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Original Article

The expression of transglutaminase 2 (TG-2) in oral squamous cell carcinoma and its clinical significance

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Abstract

Background: Glutamine has a very important role in the human body, including pH balance in an acidic environment, as well as supporting the TCA cycle in cancer cell growth. However, the expression of transglutaminase-2 (TG-2) in oral cancer growth related to renal function is unknown. Here we examined TG-2 and its expression as a prognostic tool.

Methods: Fifty-six oral squamous cell carcinoma (OSCC) tissues were collected with the inclusion of tumor in any region of oral area, and patients with creatinine (Cr) and blood urea nitrogen (BUN) results. The tissues were stained using immunohistochemistry (IHC) with a TG-2 antibody [N3C3], then observed under the microscope. The staining were calculated using Adobe Photoshop CS software and statistical analyses using SPSS ver. 21.

Results: We found that TG-2 expression showed a significant difference in the expression levels between tumor and the adjacent groups without disease-free survival, disease-specific survival, and recurrence between, with $p < 0.05$. The average staining intensity with 25th percentile of TG-2 becomes a vital score for the diagnosis. Furthermore, our study demonstrates a good prognosis outcome if the intensity score showed a difference in TG-2 expression between the adjacent and tumor tissue.

Conclusion: To our knowledge, this is the first clinical study on TG-2 expression in OSCC, and it demonstrates that TG-2 can serve as a predictor of tumorigenesis and prognosis outcome.

Keywords: Glutaminase; OSCC; Renal

1. Introduction

Oral cancer is a neoplastic lesion of the head and neck, and is one of the most common cancers in men. Each year, there are 6 million deaths worldwide due to oral cancer[1] and approximately 74% of oral cancer cases are due to the use of tobacco and alcohol. As a result, cessation of tobacco and smoking is the primary prevention method, followed by early detection of precancerous and cancerous lesions. More recently, 22 molecular biomarkers were identified that can be used as therapeutic and diagnostic tools to predict prognosis and survival in patients with oral squamous cell carcinoma (OSCC). Detecting the protein expression levels of such biomarkers using immunohistochemistry (IHC) is a well-recognized tool for identifying and providing clinical information on tumor specimens.[2,3] As a result, in this study we used IHC to detect transglutaminase 2 (TG-2) expression

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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levels in tumor tissues and its overexpression has been reported in several types of human cancer.[4]

Most of the energy in a normal cellular process comes from glucose through oxidative phosphorylation (OXPHOS). Glucose is converted to pyruvate by pyruvate dehydrogenase in the cytosol, and then enters the tricarboxylic acid (TCA) cycle, which provides acetyl Co-A to the mitochondria for rapid cell division. In tumor cells, the metabolic pathway changes and is reprogrammed. Specifically, instead of entering the TCA cycle, glucose is consumed *via* aerobic glycolysis, which is faster than OXPHOS, and results in produce more lactate. This was observed by Otto Warburg as a mitochondria defect in tumor cells. In order to achieve the energy, tumor cells need higher uptake of glucose to support their biosynthesis and redox.[5]

In addition to glucose, glutamine is another essential nutrient and an abundant amino acid for growing tumor cells. Under low glucose, glutamine serves as a metabolic intermediate that becomes converted to glutamate *via* the mitochondria enzyme glutaminase (GLS) in order to supply carbon to the TCA cycle for cell survival. Glutamine becomes a major source of energy for feeding the net production of oxaloacetate to produce acetyl Co-A *via* reductive carboxylation through alpha-ketoglutarate (AKG) metabolism, electron transport chain, OXPHOS, pre-cursor for biosynthesis of glutathione, nucleic acids, and certain amino acids.[6,7] While the precise role of glutamine in tumor cells is not completely understood, the genetic background and microenvironmental factors are believed to play an important role.[6,8,9]

In the normal human body, glutamine levels are approximately 70 g/d. The kidneys consume glutamine as an important donor to produce NH_3 by the action of phosphate-dependent glutamine (GLS1), and only 10% is metabolized by membrane-bound gamma glutamyl transferase in the lumen of the collecting tubule. This results in H^+ to form NH_4^+, which is then excreted in the urine. Approximately 70% of glutamate is then released back into the renal vein, and causes H^+ from carbonic acid to dissociate and form bicarbonate (HCO_3^-) and H^+. This HCO_3^- enters the circulation for pH regulation in the plasma. To fight the acidic stress, the production of ammonia by GLS1 is an important mechanism for neutralizing the pH environment caused by the toxic buildup of protons, and plays a vital role in cancer growth.[10,11] Conversely, glutamine also participates in gluconeogenesis from glutamate that is converted *via* the formation of 2-oxoglutarate by malate and oxaloacetate, or to phosphoenolpyruvate (or directly from malate to pyruvate). It also increases the formation of AKG to the TCA cycle, and for hepatic ureagenesis. The functions of the kidneys are for the maintenance of acid levels for processes like renal gluconeogenesis, the excretion of waste products, and the regulation of hematopoiesis.[6,12−14]

Given glutamine's important role in human physiology and its role in supporting the TCA cycle for cancer cell growth, it represents a potential early diagnostic and intervention tool. We hypothesized that glutamine would display higher expression in the progressing tumor, and would result in pH changes in the tumor microenvironment. To explore this notion, in this study we focused on the aspect of glutaminase in converting glutamine to glutamate for entry into the TCA cycle.

2. Methods

2.1. Ethic statement

The clinical study was reviewed by the Institutional Review Board (IRB) with approval number 2016-02-005BC at the Taipei Veteran General Hospital, and informed consent from patients was signed.

2.2. Tissue samples

The OSCC tissue samples were collected under surgical operation, and fixed in formalin. The samples included in the research are from patients with any region of tumor in oral area, without age limitation. Patients with creatinine (Cr) and blood urea nitrogen (BUN) results were observed for renal function. Samples were eliminated when diagnosed as renal abnormalities or failure. No data for renal electrolytes, or both Cr (0.7−1.4 mg/dL) and BUN (6−20 mg/dL) levels, were out of the normal range. Moreover, samples were not included if tissue loss under microscope observation was observed to be more than 50% after the IHC procedure. Only fifty-six patients met the requirements.

2.3. Immunohistochemistry

A standard IHC protocol was followed to stain the normal and tumor tissue samples. The normal and tumor tissue sections were deparaffinized with Xylene I and II, before treatment with 100% alcohol and double-distilled water (ddH_2O). During antigen retrieval, the slides were heated in a solution consisting of 47.5 mL ddH_2O and 2.5 mL Trilogy for 30 min at 75−100°C. Then tissue sections were washed in Tris-buffered saline and Tween-20 (TBST), and then repeated in 3% H_2O_2 for 20 min, before a final wash in Ultra V block for 5 min. Primary antibody (transglutaminase 2) was applied (1:200 in dilution buffer), and the slide was incubated overnight at 4°C in a humid chamber. The slides were washed in TBST, and biotinylated secondary antibody was applied before continuing with streptavidin-peroxidase. AEC chromogen was washed with ddH_2O, and the slides were dipped in hematoxylin solution for 10 s and carefully washed with tap water. The slides were then mounted with mounting medium (Dako Glycergel) and covered with glass cover slips.

2.4. Image acquisition

The slides were observed under a Zeiss Germany Axioskop 50 microscope with filter set 02 (G 365; FT 395; LP 420) and 5× magnification. The microscope was connected to a higher-performance camera (Evolution VF Cooled Color). The CCD sensor gives a resolution of 1.4 million pixels in a 12-bit

digital output. The Evolution VF kit comes with an Image-Pro family driver that requires one of the following Image-Pro family applications. We use the Image-Pro® Plus, the ultimate imaging software package, which includes all of the functionality of Image-Pro Discovery along with an added analysis tools and the ability to write customized macros.

2.5. Image analysis

The IHC intensity score was calculated using Adobe Photoshop CS software using the CMYK method[15] by randomly picking 10 points of the darkest staining from tumor tissue (Supplementary Fig. 1). Pilot study using normal gingiva tissues were done to determine the lowest percentage of IHC staining intensity. Then the average score was categorized into four groups, which are >75% = score 4; 51–75% = 3; 21–50% = 2, and <20% = 1. The score percentage were done using yellow (Y) as the model color for staining, which is close to brown color.

2.6. Statistical analysis

Statistical analyses were performed using the statistical package of social science (SPSS), version 20.0. Specifically, our analysis included independent two-sample t-test, paired sample t-test, Cox-regression and ROC curve from average score of tissue staining. The confidence interval was established at 95% and p-values <0.05 were considered to be significant.

3. Results

We calculated the average staining intensity of each tumor and adjacent tissues independently, and compared both groups. An independent two-sample t-test was conducted to compare the TG-2 expression between the disease-free survival (DFS-0) and no disease-free survival (DFS-1), disease-specific survival (DSS-0) and no disease-specific survival (DSS-1), with no recurrence (R-0) and recurrence (R-1) for both tumor and the adjacent group. There were no significant difference between each group in tumor and adjacent tissue.

We then compared the TG-2 expression between tumor and adjacent tissue in conditions of DFS, DSS and recurrence. Using a paired samples t-test a significant difference ($p = 0.017$) was observed for the DFS-0 between the tumor (M = 39.62, SD = 15.96) and adjacent (M = 33.52, SD = 9.97) groups. A statistically significant difference was also observed between the tumor (M = 38.73, SD = 14.57) and adjacent (M = 33.58, SD = 9.99) groups for DSS-0 ($p = 0.010$), as well as for R-0 ($p = 0.005$) between the tumor (M = 39.87, SD = 14.44) and adjacent (M = 33.74, SD = 9.31) groups (Table 1). Taken together, our results suggested that if there was a score difference between the tumor and adjacent tissue in TG-2 intensity it would predict a good prognosis in terms of no recurrence, free from symptom and disease, and a higher survival rate. On the other hand, no score difference between tumor and adjacent groups would predict a poor prognosis.

Table 1
The average stain intensity between both tumor and normal groups.

	Mean	SD	p
No disease free survival (DFS = 1)			0.451
Average stain intensity Tumor	36.15	12.32	
Average stain intensity Normal	34.52	11.73	
Disease free survival (DFS = 0)			0.017
Average stain intensity Tumor	39.62	15.96	
Average stain intensity Normal	33.52	9.97	
No disease specific survival (DSS = 1)			0.767
Average stain intensity Tumor	36.00	13.86	
Average stain intensity Normal	35.06	12.79	
Disease specific survival (DSS = 0)			0.010
Average stain intensity Tumor	38.73	14.57	
Average stain intensity Normal	33.58	9.99	
Recurrence (R = 1)			0.645
Average stain intensity Tumor	33.53	13.32	
Average stain intensity Normal	34.59	13.86	
No Recurrence (R = 0)			0.005
Average stain intensity Tumor	39.87	14.44	
Average stain intensity Normal	33.74	9.31	

Paired t-test.

According to our finding, we then examined the TG-2 range intensity in disease specific groups, and investigated whether we could detect any difference in the average score between disease specific groups for use as an early diagnosis tool. The difference between disease-specific survival among the Q1 and Q2–Q4 groups generated the best prediction based on the area under curve (AUC) for both the average stain intensity in tumor and adjacent site. Thus, we considered the 25th percentile of average stain intensity in tumor and adjacent site as the best cut-off point in following analysis (Table 2).

In terms of the study population's characteristics, including age, tumor size, lymph node status, tumor staging, survival, recurrence rate, and disease survival groups, there was no significant difference either between tumor levels in each group, or between tumor and the adjacent group. This is in agreement with our earlier finding that recurrence, disease free survival, and disease specific survival show no significant difference between tumor or normal samples. This data highlights the fact that a diagnosis cannot be made from the cancer tissue sample alone, without comparing it to the adjacent tissue. In calculating the adjacent tissue, TG-2 score intensity will help to predict tumor invasion and tumorigenesis (Fig. 1).

Table 2
The quartile of average stain intensity in tumor and adjacent site.

Variable	N	Mean	SD	Range
Average stain intensity Tumor				
Q1	16	22.19	4.79	12–28
Q2–Q4	40	44.25	11.69	29–78
Average stain intensity Normal				
Q1	15	21.40	5.05	9–26
Q2–Q4	41	38.61	8.32	27–62

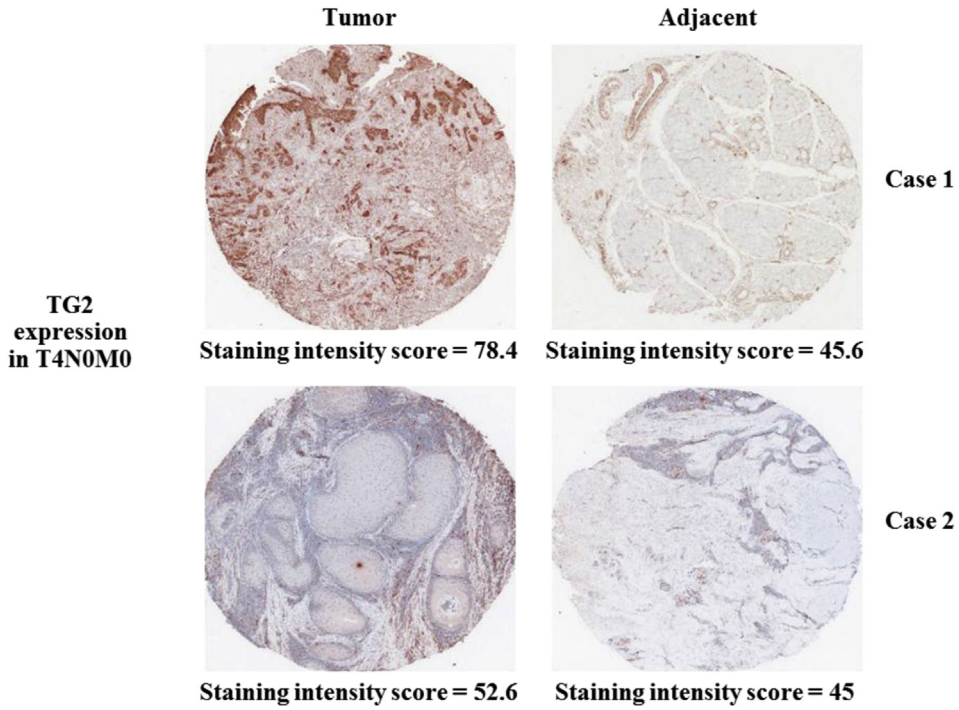

Fig. 1. Patient samples with T4N0M0 showed different TG-2 expression between tumor and adjacent tissue. This can become one of the answer to previous question why patient with similar staging but result in different outcome.

In addition, using an adjusted Cox-regression of the death rate from the disease specific survival analysis that we can infer with 96% confidence that the T4 tumor size is approximately 6.21 times, and at least 6.25 times the risk, as the T1–T3 group. Moreover, the data showed that the death rate is approximately 4.10 times with recurrence and 4.09 times in no recurrence (Table 3).

The TG-2 expression intensity score in this study showed a similar death rate between the T1–T3 and T4 groups, and also between the groups without recurrence. However, the TG-2 expression level outcome might be different due to covariance in this analysis. For example, the TG-2 intensity might be different in patients with only T1 tumor size, compared to T1 in conjunction with other systemic diseases.

Table 3
Cox regression analysis for cancer specific survival.

Variables	Unadjusted		Adjusted	
	HR	95% CI	HR	95% CI
Average stain intensity Tumor				
Q1	Reference		Reference	
Q2–Q4	1.19	0.38–3.68	1.11	0.19–6.43
Average Stain intensity Normal				
Q1	Reference		Reference	
Q2–Q4	1.06	0.60–1.86	1.04	0.49–2.17
Age group				
<45	Reference		Reference	
45 to <55	1.66	0.34–8.25	1.53	0.23–10.05
55 to <65	1.74	0.34–8.95	0.36	0.04–2.83
>65	1.17	0.19–6.99	0.48	0.06–3.74
Tumor size				
T1–T3	Reference		Reference	
T4	3.10	0.70–13.66	**6.50**	**1.04–40.37**
Lymph node status				
N0	Reference		Reference	
N1–N3	1.70	0.63–4.59	2.41	0.75–7.75
Stage				
I–III	Reference		Reference	
IV	3.10	0.70–13.66	6.02[a]	0.97–37.48
Recurrence				
No	Reference		Reference	
Yes	8.41	2.70–26.22	**15.62**	**3.81–64.06**

Bold font indicates the statistically significant results.
HR: hazard ratio.
[a] The adjusted variables for stage did not included tumor size and lymph node status due to collinearity.

4. Discussion

Glutamine is an important nutrient for human physiology. It serves as a metabolite that is exchanged between organs, helps to maintain pH balance in the blood, and can help fuel cancer cell growth. Many studies have mentioned that the kidneys are an important organ for cell survival in cancer patients, and that the relationship is often multi-factorial. For example, in a study by Humphrey et al., patients with myeloma and renal failure had a 20% shortened survival, and were diagnosed with more advance disease.[16]

There are many research about TG-2 related to cancer such as over expression of TG-2 in prostate cancer increased predict risk to metastatic, drug resistance in breast cancer and other type of cancer.[17,18] In this study, we examined TG-2 in OSCC patients with good renal function that had not been diagnosed as having renal failure. Using IHC staining we examined TG-2 expression levels, and measured the intensity score between tumor and adjacent tissue as a diagnostic tool. The outcome of our study showed that OSCC's patients will have a good prognosis if there is a difference in the intensity score between

adjacent and tumor tissue. However, the patients that showed no difference in the intensity score or small interval score between the normal adjacent tissue and the tumor had a poor prognosis.

If the adjacent tissue has nearly the same or higher intensity score over the tumor score, it would indicate that there was higher glutaminase expression. This would suggest that there was a change in the microenvironment, presumably due to a lowering of the pH, as can be seen in cancers. This acidic microenvironment has been reported to be critical for tumorigenesis, angiogenesis, and metastasis.[19] Katt et al. in their study found a similar change in TG-2 expression in response to acidic conditions caused by catalytic activities during ammonia production. Furthermore, the upregulated expression of TG-2 has been shown to protect cells from a variety of stress, as well as promoting cancer cell growth by regulating cellular pH levels.[11,20−24] In addition, it has been reported that the kidneys have a direct effect on cancer cell growth, and can inhibit malignancies, although the mechanism is still unknown.[25] However, more sample need to be collected in order to gather more information about the relationship of renal function and glutaminase expression in patients with OSCC.

This study demonstrates that it is important to analyze both the normal adjacent tissue, and the tumor tissue. One possibility is that the adjacent cells might have transformed into tumor cells, which would be missed if only the tumor sample is analyzed. Along these lines, the intensity score difference could serve as an indicator of tumor progression, as the expression of TG-2 correlates with tumor cell aggressiveness, in general.[26] According to Halin et al. (2011), the tumor cell requires the influence of the adjacent tissue to grow and spread, and they advocate for the use of adjacent normal tissue as an early diagnostic and prognostic marker in their study on prostate cancer.[27]

In this study we use the intensity score of 25 in both tumor and adjacent tissue as the lowest point of TG-2 expression for tumor progressiveness. This score of 25 actually is in the range of intensity score of normal tissue in our pilot study, which had a range from 8 to 29 score in sixteen normal tissues taken from patients undergoing dental surgery (unpublished data). We found that TG-2 with a score $\geqq 25$ in tumor tissue related to tumor size, but the results showed no statistic significant. The tumor tissue with a score <25 correlated with slower tumor growth, but we did not find a correlation with a specific outcome, presumably due to many other cofactors. Although we did not address this in our study, Antonyak et al. mentioned that microvesicles (MVs) and their cargo from cancer cells can promote tumor growth by communicating with neighboring cells.[4,20]

In the future, it will be important to study the relationship between TG-2 expression levels in patients with OSCC, and renal failure. In fact, it has already been demonstrated that renal failure can impact the plasma levels of amino acids, such as glutamine.[28,29]

In the adjacent tissue, a TG-2 expression score of <25 was considered as a normal condition, while a score $\geqq 25$ might be an indicator of altered metabolism and could serve as a predictor of tumor prognosis when combined with the TG-2 tumor score. We believe that this change in score could be caused by many factors.

In our study, we found that the T4 tumor group had a similar death rate as the T1−T3 tumor size group, and also no difference in the death rate between the R-0 and R-1 groups under normal renal function. Furthermore, we found no association between the survival rate and tumor size in our study.

The TG-2 expression not only indicates tumorigenesis, but it can also serve as a prognostic predictor for outcome. Furthermore, TG-2 promotes not only cell survival, but also drug resistance. Thus, by inhibiting TG-2 together with GLS1 it might be possible to reduce drug resistance in tumors. Combining drug therapies with treatments that inhibit both enzymes could promote intracellular acidity and offer improved therapeutic efficacies.[11,21]

To our knowledge this is the first clinical study of TG-2 expression in patients with OSCC. Our results demonstrated that TG-2 could serve as a new predictor of tumorigenesis, and a predictor of prognosis outcome, suggesting that TG-2 expression could become a diagnostic tool in patients with OSCC. A TG-2 score of 25 was a vital predictor of tumorigenesis and recurrence in this study, and an interval score between TG-2 expression in tumor and adjacent tissue provided important information for prognosis of the survival rate. Although the score of TG-2 expression is related to the renal function, it also will have different outcomes when combined with other systemic diseases. The study of TG-2 expression in patients with tumor, adjacent, and normal tissue with several conditions, such as good renal function, intrinsic renal disease and renal failure, will provide more data for better prognosis and treatment options in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2017.05.004.

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