CASE STUDY

Allelic Loss of 1Q32.1 The Kiss-1 Metastasis Suppressor Gene Locus in Iraqi Patients with Breast Cancer

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ABSTRACT

Experimental cell lines provide evidence on Kiss-1 gene as a regulator of metastasis in many kinds of cancers, including breast cancer. However, there is scarcity in data for kiss1 gene expression profile in many malignancies, and to date, no effort has been made to explore kiss-1 gene-associated microsatellite markers in many types of tumors, including breast cancer. The present study aims to investigate the microsatellite markers associated with Kiss-1 gene and the current loss of Heterozygosity LOH in Iraqi patients with breast cancer; this was further correlated with immunohistochemical Kiss-1 gene expression. One hundred and three breast carcinoma samples were collected after mastectomy. Kiss-1 gene expression was analyzed using immunostaining. Three microsatellite marker sites: SHGC-76112, SHGC-76186 intragenic, and SHGC-33412 in chromosome 1q32.1 were selected to define allelotyping of kiss-1 gene in paired DNA samples `from tumor cells and control blood cells of each patient. The loss of Kiss-1 gene expression was significantly correlated with the clinical tumor stage (p-value < 0.01). Loss of heterozygosity (LoH) occurs in 60 (60%) samples. Seven of the examined samples show LOH in two markers; none of the samples show LoH in the three markers. Seven samples show LOH for both intragenic markers (SHGC-76112 & SHGC-76186). The highest LoH percentage was 28%, which occurred within the intragenic microsatellite marker (SHGC-76186), and the lowest was 14% within the flanking region microsatellite marker (SHGC-33412). Interestingly, a statistically significant relationship was found between the LOH and Loss of Kiss-1 gene expression (p-value = 0.0005). This study convinced us to consider the vital role of this gene in the progression of the carcinoma in Iraqi patients with breast cancer.

Keywords: Breast cancer, Kiss-1 gene, Loss of heterozygosity, Metastasis.

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INTRODUCTION

The study of cancer gives an opportunity for the insight into its behavior in different cells. Cancer is a whole constellation of diseases. There are about 206 different cell types in the human body, and in every one cell type, we can find multiple types of cancer.[1] That leads to a real challenge in terms of addressing these facts therapeutically since different peoples react differently, and different cancers react differently as well. Study cancer at a molecular level and investigating genetic alteration may pave the way to figure out how to predict and prognosis.

Metastasis or generation of new tumors in distant organs is the main cause of poor prognosis in all types of cancers, including breast cancer.[2] Although metastasis is an inefficient process, 90% of cancer deaths occur by metastasis.[3] At least 50% of the patients who develop cancers of the breast will develop brain, lungs, or bone cancer.[4] Metastasis is an innate development in cancer behavior that is regulated by about (20) distinct metastasis suppressor genes one of these genes is Kiss-1 gene which was discovered in 19964 that encodes for metastin a 54-amino acid protein which is a proteolytic byproduct of main protein precursor that its name indicates its vital role in cancer metastasis.[5] The expression profile and the role of KiSS-1 gene in cancer progression are largely unknown in most of the cancers. There is scarcity in data for kiss1 gene expression profile in many malignancies, and to date, no effort has been made to explore kiss-1 gene-associated microsatellite markers in many types of tumors, including breast cancer. Chromosomal imbalance by duplications or deletions of certain allelic regions is a highlighted event in the progress of many types of tumors. The present study attempts to figure out kiss1 gene expression vis a vis the loss of heterozygosity in Kiss-1 gene-associated microsatellite markers in patients with breast carcinoma.

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MATERIALS AND METHODS

Tissue samples

One hundred and three (n = 103) breast carcinoma samples were collected (after mastectomy) from patients admitted at Baghdad medical city teaching hospital, Baghdad. Blood was collected the day before surgery and used as control samples for LOH analysis, adjacent normal lesions of tissue biopsies serve as a control for immunohistochemistry gene analysis. Every fresh tissue biopsy was divided into two parts; one was taken and kept in PBS saline and deep-frozen in –18°C to be processed for genetic studies within less than three months to guarantee good quality of extracted DNA, the other part of samples was used for histological studies as being kept in formalin and sent for pathology department for diagnosis, all the specimens were characterized as breast carcinoma. Formalin-fixed paraffin-embedded tissue sections were used for immunohistochemistry analysis after being pathologically diagnosed and confirmed. The study was approved by the Bioethics Committee of Baghdad medical city. Tumor staging was performed according to TNM Classification.[6] Informed consent was obtained orally from patients for contribution in this study. The clinicopathological parameters of the study were also reported as listed in Table 1.

Kiss-1 immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized in three repetitive washes with xylene for 5 minutes each, followed by washing in ascending concentrations of ethyl alcohol. Immunohistochemical staining was performed as described previously[7] using anti- Human KISS1/metastin Mouse Monoclonal antibody (LifeSpan BioSciences, Inc, USA). The final reaction was visualized using diaminobenzidine (DAB) primary stain followed by counterstaining with hematoxylin. Each run included a similar procedure for positive control. For negative control slides, an addition of primary antibody was omitted. Immunostaining for kiss-1 is shown in Figure 1.

DNA extraction

Previously frozen tissues and control blood samples were processed for DNA extraction using DNA extraction kit (ScienceDirect USA). The quality and quantity of extracted DNA was verified by using gel electrophoresis on 1% agarose and nanodrop spectrophotometer, respectively.

Microsatellite marker selection and PCR amplification

The flanking primer sequences used to amplify the microsatellite region were obtained from National Center for Biotechnology Information database (www.ncbi.nlm. nih.gov/) after defining three microsatellite marker sites: SHGC-76112, SHGC-76186, and SHGC-33412 in chromosome 1q32.1 were selected to define allelotyping of kiss-1 gene in paired DNA samples of tumor and control. SHGC-76112 and SHGC-76186 were within Kiss-1 gene region, whereas SHGC-33412 was on the telomeric flanking region. The sequence of primers is shown in detail in Table 1.

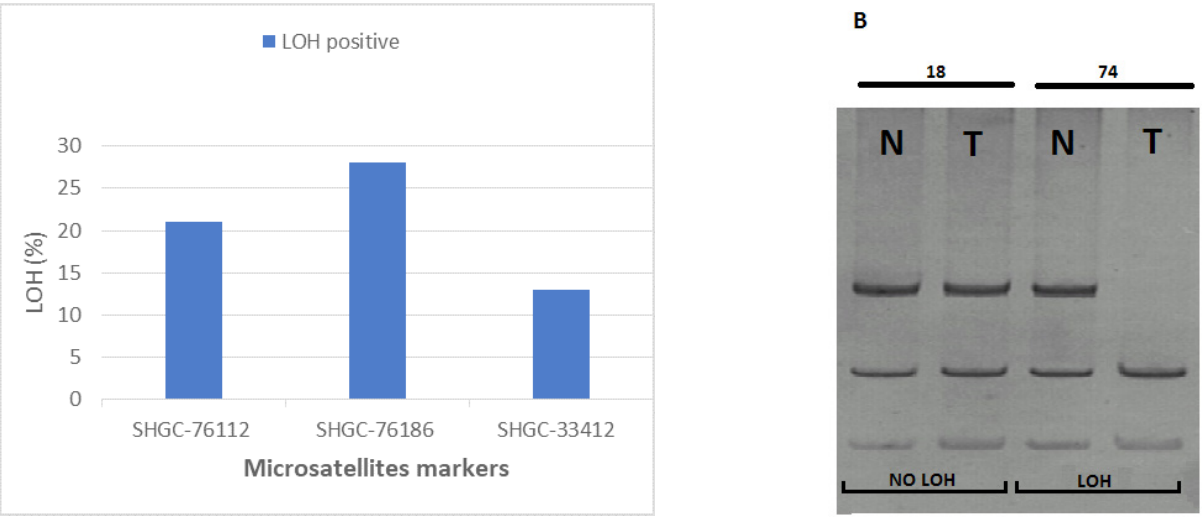

Figure 1 (A): Diagram showing a comparison in the percentage of heterozygosity of KiSS-1 gene 1q32.1 loci in breast cancer for all selected microsatellite markers of study. High LOH frequency appears in markers within the gene loci (SHGC-76112 and SHGC-76186) other than a marker of the flanking region (SHGC-33412). (B) A representative example of the loss of heterozygosity of KiSS-1 gene 1q32.1 in a tumor (T) and normal (N) tissue on polyacrylamide gel electrophoresis for SHGC-76112 microsatellite marker (samples number on the top).

Table 1: The sequence of primers used for each microsatellite marker.

Marker	*Primer sequence*	*Amplicon size*
SHGC-76112	5´ACCTGCCGAACTACAACTGG 3´CCTTTGGGGTCTGAAGTTCA	133
SHGC-76186	5´ GGTGTGATACCAGCCCC 3´ AGTCCCCTGCACTGACTC	165
SHGC-33412	5´ CTGAGGTCACCGATAAAGCA 3´ CTTATTAACCCTTGAGCTCTTTCA	125

The final volume of PCR amplification reaction was 50 µL including 50 ng DNA, 2.5 µL PCR Buffer, 1.5 mM MgCl2 200 µM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl,1 µM of each primer, 1 unit of Taq DNA polymerase (Zymo research,USA).

PCR reaction was processed under the following amplification conditions: 5 minutes initial incubation at 96°C, 28 reaction cycles at 95 °C for 1 minute each at 59 °C for first primer, 63°C for 2nd primer and 60 °C for the third primer respectively, for 1 minute each and final extension at 75°C for 3 minutes. Denaturation of amplified product was performed by heating at 95 °C for 5 minutes, followed by instant cooling in an ice bath. Produced denatured DNA was run polyacrylamide gel at a voltage of 500 V for 3–4 hours. Silver staining was done according to published protocols.[8] Loss of heterozygosity was determined by a visible shift in allele to allele quota in the tumor as compared with relative normal tissue. Allelic deletion of kiss-1 gene 1q32.1 was marked as positive. Uninformative condition (homozygous control sample) was omitted from considered data of the study.

Statistical analysis

Compiled clinicopathological parameters for the study cohort were statistically compared using the chi-square test. A $p < 0.05$ were statistically significant.[9] LoH found within Kiss-1 gene region within microsatellite markers (SHGC-76112, SHGC-76186) were statistically compared with (SHGC-33412) the microsatellite marker at the flanking region of kiss-1 gene 1q32.1 locus.

RESULTS

Study cohort and etiologic parameters

Out of 103 patients, 67 were postmenopausal, and 36 were premenopausal. Age ranged between 23 and 88 years with a median age of 58 years. Body mass index was calculated to be further correlated with etiologic parameters. BMI was within the normal limit (≤24.9) in 61 of the patients, and it was higher than normal limits in 42 out of 103. Tumor stage and evidence of distant metastasis were recorded and listed in Table 2 for statistical correlation.

Loss of heterozygosity

Out of 103 cases examined for LoH,[3] cases were uninformative for all three markers of study. One hundred samples were informative for at least one or more microsatellite markers. LOH incidence for each microsatellite marker is illustrated in Figure 1(A). The percentages of LoH positivity incidence were 21, 26, 13 and percentage of informative cases were 86, 87, 85 for the markers SHGC-76112, SHGC-76186, SHGC-33412, respectively. Out of the 100 informative cases, loss of heterozygosity occurs in 60 (60%) samples. Seven of the examined samples show LOH in two markers; none of the samples show LoH in the three markers. Seven samples show LoH for both intragenic markers (SHGC-76112 & SHGC-76186). The highest LoH percentage was (28%), which occurred within the intragenic microsatellite marker (SHGC-76186), and the lowest was (14%) within the flanking region microsatellite marker (SHGC-33412). Figure 1(B) demonstrates an example of silver stain gel for both tumor and normal control.

Kiss-1 gene expression

Out of 103 breast cancer tissue samples, 30.1% (31/103) cases showed loss of Kiss1 protein expression. Loss of Kiss1 expression was significantly correlated with clinical tumor stage [$p < 0.01$, (15.5, 30.1, 34.9, and 19.4%) cases for stages I, II, III, and IV respectively] as shown in Supplementary Table 2.

Allelotyping of Kiss-1 In relation with Kiss-1 immunohistochemical analysis

In the present study, 60% of breast cancer samples had shown Loss of heterozygosity in the 1q32.1 region. Vis, a vis 30.1% cases, showed loss of Kiss-1 gene expression. Interestingly, a statistically significant relationship was found between the LOH and Loss of Kiss-1 gene expression [P= 0.002, as summarized in Table 3. We also evaluated the correlation of Kiss-1 expression with clinical variables in LOH positive cases and we couldn't find a statistically significant relationship of menopausal status or body mass index with Kiss-1 gene expression (Table 2).

CONCLUSION

Although the role of KiSS-1 in Cancer metastasis has been reported and first discovered since 19965 the expression profiling and the chromosomal genetic alterations of KiSS-1 gene in many types of tumors are still unknown.[10] Kisspeptin KPs, a protein encoded by the Kiss-1 gene is responsible for GPR54 activation and ERK1/2 MAPK phosphorylation, a biochemical pathway essential for inhibition of cell mobilization, invasiveness, and metastasis.[11] Kiss-1 gene is the main prognostic factor used in conventional laboratory protocols for gastric cancer patients.[10,12] Downregulation of KiSS1 gene contributes to more invasive ovarian cancer and prolonged prognosis.[10] In the present study, we, therefore, investigated the loss of heterozygosity of 1q32.1 locus of Kiss-1 gene and its association with the expression level, we further analyzed the correlation of Kiss-1 gene expression and loss of heterozygosity with clinicopathological parameters. Immunostaining revealed a 30.1% (31/103) loss of Kiss-1 expression compared to controls, which is in agreement with a previous study on the Kiss-1 gene in breast cancer where upregulation of kiss-1 gene was attributed to increased tumor progression.[13] Remarkably, we found a significant correlation between loss of heterozygosity at Kiss-1 gene locus with clinical stage, distal metastasis, and immunostaining. The role of kiss-1 gene expression in tumor prognosis and invasiveness has been reported in many types of tumors. A study on patients with hepatocellular carcinoma an observed advanced stage of progression, was significantly related to overexpression of KiSS-1 gene.[14,15] The same evidence was reported in pancreatic cancer[16] and urinary bladder carcinoma.[17] Consequently, KiSS-1 gene expression has become a vital prognosticator of cancer prognosis and disease-free survival. Genetic and epigenetic alterations might be responsible for down

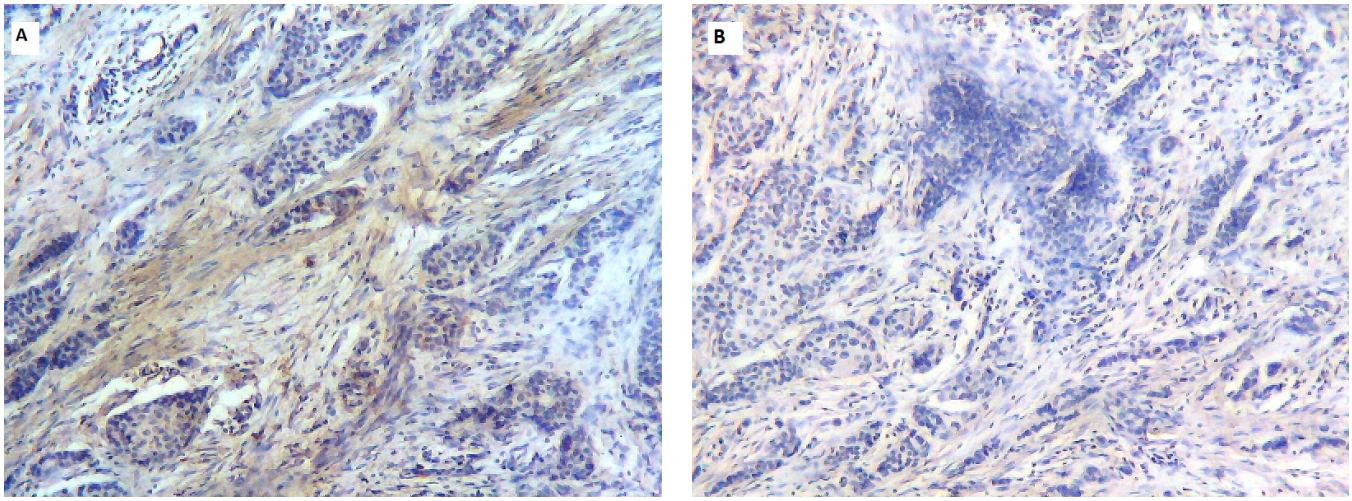

Figure 2: Immunohistochemical staining of Kiss1 protein in breast cancer. (A) Positive staining was membranous/cytoplasmic staining of carcinoma compared with negative surrounding tissue (400x). (B) negative expression of Kiss1.

Table 2: Statistical Analysis of correlation between Kiss-1 gene Expression and loss of heterozygosity with different clinicopathological parameters in Iraqi patients with Breast Cancer

		Kiss-1 gene expression (Total=103)			*Loss of heterozygosity (Total=100)*		
Etiologic parameters	*Patients*	*Positive (n = 72)*	*Negative (n = 31)*	*p- value*	*Positive (n = 60)*	*Negative (n = 40)*	*p- value*
Total	103						
Menopausal status							
Postmenopausal	67	46	21	0.70	24	23	NS
Premenopausal	36	26	10		36	17	
Median age	58						
Age range	23-88 years						
BMI Kg/m^2							
>25	42	26	16	0.14	32	19	NS
≤24.9	61	46	15		28	21	
Tumor stage							
I	16	13	3	0.01	17	09	NS
II	31	23	6		11	07	
III	36	28	10		21	16	
IV	20	8	12		11	8	
Distant metastasis (M)							
M0	66	53	13	0.002	41	15	0.002
M1	37	19	18		19	25	

Table 3: Correlation of Kiss-1 gene expression with loss of heterozygosity.

		Kiss-1 expression		
Total cases		*Positive (72)*	*Negative (31)*	*p- value*
With LOH	60	34	26	0.002
Without LOH	40	38	5	

regulation of Kiss-1 metastasis suppressor gene in tumor hence responsible for increased tumor invasiveness.[18] The present study demonstrated that out of the 100 informative cases, loss of heterozygosity occurs in 60 (60%) samples. Seven of the examined samples show LoH in two markers; none of the samples show LoH in the three markers. Seven samples show LoH for both intragenic markers (SHGC-76112 and SHGC-76186). The highest LoH percentage was (28%), which occurred within the intragenic microsatellite marker (SHGC-76186). This study convinced us to consider the vital role of this gene in the progression of the carcinoma I Iraqi patients with breast cancer. An increased evidence of LoH was also recognized within the flanking region microsatellite marker (SHGC-33412) which showed the lowest rate of

LOH among other two regions (14%) assuming that this frequently deleted region may extend beyond the 5´ of Kiss-1 gene.

The present investigation and our data propose that Kiss-1 is a presumed metastasis suppressor gene at human chromosome 1q32.1 and loss of heterozygosity of Kiss1-specific microsatellite markers affirmed that its inactivation and downregulation may contribute to progression of breast cancer and other human cancers.

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