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Original Article

High expression of podoplanin in squamous cell carcinoma of the tongue occurs predominantly in patients ≤40 years but does not correlate with tumour spread

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Abstract

More than 30% of patients with squamous cell carcinoma (SCC) of the mobile tongue have clinically undetectable lymph node metastasis. Tumour cells can spread as single cells or collectively. A protein known to play a role in both processes is podoplanin, which is expressed in endothelial cells not only in lymph vessels but also in some aggressive tumours with high invasive and metastatic potential. Here we studied samples from 129 patients with primary SCC of the tongue for expression of podoplanin using immunohistochemistry. mRNA levels were analysed in another 27 cases of tongue SCC with adjacent clinically tumour-free tongue tissue and 14 tongue samples from healthy donors. Higher levels of podoplanin were seen in tumours compared to both normal tongue and clinically normal tongue in the tumour vicinity. No association was found between levels of podoplanin, presence of lymph node metastases or other clinical factors. Patients aged 40 or less were more likely to express high levels of podoplanin protein compared to older patients ($p = 0.027$). We conclude that levels of podoplanin in primary tongue SCCs are not associated with lymph node metastases. However, tongue SCCs arising in young patients (≤40 years of age) are more likely to express high levels of podoplanin than tongue SCCs that arise in the more elderly. The data suggest that podoplanin has a distinctive role in young patients, who are known to have a poor prognosis: these patients may, therefore, benefit from podoplanin inhibitory therapies.

Keywords: squamous cell carcinoma; tongue; podoplanin

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Introduction

The majority of intraoral squamous cell carcinomas (SCC) are located in the mobile tongue. Compared to tumours in other sites of the oral cavity, tongue SCCs more frequently metastasise to cervical lymph nodes and more than 30% of patients with tongue SCC have occult lymph node metastasis at the time

Table 1. Results from scoring of podoplanin expression and number of lymph vessels in relation to age of the patient, N-status and status at the end of the study

	Age group			N-status		Lymph vessels			Status	
Podoplanin	≤40	41–65	>65	N0	N+	0	1–2	3	ADF DDF	AWD DOD DWD
QS = 0	2 (11%)	2 (4%)	4 (6%)	6 (6%)	2 (6%)	5	3	0	4	4
QS = 1–5	2 (11%)	19 (40%)	34 (53%)	41 (43%)	14 (42%)	0	38	17	32	23
QS = 6–18	14 (78%)	26 (56%)	26 (41%)	49 (51%)	17 (52%)	0	56	10	32	34
129	18	47	64	96	33	5	97	27	68	61

QS = QuickScore (see text), ADF = alive disease free, DDF = dead disease free, AWD = alive with disease, DOD = dead of disease and DWD = dead with disease, but not as first cause of death.

of diagnosis, as confirmed by histology even if clinically undetectable [1]. The high frequency of spread to lymph nodes is due to the dense lymphatic network present in the tongue, with most of the lymph fluid draining to lymph nodes in the submental and submandibular triangles (level I) and the upper jugular nodal group (level II). This rich lymphatic network also has connections across the midline, explaining why nodal metastasis can occur on both sides of the neck when the tumour is located in the midline of the tongue [1]. The frequent spread to lymph nodes from the tongue emphasises the importance of early detection for successful treatment of tongue SCC.

Tumour cells can invade either as single cells or collectively. Epithelial-to-mesenchymal transition (EMT) is important in single cell invasion. During EMT, epithelial cells lose epithelial markers such as E-cadherin and instead acquire mesenchymal characteristics including a migratory phenotype [2]. The EMT process is regulated at the transcriptional, translational and post-translational levels [3]. The molecular and pathophysiological aspects of collective invasion are less well understood. A protein known to play a role in both EMT and collective invasion is podoplanin [4].

Podoplanin is a transmembrane glycoprotein involved in formation of lymph vessels [5]. Apart from being expressed in endothelial cells in lymph vessels, podoplanin can be found in, for example, osteocytes and basal keratinocytes [5] and also in some tumours with high invasive and metastatic potential, including squamous cell carcinomas of the head and neck (SCCHNs) [5]. SCCHNs with high levels of podoplanin were reported to show significantly higher rates of lymph node metastasis, irrespective of the number of lymph vessels [6]. This finding is of clinical relevance, as diagnostic biopsies are often of limited size and accordingly the number of lymph vessels can be hard to estimate, whereas expression of podoplanin in tumour cells can be easily calculated. Restriction of podoplanin expression to the periphery of tumour nests in SCCHN has also been shown to be an indicator of good prognosis [7]. Specifically, looking at tongue SCC, the majority of tumours studied so far show high expression of podoplanin. High podoplanin expression has also been seen in the basal layer of hyperplastic and dysplastic lesions adjacent to SCCHN and interpreted as overexpression occurring early in head and neck tumourigenesis [6].

Here, we studied the prognostic impact of podoplanin in a large group of 129 tongue SCCs. We also measured the number of lymph vessels in the biopsies to clarify their impact on the clinical course of this disease.

Material and methods

Material

Formalin fixed, paraffin-embedded biopsies from 129 patients with primary SCC of the tongue available at Umeå University Hospital and Naples, were included. Sixty-five were men and 64 women. The mean age was 63.4 years, ranging from 19 to 93 years. The project was approved by the local Ethical Committee (dnr 01-057, 03-201).

The majority of patient samples had previously been analysed for expression of p16 and HPV16 [8]. Follow up ranged between 1 and 179 months, with a mean of 46.6 months. All but seven patients had a follow up of 2 years or longer.

Status at the end of the study was staged as alive disease free, alive with disease, dead of disease, dead disease free or dead with disease, but not as first cause of death. Data on status were collected from the clinical files or, when not available there, from the Swedish Death Registry. For clinical data, see Table 1.

Immunohistochemistry and scoring

An antibody detecting podoplanin (D2-40; Abcam, Cambridge, UK) was diluted 1:25. Slides were pretreated in TRIS-EDTA pH 8.0, and staining was performed in a Ventana staining machine according to the supplier's recommendations. The percentage of tumour cells expressing podoplanin as well as the intensity of staining was assessed. The percentage of tumour cells expressing podoplanin was divided into six groups, where 0–4% = 1, 5–19% = 2, 20–39% = 3, 40–59% = 4, 60–79% = 5 and 80–100% = 6. Staining intensity in turn was described as negative = 0, weak = 1, intermediate = 2 or strong- = 3. By multiplying the score for percentage of podoplanin-expressing tumour cells with the score for staining intensity, a quick score (QS) was calculated for each slide [9]. Scoring of slides was performed independently by three of the authors (NS, ELJ and KN) and cases of disagreement were reevaluated and discussed to provide a consensus score.

The number of lymph vessels in each sample was scored between 0 and 3, with 0 being no lymph vessels detectable, 1 = few, 2 = moderate numbers and 3 = many lymph vessels detectable, respectively.

qRT/PCR

Twenty-seven tongue SCCs, of which 23 had paired clinically normal tumour-adjacent tissue samples and 14 tongue biopsies from healthy controls were included in the mRNA analysis. For example, in the case of a SCC on the left border of the tongue, clinically normal tumour-adjacent tissue was taken from the right side of the tongue. For clinical data on the tumours, see Table 2. Paraffin-embedded samples from 10 of these tongue SCC were included in the protein analysis. Samples previously analysed [10] had been homogenized in trizol and total RNA was extracted using either chloroform or an RNA/protein purification kit (Norgen). The remaining samples were homogenized in lysis buffer from All Prep DNA/RNA/miRNA Universal Kit (Qiagen) using Precellys (Bertin technologies). After dilution in water, no difference in RNA quality or yield was observed between the different methods according to measurement with nano-drop and bioanalyzer. For cDNA synthesis, 500 ng of total RNA was used with RevertAid H minus first strand cDNA synthesis kit (Thermo Scientific). cDNA was diluted 5× and 2.5 µl used in each reaction with a total reaction volume of 10 µl. For PCR amplification of cDNA, IQ sybr green supermix (Bio-Rad) was used in combination with primers for podoplanin (Bio-Rad assay ID qHSaCID 0009013) and reference primers: GAPDH, UBC, LAD1, RPS12 from PrimerDesign Ltd. For GAPDH and UBC the company does not give out sequences. LAD1 (For: CCTCCCACCCGTCACACT, Rev: CTGCTGTAGGTTCGCTGTGT), RPS12 (For: TGCTGCTGGAGGTGTAATGG, Rev:GCACACAA AGATGGGCTTGG.

Cycling conditions: enzyme activation at 95°C for 3 min, denaturation at 95°C for 15 s and annealing at 60°C for 60 s. The process was run for 40 cycles.

To normalize the values, a geometric mean from the reference genes GAPDH, UBC, RPS12 and LAD1 was used in the calculation of $2^{-\Delta Cq}$.

Table 2. Clinical data on 27 tumours included in the analysis of podoplanin mRNA

Nr	Sex	Age	Localisation	TNM
20	Male	61	1	T1N0M0
35*	Female	24	2	T2N0M0
40	Female	81	3	T4N2bM0
49*	Female	52	3	T4N2cM0
51*	Male	74	1	T2N0M0
56	Female	41	3	T2N2bM0
58*	Male	61	1	T1N0M0
59*	Female	68	1	T2N0M0
61	Male	70	3	T4aN0M0
65*	Female	81	3	T2N0M0
68	Male	62	1	T2N0M0
70*	Male	71	2	T1N0M0
73	Male	81	3	T4aN0M0
76	Male	59	3	T4aN0M0
79	Male	61	2	T1N0M0
82*	Female	19	1	T4N0M0
83*	Female	64	2	T1N0M0
85*	Female	87	1	T2N0M0
92	Female	63	2	T2N0M0
98	Male	31	3	T2N0M0
105	Male	64	2	T1N0M0
111	Female	31	2	T1N0M0
119	Male	66	2	T2N0M0
124	Male	54	3	T4N2bM0
131	Female	74	2	T2N0M0
137	Female	71	2	T2N0M0
138	Male	50	1	T2N1M0

For tumour localisation 1 = tongue, 2 = border of tongue and 3 = overgrowth into floor of mouth.
Tumours labelled with * also had paraffin samples included in the protein analysis. TNM = T(umour), N(odes), M(etastasis).

Statistical analysis

SPSS version 22 was used for statistical analysis, correlating results from scoring of podoplanin to clinical data. The Chi square test was used for calculation of p-values and a p-value <0.05 was considered statistically significant. Two- and 5-year survival were used in the survival analysis. Mann-Whitney test was used to analyse mRNA levels.

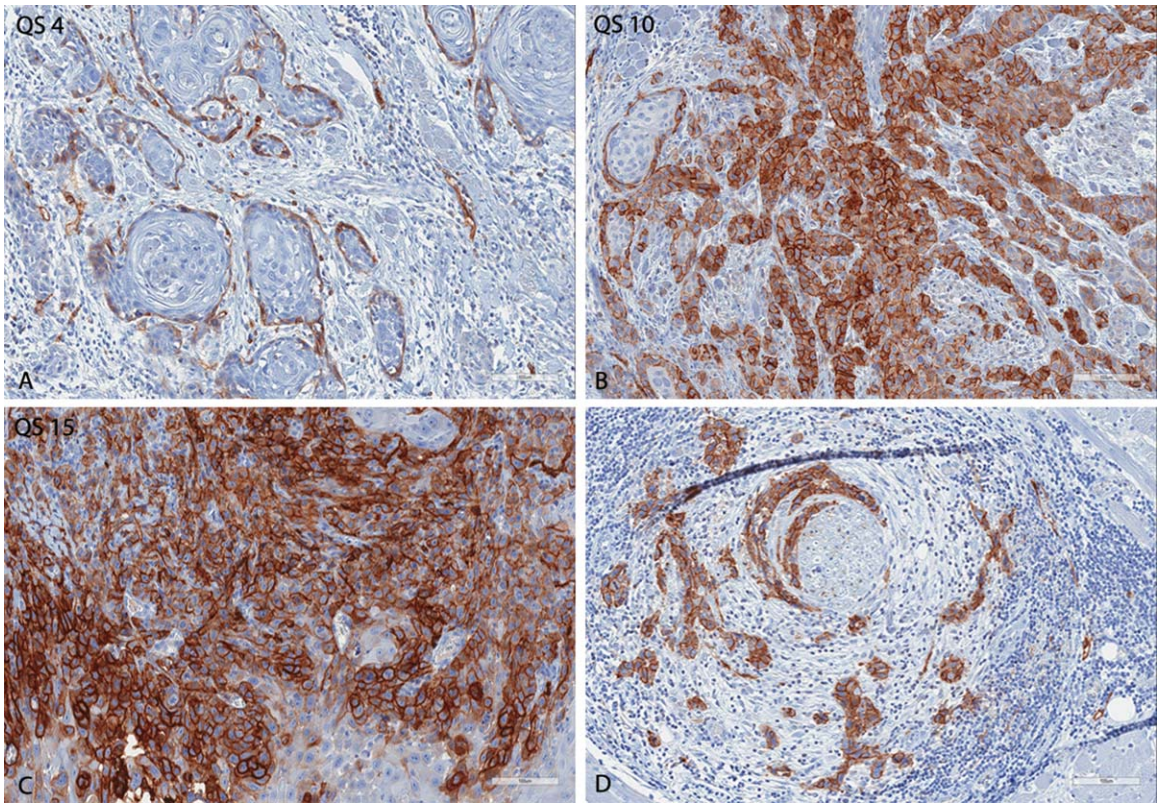

Figure 1. (A–C) Representative podoplanin immunohistochemistry in tongue SCC with different QuickScores (QS), as indicated. (D) Podoplanin-expressing tumour cells showing perineural growth.

Results

Clinical data

At 2-year follow up, which was available for 122 of the 129 patients studied, 75 were alive and 47 dead. Considering age, 56% of patients ≤40 years were alive 2 years after diagnosis, in contrast to 73% of patients aged 41–65, but similar to 54% of patients >65 years. At 5 years (follow up available for 101 patients of whom 48% were alive), 39% of patients ≤40 years were alive, in contrast to 65% of those aged 41–65 years but similar to 37% of patients >65 ($p = 0.009$). For status at the end of the study, see Table 1.

Expression of podoplanin

Of the 129 tongue SCC samples, 8 (6%) were negative for podoplanin, 55 (43%) showed low expression (QS 1–5) and 66 (51%) showed high expression (QS 6–18) (Figure 1). A significantly higher percentage of tumours in patients aged ≤40 years (78%) showed high expression of podoplanin (QS = 6–18), compared to 56% and 41% of the 41–65 and >65-year-old patients, respectively ($p = 0.027$) (Table 1).

Looking at the whole group of patients, there was no significant association between expression of podoplanin (QS), N-status, T-status, gender or localisation.

Lymph vessels were detectable in all but five samples, with 52% showing a moderate number. The five samples lacking detectable lymph vessels were also negative for podoplanin in the tumour tissue. Of the 94 samples with a lymph vessel score of 2 or 3, all but two expressed podoplanin. No significant association was seen between the presence of lymph vessels, age, gender, T- or N-stage or status at the end of the study (Table 1).

Levels of podoplanin mRNA

All 27 tumours analysed showed significantly higher levels of podoplanin mRNA compared to both healthy controls, and clinically normal tongue tissue adjacent to the tumours ($p < 0.0001$). In the group of tumour-adjacent tongue tissue, levels were also significantly higher compared to healthy normal tongue ($p = 0.005$). Inter-individual variation in podoplanin levels was seen in tumours, with the highest levels seen in two females, one 24 years old (no 35) and the other 41 years old (no 56) (Figure 2).

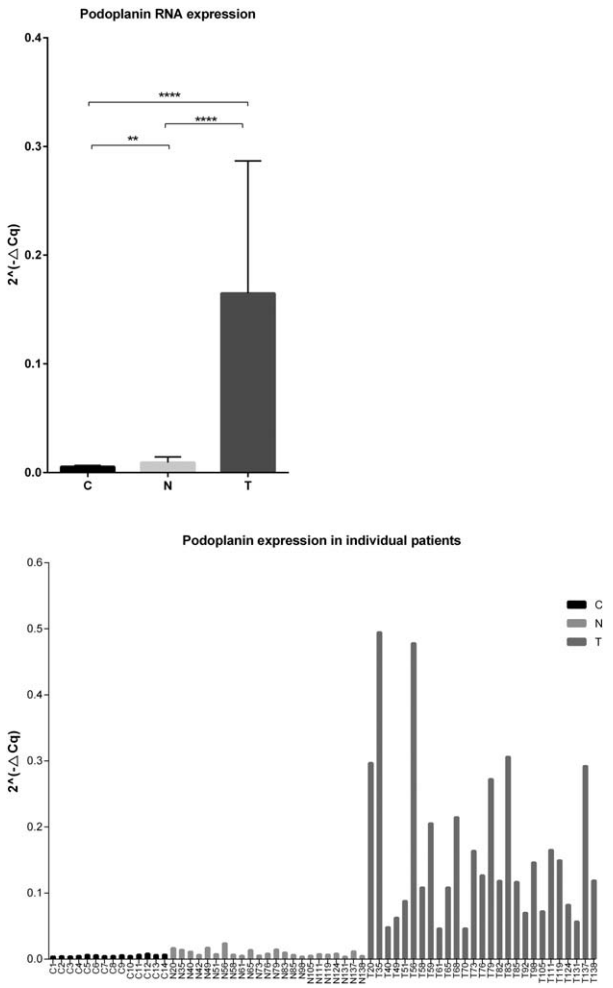

Figure 2. Top: Mean levels of podoplanin mRNA in 27 tongue SCCs (T), 23 matched clinically normal tongue samples adjacent to tumour (N) and 14 tongue samples from healthy controls (C). ****$p < 0.0001$, **$p = 0.005$. The levels of podoplanin mRNA in individual patients, matched clinically normal tongue adjacent to tumour and normal tongue from healthy controls are shown in the lower panel.

Discussion

Tongue SCC is a severe disease, which shows an increase worldwide, especially among young people and particularly women [11]. The disease may have an unpredictable outcome and the known worse prognosis for young patients [8,12] was also clearly seen in the present material, where only 39% of patients $\leq$40 years were alive after 5 years compared to 65% of those aged 41–65 years.

One of the biggest challenges with tongue SCC is that 30% of clinically node negative tumours have occult lymph node metastasis at diagnosis [1]. However, without performing a staging neck dissection it is not possible to pinpoint these 30%. The tongue, with its well-developed lymphatic network, is ideal for easy spread of tumour cells and, as N-status is a factor of known prognostic significance, it would be valuable to identify at the time of diagnosis factors that can aid in, or precede development of, nodal spread.

A factor of potential importance in this aspect is the lymph vessel-specific glycoprotein podoplanin. Previous results concerning its role in tumour spread are contradictory, where some claim that it induces EMT [13] whereas others instead show that it forms filopodia and in that way induces collective cell migration [14].

Podoplanin expression has been reported in mucosa adjacent to SCC [15] and this has been interpreted to indicate that the protein is expressed early in the tumourigenic process [16]. Our results from mRNA analysis support this interpretation as levels of podoplanin in clinically normal tongue adjacent to tumour were significantly higher than those in normal tongue taken from healthy volunteers with no evidence of SCC or other disease. Levels in tongue SCC were also significantly higher compared to both groups. At the protein level, there was no tendency for lowered expression in later stages of the tumourigenic process. On the contrary, 61% of N+ tumours showed high podoplanin expression, judged as QS 6–18, compared to 51% of N0 tumours. Although the number of cases are low, none of the eight podoplanin negative tumours was T4, whereas seven of them were T1 and T2.

Our figures for podoplanin expression with 6% negative, 43% low expressing (QS 1–5) and 51% high expressing (QS 6–18) tumours are comparable to the findings of Yuan and coworkers [6]. In contrast to their findings and also to another study [16], we found no association between high expression of podoplanin and lymph node metastasis or prediction of poorer outcome. In Yuan's material, 89% of the 28 N+ tongue SCC showed high levels of podoplanin, compared to 52% of our 33 N+ tumours. As the scales for evaluating percentage of podoplanin-expressing tumour cells differ between the two studies, it is not possible to fully compare these results. Bartuli *et al* [17] analysed a mixed group of 20 SCC, of which only 12 were located in the tongue compared to our group of 129 tongue SCCs. A limited group of 12 tumours may thus not be representative of the whole group of tongue SCCs.

As has been seen previously, patients $\leq$40 years are a specific group and, in this study, showed the significantly highest percentage of high podoplanin-expressing tongue tumours. Even if there was no difference in outcome for these young patients with

high podoplanin expression, it is noteworthy that this group, once again, differs from the majority of patients with tongue SCC, who are typically much older.

Concerning the number of lymph vessels, no association with any clinical factor was seen. It must, however, be emphasized that the calculation of lymph vessel number in diagnostic biopsy material is not optimal and not representative of all aspects of the whole tumour. Thus, the potential relevance of lymph vessel numbers cannot be excluded based on the present results.

In summary, we have measured podoplanin levels at both protein and mRNA level in tongue SCC and found significantly higher levels in tumours compared to normal tongue as well as to clinically normal tongue in the tumour vicinity. In contrast to other studies, we could not confirm the correlation seen between podoplanin levels and the presence or absence of lymph node metastases. However, we show that tongue SCC arising in patients $\leq$40 years of age has the significantly highest percentage of high podoplanin expressing tumours. The clinical value of this finding remains to be determined but suggests that targeting podoplanin with antibodies or lectins [18] could be particularly useful for the group of young patients with tongue SCC.

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Author contributions

NS collected samples, performed scoring, data analysis and writing of the manuscript; ELJ performed scoring; LB performed mRNA analysis; GC, LLM, GT, LC, GDO, RR, LL, MS, TW, KD and GL collected samples and clinical data; PJC, RF and KN supervised and coordinated the study and participated in writing of the manuscript.All authors were involved in writing the paper and had final approval of the submitted version.

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