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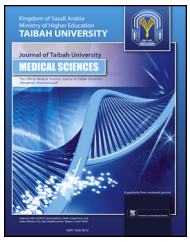

Original Article

Association of Kaiso and partner proteins in oral squamous cell carcinoma

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الملخص

أهداف البحث: تهدف هذه الدراسة لتحديد تعبير البروتين وتوطين الخلايا الفرعية لبروتينات إي-كادهيرين، ب120-كاتينين وكايسو في سرطان الفم ولدراسة التعبير البروتيني عن سايكلن-د1 وسي-ميك؛ وتمييز علاقتها وموقعها الخلوي مقارنة بالتعبير لبروتين كايسو.

طريقة البحث: تم إجراء التصنيف النسيجي وفقا لمعايير "بوردر". تم الحصول على بيانات التعبير والموقع الخلوي لبروتينات إي-كادهيرين، و ب120-كاتينين، وكايسو، سايكلن-د1، وسي-ميك، واي سي باستخدام الكيمياء الهستولوجية المناعية.

النتائج: من أصل 47 سرطان فم، أظهر 36٪ تعبيرا منخفضا عن إي-كادهيرين و 34٪ ب120-كاتينين منخفضا. تم التعرف على تعبير كايسو المنخفض في 78 ٪ من عينات الورم. شوهد الموقع الخلوي الزائغ في الهيولى لبروتين ب120-كاتينين في 80.8 ٪ من الحالات. تم تقدير الموقع الخلوي لبروتين كايسو في الهيولى في 87٪ من أنسجة الورم، بينما 29.7٪ افتقرت إلى بروتين كايسو داخل النواة. ارتبط تعبير بروتين كايسو بشكل كبير بالتعبير عن سايكلن-د1 ولكن ليس مع سي-ميك.

الاستنتاجات: حددت الدراسة الحالية تغيرا في الموقع الخلوي لبروتين كايسو في سرطان الفم. يجب التحقق من أهمية هذا فيما يتعلق بالسرطان الفموي والتشخيص بالورم مع مزيد من الدراسات باستخدام أحجام عينات أكبر وأدوات جزيئية أكثر حساسية.

الكلمات المفتاحية: إي-كادرين؛ بروتين كايسو؛ سرطان الخلايا الحرشفية في الفم

Abstract

Objectives:: 1. Identification of protein expression and subcellular localization of E-cadherin (E-cad), p120 catenin (P120ctn), and Kaiso in oral cancer (OC). 2. To study the protein expression of cyclin D1 and c-Myc (Kaiso targets) and determine their relationship with the expression and localization of Kaiso.

Methods: Histological grading was performed in accordance with Broder's criteria. Expression and localization data for E-cad, p120ctn, Kaiso, cyclin D1, and c-Myc were acquired using immunohistochemistry. Data were analyzed using SPSS version 21. The chi-square test was used to measure the statistical significance of associations, with $p < 0.05$ as statistically significant.

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Results: Of 47 OC cases, 36% showed low E-cad expression and 34% showed low p120ctn. Low Kaiso expression was recognized in 78% of tumor specimens. Aberrant cytoplasmic localization of p120ctn was seen in 80.8% cases. Cytoplasmic Kaiso localization was appreciated in 87% of tumor tissues, whereas 29.7% lacked any nuclear Kaiso. Kaiso expression was significantly associated with the expression of cyclin D1 but not with c-Myc.

Conclusion: The present study identified a change in the localization of Kaiso in OC. The significance of this in relation to OC and tumor prognosis needs to be investigated with further studies using larger sample sizes and more sensitive molecular tools.

Keywords: E-cadherin; Kaiso protein; Oral squamous cell carcinoma; ZBTB33 protein

Introduction

According to latest statistics available from the Global Cancer Observatory (GLOBOCAN 2020), 377 713 cases of oral cancer (OC) were recorded worldwide, of which 65.7% (248 360) were reported in Asia alone.[1] Mortality figures follow the same trend with a total of 177 757 deaths reported worldwide of which 131 610 were reported in Asia.[1] The burden of this disease is much greater in Asia, and in recent years a lot of research has been dedicated to identifying biomarkers that would aid in the early diagnosis and be potential molecular therapeutic targets. However due to the great genetic and epigenetic heterogeneity of this tumor, identifying a sensitive and specific biomarker has remained a challenge.[2–4]

Cancer cells utilize distinct processes to achieve invasion and metastasis. One critical step for this involves a reduction in the adhesion of cancerous epithelial cells and their detachment from both basement membrane and adjacent cells.[5] E-cadherin (E-cad) plays a central role in the maintenance of epithelial cell adhesion, and reduced expression of this protein in cancers harbinger separation and spread of cancer cells and correlates with poor survival.[5–7] p120 catenin (P120ctn) associates with E-cad at the membranous adherens junction (AJ) and stabilizes the latter.[6] In OC, reduced expression of both E-cad and p120ctn occurs with advanced clinical stage.[6] Therefore, the loss of E-cad releases various catenins (β-catenin and p120ctn) from membranous AJs, allowing p120ctn to shuttle between the nucleus and cytosol.[8] Free cytosolic or nuclear p120ctn reportedly modulates gene expression via association with Kaiso.[9]

Kaiso is a transcription factor that is principally characterized as a repressor of gene expression but possesses the capability to positively regulate its target genes.[10] Kaiso has dual modes of DNA binding, enabling it to recognize and regulate target genes by either detecting methylation marks on target gene promoters or a specific Kaiso-binding site.[9] Interaction of Kaiso is mutually exclusive with both DNA and p120ctn; therefore, binding of Kaiso with p120ctn, which occurs downstream of E-cad loss, prevents its association with DNA, resulting in the aberrant expression of Kaiso targets.[9–11] Many Kaiso target genes have been characterized with physiological expression during embryonic development as well as during pathological processes, including carcinogenesis, suggesting a role for Kaiso as a biomarker of malignancy.[12] Kaiso recruits chromatin modifiers (predominantly co-repressor complexes) to target gene promoters that either negatively or positively regulate gene expression. Occupation of Kaiso's DNA-binding domain by p120ctn therefore results in loss of Kaiso-mediated control, leading to the abnormal expression of Kaiso targets.[12,13] Aberrant expression and localization of Kaiso have been reported in several carcinomas including lung, prostate, breast, and colorectal.[14–20] The precise role of Kaiso in carcinogenesis is contextual and diverse, with some studies indicating an oncogenic role, and others indicating a tumor suppressor role.[18] In OC, however, Kaiso's potential role remains to be investigated.

In the present cross-sectional study, we investigated the potential association between expression/localization of Kaiso with the histological grade of OC and elucidated the association of the former with the protein expression of selected Kaiso targets (c-Myc and cyclin D1). We hypothesized that Kaiso is aberrantly localized in OC cells and expected to observe a change in the expression of Kaiso target proteins with former.

Materials and Methods

A total of 47 OC specimens were obtained from biopsy-proven OC patients undergoing wide tumor excision at DUHS following written informed consent. A sample size of 50 was approved by DUHS Scientific committee, since this is a pilot study, and we did not have any data on Kaiso expression in OC to use statistical tools for sample size calculation. Inclusion criteria were all patients diagnosed with OC, irrespective of age and sex, and tissue blocks with at least 70% tumor tissue were utilized for the study. Exclusion criteria were poorly fixed tissues; pregnant patients; patients who had received any prior therapy (either chemotherapy or radiation); and patients with any congenital syndrome, autoimmune disease, or other chronic illnesses. Specimens were fixed in 10% neutral buffered formalin.

Three healthy control samples were collected from the Oral Surgery Outpatient Department (DUHS) following written informed consent. Patients undergoing surgical extraction of third molars were approached for controls. Histological grading was performed by two independent pathologists (UB and HS) on hematoxylin and eosin-stained slides using Broder's system and scored I–III. Grade I = well differentiated, Grade II = moderately differentiated, and Grade III = poorly differentiated.[21]

Immunohistochemistry

Tissue sections (3–4 μM thick) were stained with anti-E-cadherin antibody (1:200, ab1416; Abcam, Cambridge, MA, USA), anti-delta 1 catenin (1:100, ab92514 [EPR357(2)]; Abcam), anti-Kaiso antibody (clone 6F/6F8) (ab12723, 1:80; Abcam, Cambrige, MA, USA), anti-cyclin D1 (clone SP4) (1:100, ABCA0079417; Abcam, Cambridge, MA USA), and anti-c-Myc (clone EP121) (1:100; Cell Marque Co., Rocklin, CA, USA) overnight at 4 °C as previously described.[22] Slides were independently reviewed by two pathologists who were blinded to tumor grade. Immunoreactive scores (IRS) were calculated for all proteins as a product of positive cell percentage and staining intensity. The percentage of positive cells was scored from 0 to 4 as follows: 0 = 0%, 1 ≤ 10%, 2 = 10–50%, 3 = 51–80%, and 4 ≥ 80%. The staining intensity was measured as follows: 0 = no staining, 1 = weak staining, 2 = intermediate staining, and 3 = strong staining. The IRS were grouped into 0 = no expression, 1–4 = low expression, 5–8 = moderate expression, and 9–12 = strong expression.[23]

Positive samples were analyzed semiquantitatively at low magnification (100×) to identify areas where proteins of interest were evenly stained. We counted 400 tumor cells and calculated the percentage of positively stained cells. Staining scores were determined by the percentage of positive cells per slide for membranous and cytoplasmic staining separately. As proposed previously,[12,35,36] normal expression was defined when more than 90% of the tumor cells showed cell membrane staining of p120ctn. When less than 90% of the tumor cells were stained for p120ctn at the cell membrane, the sample was labeled as "reduced membranous expression." When more than 10% of the tumor cells stained for cytoplasmic p120ctn expression, the sample was labeled as "ectopic cytoplasmic expression." A designation of either "reduced membranous expression" or "ectopic cytoplasmic expression" was defined as abnormal expression of p120ctn. Cases were scored as either nuclear or cytoplasmic positive when ≥1% of the cells reacted with the anti-Kaiso antibody in respective subcellular compartments.[17,24] Mean expression for both Kaiso and p120ctn was used as the cut-off to divide cases into low (≤mean) versus high (>mean) expression groups.

Statistical analyses

Frequencies and percentages were calculated for categorical variables. Continuous variables are expressed as the mean with standard deviation. Spearman rank correlation coefficient (*rs*) was calculated to determine the linear association among the expression of E-cad, p120ctn, Kaiso, cyclin D1, and c-Myc proteins. The chi-square test was conducted to identify associations with localization data. All of the analyses were performed with IBM SPSS v.21, and $p < 0.05$ was considered statistically significant.

Results

Of the 47 OC cases included in our study, a strong male predilection was recorded with a male to female ratio of 2.9:1. More than half (61.7%) of the patients were over 40

Table 1: Clinical and pathological characteristics of the tumor cases.

	Tumor parameter	N (%)
Tumor site	Buccal mucosa	35 (75.5)
	Tongue	7 (15.9)
	Lip	3 (6.4)
	Upper alveolus/maxilla	1 (2.1)
	Lower alveolus/mandible	1 (2.1)
Tumor grade	Well differentiated	16 (34)
	Moderately differentiated	15 (32)
	Poorly differentiated	16 (34)
Tumor laterality	Midline	2 (4.3)
	Right	12 (25.5)
	Left	33 (70.2)
Tumor depth/cm	≤0.5	6 (12.8)
	0.6–1	11 (23.4)
	1.1–2	18 (38.3)
	2.1–3	9 (19.1)
	3.1–4	2 (4.3)
	>4	0
Lymph node metastasis	Present	25 (53.2)
	Absent	16 (34)
	N/A	6 (12.8)
Age/years	21–30	*8 (17)*
	31–40	10 (21.3)
	41–50	12 (25.5)
	>50	17 (36.2)
Sex	Male	35 (74.5)
	Female	12 (25.5)
Stage (T)	T1	6(12.8)
	T2	0
	T3	18 (38.2)
	T4a	23 (49)
	T4b	0
Total		**47 (100)**

Values are presented as No. (%). NA, Not available.

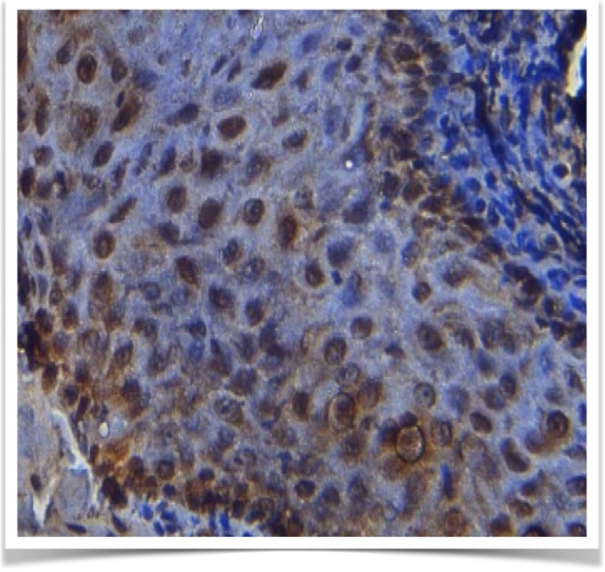

Figure 1: Immunohistochemistry (IHC) expression of Kaiso in healthy control oral mucosa showing strong nuclear expression (200×).

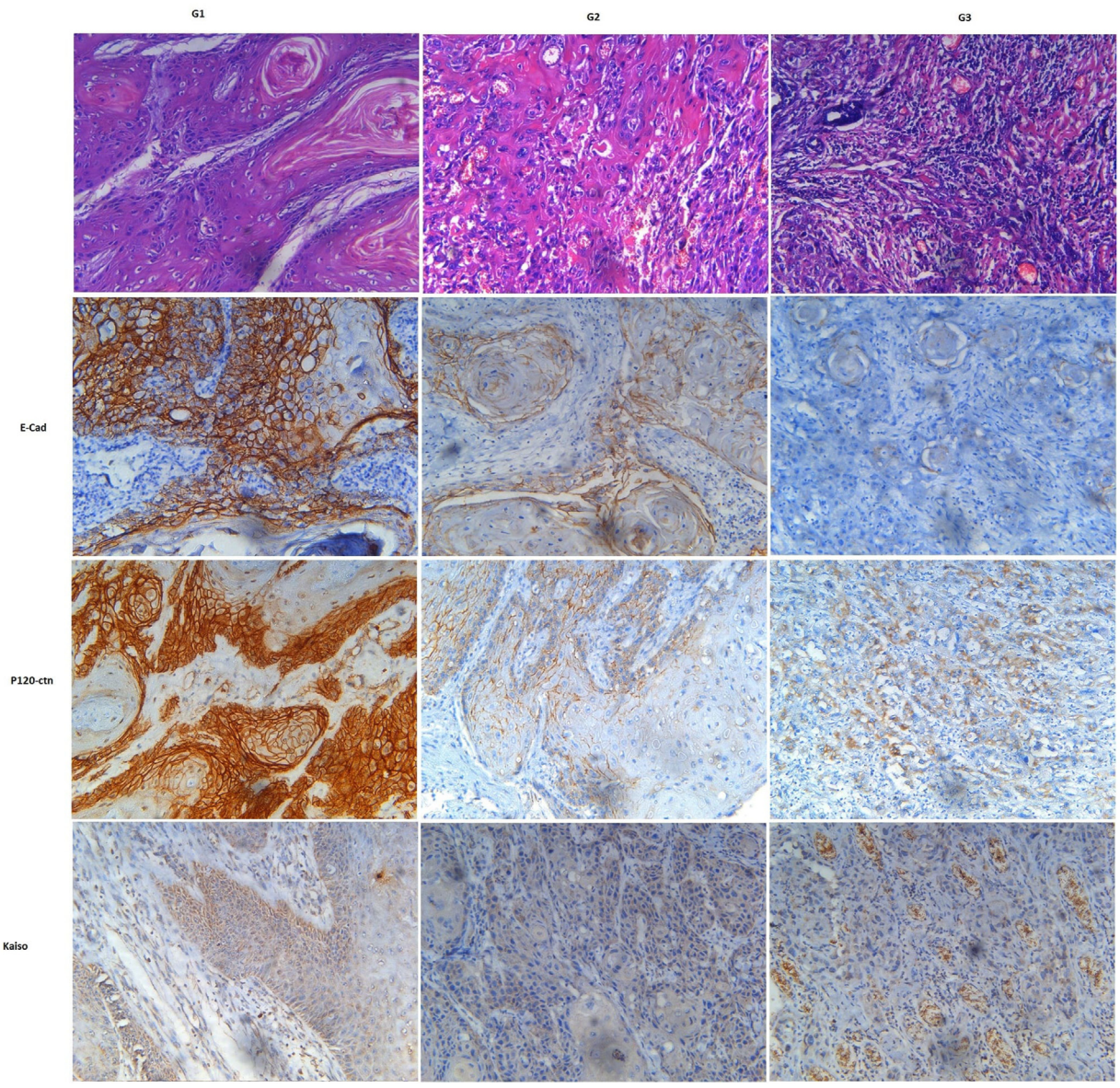

Figure 2: Hematoxylin and eosin sections showing histological grades: GI-Well-differentiated, GII- Moderately differentiated, and GIII-Poorly differentiated. IHC staining for E-cadherin, p120-catenin, and Kaiso in three histological grades. Reduced expression of all three proteins with advancing histological grade (left to right). P120ctn was increasingly expressed in the cytosol with advancing tumor grade (Magnification 200×).

Table 2: Correlation of E-cadherin expression with expression of p120ctn and Kaiso.

E-cadherin expression	p120 catenin expression				Kaiso expression				
	Low	Moderate	Strong	*p*-value	None	Low	Moderate	Strong	*p*-value
Low	11	6	0		0	13	3	1	
Moderate	4	10	5	<0.05*	1	16	1	1	0.79
Strong	1	5	5		2	5	2	2	
Total	**16**	**21**	**10**		**3**	**34**	**6**	**4**	

Table 3: Association between E-cadherin expression and Kaiso localization.

E-cadherin expression	Kaiso localization					
	Nuclear positive	Nuclear negative	*p*-value	Cytoplasmic positive	Cytoplasmic negative	*p*-value
Low	13	4	0.42	15	2	0.86
Moderate	14	5		17	2	
Strong	6	5		9	2	
Total	**33**	**14**		**41**	**6**	

Values are presented as No. Both nuclear and cytoplasmic Kaiso localization was more frequently associated with low to moderate E-cadherin expression.

years of age, with a mean age for males at the time of surgery of 43.5 ± 2 years, nearly a decade younger than the mean age for females (56 ± 5 years). A strong preponderance was noted for involvement of buccal mucosa (75.5%) and the left side of the oral cavity (70.2%). The clinical and pathological characteristics of the 47 cases included in the study are summarized in Table 1.

We first determined the expression/localization of Kaiso in healthy samples using immunohistochemistry (IHC). Strong nuclear expression of Kaiso in oral mucosal epithelium was noted in accordance with Kaiso's known role as a transcription factor (Figure 1).

OC tissues, on the other hand, have prominent cytoplasmic Kaiso localization, indicating a nuclear-cytoplasmic shift during carcinogenesis. With advancing tumor grade, the overall expression of all three proteins (E-cad, p120ctn, and Kaiso) diminished, and the localization of both p120ctn and Kaiso became more prominently cytoplasmic (Figure 2).

With the relationship between expression of E-cad and p120ctn already established, we expanded our analysis to study whether there was any significant change in the expression of Kaiso with the expression of p120ctn. As expected, a significant correlation between the expression of E-cad and p120ctn was observed ($rs = 0.549$, $p = 6 \times 10^{-5}$); however a significant association was not observed between the expression of Kaiso with both E-cad ($rs = 0.037$, $p = 0.79$) and p120ctn ($p = 0.103$; results not shown), even though half of cases showing strong Kaiso expression also had strong E-cad expression and nearly half (n = 15) of cases with low Kaiso expression also had low to moderate E-cad expression (n = 34) (Table 2).

The primary objective of this study was to determine the localization of Kaiso, with an emphasis on expression planned in future studies to explore exact role of Kaiso on oral carcinogenesis using cell-culture-based models and animal models. Analysis of the localization of Kaiso in OC revealed

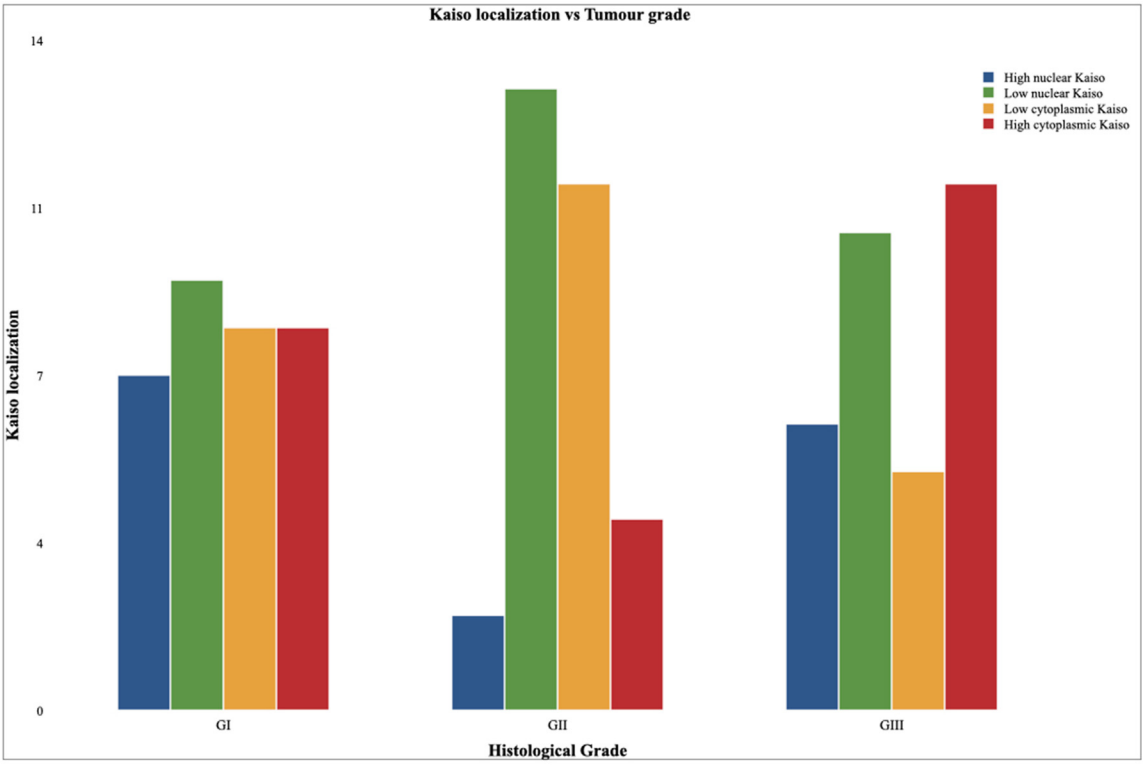

Figure 3: Association between histological tumor grade and Kaiso localization. All grade groups showed stronger association with low nuclear Kaiso localization ($p = 0.06$). Low histological grade showed no difference in cytoplasmic Kaiso localization, whereas moderate grade group (GII) showed stronger association with low cytoplasmic Kaiso, and high-grade group (GIII) showed stronger association with high cytoplasmic Kaiso ($p = 0.16$).

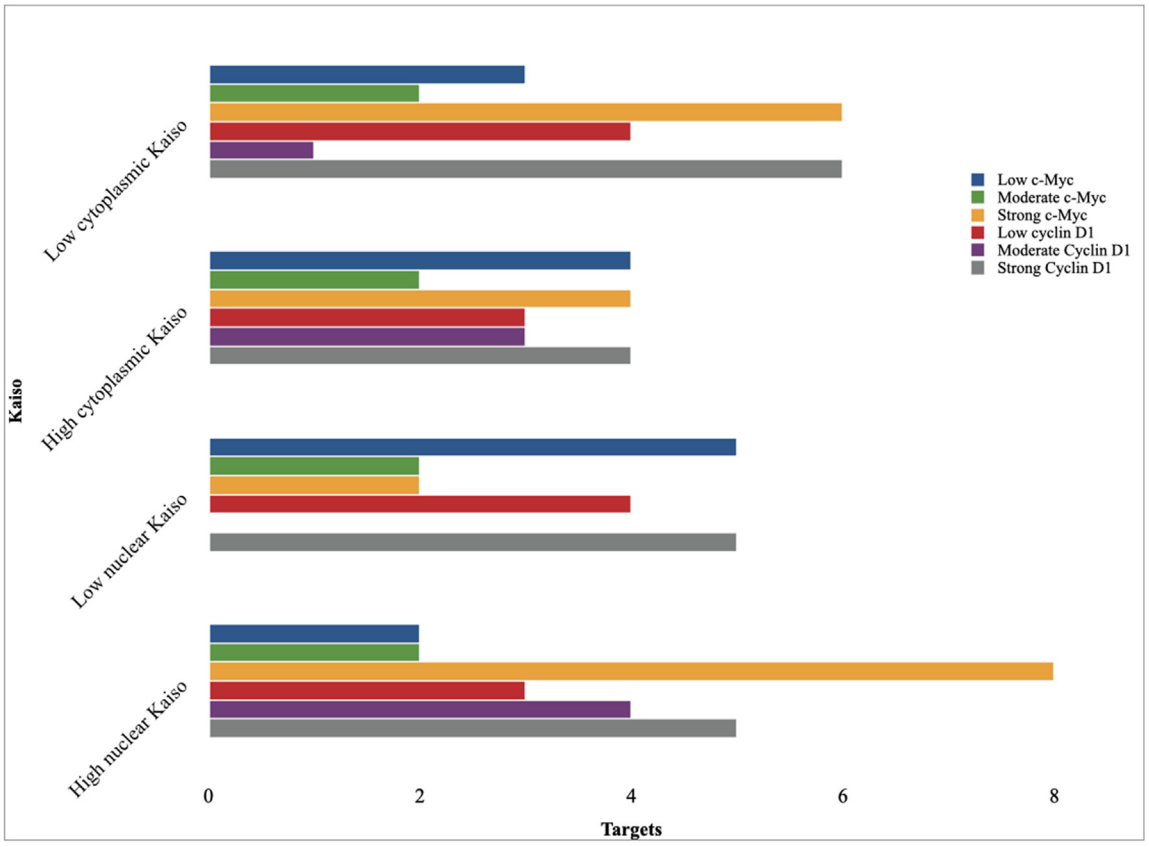

Figure 4: Association of Kaiso localization with target protein expression (c-Myc and cyclin D1). Strong c-Myc expression was noted more frequently in the high nuclear Kaiso expression group and the low Kaiso cytoplasmic expression group. Strong cyclin D1 expression was slightly more frequently noted in the low cytoplasmic Kaiso expression group than in the high cytoplasmic Kaiso expression group, whereas no difference was noted in the low versus high nuclear Kaiso group.

that 87.2% (n = 41) of OC cases had positive cytoplasmic Kaiso, whereas 70.2% (n = 33) of cases also had positive nuclear expression. Positive nuclear Kaiso expression was significantly associated with age ($p = 0.036$*), whereas a greater percentage of nuclear-negative cases was seen in the younger (21−30 years) age group (n = 4) than in the older (>50 years) age group (n = 1). No significant association with prognostic tumor characteristics, including histological grade ($p = 0.52$) and lymph node metastasis ($p = 0.442$), was noted. Notwithstanding the lack of statistical significance, grade III tumors had a greater propensity to be negative for Kaiso (n = 6) than grade I tumors (n = 3).

Based on our knowledge of the relationship between E-cad loss and delocalization of p120ctn from the membrane to cytoplasm and the ability of p120ctn to bind with Kaiso, our next objective was to determine whether Ecad expression had any effect on Kaiso's localization in OC. Our analysis, however, yielded no statistically significant association between Ecad expression and Kaiso localization (Table 3).

To further explore potential role of Kaiso's localization in OC, we calculated the mean cytoplasmic and nuclear positive cell percentages for Kaiso in OC cases and used these values as cut-offs to stratify cases into low cytoplasmic Kaiso (<42% cytoplasmic Kaiso expression) versus high cytoplasmic (≥42%) and low nuclear Kaiso (<11% nuclear Kaiso expression) versus high nuclear Kaiso (≥11%) groups.

Using these threshold values, we analyzed the relationship of Kaiso's localization with histological grades. With advancing tumor grade, Kaiso's expression became more cytoplasmic and less nuclear (Figure 3). A similar trend was observed when we calculated the correlation between tumor stage and Kaiso localization with no significant association observed with either nuclear ($p = 0.71$) or cytoplasmic Kaiso localization ($p = 0.29$).

Our last objective was to study the expression of selected Kaiso targets (cyclin D1 and c-Myc) and how it correlates with the expression/localization of Kaiso. Kaiso expression was significantly associated with the expression of cyclin D1 ($p = 0.007$), but no association was observed for Kaiso expression with c-Myc ($p = 0.85$). Strong c-Myc expression was observed with greater frequency in high nuclear Kaiso and low cytoplasmic Kaiso groups, whereas low nuclear Kaiso and high cytoplasmic Kaiso groups had low c-Myc expression in a greater percentage of tumor tissues. A similar trend was noted when Kaiso's localization was plotted against cyclin D1 expression, with strong cyclin D1 expression observed more frequently in the high nuclear and low cytoplasmic Kaiso groups. However, a statistically

significant association between Kaiso localization and target expression, however, was not recorded (Figure 4).

Discussion

Kaiso is a relatively newly discovered transcription factor capable of both, a tumor suppressor and oncogenic role depending on its localization and methylation status of its target gene promotes in different malignancies.[12] By virtue of this and its potential to sensitize tumor cells to chemotherapeutic drugs, Kaiso has emerged as an interesting biomarker for malignancy as well as potential molecular therapeutic target. Therefore, with the background of what we know about reduced E-cad expression in OC and its contribution to OC progression, as well as the established relationship of E-cad loss with aberrant localization of p120ctn and latter's association with deviant Kaiso localization in several epithelial malignancies, we conducted the present study to identify potential role for Kaiso in OC.[25–27]

Before studying the localization of Kaiso in OC, it was important to establish localization in healthy oral mucosa (Figure 1). Primarily nuclear Kaiso localization was noted in healthy oral mucosa, which was expected due to Kaiso's known function as a nuclear transcription factor with ubiquitous expression in most body tissues. Nuclear expression has also been reported in healthy oral mucosal epithelium by The Human Protein Atlas project.[28]

An increase in the cytoplasmic localization of Kaiso in OC tissues was noted whereas nuclear expression was reduced (Figure 2). This pattern of increased cytoplasmic and reduced nuclear localization in tumor tissues is similar to the pattern observed in CML, pancreatic ductal adenocarcinoma and lung cancer.[16,29,30] In lung cancer cells, p120ctn facilitates cytoplasmic shuttling of Kaiso and latter is associated with increased expression of oncoprotein cyclin D1.[16] In this study, 80.8% (n = 38) of cases showed reduced membranous/ectopic cytoplasmic expression of p120ctn. A significant association was not observed between cytoplasmic Kaiso and p120ctn localization in this study. This could either be due to a separate mechanism for the cytoplasmic shift of Kaiso in OC independent from p120ctn or failure of the present study to detect an association due to a small sample size.

With advancing tumor grade Kaiso became more cytoplasmic and less nuclear (Figure 2). We also assessed the correlation of Kaiso localization with tumor stage and failed to observe any significant association; however, a major limitation was the smaller group size with only six cases of stage T1 and most of the cases (n = 41) from stage T3 and stage T4 (Table 1). This observation is in contrast to what has been reported in breast, prostate, and colorectal cancers, where the potentiation of tumor progression is attributed to nuclear enrichment of Kaiso.[18,19,31] Our findings indicate that nuclear Kaiso could play a tumor suppressor role in OC, with cytoplasmic shuttling of Kaiso contributing to tumor progression via thus far unknown mechanism.

We studied the expression/localization of Kaiso in relation to its partner proteins, p120ctn and E-cad. We first analyzed the expression of E-cad in OC and distinguished moderate-to-low E-cad expression in 76% (n = 36) of cases, in accordance with previous studies on OC.[32–35] An interesting finding of the present study was a statistically significant difference in E-cad expression between men and women, with low expression observed in 66.7% (n = 8) of female cases compared with 25.7% (n = 9) of males and strong E-cad expression observed in 8.3% (n = 1) of females compared with 27% (n = 10) of males ($p = 0.036$). This sex disparity in E-cad expression has not been reported previously and warrants further investigation. Lopez et al. reported slightly higher mean mRNA expression of E-cad in male patients with OC compared with females, but the difference was not statistically significant.[33,35] We failed to note a statistically significant association of E-cad expression with tumor grade or lymph node metastasis, even though E-cad expression has been reported to significantly correlate with both.[35] This could be due to the smaller sample size in the present study as well as subjective scoring of E-cad expression as well as histological grading of tumors.

There was a significant association between the expression of E-cad and p120ctn, in agreement with previous studies.[6,36] Although we did not observe a significant association between the expression of E-cad and Kaiso, we did find that strong Kaiso expression was more frequent in the strong E-cad expression group (50%), indicating that a significant difference might be elucidated with a larger sample size.

Previous studies in OC have reported reduced membranous p120ctn expression as a prognostic indicator, present study however failed to observe this.[6,37,38] Nuclear localization of Kaiso was significantly more positive in the higher age group (>50 years); however an association with other pathological parameters could not be established. This observation may be attributed to histological grade of tumor at presentation, whereby a greater proportion of cases in the high age group (>50 years) presented with lower grade tumor (52.9%, n = 9) compared with patients under 50 years of age in whom only 23.3% (n = 7) presented with Grade I tumor. This suggests that cytoplasmic delocalization of Kaiso may play a role in tumor progression to higher grade, however this needs to be established with greater sample size. In addition, both age and genetics could serve as confounding variables in present study and more controlled studies with larger study groups are warranted to clearly elucidate Kaiso's potential as a biomarker for OSCC.[39] We also did not observe a significant association between Kaiso localization and E-cad expression, even though we expected lower E-cad expression to be more frequently associated with cytoplasmic Kaiso, as observed in studies in other tumors.[18,31]

Kaiso's role in carcinogenesis is dynamically capable of both upregulating and downregulating gene expression.[10,12] For the present study, two known Kaiso targets were chosen: cyclin D1 and c-Myc. Kaiso expression was significantly associated with the expression of cyclin D1 ($p = 0.007$), whereby there was an inverse relationship between the expression of Kaiso and cyclin D1 protein was observed. An inverse relationship was also noted with c-Myc protein expression, but the difference was not significant. We selected known target proteins from published data due to the limited budget.[12] It is pertinent

for future studies to identify target gene promoters that Kaiso specifically interacts with in OC using chromatin immunoprecipitation (ChIP). OC is a heterogeneous tumor with wide-ranging genetic and epigenetic factors that influence the expression of target proteins including human papillomavirus (HPV) status, genetic mutations, and methylation status of promoter operator sequences to name a few.[40] Controlled experiments using cell culture-based methods and/or animal models could better identify the interaction between Kaiso and its target genes in OC cells specifically using sensitive and specific molecular tools such as ChIP and assays using Kaiso knockdown and Kaiso-expressing cells. The lack of statistical significance in this study, therefore, does not exclude Kaiso's role in influencing their expression in OC but instead gives direction for future research into potential diagnostic and prognostic significance of Kaiso as well as its potential role in molecular cancer therapy.

This study had several limitations including the following.

1. Relatively small sample size. We failed to detect a significant association between Kaiso expression and localization with most of our study parameters as well as with Kaiso target proteins, even though variation was noted in the data obtained and could be due to small sizes of various grade groups.
2. Semi-quantitative and subjective methods used for protein quantification. We have utilized IHC for protein expression and localization analysis and measured using subjective scoring, which is not as accurate as other molecular techniques such as western blot analysis or quantitative PCR.
3. Variables such as HPV status, methylation and mutation profiles, which could influence results but were not studied due to time and budget constraints.
4. Selection of target proteins from published data instead of identifying specific targets in OC using ChIP.
5. Cyclin D1 and c-Myc expression in OC could be influenced by a variety of factors other than Kaiso including transcriptional upregulation, gene rearrangement, and amplifications.[41]

We established that Kaiso undergoes a shift in localization from predominantly nuclear to largely cytoplasmic during malignant transformation of oral mucosal epithelium. The implications of this cytoplasmic shuttling need to be determined with further studies utilizing cell culture and/or animal models to identify Kaiso targets using ChIP analysis. It is also essential to identify the methylation status of Kaiso targets and how Kaiso's transcriptional control is influenced by epigenetic factors. Kaiso knockdown study will also allow us to delineate its role in OC progression. Because HPV-positive OC has distinct molecular mechanisms for tumor progression than HPV-negative tumors, we also need to study Kaiso's role in relation to HPV status.

Conclusions

There is a shift in Kaiso's localization in OC tissues compared to healthy oral mucosal epithelium, and even though a statistically significant difference could not be established with prognostic pathological factors, a trend has been observed whereby increasing cytoplasmic localization and decreasing nuclear localization of Kaiso is noted with increasing histological grade of tumor and warrants further validation with a larger sample size and more sensitive identification tools. Furthermore, Kaiso's specific targets in OC need to be ascertained in future studies to better understand its carcinogenic role in this tumor as we failed to notice any strong association with either of known targets (cyclin D1 or c-Myc).

Abbreviations: OC, Oral cancer; HPV, Human papilloma virus; SES, Socioeconomic status; TNM, Tumor; node, metastasis; E-cad, E-cadherin; p120ctn, p120-catenin; DNA, Deoxyribonucleic acid; KBS, Kaiso-binding site; AJ, Adherens junction; IHC, Immunohistochemistry; qPCR, Quantitative polymerase chain reaction; ZF, Zinc finger; MBP, Methyl CpG DNA-binding proteins; BTB/POZ, Broad complex; Tramtrack, and Bric a brac/poxvirus and zinc finger; DDRRL, Dow Diagnostic Research and Reference Laboratory; DUHS, Dow University of Health Sciences; FFPE, Formalin-fixed paraffin embedded; H&E, Hematoxylin and eosin; c-Myc, Cellular Myc proteins; ChIP, Chromatin immunoprecipitation.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The present study was conducted after obtaining approval from the institutional review board (IRB) of Dow University of Health Sciences (IRB-997/DUHS/Approval/2018/43) on 10th February 2018. Informed written consent was obtained from participants. The authors hereby confirm that all methods were carried out in accordance with relevant guidelines and regulations in the Declaration of Helsinki.

Consent for publication

NA.

Authors' contributions

HS contributed to the conception, design, data acquisition, and interpretation and drafted and performed all statistical analyses. SA contributed to the design, data acquisition and interpretation and critically revised the manuscript. FB contributed to the conception, design, interpretation, and critically revised the manuscript. UB contributed to the conception, design, data acquisition and interpretation; performed all statistical analyses; and drafted and critically revised the manuscript. GH contributed to the data acquisition and drafted and revised the manuscript. SN contributed

to the interpretation, statistical analyses, and manuscript review. NM contributed to the conception, design, interpretation, and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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