

Antigen peptide transporters are upregulated in squamous cell carcinoma of the oral tongue and show sex-specific associations with survival

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Abstract. Transporter associated with antigen processing 1 (TAP1) and TAP2 serve pivotal roles in adaptive immunity. Tumor cells often show reduced antigen presentation on their surface as one mechanism to escape immune recognition. Whether downregulation of TAPs is a common mechanism of tumor immune evasion in squamous cell carcinoma of the oral tongue (SCCOT) is unclear. In the present study, samples from 78 patients with SCCOT and 17 patients with benign hyperplastic tongue lesions were analyzed for TAP1 and TAP2 expression by immunohistochemistry. The percentage of positive cells and staining intensity were scored. Associations with clinicopathological variables and survival outcome were also investigated. The results demonstrated that TAP1 and TAP2 levels were highly associated with each other in individual samples and were upregulated in SCCOT compared with benign lesions ($P < 0.001$). The proportion of TAP1- or TAP2-positive tumor cells was $>80\%$ in all but two of the tumors, whereas 25.6 and 23.0% of the tumors showed weak intensity of TAP1 and TAP2, respectively. There were no significant associations with clinicopathological variables or survival outcomes between TAP-intermediate/strong and TAP-weak tumors. However, in patients <70 years old and with early stage SCCOT, male patients had better outcomes than female patients (log-rank $P < 0.05$), and the best outcome was observed in male patients with intermediate/strong TAP expression. In conclusion, loss of TAP was not a frequent event in SCCOT and stronger TAP expression in male patients was

associated with improved survival, providing further evidence for sex-specific immune modulation in cancer.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) arises from the mucosal epithelium of the oral cavity, pharynx and larynx and is the most prevalent malignancy involving the head and neck region (1,2). The incidence of this cancer type is increasing globally and is expected to rise by 30% by 2030, corresponding to 1 million new cases annually (1). Despite improvements in treatment modalities resulting in improved local control, the long-term overall survival rates have only improved modestly over the last three decades (1,2).

Cancer is driven by the accumulation of mutations and subsequent clonal selection (3). Peptides derived from cancer-specific mutations can be loaded onto human leukocyte antigen class I molecules (HLA-I) and displayed on the cell surface as cancer antigens, or neoantigens. The recognition of neoantigens by CD8⁺ T-cells elicits an anticancer immune response. To evade immune recognition and destruction, tumor cells develop escape mechanisms that inhibit anti-tumor cell immunity, such as activating the PD-1/PDL-1 immunosuppressive system, altering the functions of T-cell-priming dendritic cells, depleting antigens, or downregulating the class I antigen presentation machinery (APM) (4). The APM consists of molecules involved in the multistep peptide-presentation process (5) and deficiency in APM components has been reported in many cancer types, including SCCHN. However, the frequency of APM component reduction varies with cancer type and between studies, ranging from 0 to 93% (6).

One of the most extensively studied APM components is the transporter associated with antigen processing (TAP) (7). TAP is a heterodimer comprised of TAP1 and TAP2, and is responsible for transporting short peptides from the cytosol into the endoplasmic reticulum, where together with other chaperones it forms the peptide loading complex. TAP is also involved in transporting peptides into phagosomes and endosomes during cross-presentation in dendritic cells (8). There is evidence that deficiency of TAP in tumor cells allows evasion

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of immune surveillance and increases tumorigenesis (9). In patients with SCCHN, loss or downregulation of TAP in tumor cells occurs with a frequency ranging from 28 to 71% for TAP1 and 9 to 88% for TAP2 (10-13). Limited and conflicting results have also been reported for TAP levels and clinicopathological factors or survival outcome. Ferris *et al* (14) showed that the frequency of APM defects in maxillary carcinoma is higher than that in laryngeal and tonsillar carcinoma. Therefore, it is likely that in addition to the differences in staining/scoring methods and/or the investigated patient cohort, the anatomic subsite of tumors also accounts for the varying frequencies of TAP defects reported.

SCC of the oral tongue (SCCOT) is the most common intraoral subsite for SCCHN. Previously, we showed that *TAP1* mRNA is progressively upregulated from control tissue to tumor-free tissue in SCCOT patients to SCCOT tumor samples (15). Notably, low levels of *TAP1* mRNA in tumor-free samples taken contralateral from the SCCOT associated with better patient survival, whereas no correlation to survival was seen for *TAP1* levels in tumors (15). Given that TAP is constitutively expressed and that levels are relatively high in many hematopoietic cells (6), our whole tissue-based findings are insufficient for evaluating the role of TAP in SCCOT cells for shaping immune evasion. As immunotherapy has emerged as a prominent treatment option (16,17), understanding immune evasion mechanisms is important for the design and selection of appropriate immune-restoring strategies. Therefore, we set out to measure protein levels of TAP1 and TAP2 in patients with SCCOT and benign tongue lesions to gain further insight into TAP expression in tumor cells to clarify whether downregulation of TAP is a frequently adopted strategy of tumor cells to reduce their neoantigen presentation.

Materials and methods

Patient material and ethical approval. The current study comprised tongue tissue samples from 95 patients, of whom 17 had benign hyperplastic lesions and 78 had SCCOT. Of the SCCOT patients, 37 were men and 41 women with a median age of 64 years at diagnosis (range 19-89 years). All patients were treated at Otorhinolaryngology and Head & Neck Surgery and Oncology at Umeå University Hospital in Umeå, Sweden. The clinicopathological characteristics of SCCOT patients are presented in Table I. The minimum follow-up time was 5 years for SCCOT patients with status 'alive disease-free'. Archived formalin-fixed, paraffin-embedded (FFPE) tissue from Clinical Pathology, Umeå University Hospital was used. The project was performed according to the principles of the Declaration of Helsinki after approval by the Regional Ethics Review Board, Umeå, Sweden (Dnr 03-201 and Dnr 08-003 M). Patient samples were anonymized and no written consent was required as archived tissue was used in this study.

Immunohistochemistry and scoring. Tissue sections (4 μ m) were stained with a TAP1 polyclonal antibody (1114-1-AP, Proteintech, Chicago, IL, USA) or a TAP2 polyclonal antibody (ASJ-BB69A9; Nordic BioSite, Sweden) at a dilution of 1:100 and 1:50 respectively in an automated Ventana Benchmark Ultra staining machine with the ultra-VIEW DAB Detection

Kit for visualization (Ventana Medical Systems, Inc., Tuscon, AZ, USA) according to the supplier's recommendations. Lymphocytes were used as internal positive controls. The stained samples were photodocumented using a Panoramic 250 Flash III scanner (3DHISTECK Ltd., Hungary).

The proportion of epithelial cells stained with each antibody was categorized into six groups: 1=0-4%, 2=5-19%, 3=20-39%, 4=40-59%, 5=60-79%, and 6=80-100%. Staining intensity was divided into four levels: 0=negative, 1=weak, 2=intermediate, and 3=strong. The QuickScore (18) was calculated by multiplying the proportion and the intensity scores (range 0 to 18). Cells were scored independently by three of the authors (N.A., X.G. and K.N.). Cases with discrepant scoring were discussed to provide a consensus score.

Worst pattern of invasion (WPOI) and lymphocytic response (LR) at the host/tumor interface were evaluated according to Brandwein-Gensler *et al* (19). WPOI was divided into five grades: 1=Broad pushing invasive front; 2=Broad pushing fingers; separate tumor islands; 3=Invasive islands (>15 cells/island); 4=Invasive islands (<15 cells/island), including single cell invasion; 5=Tumor satellites \geq 1 mm distance from tumor. LR was divided into three grades: 1=Continuous dense rim of lymphoid tissue; 2=Patches of discontinuous dense lymphoid infiltrate; 3=Limited or no response.

Statistical analysis. Correlations between categorized clinicopathological variables and categorized TAP levels were determined by Fisher's exact test. For continuous variables, correlations were studied using nonparametric Spearman correlation analysis and the correlation coefficient (ρ) was calculated to evaluate correlation strength. Nonparametric Mann-Whitney U test was used to study the difference between two groups of continuous variables. The Kaplan-Meier method with log-rank test was performed to estimate the impact of TAP levels on patient survival. Three survival measures were analyzed: overall survival (time from date of diagnosis to death from any cause), cancer-specific survival (time from diagnosis to death due to SCCOT) and disease-free interval (time from completion of treatment to date of relapse or death). All tests were conducted in IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). A two-sided P-value <0.05 was considered significant.

Results

TAP1 and TAP2 levels in SCCOT and benign lesions. Cytoplasmic TAP1 was detectable in all SCCOT specimens (n=78). The proportion of TAP1 expressing tumor cells was graded 6 (80-100% of the tumor cells expressed TAP1) in 76 cases and 5 (60-79% of the tumor cells expressed TAP1) in 2 cases. Staining intensity of TAP1 was scored as weak, intermediate and strong in 20 (25.6%), 52 (66.7%) and 6 (7.7%) SCCOT, respectively. In benign hyperplastic lesions (n=17), the proportion of TAP1 expressing cells varied from 1 to 4, and intensity was graded as weak in all but one of these benign lesions. The resulting QuickScores for TAP1 were compared between SCCOT and benign lesions. The majority of SCCOT lesions had a QuickScore of 12 (51 samples, 65.4%), whereas most benign lesions had a QuickScore of 3 (9 samples, 52.9%),

Table I. Clinicopathological features of the patients with squamous cell carcinoma of the oral tongue (n=78).

Clinicopathological features	Frequency (%)
Age, years	
19-40	8 (10.3)
41-69	38 (48.7)
70-89	32 (41.0)
Sex	
Female	41 (52.6)
Male	37 (47.4)
T stage	
1	20 (25.6)
2	30 (38.5)
3	12 (15.4)
4	16 (20.5)
Nodal status	
Negative	61 (78.2)
Positive	17 (21.8)
TNM stage	
I	20 (25.6)
II	27 (34.6)
III	10 (12.8)
IV	21 (26.9)
Worst pattern of invasion	
Broad pushing fingers	1 (1.3)
Invasive islands (>15 cells/island)	7 (9.0)
Invasive islands (<15 cells/island)	70 (89.7)
Degree of differentiation	
Poor	6 (7.7)
Poor-moderate	24 (30.8)
Moderate	27 (34.6)
Moderate-high	20 (25.6)
High	1 (1.3)
Lymphocytic response	
Dense rim	26 (33.3)
Patches	39 (50.0)
Limited	13 (16.7)
Treatment	
Surgery only	8 (10.3)
Radiotherapy only	15 (19.2)
Postoperative radiotherapy	12 (15.4)
Preoperative radiotherapy	38 (48.7)
No treatment	5 (6.4)
Response to RT	
Complete response	31 (39.7)
Partial response	11 (14.1)
No response	7 (9.0)
No radiotherapy	12 (15.4)
Unknown	17 (21.8)
Overall survival	
Alive >5 years	20 (25.6)
Dead (<5 years)	42 (53.8)
Dead (>5 years)	16 (20.5)

RT, radiotherapy.

showing a significant increase in TAP1 levels in SCCOT compared to benign lesions ($P<0.001$, Mann-Whitney U test, Fig. S1A).

Due to the limited volume of some samples, TAP2 staining could not be performed on four cases of SCCOT and three benign lesions. Similar to TAP1, cytoplasmic TAP2 was detected in all SCCOT specimens, and the proportion of TAP2-positive tumor cells was graded as 6 in 72 lesions and 5 in 2 lesions. Weak, intermediate and strong TAP2 expression was seen in 17 (23.0%), 51 (68.9%) and 6 (8.1%) SCCOT lesions, respectively. In the 14 benign lesions analyzed, the proportion of TAP2-expressing cells varied from 2 to 6 and the intensity was weak in all but one of the lesions. Histograms of TAP2 QuickScore (Fig. S1B) showed that the majority of SCCOT lesions had a QuickScore of 12 (50 samples, 67.6%) and the majority of benign lesions a QuickScore of 3 (6 samples, 42.9%). Mann-Whitney U test showed that TAP2 was significantly upregulated in SCCOT compared to benign lesions ($P<0.001$). A close correlation between TAP1 and TAP2 QuickScores was identified ($\rho=0.640$, $P<0.001$). Representative staining results for TAP1 and TAP2 are shown in Fig. 1.

Correlations between clinicopathological factors and TAP intensity. Due to the variance in TAP1 intensity between SCCOT lesions we continued to investigate whether there were associations between clinicopathological factors and staining intensities. Patients were divided into two groups: TAP1-intermediate/strong and TAP1-weak. TAP1 intensity did not associate with clinicopathological factors such as age, sex, T stage, nodal status, TNM stage, WPOI, LR or degree of differentiation. Similarly, no correlations between clinicopathological factors and TAP2 intensity were found (Table II, $P>0.05$ for all comparisons).

Impact of TAP intensity on patient survival. Kaplan-Meier curves with log-rank test was performed to compare survival between patients with intermediate/strong and weak TAP1 expression. No significant differences were observed for overall survival ($P=0.530$), cancer-specific survival ($P=0.888$) or disease-free interval ($P=0.657$) (Fig. 2). Similar results were observed for TAP2 on overall survival ($P=0.084$), cancer-specific survival ($P=0.278$) and disease-free interval ($P=0.428$).

Next, we investigated whether there was a TNM stage- or sex-specific impact of TAP. In our cohort, there was a significant difference in age between male and female patients with early-stage tumors (stage I and II) (Table SI, $P=0.001$). To have age-wise comparable groups of females and males, only patients under 70 years were analyzed, showing significant differences in overall survival ($P=0.002$), cancer-specific survival ($P=0.005$) and disease-free interval ($P=0.011$) between males ($n=19$) and females ($n=11$) (Fig. 3).

Further division of patients into four groups according to sex and TAP1 intensity, varying degrees of differences in overall survival ($P=0.009$), cancer-specific survival ($P=0.046$) and disease-free interval ($P=0.066$) were identified (Fig. 4A, C and D). The best overall survival was seen in males with intermediate/strong TAP1 ($n=17$) which is distinct to TAP1-weak males ($n=2$), TAP1-intermediate/strong

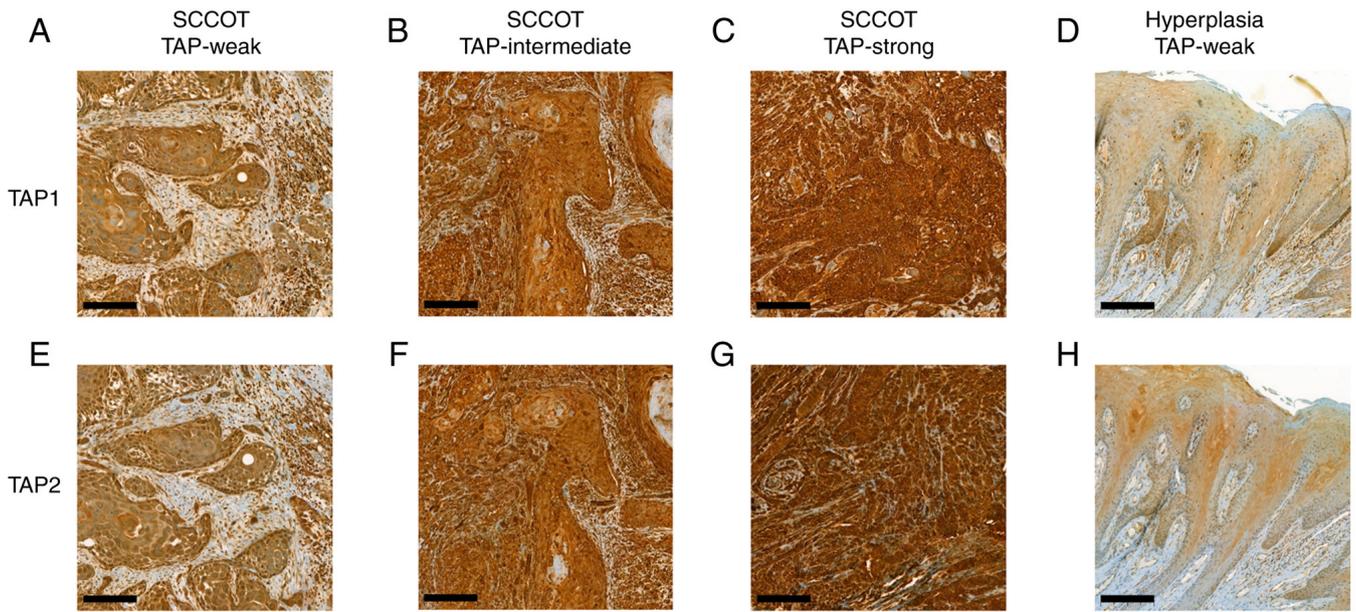


Figure 1. Immunohistochemical staining of TAP1 and TAP2 in tumors and benign lesions. Representative results of (A) weak, (B) intermediate and (C) strong staining intensity of TAP1 in SCCOT, and (D) weak staining intensity of TAP1 in hyperplastic lesion. Corresponding staining results for TAP2 are (E) weak, (F) intermediate and (G) strong in SCCOT and (H) weak in hyperplastic lesion. SCCOT, squamous cell carcinoma of the oral tongue; TAP, transporter associated with antigen processing.

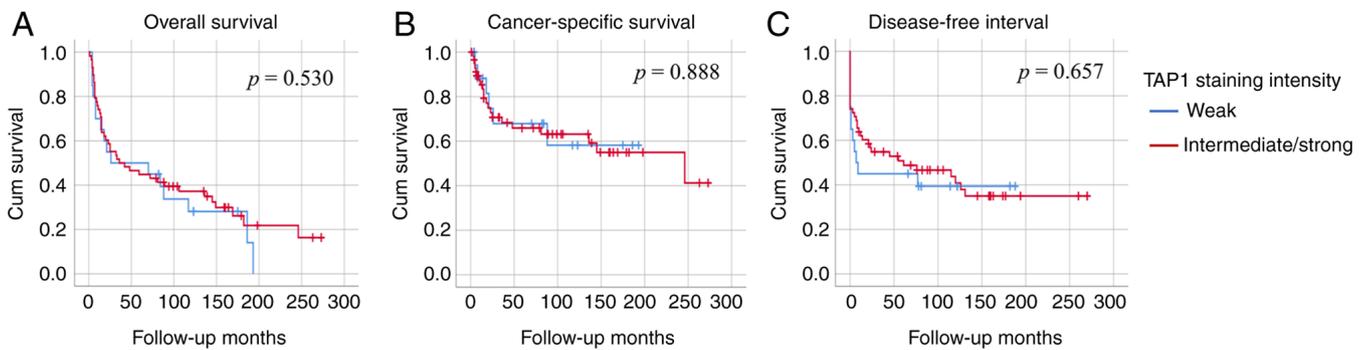


Figure 2. Effect of TAP1 levels on patient survival. Kaplan-Meier curves showing no difference in (A) overall survival, (B) cancer-specific survival or (C) disease-free interval between TAP1-weak (n=20) and TAP1-intermediate/strong (n=58) patients. The P-values for the log-rank tests are shown. Cum, cumulative; TAP, transporter associated with antigen processing.

females (n=7) and TAP1-weak females (n=4). Similarly, the best overall survival was seen in males with intermediate/strong TAP2 (n=13) compared to TAP2-weak males (n=4), TAP2-intermediate/strong females (n=9) and TAP2-weak females (n=1) (Fig. 4B and F). No correlations between sex and other clinicopathological factors were found (data not shown).

Discussion

The immune system has the capacity to fight cancer, however, cancer cells can evade immune elimination through a wide range of mechanisms. Loss of antigen presentation due to mutations, transcriptional downregulation, hypermethylation and/or loss of heterozygosity (LOH) in APM components have been reported as immune escape mechanisms in cancers (4). In the current study, we showed that TAP1 and TAP2 levels are closely correlated and that SCCOT contained

higher levels of TAP than hyperplastic tongue lesions. Criteria established by the HLA and cancer component of the 12th International Histocompatibility Workshop suggest that proteins with a percentage of stained tumor cells <25%, 25-75% and >75% are scored as negative, heterogeneous or positive, respectively (20). According to this criterion, we conclude that TAP in all but two of our investigated SCCOT samples are 'positive'. Considering staining intensity, no distinct clinicopathological characteristics or survival outcome were found between TAP-intermediate/strong and TAP-weak patients. Taken together, it seems that loss of TAP expression is not an underlying mechanism of tumor immune evasion in SCCOT.

In their analysis of 89 patients with SCCHN, Feenstra *et al.* (10) reported that decreased TAP1 and TAP2 staining intensity in tumor cells compared to the surrounding stromal cells was seen in 28 and 9% of patients, respectively. In another 25 patients with SCCHN, loss or downregulation

Table II. Associations between TAP intensity and clinicopathological factors.

Clinicopathological features	TAP1			TAP2		
	Weak	Intermediate/ strong	P-value	Weak	Intermediate/ strong	P-value
Age, years						
19-69	11	35	0.793	8	35	0.402
70-89	9	23		9	22	
Sex						
Female	11	30	>0.999	9	30	>0.999
Male	9	28		8	27	
T stage						
1, 2	13	37	>0.999	9	38	0.391
3, 4	7	21		8	19	
Nodal status						
Negative	16	45	>0.999	13	44	>0.999
Positive	4	13		4	13	
TNM stage						
I, II	11	36	0.605	7	37	0.097
III, IV	9	22		10	20	
Worst pattern of invasion						
Broad pushing fingers or Invasive islands (>15 cells/island)	1	7	0.672	2	5	0.657
Invasive islands (<15 cells/island), including single cell invasion	19	51		15	52	
Lymphocytic response						
Continuous dense rim of lymphoid tissue	6	20	0.789	4	19	0.558
Patches of discontinuous dense lymphoid infiltrate or limited/no response	14	38		13	38	
Degree of differentiation						
Poorly, poorly-moderately differentiated	8	22	>0.999	8	21	0.573
Moderately, moderately-well or well differentiated	12	36		9	36	
Recurrence						
No	8	27	0.765	6	26	>0.999
Yes	7	18		5	19	
5-year overall survival status						
Alive	10	26	0.796	6	26	0.580
Dead	10	32		11	31	

TAP, transporter associated with antigen processing.

of TAP1 and TAP2 (<75% tumor cells) was found in 52 and 88% of the lesions, respectively (11). In a study of 25 patients with SCCHN, loss or downregulation of TAP1 and TAP2 was found in 68 and 64% of the lesions, respectively (12). In 51 patients with laryngeal SCC, decreased expression of TAP1 was found in 71% of patients (13). Obviously, our results from 78 patients with SCCOT are inconsistent with most previous SCCHN studies. Even if differences in staining methods and evaluation could lead to variations between different studies, the most likely explanation is that we studied tumors and benign tissues located in the oral tongue only, based on

previous findings of subsite-based differences in protein and RNA expression clearly indicating wide differences in tumor phenotypes at different subsites in the head and neck and even within the oral cavity (14,21,22).

Earlier small-cohort studies have shown that LOH at the HLA-I loci on chromosome 6 is a frequent event in cancers (4). Recently, investigation of 83,644 cancer samples revealed that HLA-I LOH was present in 17% of patients, with a range of 2-42% across different tumor types (23). Within the 1,134 patients with SCCHN included in that study, the prevalence of HLA-I LOH was 27.2% (23). More recently,

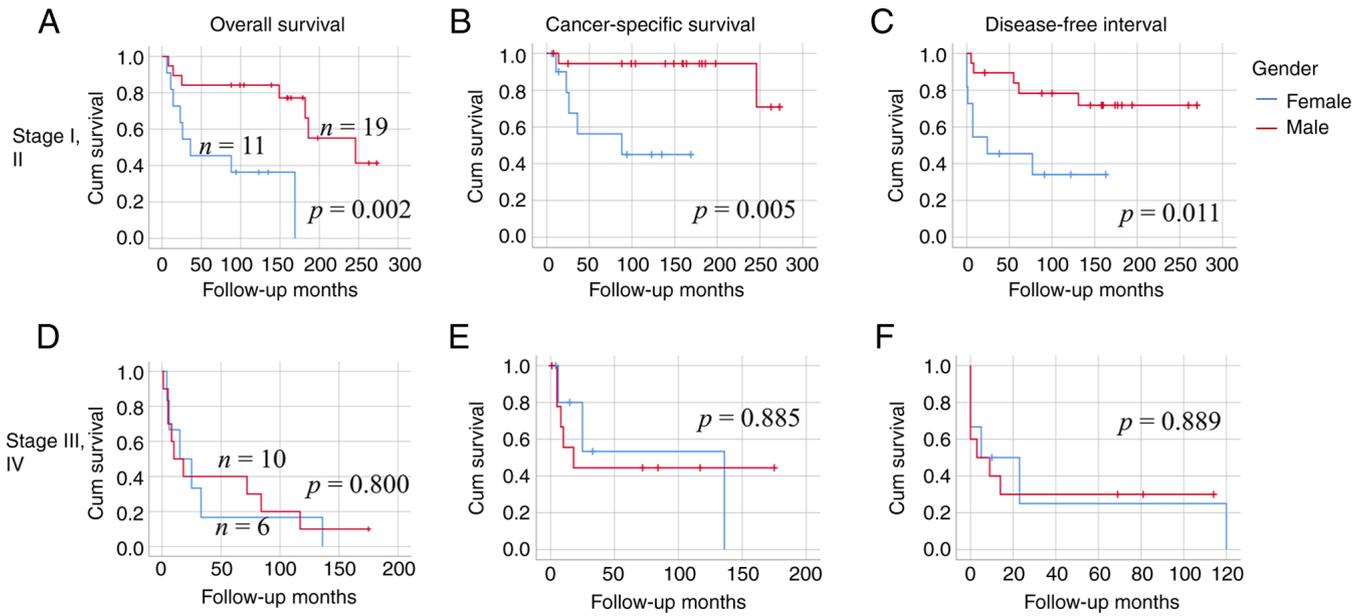


Figure 3. Effect of sex on survival outcome in patients under 70 years of age. For patients with early stage SCCOT, Kaplan-Meier curves indicate the effect of sex on (A) overall survival, (B) cancer-specific survival and (C) disease-free interval. For patients with late-stage SCCOT, there is no effect of sex on (D) overall survival, (E) cancer-specific survival and (F) disease-free interval. The P-values for the log-rank tests are shown. Cum, cumulative; SCCOT, squamous cell carcinoma of the oral tongue.

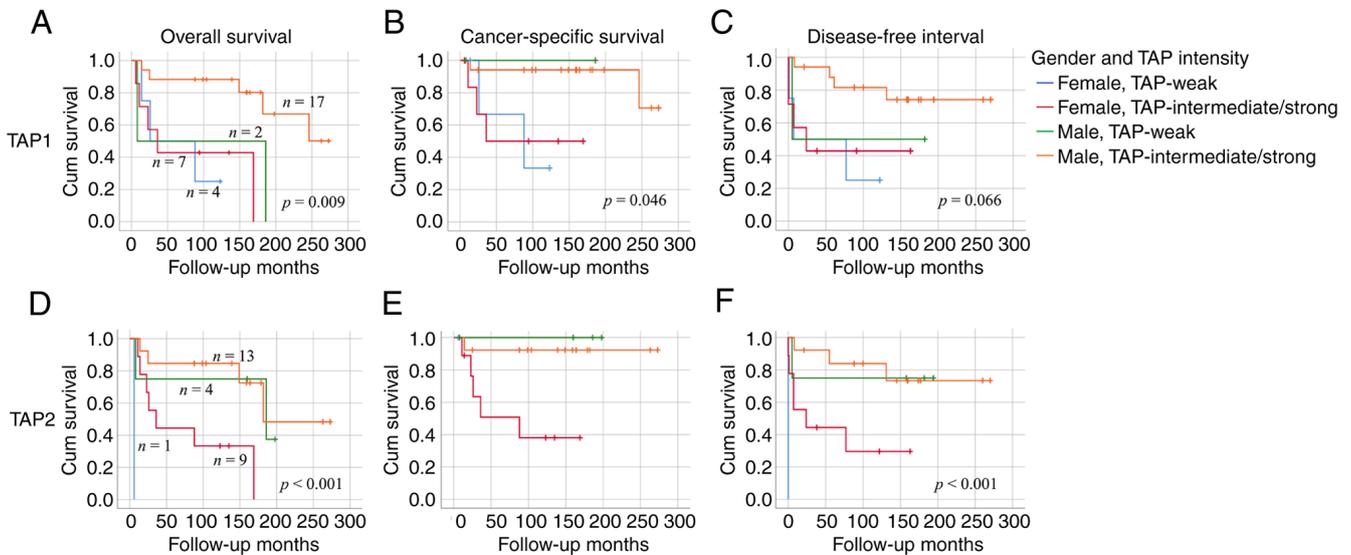


Figure 4. Effect of sex and TAP on survival outcome in patients under 70 years and with early-stage squamous cell carcinoma of the oral tongue. Kaplan-Meier curves showing the impact of sex and TAP1-intensity on (A) overall survival, (B) cancer-specific survival and (C) disease-free interval. Impacts of sex and TAP2-intensity on (D) overall survival, (E) cancer-specific survival and (F) disease-free interval. The P-values for the log-rank tests comparing survival among the four groups are shown. For TAP2 and cancer-specific survival, no statistics were calculated because all cases are censored. Cum, cumulative; TAP, transporter associated with antigen processing.

Garrido *et al.* (24) reported that copy-neutral LOH affecting the entire chromosome 6 is a frequent mechanism of HLA-I alterations in cancer. On the other hand, analysis of The Cancer Genome Atlas (TCGA) database for 10,967 patients with different types of cancer revealed that approximately 5% harbor homozygous deletions or truncating mutations in APM components (4). Therefore, LOH at HLA-I, which could reduce the diversity of the presented peptide repertoire, seems to be a more common mechanism for cancer immune escape (4). Importantly, in addition to HLA-I, genes encoding

class II and class III HLA, and several components of APM are also located on the short arm of chromosome 6, including TAP1 and TAP2. Ciani *et al.* (25) analyzed allele-specific genomic data derived from TCGA, including 334 patients with SCCHN, to elucidate the role of copy-neutral LOH and somatic gain in cancer. From their results of HLA-I and TAP, concordant copy-neutral LOH occurs in 14% and concordant gain of copy number in 29% of tumors without sex difference. Therefore, during the evolutionary process of cancer development, alterations in haplotype and copy numbers in

chromosome 6 might provide selective advantage to the tumor cells to escape immune recognition. Even though the levels of TAP proteins remain 'positive' in our cohort of patients with SCCOT, their ability to present cancer antigens on the cell surface might be compromised due to chromosome 6 LOH or copy number alterations.

Overall, no significant correlations between TAP levels and clinicopathological features were found in this study. Comparing TAP-weak to TAP-intermediate/high patients in survival outcome in general, we failed to identify any differences. This result is consistent with our previous findings that *TAP1* mRNA levels in tumor samples are not associated with patient survival. However, focusing on patients under 70 years with early stage SCCOT, we found that males had better survival than females. Garavello *et al* (26) reported that sex does not influence prognosis in patients with oral tongue cancer and Roberts *et al* (27) found no evidence of sex-related survival disparities among SCCHN patients, even when the analysis was restricted to individual anatomic sites. In a recent large cohort study, Mazul *et al* (28) showed that females with non-oro-pharyngeal HNSCC had better five-year overall survival than males. Therefore, our finding that males had better survival than females is surprising. As male patients with intermediate/strong TAP intensity displayed distinct survival outcome compared to female patients, we suggest a sex-specific impact of TAP on survival. It seems that males could gain from higher expression of the TAP proteins. Sex differences in immune response have long been recognized (29). Recently, Castro *et al* (30) found that younger and female patients accumulate driver mutations in their tumors that are less readily presented compared to males and older patients, suggesting that the strength of immune selection during tumor development varies with sex and age. Considering TAP, more patients should be studied to elucidate its sex-specific impact on patient survival.

In summary, this is the first study of TAP proteins in a cohort of patients with SCCOT. Results showed an increase of TAP in tumor cells and provide further evidence for sex-specific immune modulation in cancer. With immunotherapy being more and more used in cancer treatment one could thus speculate whether increasing TAP expression could be a useful immune-restoring strategy at least in male patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NA performed experiments, analyzed data and wrote the manuscript. PJC interpreted data, wrote and edited the manuscript. KZ, BE and MM provided medical materials, interpreted data and reviewed the manuscript. NS interpreted data and reviewed the manuscript. KN supervised the project, performed experiments, and wrote and edited the manuscript. XG supervised the project, performed experiments, analyzed data, and wrote and edited the manuscript. All authors read and approved the final manuscript. NA, KN and XG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical permission for the study has been granted by the Regional Ethics Review Board, Umeå, Sweden (DNR 03-201, 08-003M) and the project was performed according to the principles of the Declaration of Helsinki. Written consent is not required for the use of surplus archived tissue samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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