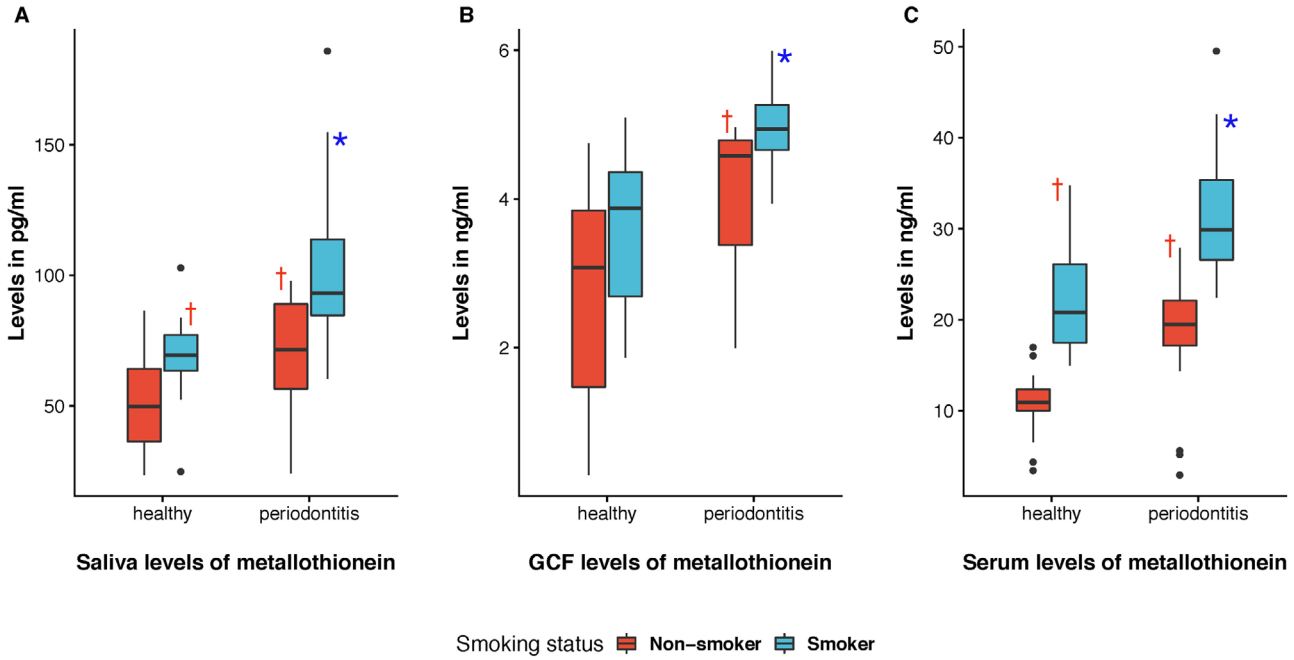

FIGURE 1 Levels of MT in (**A**) saliva (**B**) GCF, and (**C**) serum of periodontally healthy (PH) and chronic periodontitis (CP) smokers (S) and non-smokers (NS). *Statistically significant difference compared with CP+NS, PH+S, and PH+NS.[†]Statistically significant difference compared with PH+NS

TABLE 3 Comparative evaluation of CP+S with other groups for MT levels in serum, saliva, and GCF

	CP+NS		PH+S		PH+NS	
Parameter	**Mean Difference**	***P* value**	**Mean Difference**	***P* value**	**Mean Difference**	***P* value**
Serum	12.988	.001[a]	9.537	.001[a]	20.69	.001[a]
Saliva	30.88	.001[a]	32.27	.001[a]	51.47	.001[a]
GCF	0.976	.001[a]	1.49	.001[a]	2.39	.001[a]

[a]Statistically significant.

revealed a significant difference ($P = .001$). Higher mean values were found in CP+S group in comparison to other groups (Table 3).

Correlation between clinical parameters and serum, saliva and GCF MT levels was analyzed (pooled data). A positive significant correlation was found between MT levels in serum, saliva and GCF and clinical periodontal parameters (Table 4).

4 | DISCUSSION

Quantification of MT in body fluids may be a useful biological marker in individuals exposed to oxidative stress and heavy metals intoxication. The present cross-sectional study determined the levels of MT in serum, saliva and GCF of smokers and non-smokers with chronic periodontitis by sandwich ELISA as it is the simple and convenient method.[21,30] Moreover, ELISA measures both major isoforms (MT-1 and MT-2) of protein; it was assumed that the determined levels are depictive of total MT.

The levels of MT were significantly raised in investigated biofluids of chronic periodontitis (CP+S and CP+NS) patients and periodontally healthy smokers (PH+S) in comparison to control subjects (PH+NS). There is sufficient direct and indirect evidence to support these observations. Because both periodontitis and smoking are associated with overproduction of oxygen free radicals and release of pro-inflammatory cytokines;[1,5] these stimuli have been reported to increase the synthesis of MT[14] and therefore may contribute to its raised levels. Moreover, smokers are exposed to high amounts of cadmium[8,31] and several chemical compounds (hydrocarbons, volatile aldehydes, phenols, alkylating agents, nitric oxide etc.)[7] which are potent inducers of MT.[14] A significantly higher plasma concentration of MT in smokers has also been observed in

TABLE 4 Correlation among clinical parameters and MT levels in serum, saliva and GCF (r [*P* value])

Parameter	Serum MT	Saliva MT	GCF MT
PI	0.534 (.000)	0.542 (.000)	0.543 (.000)
GI	0.269 (.013)	0.336 (.002)	0.375 (.000)
SBI	0.340 (.001)	0.395 (.000)	0.424 (.000)
PD	0.481 (.000)	0.511 (.000)	0.593 (.000)
CAL	0.543 (.000)	0.614 (.000)	0.585 (.000)
Serum MT		0.646 (.000)	0.604 (.000)
Saliva MT			0.567 (.000)

r: Spearman's correlation coefficient.

previous studies.[21,22] These findings suggest that tobacco smoking and/or chronic inflammation influence the extracellular (serum, saliva, and GCF) concentration of MT. Further it should be noted that smokers with CP (CP+S) showed a significantly higher concentration of MT in serum, saliva, and GCF than PH+S and CP patients. This can be attributed to the cumulative effect of the raised oxidative burden and heavy metal toxicity because of these two conditions. Similar results of increased MT expression in gingival tissues of smokers compared to non-smokers with advanced periodontitis have been reported in a previous study.[20]

Increased expression of MT in response to heavy metal intoxication is well documented[21,22] but recent studies have focused on its potential role as a radical scavenger evident by increased expression in various chronic inflammatory diseases such as RA,[15] IBD[16], atherosclerosis,[17] and pulmonary diseases (asthma, oxidative and lipopolysaccharide related lung injury).[32] In present study, raised levels of MT in CP+NS further support the role of MT in oxidative stress induced by chronic inflammatory disease per se periodontitis. Several direct and indirect mechanisms have been proposed by which ROS induce the synthesis of MT, however exact mechanism is still unclear. One possible mechanism is that ROS generated during inflammatory course increases MT transcriptional responses in a dose-dependent manner through metal regulatory transcription factor 1 (MTF-1) which in the presence of Zn binds to the metal-responsive element (MRE) in the promoter region of the MT gene to initiate its transcription.[33] Nucleotide sequences other than MREs have also been found to respond to hydrogen peroxide.[34] Another possible pathway may involve the events associated with various second-messenger protein kinase pathways.[35] In addition, Chan and Weiss suggested ROS (singlet oxygen [$^1O_2$], O_2^-, OH^-) as direct inducers of MT synthesis.[36]

A close observation of the results shows higher levels of MT in GCF and saliva of CP+NS in comparison to PH+S, though the difference was non-significant. These results can be explained by the pathogenic mechanism of periodontitis. The pocket epithelium and subjacent connective tissue is densely infiltrated with leukocytes, primarily neutrophils, and monocytes. Further progression of inflammatory process is associated with lymphocytic predominance (~ 40% of the inflamed periodontal tissue volume) in the underlying connective tissue.[37] These cells and their products pass through the pocket epithelium into periodontal pocket. Because MT is synthesized in the nucleated blood cell such as peripheral blood lymphocytes and monocytes,[38,39] it is hypothesized that increased levels of MT in GCF and subsequently in saliva of CP patients is the result of their increased release from the inflammatory cells in inflamed periodontal tissues and reflects the local tissue activity. Assessment of GCF levels of MT may reflect the ongoing periodontal tissue destruction in periodontitis.

There is abundant literature on deleterious effects of smoking on periodontium.[5,9–11] Although exact mechanism of tissue damage is not fully understood, depletion of antioxidant enzymes in response to smoking induced oxidative stress may be one of the mechanisms.[40] SOD is a primary antioxidant enzyme containing metal ions (Zn^{2+}, Cu^{2+}, Mn^{2+}) in active center and involved in sequestration of superoxide radicals. There is strong evidence to show reduction in levels of SOD in a dose-dependent manner in smokers[4,29], though conflicting data exists. Cadmium in tobacco smoke displaces the bivalent metals resulting in conformational changes and inhibition of enzyme activity.[41] Research has shown that lack of expression of Cu^{2+}/ Zn^{2+} SOD is associated with an increased synthesis of MT as a compensatory mechanism.[22,42] Although SOD levels were not determined in present study, it is reasonable to assume that accentuated levels of MT in smoker groups can be attributed to decreased or inhibited SOD activity, suggesting the critical role of MT as an antioxidant in extracellular environment. Further research evaluating the levels of both MT and SOD in smokers and non-smokers with CP should validate this mechanistic hypothesis.

It has been suggested that endotoxins induce MT synthesis through cytokines such as IL-1, IL-6 and TNF-α.[43] Transforming growth factor- α[44] and macrophage-derived heat-stable protein factor[45] released from macrophages

may also be involved in endotoxin induced MT synthesis. An increased plasma MT concentration has also been reported in response to circulating endotoxins.[46] Studies have shown that pro-inflammatory bacterial components such as endotoxins (lipopolysacchrides) are released in to the blood stream from periodontal pocket and this resultant endotoxemia is directly correlated to the extent and severity of periodontitis.[47] These evidences, though indirect, further supports the increased levels of MT in patients with CP.

A positive correlation was observed between MT levels and clinical parameters of periodontal disease activity. These correlations suggest that local and systemic levels of MT are indicative of inflammatory burden in periodontal tissues and therefore can emerge as a novel oxidative stress biomarker in periodontitis.

In present study, participants were not age and gender matched in all groups. This may be related to the low prevalence of female smokers ($\leq$ 4%) and higher prevalence of periodontitis in aged population in our country.[48,49] It has been shown that MT levels are raised with increase in age.[50] Further studies in periodontitis affected individuals are warranted to establish the age-related differences in MT levels. Further it should be noted that method of ELISA used and sensitivity of the different commercially available ELISA kits may also influence the results.

Oxidative stress, smoking and periodontal disease severity are strongly related. In future studies, MT levels can be investigated independently in large group of patients presenting with different stages and grades of periodontitis. A potential limitation of the study is that smoking status of the participants was based on the self-reported smoking behavior rather than estimating the serum and/or salivary cotinine levels. Further research should correlate MT levels with cotinine and a well-established marker such as high sensitivity C-reactive protein (hsCRP) to better define its role in relation to smoking status and periodontal disease. Moreover, by knowing the effects of smoking cessation and certain surgical or non-surgical procedures to alleviate the inflammatory changes in terms of improvements in the levels of MT in cases of CP and smokers would further consolidate the effectiveness of such treatments in relieving oral co-morbid pathologies.

5 | CONCLUSIONS

Within the limitations, results of present study suggest that both smoking and periodontitis are associated with an increase of MT levels in serum, saliva and GCF in a cumulative and independent manner. Additional studies with a large sample size and an intervention arm are needed to further substantiate the role of MT in periodontal disease. Future research aimed at exploring the potential of MT as a reliable biomarker in periodontitis could expand the horizons of periodontal diagnostic tools.

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AUTHOR CONTRIBUTIONS

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