Expressional Variations of Kaiso: An Association with Pathological Characteristics and Field Cancerization of OSCC

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Abstract

Background:

In patients diagnosed with cancer it is considered that there is a field of tissue in the diagnosed organ containing cells with genetic aberration which pose a higher risk of transforming in to premalignant and malignant lesions. This field of altered cells is known as field of cancerization and is considered responsible for frequent recurrence of the cancer. Various molecules are being investigated for their significance in development of field cancerization and hence malignant diseases. Various members of POZ-ZF (poxvirus and zinc finger (POZ)-zinc finger) family of transcription factors are known to play a part in pathogenesis of neoplasm, one such protein of this family is Kaiso also known as ZBTB33 (Zinc Finger and BTB Domain containing 33. Kaiso is a transcription factor known for having two modes of binding with DNA. The protein belongs to POZ-ZF family of transcription factors and may have functional tasks similar to its other siblings such as growth and development of vertebrates and pathogenesis of neoplastic diseases. Nevertheless, its role in field cancerization, pathogenesis and progression of oral cancer still needs exploration. Hence, this study was designed to explore the expressional differences between the mucosa of controls and those diagnosed with OSCC.

Methods:

Soft tissue samples were obtained from main tumor, tumor periphery and opposite buccal mucosa of 50 oral cancer patients, whereas normal mucosa was taken from 50 volunteers undergoing elective tooth removal. The acquired samples were then subjected to Immunohistochemical exploration for expression of Kaiso. The expression was measured using Image-J IHC profiler and summed as Optical density. The Optical density values were then subjected to statistical analysis.

Results:

Results revealed a significant differential expression of Kaiso between the mucosal tissues taken from oral cancer patients and controls (p-value: <0.0001) showing almost 50% down-regulation of Kaiso in all three tissue samples taken from oral cancer patients as compared to normal mucosa.

Conclusion: Kaiso have significant difference of expression in the mucosa of oral cancer patients than the normal mucosa making it a probable contributor to disease pathogenesis and field cancerization.

Background

Neoplasms arise due to compound genetic and epigenetic aberrations that consequentially transmute cells of a particular organ initiating an advanced invasive disease. It is hypothesized that these genetic and epigenetic aberrations are not localized to a specific group of cells, rather they involve all the daughter cells residing in a particular field or surrounding tissues of the tumor. Although, not currently transformed completely in an invasive disease these resident cells harbor enough genetic anomalies

which may transform them in to malignant tumor at any given time in future. This populace of daughter cells residing in an organ, which harbor genetic aberrations but don't display morphological transformation consistent with malignant neoplasm, corroborate with the concept of field cancerization, initially presented in 1953 by Slaughter *et al.*[1] Scientific studies are being conducted to pinpoint molecular signatures which may help identify these genetically anomalous cell population which haven't adapted to pathologic morphology of invasive carcinoma. When identified, these molecular markers may provide remarkable utility in terms of screening, diagnostics and targeted therapeutics. Some of the molecular markers found associated with oral field cancerization include cytokeratins 7, 8, 13, 16, and 19,[2] type 2 chain ABH antigen,[3] cyclin D1,[4,5] EGFR,[6–8] TGF-α,[8] Ki-67,[9] Bcl-2,[10] vascular markers (VwF, CD31, αVβ3, α-SMA),[11] and p53.[12–17] In this study an effort is done to find an association between field cancerization in oral cavity and a protein known as Kaiso. Kaiso is a member of broad-complex, tramtrack and bric-a-brac/poxvirus and zinc finger (BTB/POZ) family with subfamily of zinc finger proteins (POZ-ZF).[18] Transcription factors belonging to this family are known to play part in growth and developmental aspects of vertebrates, which indicates that Kaiso might have a comparable functionality.[19] Kaiso's functional preferences are described to change in context to its interactions with different proteins.[20] For instance, it has been demonstrated that Kaiso activates BCL2 a protein that inhibits apoptotic death of the cell while deactivating the two pro-apoptotic proteins BAX and BIK hence leading to apoptotic diffidence.[21] Another example of context specific functionality of Kaiso is demonstrated by its binding with wild type and mutated p53 where it promotes apoptosis when bonded with former and suppresses programmed cell death when bonded with later, respectively.[22,23] Considering the examples given previously Kaiso may be a vital part of pathogenic pathways leading to development of neoplastic diseases. To date, ample scientific literature have verified Kaiso's involvement in different types of cancers such as breast CA,[22,24–27] Lung cancer (NSCLC),[28] prostate CA,[29,30] pancreatic cancer (PDAC).[31] Though, depicting Kaiso as a tumor suppressor or tumor promoter with certainty has been a challenging task. Furthermore, scientific literature is also deficient regarding Kaiso's functional role in pathogenesis of oral mucosal cancer. Also, it is yet to be explored that whether the exposure of oral mucosa to known carcinogens such as tobacco, betel quid, betel nuts and other combination products have any effect on expressional values of Kaiso. Consequently, this study was directed at exploring expressional changes of kaiso in mucosal specimens taken from Tumor, periphery of the tumor, and opposing mucosa of patients diagnosed with oral squamous cell carcinoma compared with subjects who were disease free and were not exposed to any of the chemical carcinogens associated with the disease.

Methodology

Study Design and Study Setting

The study design was analytical cross-sectional. A total Soft tissue specimens from 50 biopsy proven OSCC patients and 50 Controls were obtained who were attending the Department of Oral and

Maxillofacial Surgery at Dow International Dental College, Dow University of Health Sciences, Karachi, Pakistan. The samples were collected during patient's therapeutic surgery from OSCC cases and during elective tooth removal from controls, respectively. Cognizant patient consent and endorsement from Institutional Review Board of Dow University (IRB-1319/DUHS/Approval/2019) was obtained for the study. Patients' biographic information, medical history, extent and characteristics of the disease were documented in pre-designed proformas. Small tissue samples were taken from Tumor (Labeled T), periphery of the tumor (Labeled P), and opposing mucosa (Labeled O), of OSCC patients who matched the inclusion and exclusion criteria (Table 1). From controls who matched the inclusion and exclusion criteria (Table 1) a small tissue specimen was collected during elective surgical removal of wisdom teeth, Labeled as C. All samples were immediately placed in 10% buffered Neutral formalin.

Table 1: Inclusion and Exclusion Criteria for Cases and Controls

CASES	Inclusion Criteria	Exclusion Criteria
	Biopsy proven cases of OSCC regardless of age/gender.	-Recipients of prior chemotherapy or radiotherapy. - Patients with any congenital syndrome, autoimmune diseases, chronic inflammatory diseases, and any other chronic illness - Poorly fixed tissue.
CONTROLS	**Inclusion Criteria**	**Exclusion Criteria**
	Adult patients undergoing elective surgical tooth extractions for wisdom teeth.	-Patients with infected teeth -Patients with any congenital syndrome, autoimmune diseases, chronic inflammatory diseases, and neoplastic diseases
	Patients never exposed to any chemical carcinogens such as betel quid, betel nut, and any form of tobacco	-Patients with habits of: tobacco use in any form, Betel quid use, betel nut use, Alcohol, or any combinations of these products.

Tissue Processing and Immunohistochemical Staining

The tissues obtained were embedded in paraffin to form blocks. Ultra-thin consecutive sections of 4µm were incised from every single block. To scrutinize histopathological physiognomies, all sections from each sample were stained with Hematoxylin & Eosin stains, after analyzing histologic characteristics the tissues were then probed for expression of Kaiso using Immunohistochemical Staining. Briefly, the tissue sections were deparaffinized by xylene, after which tissue hydration followed by heating in microwave with citrate buffer (pH = 6) for retrieval of antigens was performed. Afterward the tissues were subjected

to incubation in hydrogen peroxide for 20 mins to mollify endogenous peroxidase interactions. Subsequently, tissue specimens were exposed to bovine serum albumin for an hour to terminate non-specific binding of antibodies. Next, tissues were incubated with polyclonal rabbit anti-kaiso (Thermo Fischer Scientific; PA5-81890) antibodies after which they were washed using Phosphate Buffered Saline. Then, EnVision FLEX, High pH (Link) (DAKO, Agilent Tech.) was used for detecting primary antibodies. Finally, the tissues were incubated with diaminobenzidine (DAB) + chromogen followed by hematoxylin stain. After completing the chemical treatment tissues slides were mounted using DPX and cover slip.

Imaging and Expressional Analysis

IHC slides from both cases and controls were photographed using Nikon Eclipse 80i at 40x. To quantify protein expression images were then analyzed using ImageJ IHC profiler software, which showed the protein expression as High positive (HP), positive (P), low positive (LP) and negative (N) signals. The four values obtained from ImageJ were then converted to optical density score, via formula given by S.Jafari *et al*[32]:

(4HP+3P+2LP+1N) / 100

The optical density values were then used for statistical analysis.

Statistical Analysis

Data normality was analyzed using Kolmogorov-Smirnov test. Differential expression of the Kaiso protein among the cases and controls, different tumor sizes and tumor grades was analyzed using one way Anova. Also, Pearson's r test was done to find correlation between Expression of Kaiso and all the histological features recorded. The statistical analysis was performed by means of Graph Pad Prism software and a p-value of less than 0.05 was considered significant.

Results

Out of total 50 cases 41 were obtained from males, whereas 9 were obtained from the females. The mean age of OSCC case group was 50.8 ± 11 years. Further stratification of the cases was done according to Broders grading System **Table 2,** and according to tumor size **Table 3.**

Table 2: Distribution of Cases According to Broders Grading System

Grade	No. of Cases	Percentage
Well Differentiated	18	36
Moderately Differentiated	20	40
Poorly Differentiated	12	24

Table 3: Distribution of Cases According to Tumor Size

Tumor Size	No. of Cases	Percentage
T1	7	14
T2	9	18
T3	6	12
T4	28	56

Differential Analysis

Differential expression of Kaiso was assessed between tissue specimens taken from controls and tissue specimen taken from OSCC patients to determine expressional variations between the two and to assess if there is any kind of evidence present regarding field cancerization. Furthermore, expressional variations were assessed in different grades and sizes of tumors to determine Kaiso's role in disease progression.

KAISO Expression in Controls and Tumor, Periphery and Opposing Buccal Mucosa of OSCC Patients

When expression of Kaiso was compared among the two groups specimens taken from tumor (T), periphery (P), and opposite buccal mucosa (O); no significant differential expression was seen among the three groups in OSCC cases of Kaiso (p-value 0.1646), **Figure 1(a)**. But when all three values were analyzed against the expressional values of Control group a significant difference of expression was observed between tissues of controls and cases with a P-value of <0.0001, **Figure 1(b).** Mean expression values of Kaiso protein in all specimen are given in **Table 4** showing a drastic decrease in kaiso expression in all tissues obtained from OSCC group.

Table 4: Mean Expression of Kaiso in Cases and Controls

Controls (C)	*OSCC CASES*		
	Tumor (T)	Periphery (P)	Opposite (O)
3.443 ± 0.3743	1.516 ± 0.2790	1.623 ± 0.3068	1.526 ± 0.3397

KAISO Expression in Different Grades and Sizes of OSCC:

On comparison between the different grades of tumor, no significant alteration could be observed among the mean expression values of Kaiso among different grades of OSCC, as confirmed by p-value 0.4042, **Figure 2(a)**. Similarly, no significant mean expressional variation could be found in different tumor (T) sizes (p-value: 0.3762), **Figure 2(b).** Mean expression values of Kaiso in different grades and T sizes are given in **Table 5.**

Table 5: Mean Expression of Kaiso in Different Tumor Grades and Sizes

Kaiso Expression in Different Grades of OSCC			
Well Differentiated OSCC	**Moderately Differentiated OSCC**		**Poorly Differentiated OSCC**
1.471 ± 0.3016	1.582 ± 0.3016		1.474 ± 0.1899
Kaiso Expression in Different Tumor Size			
T1	**T2**	**T3**	**T4**
1.401 ± 0.1532	1.559 ± 0.2998	1.375 ±0.2607	1.561 ± 0.2938

Correlation Analysis

Expression of kaiso was correlated with various histological features such as histological features defined in bryne's TIF scoring system and also total malignancy score(33)**(Table 6); Figure 3**, number of positive lymph nodes **Figure 4(a),** tumor depth **Figure 4(b),** and tumor budding scores(34) **(Table 7), Figure 4(c).** No significant positive or negative correlation could be found for any of the physio-pathological characteristics as depicted by the straight correlation lines of the graphs and p-values mentioned in the respective figures.

Table 6: Bryne's Tumor Invasive Front (TIF) Grading System(33)

Morphological Features	SCORE			
	1	2	3	4
Degree of keratinization	Highly keratinized (>50% of the cells)	Moderately Keratinized (20-50% of cells)	Minimal Keratinization (5-20% of cells)	No Keratinization (0-5% of cells)
Nuclear Polymorphism	Little Nuclear Polymorphism (>75% mature cells)	Moderate Nuclear Polymorphism (50-75% mature cells)	Abundant Nuclear Polymorphism (25-50% mature cells)	Extreme Nuclear Polymorphism (0-25% mature cells)
Number of Mitosis (HPF)	0-1	2-3	4-5	>5
Pattern of Invasion	Pushing well delineated	infiltrating cords/ bands strands	small cell group or chords of infiltrating cells n>15	Marked and widespread cellular dissociation in small groups of cell (n<15) and/or in single groups
Plasma Lymphocytic Infiltrate	Marked	Moderate	Slight	None

Table 7: Tumor Budding Score[34]

No. of Tumor Buds per 10 *HPF x40	Score
0 Tumor Buds = No Budding	1
1-14 tumor buds = low Budding	2
>15 tumor buds = high Budding	3

Discussion

Concept of field cancerization was first suggested by Slaughter et al. after extensive histological examination of 783 cases of oral cancer, where he observed multiple centers of abnormal epithelium beyond the boundaries of clinically evident malignant tumor. He observed that there was evidence of multiple microscopic foci of abnormal epithelial cells which later enlarged and coalesce to form clinical picture of invasive carcinoma. [1] Later on, the term “Field Cancerization” was coined for this phenomenon and extensive scientific investigations on molecular level were started regarding this concept to determine subsequent genetic aberrations which make the driving force towards malignant transformation of cells.

Several genes and their expressional abnormalities have been implied as markers for identification of field cancerization. These include, tumor suppressor genes such as p53,[35–37] p16,[38] Cyclin D1,[39] p21, [37]; proto-oncogenes like, Ras[40] and ErbB1[41]; growth factor receptors such as EGFR[42], VEGF[43], TGF-α[8], and CD34[44]. However, in this study we have made an effort to determine whether expressional dysregulation of Kaiso has any role in field cancerization.

Kaiso is a member of BTB/POZ-ZF family of site specific transcription factors. Functional activity of Kaiso as a transcription factor has been considered vacillating due to its inclination towards context specific suppression and/or activation of various genes in different types of cells.[20] Various factors are anticipated as game changers in Kaiso's functional specificity towards a tumor suppressor or activator role. One of the factors may be Kaiso's ability to bind both methylated and non-methylated sequence specific sites (bi-modal DNA binding).[45,46] Second factor that should be taken into account is its SUMOylation which may play a role in bimodal functional abilities of the protein.[47] Another factor that can be taken in to account is that whether Kaiso binds with wild-type p53 or mutated p53 which determines the final functional path chosen by the protein.[22,23] Here in this study we compared expression of kaiso in mucosa of subjects who were never exposed to chemical carcinogens known to predispose to oral cancer, to the expression of kaiso in biopsy proven tumors and seemingly normal mucosa taken from the tumor periphery and opposing cheek of the same patient. The idea was to determine what path this protein takes in case of oral squamous cell carcinoma in terms of expression. Findings, astonishingly show an almost 50% decreased expression of Kaiso in the mucosa of OSCC patients in comparison to controls with p-value of <0.0001 (Figure 1b). Also, it can be appreciated that pure tumor tissue, peripheral mucosa and opposing mucosa of OSCC cases, all show similar mean expression of kaiso (Table 4), indicating a decreased kaiso expression in entire oral cavity and not only the tumor site. These findings highlight that Kaiso might have a vital role in defining field of cancerization in cases of oral squamous cell carcinoma. Furthermore, a comparison of expressional variations of kaiso was done between different tumor grades and tumor sizes which turned out to be insignificant (Figure 2a & 2b). This outcome indicates that kaiso's expressional vicissitudes are more likely involved in incidence of OSCC rather than its progression of the malignancy. To further explore this notion, correlation analysis were done employing Kaiso expression in tumor tissues and the histological features of these specimens, that are frequently used in terms of prognostic determinants of OSCC (Figures 3 and 4), namely number of positive lymph nodes, depth of tumor, number of tumor buds, degree of keratinization, number of mitotic figures, nuclear polymorphism, pattern of invasion and extent of plasma-lymphocytic infiltrate. No significant correlation could be observed between expression of Kaiso and histological features mentioned, which further strengthens our impression of kaiso acting as a match stick to a hay stack in case of OSCC. A study by Cofre et al. demonstrated that diminution of kaiso expression resulted in augmented proliferation and reduced expression of differentiation markers; supporting our findings.[48]In contrast to this study several studies have reported a higher expression of kaiso associated with different types and pathological features of cancers. For instance, Pierre *et al. reported a* greater Kaiso expression in primary & metastatic tumor tissue specimens of colon, in comparison to normal.[49] Whereas, Jones *et*

al. observed *a* higher Kaiso expression in malignant tumors of the prostate than benign prostatic hyperplasia. Also, he reported that higher expression of kaiso had a correlation with higher tumor grade.[29] In another scientific paper, Jones *et al*. reported similar associations between high kaiso expression in pancreatic ductal carcinomas and higher tumor grades and sizes.[31] Analogous findings are reported in case of breast carcinoma in various studies.[5, 7, 22, 23] Differences which might be responsible for such contrasting results might include exposure to chemical carcinogens specific to oral cavity such as betel nut and betel quid promoting a factor specific functioning of Kaiso in oral mucosa.

What mechanism and/or factors are behind this striking decrease in Kaiso's expression in the mucosal of oral cancer patients still needs to be explored, giving us a future direction for scientific research that is much needed.

Limitations of the study

Like most studies, there were some limitations to this study. The sample size employed in this study is small. The measurement of protein was done only by one method and may be performed with other methods of protein estimation to cross check the results. No specimens were included from patients with precancerous conditions, inclusion of which may help determine a threshold level of Kaiso that may be used to preempt conversion of premalignant state into a malignant state before appearance of physiopatholigcal characteristics. Hence, it is required that the research is validated with an improved sample size and multicenter study, with populations from different ethnicities, and with groups including oral premalignant states and lesions in future.

Conclusion

In conclusion, it can be safely assumed that Kaiso has a role in genesis of Oral cancer indicated by its expressional dysregulation in OSCC patients. Also, Kaiso may serve as a marker for field cancerization in OSCC patients. These findings make Kaiso a suitable candidate for targeted therapy. Therefore, it is recommended that other aspects of Kaiso's functionality should be assessed to further elaborate its role in pathogenesis of oral squamous cell carcinoma and field cancerization.

Abbreviations

OSCC: Oral Squamous Cell Carcinoma, IHC: Immunohistochemistry, PBS: Phosphate Buffered Saline, BNF: Neutral Buffered Formalin

Declarations

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Authors contribution

SA: Contributed in conceptualizing the overall study, data collection, sample processing and drafting of manuscript. SK: Supervised, drafting and editing of manuscript.

AQ: Co-Supervised the study, drafting and editing of the main manuscript.

UB: Contributed and helped in assessments of patients of Oral Squamous Cell Carcinoma.

NM: Co-Supervised the study, contributed in conceptualizing the overall study.

MA: Contributed in analyzing the results of the study.

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Availability of data and materials

The data collected and used for this research is the property of Dow University of Health Sciences, and is available upon request from the corresponding author

Ethics approval and consent to participate

This study is conducted after taking endorsement from Institutional Review Board of Dow University of Health Sciences IRB No: IRB-1319/DUHS/Approval/2019. All techniques employed in the study were in agreement with 1964 Helsinki Declaration and the standards approved by the institutional and/or national research committee. Informed consent was obtained in writing from all the research participants.

Consent for publication

The current study does not contain any images or videos related to any research participant. The consent for publication is obtained from all research participants included in the current study.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

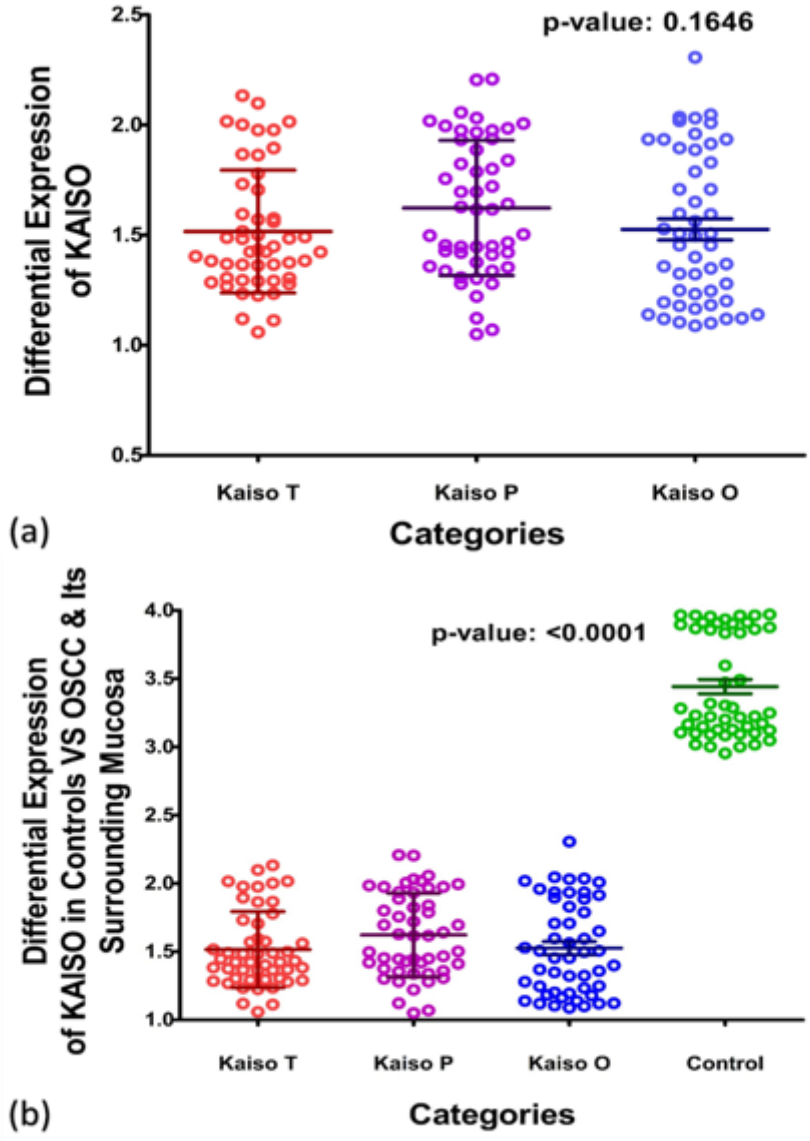


Figure 1

Differential Expression of Kaiso in OSCC (a) and Controls against OSCC (b). The graph in figure **(a)** demonstrate the difference of expression in Kaiso among tissue specimens taken from OSCC cases; whereas figure **(b)** demonstrates differential expression between the Controls and OSCC tissue

specimens. All values plotted here are in the form of optical density (OD). The large central line represents mean, whereas small horizontal lines above and below represent the standard deviation.

Figure 2

Differential Expression of Kaiso in Different Tumor Grades (a) and Tumor Sizes (b). The graph in figure **(a)** demonstrate the difference of expression in Kaiso among different Tumor Grades; whereas figure **(b)** demonstrates differential expression of Kaiso among different tumor sizes. All values plotted here are in the form of optical density (OD). The large central line represents mean, whereas small horizontal lines above and below represent the standard deviation.

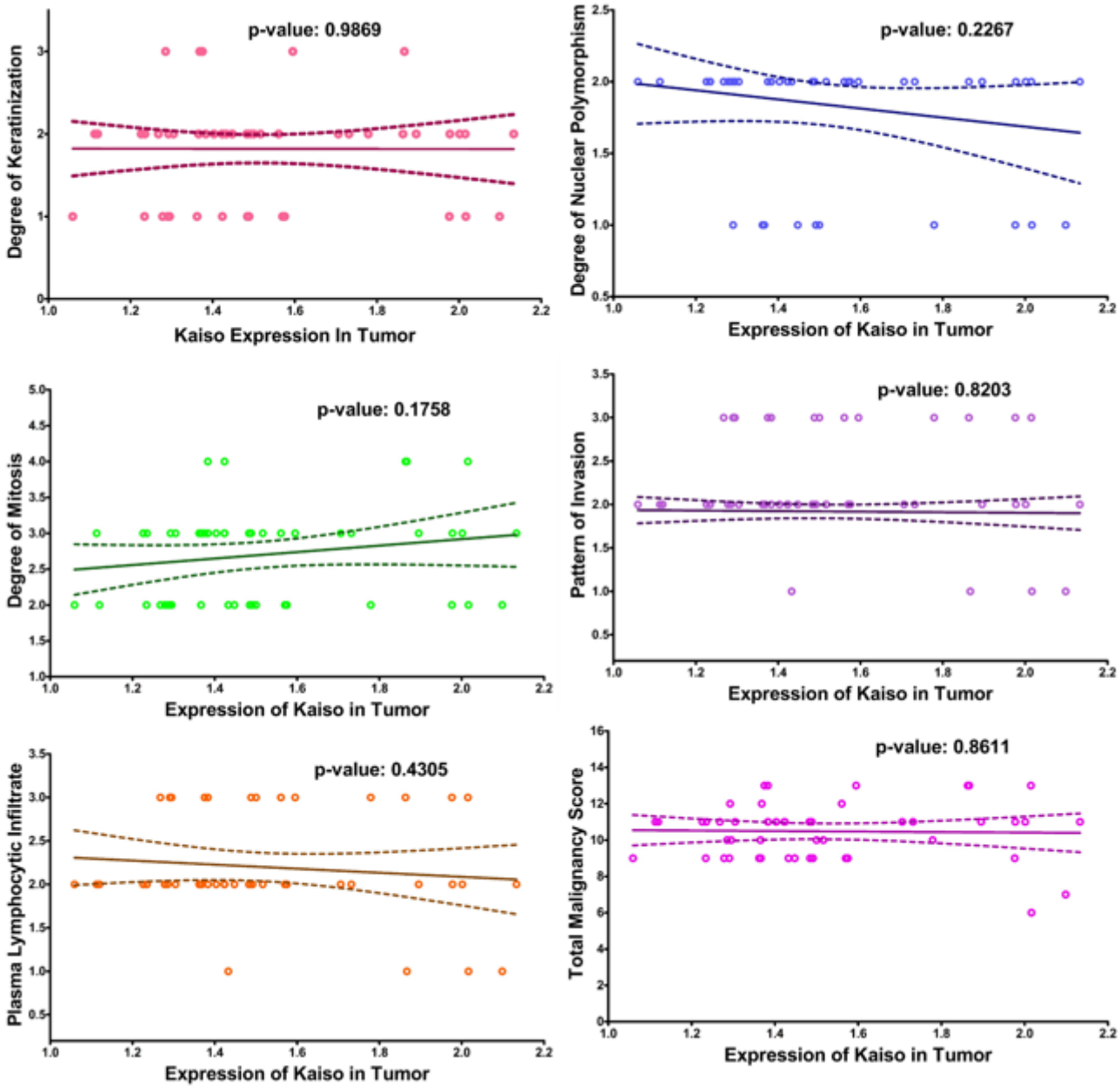
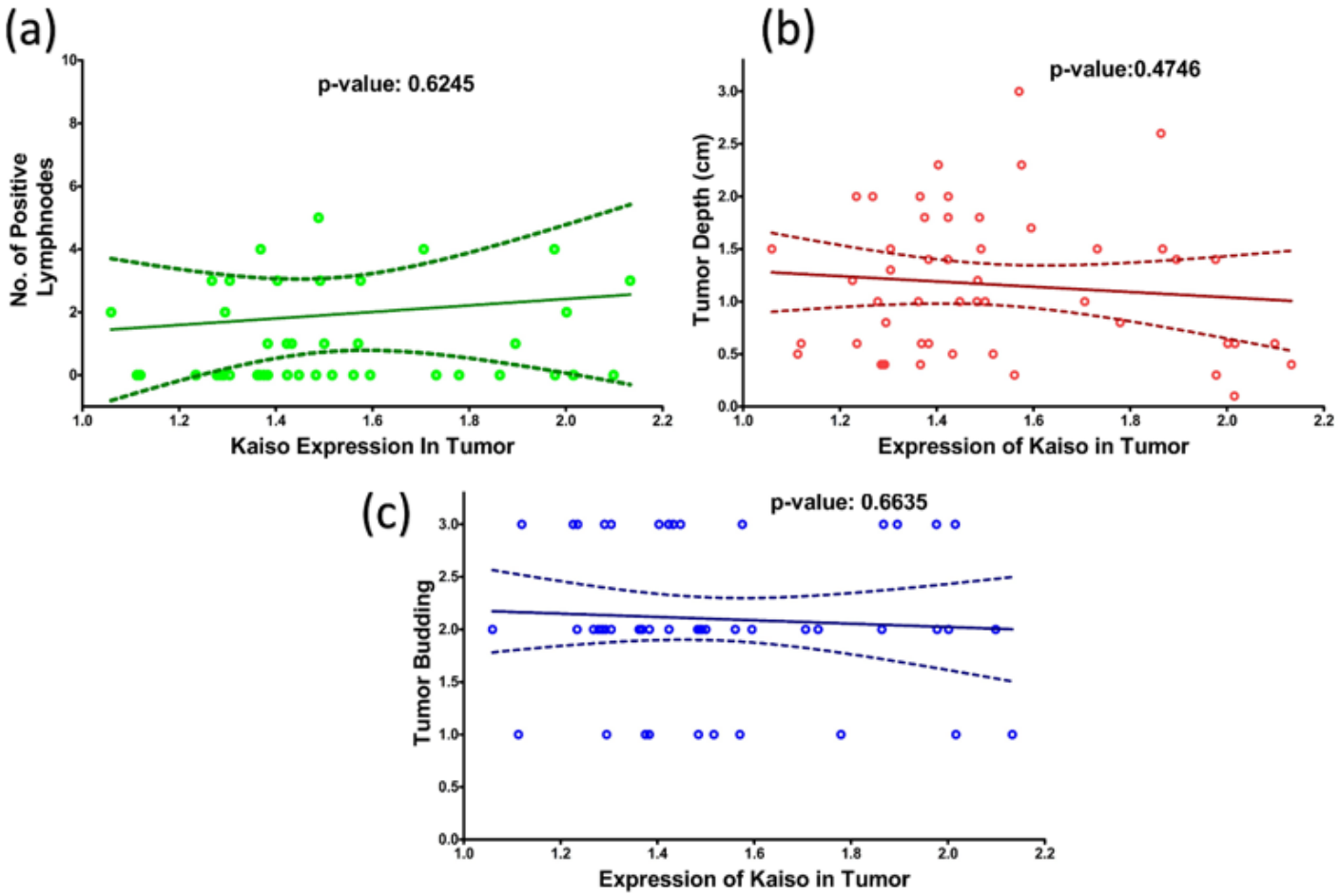


Figure 3

Correlation between Expression of Kaiso and Histological Feature Scores from Bryne's TIF Grading System. The graphs here demonstrate the correlation between expression of Kaiso in OSCC compiled as Optical Density and scores given to each histological feature according to Bryne's scoring system. Each histological feature is correlated separately followed by the correlation with the total malignancy score.



*HPF: High Powered Field

Figure 4

Correlation between Expression of Kaiso with Tumor Positive Lymphnodes (a), Tumor Depth (b) and Tumor Budding Score (c). The graphs here demonstrate expression of Kaiso in OSCC compiled as Optical Density, correlated with number of tumor positive lymphnodes, tumor depth measured in centimeters and tumor budding score.