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Treponema denticola chymotrypsin-like proteinase is present in early-stage mobile tongue squamous cell carcinoma and related to the clinicopathological features

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Background: Certain periodontopathogenic bacteria have been linked to cancers. *Treponema denticola* (*Td*) is associated with severe periodontitis. Chymotrypsin-like proteinase (CTLP), a major virulence factor of *Td*, can degrade various host proteins and peptides, and modulate inflammatory responses. However, the role of *Td* in the tongue carcinogenesis remains unknown. This study aimed to investigate the presence of *Td*-CTLP in early-stage mobile tongue squamous cell carcinoma (MTSCC) and its relation to clinical and pathological characteristics.

Methods: The immunopositivity of *Td*-CTLP was assessed in samples obtained from 60 patients with MTSCC and associated with their clinicopathological data. Additionally, *Td*-CTLP expression was compared with immunoexpression of matrix metalloproteinases (MMP-8 and MMP-9), toll-like receptors (TLR-2, TLR-4, TLR-7 and TLR-9), c-Myc, Ki-67, Bmi-1 and Snail.

Results: *Treponema denticola*-chymotrypsin-like proteinase was present in 95% of MTSCC tumours of which many (40.4%) showed high immunopositivity. *Td*-CTLP positivity was significantly associated with invasion depth, tumour diameter and the

Sorsa and Hagström equally contributed to this study.

expression of TLR-7, TLR-9 and c-Myc. High *Td*-CTLP immunopositivity in younger patients (≤ 60 years old) predicted early relapse.

Conclusion: Our data indicate that *Td* and its CTLP are present in early-stage MTSCC carcinoma and may contribute to carcinogenesis, and therefore provide novel perspectives into intervention and therapeutic measures of MTSCC.

KEYWORDS

chymotrypsin-like proteinase, matrix metalloproteinase, mobile tongue squamous cell carcinoma, toll-like receptor, *Treponema denticola*

1 | INTRODUCTION

Squamous cell carcinoma (SCC) of the head and neck region is the sixth most common cancer globally. Its incidence and mortality have increased steadily worldwide. SCC can emerge in any anatomical site of the head and neck region. Tongue squamous cell carcinoma is the most common cancer of the oral cavity. Despite advancements in cancer therapies and treatment protocols, the prognosis of the MTSCC has remained relatively poor with 5-year survival rate presenting as 62%.[1] Therefore, there is a need for studies that explore the pathogenesis of MTSCC and clarify the phenomena behind it.

Periodontitis, chronic inflammatory disease of the tooth-supporting tissues, is driven by dysbiotic oral microbiome. Without treatment, it can cause severe and even fatal systemic implications. Various studies have linked periodontitis with several types of cancers.[2] Certain pathogenic bacteria in the oral microbiome, the so-called periodontopathogens, have a key role in the pathophysiology of periodontitis. In addition to chronic periodontal inflammation, these bacteria have been associated with carcinogenesis.[3] The strongest evidence has been established on *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.[4] *Treponema denticola* (*Td*) is a periodontopathogen and the most common spirochaete in the oral microbiome that is associated with aggressive forms of periodontal disease.

Together with *Porphyromonas gingivalis* and *Tannerella forsythia*, *Td* forms the so-called red complex that increases the severity of tissue destruction in periodontal disease. *Td* has plethora of virulence factors that enhance its capability to neutralise the host defence systems, penetrate the tissue, stimulate inflammatory processes and cause tissue destruction.[5] Chymotrypsin-like proteinase (CTLP) is one of the most well-studied factors. *Td*-CTLP is a membrane-bound proteinase with a multitude of functions. It promotes *Td* adherence to epithelial cells, and it exerts cytotoxicity by degrading the link between actin cytoskeleton and cell membrane and through interference of intracellular signalling.[6] *Td*-CTLP is a catalytically competent proteolytic enzyme that can fragment various extracellular matrix proteins as well as activate human pro-matrix metalloproteinases. Therefore, *Td*-CTLP can contribute to periodontal tissue invasion and thus may have a role in tumour invasion.

The literature exploring the presence and role of *Td* in carcinogenesis is still limited. Previously, the presence of *Td* in oesophageal and oral SCC has been shown,[7,8] and recently, our group demonstrated the presence of *Td*-CTLP in various oral and gastrointestinal malignancies ex vivo with critical immunomodulatory and tissue-destructive mechanisms of *Td*-CTLP in vitro.[9]

Matrix metalloproteinases (MMPs) are one of the key players contributing to cancer progression. MMPs can promote angiogenesis, tumour growth and metastasis by influencing tumour environment. Toll-like receptors (TLRs) can regulate the expression of certain MMPs. TLRs are the most important pattern recognition receptors of innate immunity and are able to recognise microbial agents as well as endogenous macromolecules released by injured tissue. Expression of TLRs and their role in various tumours have been reported by numerous studies.

Bmi-1 is a key epigenetic regulator involved in cell cycle regulation, immortalisation and senescence. It is overexpressed in various cancers and indicated to play an important role in cancer initiation and progression and to correlate with poor prognosis. Bmi-1 overexpression in oral cancer has been shown to influence cancer cell replication and survival.[10] In addition, c-Myc proto-oncogene has a similar function as Bmi-1 in cancer cells and is assumed to participate in oral carcinogenesis.[11] Snail, a transcription factor, is able to downregulate the expression of cell adhesion and basement membrane proteins, and it is essential for epithelial-mesenchymal transition. It is expressed in several types of cancers of the head and neck.[12]

Considering the virulence properties of *Td*-CTLP and the recent data of its immunomodulatory activities and presence in cancer tissues, we aimed to explore its expression in mobile tongue squamous cell carcinoma (MTSCC) and to examine its correlation with various clinicopathological characteristics of these tumours. In addition, we also correlated *Td*-CTLP expression with the expression of MMP-8, MMP-9, TLR-2, TLR-4, TLR-7, TLR-9, c-Myc, Ki-67, Bmi-1 and Snail studied and reported before.[13-15] We hypothesised that *Td*-CTLP is present in MTSCC and the degree of its expression is related to deep invasion and poor prognosis.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Clinicopathological data of 141 patients with MTSCC treated at Helsinki University Hospital between 1992 and 2002 have been

described in more detail previously.[14] Altogether, 73 patients fulfilled the inclusion criteria defined as T1 or T2, N0 with a clinical follow-up data of at least 24 months or until death. The 5-year survival rate of the patients was 62%. Tumour samples from 60 patients with early-stage MTSCC (30 males and 30 females, median age 60 years, range 23-95) with clinically T1N0M0 or T2N0M0 disease treated with curative intent were available for immunohistochemistry. According to clinical staging, 24 (40%) tumours were classified as T1 and 36 (60%) as T2. The neck lymph node status was confirmed using different combinations of clinical and radiological approaches, including palpation, computed tomography (CT), ultrasonography, magnetic resonance imaging and fine-needle aspiration biopsy (microscopically). All patients were clinically staged N0.

After histopathological evaluation, 40 (67%) cases were classified as pT1 and 20 (33%) as pT2 tumours. Thirty-seven (62%) patients had elective neck dissection, and 15 of these had pathological node positivity. Demographic data of the patients are presented in Table 1.

TABLE 1 Demographic and clinicopathological features of 60 patients with early-stage mobile tongue squamous cell carcinoma

Clinicopathological variable	No. of patients (%)
Sex	
Male	30 (50)
Female	30 (50)
Age, y	
≤60	32 (53)
>60	28 (47)
Range	23-95
Median	60
Clinical T class (mm)	
cT1 (≤20)	24 (40)
cT2 (21-40)	36 (60)
Pathological T class (mm)	
pT1 (≤20)	40 (67)
pT2 (21-40)	20 (33)
Pathological node positivity[a]	
N0	22 (37)
N+	15 (25)
Stage (TNM)	
I	34 (57)
II	11 (18)
III	12 (20)
IV	3 (5)
Grade	
I	19 (32)
II	30 (50)
III	11 (18)
Invasion depth (mm)	
≤4	18 (30)
>4	42 (70)

T, tumour; N, nodal; c, clinical; p, pathological.
[a]37/60 (62%) of the patients had elective neck dissection.

All patients in this present study were treated according to the Finnish national guidelines for the treatment of head and neck cancer. Patients with deeply invasive tumours and nodal metastases received post-operative radiation therapy. Consequently, 22 patients received no further primary treatment for the neck (follow-up only). Elective neck dissection was performed on 37 patients, and 1 patient received radiotherapy without surgery. Thirty patients received post-operative radiotherapy, including the neck area in 29 patients. Twenty-two patients (37%) had occult neck metastases, including lymph node metastases in the elective neck dissection specimen (n = 15) and neck metastasis during follow-up without failure at the primary site (n = 7). Ten patients had local recurrence during follow-up. Two patients were diagnosed with distant metastases, both after locoregional recurrence. Median follow-up time was 7.02 years (range 0.33-17.24), during which 18 patients died of tongue cancer and 19 of other causes. National agency of population statistics, Statistics Finland, provided the dates and causes of death. Three different tissue areas of the tumour (surface, centre and invasive front) were taken from each patient and placed into microarray paraffin blocks. The study design complied with the Declaration of Helsinki and was approved by the Ethics Committee of Helsinki University Hospital.

2.2 | Immunohistochemical analysis

Tissue microarray slides of 4 μm thickness were immunohistochemically stained as described previously.[9,13,15] The specific antibodies used are represented in Table S1. Negative control was performed with non-immune species-specific immunoglobulin or by omitting the primary antibody. Periodontitis tissues verified positive for *Td* by PCR were used as positive controls.

Immunostainings were evaluated by 2 independent observers with single-blind method. The percentage of the positive *Td*-CTLP tumour cells in each cylindrical TMA was graded as 0 (negative), 1 (low, up to 29%), 2 (moderate, 30%-49%), 3 (high, 50%-80%) and 4 (strong, over 80%) (Table 2). MMP-8, MMP-9, TLR-2, TLR-4, TLR-7, TLR-9, c-Myc, Ki-67, Bmi-1 and Snail were scored as described in previous studies.[13-15] The highest score from 3 different tissue areas in each patient was used. In the survival analysis, negative to mild positive *Td*-CTLP immunoreactivity (score 0-2) was regarded as low immunopositivity, whereas moderate to high positive *Td*-CTLP (score 3-4) as high immunopositivity. Furthermore, we analysed the association between the immunopositivity of *Td*-CTLP and the clinicopathological data and tumour-related protein markers.

2.3 | Statistical analysis

IBM SPSS Statistic 21 software was used for data analysis. Cohen kappa coefficient was used to analyse interobserver scoring agreement in immunostaining evaluation. The association between immunopositivity score of *Td*-CTLP with clinicopathological variables

TABLE 2 Association of *Td*-CTLP immunoexpression with demographic and clinicopathological features of early-stage mobile tongue squamous cell carcinoma

Variables	N	*Td*-CTLP immunoexpression					*P*-value ($P < .05$)	Correlation coefficient rs
		Negative	1	2	3	4		
All cases	60	3	9	9	16	23		
Age, y (mean)	60	67.33	57.44	51.33	60.94	64.26	.281	0.141
Sex	60						.669	0.056
Location	60						.285	−0.14
Upper surface	1	0	0	0	0	1		
Tip	2	0	0	1	1	0		
Edge	51	2	8	7	13	21		
Lower surface	6	1	1	1	2	1		
Grade	60						.213	−0.163
I	19	0	1	4	5	9		
II	30	1	6	4	10	9		
III	11	2	2	1	1	5		
Node positivity (pN)	37						.750	0.054
N0	22	0	4	5	4	9		
N+	15	2	1	1	4	7		
Invasion depth, mm (mean)	60	6	5.97	5.98	6	9.07	.01*	0.331
Tumour diameter, mm (mean)	60	17.67	9.89	14.33	14.44	18.3	.019*	0.302
MMP-8, %	60						.065	0.240
0 = no positivity	1	0	0	0	1	0		
1 = up to 10% (low)	3	1	1	1	0	0		
2 = 11%-50% (moderate)	6	0	1	2	2	1		
3 = 51%-90% (high)	28	1	3	4	10	10		
4 = over 90% (strong)	22	1	4	2	3	12		
MMP-9, %	60						.123	0.202
0 = no positivity	8	0	1	5	1	1		
1 = up to 10% (low)	20	1	5	1	6	7		
2 = 11%-50% (moderate)	27	1	3	3	6	14		
3 = 51%-90% (high)	5	1	0	0	3	1		
4 = over 90% (strong)	0	0	0	0	0	0		
TLR-2 cytoplasmic, intensity	58						.877	−0.021
0 = negative	2	1	0	1	0	0		
1 = mild	11	0	1	3	1	6		
2 = moderate	23	1	4	3	6	9		
3 = strong	22	1	4	2	7	8		
TLR-2 nuclear, %	58						.530	0.084
0 = no positivity	1	0	0	1	0	0		
1 = up to 10% (low)	1	1	0	0	0	0		
2 = 11-50% (moderate)	2	0	0	1	0	1		
3 = 51-80% (high)	3	0	1	0	0	2		
4 = over 80% (strong)	51	2	8	7	14	20		
TLR-4 cytoplasmic, %	60						.731	−0.045
0 = no positivity	1	0	0	1	0	0		
1 = up to 10% (low)	4	0	0	2	0	2		
2 = 11%-50% (moderate)	1	0	0	0	0	1		

(Continues)

TABLE 2 *(Continued)*

Variables	N	*Td*-CTLP immunoexpression					*P*-value ($P < .05$)	Correlation coefficient rs
		Negative	1	2	3	4		
3 = 51%-80% (high)	15	1	2	2	4	6		
4 = over 80% (strong)	39	2	7	4	12	14		
TLR-7, %	60						.021*	0.297
0 = no positivity	4	1	0	2	1	0		
1 = up to 10% (low)	17	2	2	4	4	5		
2 = 11%-50% (moderate)	28	0	5	2	10	11		
3 = 51%-80% (high)	7	0	1	1	1	4		
4 = over 80% (strong)	4	0	1	0	0	3		
TLR-9, intensity	60						.013*	0.320
0 = negative	2	1	0	0	1	0		
1 = mild	18	2	4	4	3	5		
2 = moderate	35	0	5	4	11	15		
3 = strong	5	0	0	1	1	3		
c-Myc cytoplasmic, %	60						.033*	0.276
0 = no positivity	16	1	3	6	9	4		
1 = up to 29% (low)	23	2	5	2	5	17		
2 = 30%-49% (moderate)	9	0	1	0	1	1		
3 = 50%-80% (high)	10	0	0	1	0	1		
4 = over 80% (strong)	2	0	0	0	0	0		
Ki-67, %	60						.896	−0.017
0 = no positivity	0	0	0	0	0	0		
1 = up to 29% (low)	12	0	2	2	6	2		
2 = 30%-49% (moderate)	21	0	1	5	5	10		
3 = 50%-80% (high)	13	1	3	1	4	4		
4 = over 80% (strong)	14	2	3	1	1	7		
Bmi-1, %	60						.974	0.009
0 = no positivity	12	1	0	1	5	5		
1 = up to 29% (low)	22	1	4	6	3	8		
2 = 30%-49% (moderate)	13	1	3	1	4	4		
3 = 50%-80% (high)	9	0	2	1	2	4		
4 = over 80% (strong)	4	0	0	0	2	2		
Snail, %	60						.062	0.243
0 = no positivity	0	0	0	0	0	0		
1 = up to 29% (low)	0	0	0	0	0	0		
2 = 30%-49% (moderate)	3	0	0	3	0	0		
3 = 50%-80% (high)	17	1	2	3	8	3		
4 = over 80% (strong)	40	2	7	3	8	20		

Td-CTLP, *Treponema denticola* chymotrypsin-like proteinase; N, nodal; MMP, matrix metalloproteinase; TLR, toll-like receptor.
Spearman's analysis, $^*P \leq .05$.

(age, sex, tumour location, grade, tumour size, node positivity and invasion depth) and with previously studied markers (MMP-8, MMP-9, TLR-2, TLR-4, TLR-7, TLR-9, c-Myc, Ki-67, Bmi-1 and Snail) was assessed using Spearman's correlation coefficient. Univariate analyses on the 3 reliable prognostic factors for overall survival and one for cancer-specific survival were performed using Cox regression. Kaplan-Meier estimator was used to analyse the relationship between the immunopositivity of *Td*-CTLP and the survival rate of patients with MTSCC, and this relationship was further analysed by log-rank test. As the sample size in the group under 40 years old is

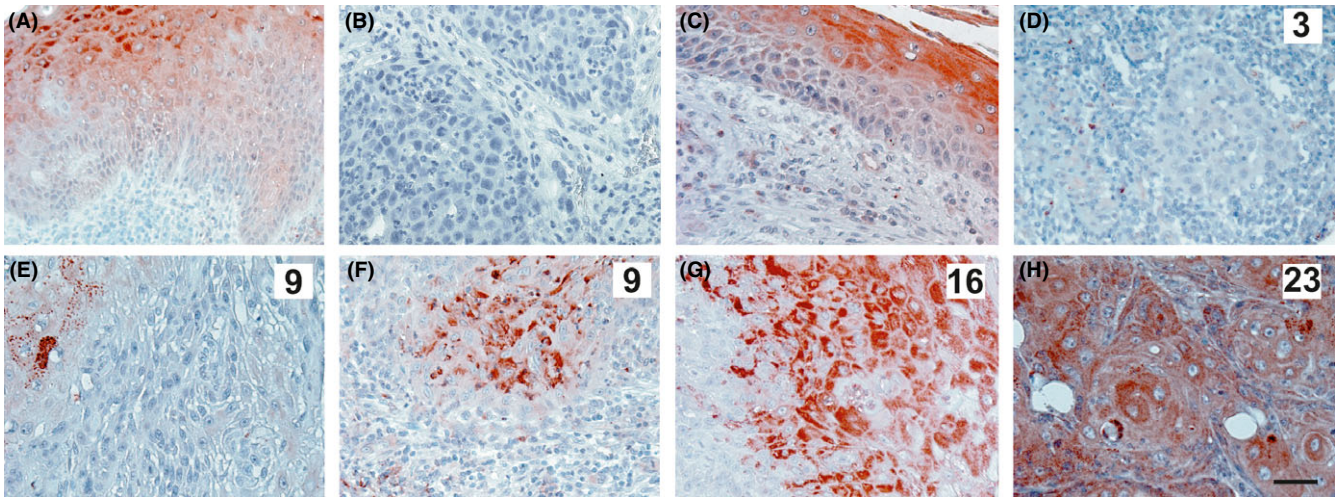

FIGURE 1 Immunoexpression of *Td*-CTLP. Positivity shown in the epithelium of periodontitis gingival tissue (positive control tissue; A), in the morphologically normal epithelium adjacent to tumour site (B), in the non-orodigestive tissue (C) and in tumour site of early-stage MTSCC (D-H). Immunohistochemical stainings of the tumour tissues were scored as (D) negative 0%, (E) very mildly positive <30% (scored 1), (F) mildly positive 30%-50% (scored 2), (G) moderately positive 50%-80% (scored 3) and (H) strongly positive >80% (scored 4). The number of cases for each level of positivity is presented at the upper right. 3-amino-9-ethylcarbazole (AEC) was used as a chromogen (red) and haematoxylin as a counterstain. All red-stained areas on each tissue section indicate specific detection of *Td*-CTLP. Magnification 200×. Scale bar is 50 μm, relevant for all panels

TABLE 3 Univariate analysis of overall survival and cancer-specific survival

	Overall survival			Cancer-specific survival		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Invasion depth (for 1 mm increase)	1.041	0.974-1.113	.233			
Tumour diameter (for 1 mm increase)	1.051	1.001-1.102	.045*			
Td-CTLP immunoexpression	1.190	0.605-2.340	.614	1.786	0.637-5.011	.271

*$P < .05$.

very low, we chose to use 60 years old as a cut value for age and divided the samples into 2 groups (≤60 years old as younger and >60 as older group) to make the study group size reliable to statistical analyses for survival. Survival time was calculated from (i) primary surgery to death for any reason (overall survival, OS), (ii) primary surgery to death from MTSCC (disease-specific survival, DSS) and (iii) primary surgery to local, locoregional or distant recurrence (disease-free survival, DFS).

3 | RESULTS

Majority (95%) of tumour tissues (57 of 60 patients) were positive for *Td*-CTLP, and 40.4% (23 of 57 patients) of these positive tissues had high immunopositivity (Figure 1). There was a strong agreement in immunopositivity scoring between the 2 observers ($\kappa = 0.818$, $P < .0005$) in all 60 patient samples. Immunopositivity of *Td*-CTLP was present in the cytoplasm of neoplastic epithelial cells, and granular pattern was observed. Additionally, certain leucocytes showed *Td* immunoexpression in the cytoplasm and some positive signals were overlapping with nucleus. Furthermore, part of the normal epithelium adjacent to the tumour site expressed also *Td*-CTLP (Figure 1).

3.1 | Association between Td-CTLP immunopositivity and clinicopathological variables of MTSCC and tumour-related markers

Treponema denticola-chymotrypsin-like proteinase immunopositivity showed significant positive correlation with invasion depth ($P = .01$) and tumour size ($P = .019$) (Table 2). There was no association between *Td*-CTLP and the following clinicopathological characteristics: age, sex, tumour grade, nodal metastasis or tumour localisation. Most of the MTSCCs (51 of 60 patients) that localised to the lateral side of tongue showed high *Td*-CTLP positivity. When comparing the immunopositivity of *Td*-CTLP with other MTSCC-related protein markers, we found a significant positive correlation with TLR-7, TLR-9 and cytoplasmic c-Myc. However, no significant correlation was found between the immunopositivity of *Td*-CTLP and the markers MMP-8, MMP-9, TLR-2, TLR-4, Ki-67, Bmi-1 or Snail (Table 2).

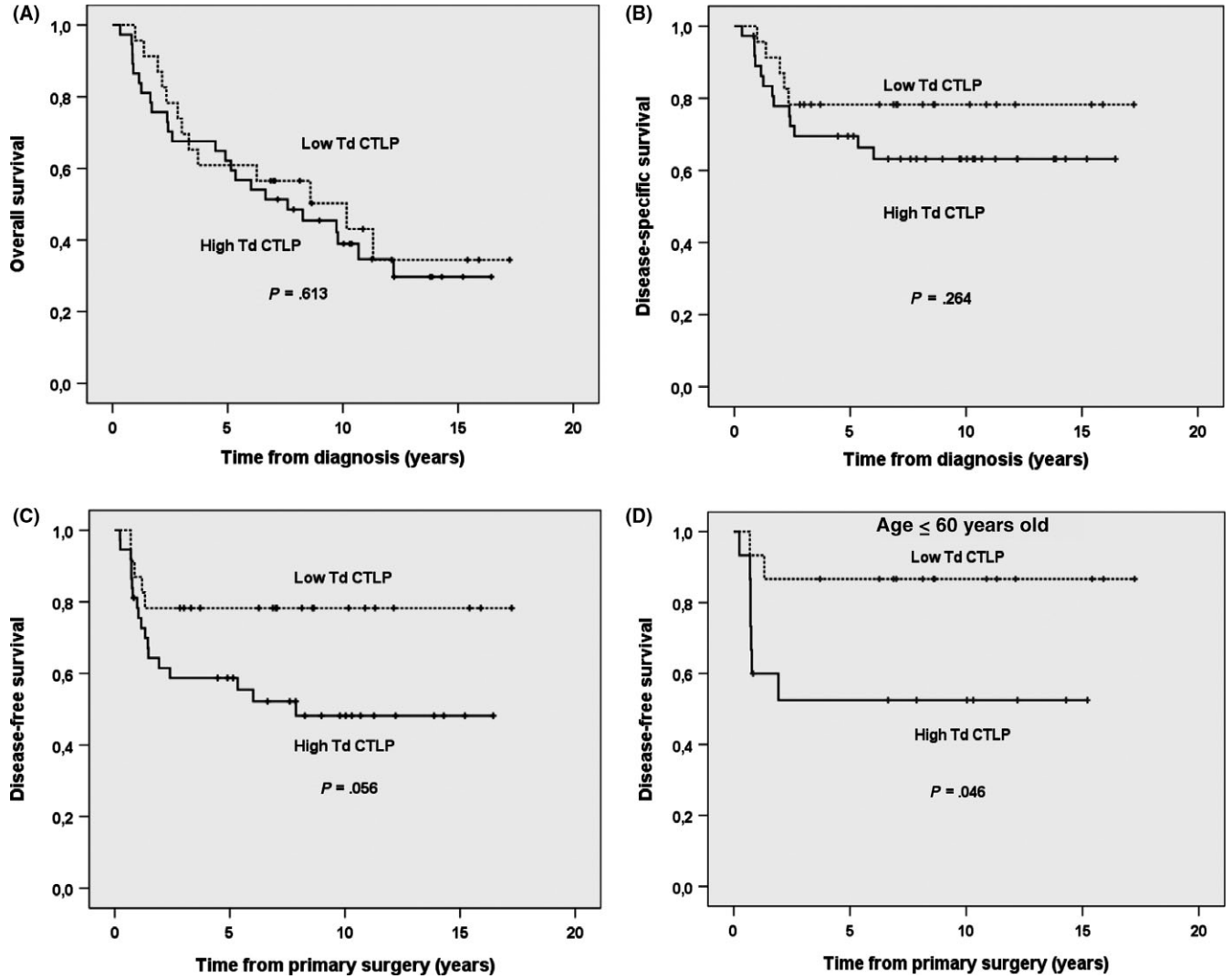

FIGURE 2 Kaplan-Meier analysis for survival of patients with early-stage MTSCC with either low or high *Td*-CTLP immunoexpression. (A) Patients with early-stage MTSCC with high immunopositivity of *Td*-CTLP had shorter overall survival outcome ($P > .05$), (B) shorter disease-specific survival outcome ($P > .05$) and (C) shorter relapse time ($P > .05$). (D) The relapse time of the patients with early-stage high immunopositivity MTSCC ≤ 60 years old at diagnosis was significantly shorter compared with low immunopositivity patients ($P < .05$)

Univariate analysis showed only one significant predictor for overall survival (tumour diameter); therefore, the multivariate model was not further analysed (Table 3).

3.2 | Td-CTLP immunopositivity in MTSCC and patient survival

Immunopositivity of *Td*-CTLP did not correlate with OS, DSS or DFS (Figure 2A,B,C) in Kaplan-Meier survival analysis, nor with pT-stage. However, in patients under 60 years of age, high *Td*-CTLP expression correlated with shorter DFS (log-rank, $P = .045$) (Figure 2D).

4 | DISCUSSION

In this study, we examined the potential link between the presence of the periodontopathogenic bacterium *Td* and early-stage MTSCC. *Td* is anaerobic and highly motile spirochaete, which is difficult to isolate and cultivate due to its low oxygen tolerance and complex nutritional requirements. Therefore, it has been often overlooked in scientific experiments and studies. Due to these difficulties, we used *Td*-CTLP immunoexpression to detect *Td* presence and localisation in the tissue as *Td*-CTLP is a membrane-bound virulence factor, and its existence represents the location of *Td*. CTLP is expressed by *Td* during tissue and cell invasion. However, the main intracellular biological action of *Td*-CTLP is not yet completely recognised, but it is presumed that it affects the protein synthesis of the cell.[16]

We analysed early-stage MTSCC samples from 60 patients and found that majority of the tumours were strongly positive for *Td*-CTLP, a large spectrum proteinase and critical virulence factor of *Td*. Furthermore, we investigated the association between the expression of *Td*-CTLP and different clinicopathological characteristics. Our data indicate that *Td*-CTLP immunopositivity significantly correlates with invasion depth and tumour size. Moreover, we found that high

Td-CTLP immunoexpression correlated with shorter relapse time in patients under the age of 60. Our findings provide evidence that *Td*-CTLP is present in MTSCC and indicate its potential role in tongue cancer. In addition, our findings support and further extend the various reports that show a link between periodontopathogenic bacteria and oral cancer.

Chymotrypsin-like proteinase is an outer membrane-associated proteolytic enzyme of *Td*. This proteinase has been associated with promotion of *Td* attachment to epithelial cells, degradation of host proteins and protease inhibitors and activation of latent proMMPs, thus facilitating *Td* migration through the epithelium down to the basement membrane.[6,9] The role of *Td* and *Td*-CTLP in cancer may be related to its ability to cause structural damage to epithelial cells[6] and to activate mitogen-activated protein kinase (MAP kinase) signalling pathways controlling cell proliferation and survival,[17] as well as to immunomodulation.[9]

The high prevalence and high immunopositivity of *Td*-CTLP in patients with MTSCC in this study may indicate a potential contribution of *Td*-CTLP to MTSCC. Majority of the tumours with *Td*-CTLP expression were located in the edge of the tongue. This region comes into frequent contact with the dentogingival area, the place for accumulation of dental biofilm containing periodontopathogenic bacteria including *Td*. This may aid *Td* translocation from dentogingival region into tongue epithelium. To strengthen our findings, recent article by Shin et al[8] examined the microbiome of head and neck tumours and discovered significantly higher abundance of *Treponema* species in the primary tumour samples of the oral cavity in comparison with healthy samples.

Tumour size (diameter) and invasion depth are risk factors for metastasis. A significant positive correlation between the presence of *Td*-CTLP with invasion depth and tumour diameter observed in this study may indicate contribution of *Td* to tumour aggressiveness. *Td* via its CTLP is capable to degrade various type of proteins and cause activation or inactivation of tissue regulatory proteins. Activation of tissue-destructive MMPs might allow the cancer cells to invade deeper into tissues and even metastasise via lymphatic or blood vessels. At moderate concentration, *Td* infection leads to epithelial cell proliferation and survival through activation of ERK1/2.[17] The significant positive correlation between *Td*-CTLP and c-Myc expression supports these findings as c-Myc has been reported to activate cell proliferation and to immortalise epithelial cells.[18,19] In contrast, *Td*-CTLP did not significantly correlate with the proliferation marker Ki-67 expression.

The correlation of the *Td*-CTLP immunopositivity with other markers linked to MTSCC reported previously by our group showed varying results. No correlation between *Td*-CTLP immunopositivity and MMP-8 or MMP-9 expression was found, although it has been reported that *Td*-CTLP can activate proMMP-8 and proMMP-9 and that these MMPs could serve as prognostic markers in oral SCC.[20] Previously, TLR-2, TLR-4 and TLR-9 but not TLR-7 have been reported to have a correlation with deeper tumour invasion in MTSCC.[14] We found a significant association between *Td*-CTLP and TLR-7 and TLR-9 expressions. Supporting our finding, TLR-9 expression has previously been reported to associate with tumour size in MTSCC.[21] Expression of TLR-9 in cancer is related to its role in cell proliferation and differentiation, and TLR-9 stimulation may lead to tumour progression and increasing cell survival.[22] Both TLR-7 and TLR-9 are cytoplasmic/endosomal receptors activated by viruses or intracellular bacteria such as *Td*. Furthermore, TLR-9 promotes cellular migration via upregulating MMP-2 and increase invasiveness in oral cancer,[23] while *Td*-CTLP may have the same impact via MMP-8 and MMP-9.[9] Ligation of TLR-7 and TLR-9 induce proliferative and prosurvival effect on tumour cells.[24] However, previous studies on TLR-7, TLR-9 and other TLR's role in cancer have shown some opposite effects which are likely dependent on tumour microenvironment.[25]

Bmi-1, c-Myc and Snail are associated with the progression and prognosis in several malignancies.[26,27] In MTSCC, only Bmi-1 has been reported to correlate with recurrence, while Snail has been associated with histopathological grade and depth of invasion. Vora et al[28] showed patients with early-stage disease had higher expression of c-Myc compared to patients at more advanced stage. It is presumed that c-Myc takes part in early oral carcinogenesis. In our study, only cytoplasmic c-Myc correlated with *Td*-CTLP immunopositivity. In colon cancer, c-Myc has been suggested to induce cell survival and motility with further contribution to cancer progression and metastasis.[29] C-Myc expression in MTSCC may have a synergistic contribution with *Td*-CTLP to promote metastasis.

Previous study has reported that younger patients with MTSCC tend to have higher recurrence rate and highest recurrence death rate.[30] However, the underlying factors that determine this difference between young and old patients are largely unknown. Therefore, more concern should be paid to the MTSCC in younger patients to understand better the possible factors behind these tumours and find better prognostic markers in this patients group. Our results propose a role for *Td*-CTLP as a prognostic marker to predict relapse time of MTSCC in younger patients. In addition, for younger patients, the usual causative factors (ie alcohol and tobacco) may not be so significant. Therefore, the presence and actions of certain microbes may play more significant role.

Treponema denticola infection could act as an aetiologic factor in MTSCC and provide prognostic potential in the future. However, our study seems to be the first to show the presence of *Td*-CTLP in oral cancer. It would be important to have further studies with larger patient materials to validate our results.

In summary, our study brought evidence for the possible role of *Td*-CTLP in MTSCC. In addition, our data further support the link between periodontitis and/or periodontopathogenic bacteria and oral carcinogenesis potentially contributing to tumour growth and invasion. Future studies using cell culture and animal models are essential to further elucidate the contribution of *Td* and its virulence factor CTLP in MTSCC.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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