Lack of Association between LCS6 Variant in KRAS Gene with the Occurrence of Breast Tumors in Iraqi Women

Roya H. Al-Haddad\textsuperscript{1}\textsuperscript{*}  Hameed M. Jasim\textsuperscript{1}  Wieeam Abdulfattah Saleh\textsuperscript{2}

Received 22/6/2019, Accepted 27/10/2019, Published 1/6/2020

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Abstract:
Breast cancer is the most commonly diagnosed cancer and remains one of the main reasons of cancer-related mortality in women worldwide. KRAS variant rs61764370 (T>G) is associated with an increased risk of occurrence of many cancers, Here The case-control study was accomplished on 135 women including 45 women with breast cancer patients, 45 women with benign breast lesions and 45 healthy women to analyze the association of KRAS variant rs (61764370 T>G) with breast cancer. LCS 6 variant in KRAS gene was amplified by using specific primers, then genotype was detected after sequencing the PCR products. The results showed that the genotype and allele frequency of TT and GT allele of KRAS gene were statistically non-significant (p< 0.01) among breast cancer patients and breast benign lesions compared with healthy controls in Iraqi women.

Key words: Breast benign lesions, Breast cancer, KRAS, rs 61764370.

Introduction:
Breast cancer is the main lethal cancer in females all over the world, most of the cases occur in postmenopausal women, but a great number of younger women are afflicted (1). The genetic type of cancer development is based on that the transformation of a normal breast cell into a tumor depends on the change of genes found in the normal cell (2). KRAS is one of the most important oncogenes tumorigenesis. It encodes protein and the product can bind both guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and plays an important role in the regulation of signal transduction (3). Latest evidence has displayed that single nucleotide polymorphisms (SNPs) which reside in KRAS 3'-untranslated regions (UTR) of human contains multiple supposed tumor suppressor lethal-7 (let-7) complementary sites (LCS). These SNPs can regulate the activity of KRAS and prevent the let-7 miRNA from binding to KRAS in order to adjust the expression of its protein (4).

A number of studies have analyzed the influence of KRAS rs61764370 T>G polymorphism on the risk of various cancers. Among these are, Christensen et al., who showed that patients with head and neck squamous cell carcinoma, who carry the KRAS-LCS6 variant allele had a reduced survival time compared to individuals with wild type (5). Ratner et al., collected DNA using standard isolation methods from tissue, blood, buccal cell samples, or saliva, to demonstrate that KRAS variant is associated with the risk of developing epithelial ovarian cancer (6), Kjersem et al., found out that the LCS6 variant allele does not occur to be a risk factor for occurrence of colorectal polyps or colorectal cancer (7). In another study conducted by Cerne et al., it was shown that extracted DNA from whole blood in familial breast cancer cases and controls, had no significant correlation between the KRAS variant and risk of (sporadic and familial) breast cancer (8). Also another study by Uvirovaa et al., mentioned that KRAS-LCS 6 (rs61764370) was non-significantly associated with breast cancer (9). Dai et al., proposed that G allele of rs61764370 has a shorter life and a higher risk of reappearance or metastasis of colorectal cancer (10). Zhang and Shi showed that genotype GG, GT of (rs61764370) was not a factor for estimating cancer risk in Caucasian people (11).

This study was carried out in Iraq to determine the influence of KRAS gene polymorphism (rs61764370 T>G) on breast cancer and breast benign lesions compared to healthy women.
Materials and methods

**Subjects:**
A total of 135 women were enrolled in this study, forty five of them were had a breast cancer with mean age of 51 years, forty five of them with breast benign lesion with mean age of 32 (all these women attended oncology teaching hospital in Baghdad from different governorates of Iraq) in addition to forty five healthy control women with mean age 50 years. Details of clinical data and demographic characteristics for the patients with breast cancer only were collected by medical record review according to American joint committee on Cancer (AJCC) (12), which includes: the age, smoking status, tumor site and size, grade, recurrence/metastasis status, estrogen, progesterone receptor, HER2 neu status and family history.

**Genomic DNA isolation**
Genomic DNA was isolated from the EDTA-coated venous blood samples using ReliaPrep™ Blood gDNA Miniprep System (Promega/ USA) according to the manufacturer's instructions. The DNA concentration was measured by Quantus™ Fluorometer (Promega/ USA).

**Amplification of LCS6 variant in KRAS gene**
Polymerase chain reaction (PCR) was used for the identification of LCS6 variant in 3'UTR of KRAS gene by using specific primers mentioned by Sanaei et al., (13) (rs 61764370) the forward primer: 5'- GTGTCAGAGTCTCGCTCTTGTC - 3' and reverse primer: 5'- AGACCACACTAGCACTACCTAAGGA-3, the reaction mixture (25 µl) was prepared by adding 12.5 µl of 2X GoTaq Green master mix, 1 µl of each primer, 8.5 µl of Nuclease-Free Water and 2 µl of DNA template. The PCR condition was: 30 cycle of 95 ºC for 30s, 57 ºC for 30S, 72 ºC for 30s followed by a final extension step for 7 min at 72 ºC. After amplification, agarose gel electrophoresis was used to approve the presence of amplification products.

**Sequencing of the amplified products**
PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer used by Macrogen Corporation – Korea. Results of sequencing were analyzed by using Geneious software.

**Statistical analysis**
SPSS (statistical package for social sciences) version 24 was used for the statistical analysis (14), to determine the significant difference (P<0.01) between odds ratio and allele frequencies of T>G polymorphism in KRAS gene among each case study.

**Results and Discussion:**
Patients with invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) accounted 75.5 % and 15.5 % respectively. It was found that the breast cancer patients who expressed the estrogen receptor (ER) (82.2%) and the progesterone receptor (PR) were (75.5%). According to the tumor-node-metastasis (TNM) status, it was found that 51.1% of the breast cancer patients were at stage II; other patients (48.9%) were at other stages (I, III, IV). The medical files showed that 60 % of breast cancer patients were with lymph node metastasis as shown in Table (1).

**Table 1. Clinical and histological characteristics of Iraqi women having breast cancer**

| Characteristics | Cases (45) No (%) |
|-----------------|------------------|
| **Histological type** | |
| IDC             | 34 (75.5)        |
| ILC             | 7 (15.5)         |
| Others          | 4 (8.9)          |
| **Grade**       |                  |
| I               | 6 (13.3)         |
| II              | 26 (57.8)        |
| III             | 9 (20)           |
| IV              | 4 (8.9)          |
| **Tumor size**  |                  |
| Stage I         | 13 (28.9)        |
| Stage II        | 23 (51.1)        |
| Stage III       | 6 (13.3)         |
| Stage IV        | 3 (6.7)          |
| **Lymph node**  |                  |
| (+)             | 27 (60)          |
| (-)             | 18 (40)          |
| **ER status**   |                  |
| (+)             | 37 (82.2)        |
| (-)             | 8 (17.7)         |
| **PR status**   |                  |
| (+)             | 34 (75.5)        |
| (-)             | 11 (24.4)        |
| **HER2 neu status** |       |
| (+)             | 12 (26.6)        |
| (-)             | 33 (73.3)        |

To ascertain if the KRAS single nucleotide polymorphisms (rs61764370 T>G) was associated with the incidence breast cancer we first amplify the Let-7 complementary site 6 of KRAS gene in each case study by using specific primers with product size of 385bp obtained and visualized after Agarose gel electrophoresis by UV light, then size of fragment was calculated by using gel documentation system as shown in Fig. (1).
The PCR products were sequenced and analyzed to evaluate the frequency of this polymorphisms in patients with breast cancer as shown in Fig. (2).

The genotype and allele frequency of TT, GT are indicate in the Table-2. Results showed that the allele frequency of T and G in women with breast cancer was 0.93%, 0.07% respectively, while the allele frequency for T and G alleles for women with benign lesion was 0.94%, 0.06% respectively and the allele frequency for the same alleles in healthy women was 0.97%, 0.03% respectively. The genotype frequency of the TT, TG show no significant (NS) association in the three groups (malignant, benign and control) among Iraqi population.

| Polymorphism (rs61764370) | Malignant (G1) | Benign (G2) | Control (G3) |
|---------------------------|---------------|-------------|--------------|
| Genotype frequency, No (%) |              |             |              |
| TT                        | 39 (86.67%)   | 40 (88.89%) | 42 (93.33%)  |
| TG                        | 6 (13.33%)    | 5 (11.11%)  | 3 (6.67%)    |
| GG                        | 0 (0.00%)     | 0 (0.00%)   | 0 (0.00%)    |
| Allele frequency (%)      |              |             |              |
| T                         | 0.93          | 0.94        | 0.97         |
| G                         | 0.07          | 0.06        | 0.03         |

Figure 1. PCR products for rs61764370 (T>G) of KRAS gene for human samples was fractionated on 1.5 % agarose gel electrophoresis (100 volt for 75 minutes) Lane (1): 1000 bp DNA marker lane (2-8) patients having breast cancer, lane (9-14) patients having breast benign lesions, and lane (15-19) control.

Figure 2. The sequence of KRAS (rs61764370 T>G) gene when alignment with reference sequence (showed substitute nucleotide from TT to TG).

Table 2. Genotype and allele frequency of (rs61764370 T>G) polymorphisms in breast cancer patients, breast benign lesions patients and control
In this case-control study it was found that there are no significant differences in allele and genotype frequency of rs61764370 T>G in breast cancer patients compared with benign lesions and healthy controls, similar results reported that the occurrence of the KRAS-LCS6 (rs61764370) TG genotype was associated with non-significantly with the risk of breast cancer by Cerne et al., (8) and Uvirova et al., (9). Contrary, Hollestelle et al., stated that KRAS variant (rs61764370 T>G) frequencies might be increased among BRCA1 carriers but not BRCA2 or non BRCA1/BRCA2 families among controls (15). On the other hand, Paranjape et al., (16) and Ustinova et al., (17) found that the KRAS variant was significantly associated with breast cancer at menopausal age. Another study by Pilarski, et al., (18), found that KRAS variant is considered as a genetic marker for hereditary breast ovarian cancer families. In this study the homoygous genotype GG was not found in patients with breast cancer, patients with benign breast disease and controls in Iraqi women.

Chin et al., (19) found that LCS6 variant in the KRAS 3'-UTR varied across geographic populations, it was infrequently in Asian (like Iraq in this research) and Native American populations while in African populations were less frequently, however, it occurs most frequently in European populations.

Conclusion:
This work indicates that the genotype frequency of GT (heterozygous) and TT (wild type) in single nucleotide polymorphism (rs61764370 T>G) of KRAS gene is not associated with the incidence of breast cancer and breast benign lesions as a risk factor compared with normal Iraqi women.

Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in Al-Nahrain University.

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فقدان العلاقة بين التباين الوراثي LCS6 في جين KRAS في النساء العراقیات

رویا هادي الحداد1
حمید مجيد جاسم2

1كليه التقنيات الاحیائية، جامعة النهرين، بغداد، العراق.
2مستشفى الاورام التعليمي، وزارة الصحة، بغداد، العراق.

الخلاصة:
سرطان الثدي هو أكثر أنواع السرطان شيوعًا ولا يزال أحد الأسباب الرئيسية للوفيات المرتبطة بالسرطان لدى النساء في جميع أنحاء العالم. يرتبط التباين الوراثي (T>G rs61764370) بال%^5% درجة خطر الإصابة بالعديد من أنواع السرطانات. أجريت هذه الدراسة على 135 امرأة، حيث استخدمت 45 عينة من النساء بسرطان الثدي و45 عينة بمرض الأورام الغدي، و45 من النساء الاصحاء لتحليل العلاقة بين التباين الوراثي LCS6 وKRAS وسارطان الثدي. تم استخدام الوراثي الفرعي المستخدم في الوراثي المستخدم لتحديد النمط الوراثي. وتم التكشف عن النمط الوراثي عند استخدام التفاعلات الفردية للتسلسل المتبلمر. وقد أظهرت النتائج أن النمط الوراثي GT لجين KRAS K5 تشتمل على تأثيرVD 0.01 بين مرضى سرطان الثدي والأورام الغدي. وتم كشف السلسلة المستمر من المحتمل أن يكون GT بين امرأة سرطان الثدي وكلا الجنسين.

الكلمات المفتاحية: أورام الثدي الحميدة، سرطان الثدي، KRAS، LCS6.