Association of Kaiso and Partner Proteins in Oral Squamous Cell Carcinoma

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Abstract

Background

E-cadherin loss liberates associated catenins, including p120 catenin, from adherens junctions. Liberated p120 binds a transcription factor, Kaiso, resulting in aberrant expression of the latter's target genes, several of which are known oncogenes and tumor suppressor genes; therefore, Kaiso plays a dynamic role in various malignancies; however, its role in oral cancer remains to be investigated.

Objectives

1. To identify the protein expression and intracellular localization of E-cadherin, p120 catenin, and Kaiso, in oral cancer tissues using Immunohistochemistry
2. To study the protein expression of selected Kaiso targets (Cyclin D1 and c-Myc) and to correlate their expression with the expression and localization of Kaiso
3. To correlate the expression of the aforementioned proteins with clinicopathological parameters

Materials and Methods:

Histological grading was performed using Broder's criteria. IHC staining was used to study the expression and localization of E-cadherin, p120-catenin, Kaiso, Cyclin D1, and c-Myc. Data was analysed using SPSS version 21. The chi-square test was used to measure the statistical significance of associations, keeping a p -value <0.05 as significant.

Results:

Out of 47 oral cancer cases, E-cad expression was low in 36%, p120-ctn expression was low in 34%, and Kaiso expression was low in 78% tumor specimens. P120ctn was delocalized from membrane in 80.8% cases. Kaiso was expressed in the cytoplasm of 87% of tumor tissues, whereas 29.7% of cases had negative nuclear Kaiso expression. 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 subcellular localization of Kaiso in oral cancer tissues. The important implications of this aberrant localization on oral carcinogenesis and tumour prognosis need to be investigated with further studies using larger sample sizes and more sensitive molecular tools.

Background

Distinct pathological processes assist cancer cells in achieving invasion and metastasis. An early fundamental step of this operation is reduction in adhesion of transformed epithelial cells and their

detachment from the subjacent basement membrane as well as adjacent cells(Sasahira 2018). Adhesion protein E-cadherin (E-cad) plays a central role in maintenance of cell-cell adhesion in normal epithelia, and either loss or downregulation of this protein harbingers detachment of cancer cells, which correlates with poor survival(Jiang 2015, Luo 2014, Sasahira 2018). P120-catenin (p120ctn) colocalizes with E-cad at the membranous adhesion complex (adherens junction) and stabilizes the latter(Jiang 2015). Reduced expression of both E-cad and p120ctn has been correlated with advanced clinical stage of oral cancer (OC)(Jiang 2015). Loss of E-cad liberates various catenins (β -catenin and p120ctn) from membranous adherens junctions (AJs), allowing the latter to shuttle between the nucleus and cytoplasm (Bellovin 2005). Free cytosolic or nuclear p120ctn is capable of modulating gene expression via interaction with its nuclear binding partner, Kaiso(Daniel 2002).

Kaiso is a transcription factor that is predominantly characterized as a repressor of gene expression but is also capable of positively regulating target genes(Van Roy 2005). Kaiso has dual DNA-binding specificity, enabling it to recognize and regulate target genes by either detecting methylation marks on nucleotides or a sequence-specific Kaiso binding site (KBS)(Daniel 2002). Kaiso's interaction with DNA and p120ctn is mutually exclusive; therefore, the association of Kaiso with p120ctn, which occurs downstream of E-cad loss, impedes its association with DNA and results in deregulation of Kaiso-regulated genes(Daniel 1999, Daniel 2002, Van Roy 2005). Many Kaiso target genes have been delineated with physiological expression during embryonic development as well as during pathological processes, including carcinogenesis, insinuating a role for Kaiso as a biomarker of malignancy(Pierre 2019). Kaiso interacts with target gene promoters and recruits chromatin modifiers (predominantly corepressor complexes) that either negatively or positively modulate gene expression. Engagement of Kaiso's DNA-binding domain by p120ctn therefore relieves target genes from this control, resulting in deviant expression(Kourtidis 2017, Pierre 2019). Both aberrant expression and localization of Kaiso have been reported in several malignancies, including lung, prostate, breast, and colorectal cancers(Bassey-Archibong 2016, Bassey-Archibong 2017, Dai 2009, Dai 2010, Jones 2012, Lopes 2008, Wang 2016). The exact role of Kaiso in carcinogenesis is contextual and diverse, with some studies having indicated a pro-oncogenic role as opposed to others that have indicated a tumour suppressor role(Jones 2012). While aberrant expression and localization of Kaiso has already been established in several epithelial malignancies, its potential role in oral cancer remains to be investigated. With the present cross-sectional study, we aimed to investigate the potential association of Kaiso expression and localization with the histological grade of oral squamous cell carcinoma and to elucidate the association of the former with the expression of selected Kaiso target proteins (cMyc and cyclinD1).

Methods

The present study was conducted after obtaining ethical approval from the Institutional Review Board (IRB) of Dow University of Health Sciences (DUHS). A total of 47 OC specimens were obtained from biopsy-proven oral squamous cell carcinoma patients undergoing wide tumour excision at DUHS, following written informed consent. Specimens were immediately fixed in 10% neutral buffered formalin.

Three healthy control samples were collected from the Oral Surgery outpatient department (DUHS) after written informed consent was obtained from patients undergoing surgical extraction of third molars.

Histological grading was performed on H&E-stained slides using Broder's system and scored I-III. Grade I = well differentiated, Grade II = moderately differentiated, and Grade III = poorly differentiated (Akhter 2011).

Immunohistochemistry

Tissue sections (3-4 μ M thick) were stained with anti-E-cadherin antibody (1:200; Abcam: ab1416), anti- δ 1 Catenin (1:100; Abcam: ab92514, [EPR357(2)]), anti-Kaiso antibody [clone 6F/6F8] (1:80; Abcam: ab12723), anti-Cyclin D1 [clone SP4] (1:100; ABCA0079417), and anti-c-Myc [clone EP121] (1:100; Cell Marque)] overnight at 4 °C as previously described().

Immunoreactive scores (IRS) were calculated for all proteins as a product of positive cell percentage and staining intensity. The percentages of positive cells were scored from 0-4 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 was grouped into 0= no expression, 1-4= low expression, 5-8= moderate expression, and 9-12= strong expression (Koensgen 2010).

Positive samples were analyzed semiquantitatively at low magnification ($\times 100$) to identify areas where proteins of interest were evenly stained. We counted 400 tumour 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 over 90% of the tumour cells showed cell membrane staining of p120ctn. When less than 90% of the tumour cells were stained for p120ctn at the cell membrane, the sample was labelled with "reduced membranous expression". When more than 10% of the tumour cells stained for cytoplasmic p120ctn expression, the sample was labelled with "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 $\geq 1\%$ of the cells reacted with the anti-Kaiso antibody in respective subcellular compartments (Dai 2010, Vermeulen 2012). Mean expression for both Kaiso and p120ctn was used as the cut-off to divide cases into low ($\leq$ mean) versus high ($>$ mean) expression groups.

Statistical Analysis

Frequencies and percentages were calculated for categorical variables. Continuous variables are expressed as the mean with standard deviation. The chi-square test was executed when finding associations between expression and localization data in relation to each other and clinical variables. All the analyses were performed in IBM SPSS v. 21, and a p -value less than 0.05 was set as the threshold to show a significant difference.

Results

Among the 47 OC cases included in our study, a strong male preponderance was noted with a male to female ratio of 2.9:1. More than half (61.7%) of the patients were greater than 40 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). Tumors showed a strong predilection for involvement of the buccal mucosa (75.5%) and left side of the oral cavity (70.2%). The clinicopathological characteristics of the 47 patients included in the study are summarized in Table 1.

Table I. Histopathological characteristics of tumour cases.		
	Tumor parameter	N(%)
Tumor site	<i>Buccal mucosa</i>	35 (75.5)
	<i>Tongue</i>	7 (15.9)
	<i>Lip</i>	3 (6.4)
	<i>Upper alveolus /Maxilla</i>	1 (2.1)
	<i>Lower alveolus/ Mandible</i>	1 (2.1)
Tumor grade	<i>Well-differentiated</i>	16 (34)
	<i>Moderately differentiated</i>	15 (32)
	<i>Poorly-differentiated</i>	16 (34)
Tumor laterality	<i>Midline</i>	2 (4.3)
	<i>Right</i>	12 (25.5)
	<i>Left</i>	33 (70.2)
Tumor depth /cm	≤ 0.5	6 (12.8)
	<i>0.6-1</i>	11 (23.4)
	<i>1.1-2</i>	18 (38.3)
	<i>2.1-3</i>	9 (19.1)
	<i>3.1-4</i>	2 (4.3)
	<i>>4</i>	0
Lymph node metastasis	<i>Present</i>	25 (53.2)
	<i>Absent</i>	16 (34)
	<i>N/A</i>	6 (12.8)
Age / years	<i>21-30</i>	8 (17)
	<i>31-40</i>	10 (21.3)
	<i>41-50</i>	12 (25.5)
	<i>>50</i>	17 (36.2)
Gender	<i>Male</i>	35 (74.5)
	<i>Female</i>	12 (25.5)
Total		47 (100)

Table I. Histopathological characteristics of tumour cases.

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

As a pilot study of Kaiso's role in OC, we first ascertained Kaiso's localization in healthy control oral mucosal samples using IHC. Strong nuclear expression of Kaiso in healthy oral mucosal epithelial cells was observed in concordance with Kaiso's known role as a regulator of gene expression (N=3) (Figure 1).

In contrast, IHC analysis in OC tissues showed prominent cytosolic Kaiso expression, indicating a shift in localization during carcinogenesis. With advancing tumour grade, the overall expression of all three proteins diminished. Additionally, the expression of both p120ctn and Kaiso became more cytoplasmic with advancing tumour grade (Figure 2).

The relationship between the expression of E-cad and p120ctn has already been well established, and we expanded our analysis to check whether there was any significant alteration in the expression of Kaiso with a change in the expression of the former. A significant correlation between the expression of E-cad and p120ctn was recorded, but a statistically significant association was not observed between the expression levels of E-cad and Kaiso, even though half of cases showing strong Kaiso expression also had strong E-cad expression and 85% of cases with low Kaiso expression also had low to moderate E-cad expression (Table 2).

Table 2. Correlation of E-cadherin expression with expression of p120ctn and Kaiso									
E-cadherin expression	P120 catenin expression			<i>p-value</i>	Kaiso expression				<i>p-value</i>
	Low	Moderate	Strong		None	Low	Moderate	Strong	
<i>Low</i>	11	6	0	0.003*	0	13	3	1	0.19
<i>Moderate</i>	4	10	5		1	16	1	1	
<i>Strong</i>	1	5	5		2	5	2	2	
	16	21	10		3	34	6	4	

The focus of the present study was Kaiso's localization more than its expression. Analysis of Kaiso's localization in OC relative to clinicopathological tumour parameters revealed that 87.2% (n=41) of OC cases had positive cytoplasmic Kaiso localization and 70.2% (n=33) of cases also had positive nuclear expression. Positive nuclear expression of Kaiso was significantly associated with age, whereby a greater percentage of nuclear-negative cases was seen in the 21-30 year age group (50%) than in the >50 year

age group. No significant association with prognostic tumour characteristics, including histological grade and lymph node metastasis, was seen. Notwithstanding the lack of statistical significance, the percentage of tumour cases that were negative for nuclear Kaiso was higher in GIII tumours (42.8%) than in GI tumours (21.4%). Similarly, negative cytoplasmic Kaiso expression was more frequent in GI (33.3%) than in GIII (16.6%).

In keeping view of the relationship between E-cad loss and delocalization of p120ctn from the membrane to the cytoplasm and the latter’s ability to potentially interact with Kaiso, we wanted to check whether Ecad expression had any bearing on Kaiso’s localization in OC. Our analysis, however, yielded no statistically significant association between Ecad expression and Kaiso localization (Table 3).

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	
	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.						

To delve deeper into Kaiso’s localization and its role 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 tumour grade, Kaiso’s expression was more cytoplasmic and less nuclear; however, a statistically significant association was not recorded (Figure 3).

Our last objective was to identify any variation in the expression of selected Kaiso target proteins (Cyclin D1 and cMyc) with the expression and localization of Kaiso in OC. Kaiso expression was significantly associated with the expression of cyclin D1 (p-value 0.007), but no association was recorded for Kaiso expression with the expression of c-Myc (p-value 0.85).

Finally, we analyzed how Kaiso’s subcellular localization influences the expression of selected target proteins. Strong c-Myc expression was noted with greater frequency in the high nuclear Kaiso and low

cytoplasmic Kaiso groups, whereas the low nuclear Kaiso and high cytoplasmic Kaiso groups had low c-Myc expression in a greater percentage of tumour tissues, even though a statistically significant difference was not recorded. 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, but a statistically significant difference between groups was not seen. (Figure 4).

Discussion

Kaiso is a relatively newly discovered transcription factor with heterogeneous implications in various cancers(Pierre 2019). Our thorough review of scientific literature has yielded, to the best of our knowledge, no studies to date on the localization and expression of Kaiso in oral cancer(Pierre 2019). Therefore, with the background of what we know about reduced E-cadherin expression in OC and its contribution to OC progression, as well as the established relationship of E-cadherin loss and subsequent p120-catenin delocalization with aberrant Kaiso localization in several epithelial malignancies, we conducted the present study to identify Kaiso's potential role in OC(Peng 2018, Rivera 2017, Silva 2017).

Before studying the localization of Kaiso in OC, it was important to establish localization in healthy oral mucosa, so we analyzed Kaiso localization using IHC in healthy control tissues (Figure 1). Kaiso was localized in the nucleus of healthy oral mucosal epithelial cells, which was expected owing to Kaiso's 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().

Interestingly, we observed a striking increase in the cytoplasmic localization of Kaiso in OC tissues, with 87% (n=41) of cases showing positive cytoplasmic Kaiso expression, whereas nuclear expression was reduced, with 29.7% (n=14) of cases showing the absence of nuclear Kaiso. This pattern of increased cytoplasmic localization with reduced nuclear localization in tumour tissues is similar to the pattern observed in lung cancer, pancreatic ductal adenocarcinoma and CML(Cofre 2012, Dai 2009, Jones 2016). In lung cancer, cytoplasmic shuttling of Kaiso is facilitated by p120ctn and associated with increased expression of oncoprotein CyclinD1(Dai 2009). In the present study, 80.8% (n=38) of cases showed reduced membranous/ectopic cytoplasmic expression of p120ctn. A statistically significant association could not be established between Kaiso and p120ctn localization in the present study. This could either be due to a separate mechanism for cytoplasmic shuttling of Kaiso in OC independent of p120ctn or failure of our study to detect an association due to the relatively small sample size and lack of colocalization study, which would provide better insight into the relationship between these proteins in OC.

The overall expression of Kaiso was none to low in 78.7% (n=37) of cases in the present study. As histological grade increased, Kaiso expression became more cytoplasmic and less nuclear, even though the difference was not statistically significant. This observation is in contrast to what has been reported

in colorectal, prostate and breast cancers, where nuclear enrichment of Kaiso has been reported to potentiate tumour progression(Jones 2012, Lopes 2008, Vermeulen 2012). These findings indicate that nuclear Kaiso might play a tumour suppressor role in oral cancer, with cytoplasmic shuttling of Kaiso contributing to tumour progression. However, this is a very limited study, and further evidence needs to be elucidated before concluding Kaiso's exact role in oral cancer progression.

We studied the expression and localization of Kaiso in relation to the expression and localization of its partner proteins p120ctn and E-cad.

We first analyzed the expression of E-cad in OC tissues and identified moderate to low E-cad expression in 76% (n=36) of cases. Diminished E-cad expression is in concordance with previous studies in OC that have reported reduced E-cad to be a prognostic marker for OC(Diniz-Freitas 2006, López-Verdín 2019, Zhao 2012, Zhou 2015). Interestingly, we observed a statistically significant difference in E-cad expression between men and women, with low expression noted in 66.7% of female cases compared with 25.7% of males and strong E-cad expression noted in 8.3% of females compared with 27% of males (p-value = 0.036). This sex disparity in E-cad expression has not been noted in previous similar studies and warrants further investigation with a larger sample size, even though a recent study by Lopez et al. showed slightly higher mean mRNA expression of E-cad in male OC patients than females, but the difference was not statistically significant(López-Verdín 2019, Zhou 2015). We were unable to find a statistically significant difference in E-cad expression with respect to various clinicopathological parameters, including histological tumour grade or lymph node metastasis, even though previously E-cad expression has been reported to significantly correlate with histological tumour grade as well as lymph node metastasis(Zhou 2015). 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 concordance with previous studies(Jiang 2015, Sasaya 2013). Although we did not observe a significant association between the expression of E-cad and Kaiso, we did observe strong Kaiso expression to be more frequent in the strong E-cad expression group (50%) and low Kaiso expression in the low to moderate E-cad expression group (85%), indicating that a significant difference might be elucidated with studies using larger sample sizes.

We failed to observe a significant association between p120ctn localization and pathological parameters of the study, even though an association has been previously reported(Jiang 2015, Ma 2012). Nuclear localization of Kaiso was significantly more positive in the higher age group (>50 years), but the association of Kaiso's localization with other pathological parameters could not be ascertained. 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 tumours(Jones 2012, Vermeulen 2012).

Kaiso plays a dynamic role in cancers as well as healthy tissues and is known to be capable of both upregulating and downregulating gene expression(Pierre 2019, Van Roy 2005). We selected two known

Kaiso targets and evaluated their protein expression using IHC in relation to the expression and localization of Kaiso. Cyclin D1 and c-Myc. Kaiso expression was significantly associated with the expression of Cyclin D1 (p-value = 0.007), whereby there was an inverse relationship between the expression of Kaiso and CyclinD1 protein. An inverse relationship was also noted with c-Myc protein expression, but the difference was not statistically significant. Low expression of both target proteins, CyclinD1 and c-Myc, was also noted more frequently in the low Kaiso expression group, indicating that an association cannot be established without further studies with more sensitive techniques. We selected known target proteins from published data owing to limitations in the available budget(Pierre 2019). It is important to also establish which target gene promoter Kaiso interacts with in oral cancer using chromatin immunoprecipitation (ChIP) and quantification of target gene expression using quantitative polymerase chain reaction (qPCR). OC is a very heterogeneous tumour with many genetic or epigenetic factors that could influence target protein expression, such as HPV status, mutations, and promoter methylation(Jadhav 2013). Controlled experiments using cell lines/animal models could better identify the interaction between Kaiso and its target genes in oral cancer. The lack of association in the present study, therefore, does not exclude Kaiso's role in influencing their expression in oral cancer but instead gives direction for future research.

Conclusion

There is a shift in Kaiso's localization in oral cancer tissues compared to healthy oral mucosal epithelium, and even though a statistically significant difference could not be established with histological grade or other pathological parameters, a trend has been observed and warrants further validation with a larger sample size and more sensitive identification tools.

Declarations

Ethics approval and consent to participate

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). Informed written consent was obtained from participants. 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

Availability of data and materials

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

Conflict of interest

Authors declare no conflicts of interest

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Authors' contributions

Dr. Hira Salam contributed to conception, design, data acquisition and interpretation and drafted and performed all statistical analyses.

Dr. Shaheen Ahmed: Contributed to conception, design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript.

Dr. Furqan Bari: Contributed to conception, design, interpretation, and critically revised the manuscript

Dr. Uzma Bukhari: Contributed to conception, design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript.

Mr. Ghulam Haider contributed to data acquisition and drafted and revised the manuscript.

Dr. Nouman Mughal: Contributed to 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.

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NA

Abbreviations

OC	Oral cancer
HPV	Human Papilloma Virus
SES	Socio-economic 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
EMT	
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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Figures

Figure 1

Immunohistochemical expression of Kaiso in healthy control oral mucosa showing strong nuclear expression (x200)

Figure 2

H&E 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 becomes increasingly expressed in the cytosol with advancing tumour grade (Magnification x 200)

Figure 3

Association between histological tumour grade and Kaiso localization. All grade groups showed stronger association with low nuclear Kaiso localization (p -value = 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 -value = 0.16)

Figure 4

Association of Kaiso localization with target protein expression (cMyc 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 vs high nuclear Kaiso groups

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