RESEARCH ARTICLE

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The Relevance of Common *K-RAS* Gene Mutations and K-RAS mRNA Expression with Clinicopathological Findings and Survival in Breast Cancer

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Abstract

Introduction: Studies have shown the role of mutation and gene expression of K-RAS in luminal breast cancer. In the current study, the status of common K-RAS mutations and mRNA expression in breast cancer were investigated. The aim of this research was determining the relationship of these molecular finding with clinicopathological features and 5 year overall survival. **Material and Methods:** In this case control study, we examined tumor tissue obtained from patients who had breast surgery which their paraffin tissue samples were available in the pathology department. Samples who had codon 12 and 13 mutations in exon 2 of K-RAS gene were considered as case group and tumor tissues without these mutations were considered as control group. The expression of K-RAS mRNA was explored by real-time polymerase chain reaction (RT-PCR) and the results were reviewed with clinicopathological features and survival of patients. **Results:** The results of the present study showed that 14% and 10% of patients had K-RAS mutations in codons 12 and 13, respectively. There was a significant relationship between K-RAS mutations with T staging and PR positivity in tumors. Five years overall survival was 8% in case group compare to control group who had 69% 5y OS. Furthermore, K-RAS mRNA expression had a significant relationship with T and N staging and 5 year survival. In conclusion, it seems that two molecular markers of mutation and K-RAS gene expression may be used simultaneously to estimate the prognosis of breast cancer. **Conclusion:** It seems that two molecular markers of mutation and K-RAS gene expression may be used simultaneously to estimate the prognosis of breast cancer.

Keywords: Survival- breast neoplasm- K-RAS mRNA- KRAS protein- human

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Introduction

Breast cancer is known as one of the most common cancers among women, Breast cancer accounts for 22.9% of all cancers in women, and in 2016, approximately 3.5 million women were living with a history of breast cancer in the United States (Bertucci et al., 2012; Marriotto et al., 2017). According to the latest statistics of the Iranian Cancer Research Center, about 8,500 new cases of breast cancer are reported every year, of which 1,400 deaths are caused by breast cancer. Also, currently about 40,000 people in the country are suffering from this disease (Tahergorabi et al., 2014).

Over the years, many genes and proteins have been discovered that play different roles in carcinogenesis of breast cancer (Khan et al., 2019). One of the most commonly mutated human carcinogens are RAS proteins which remain a critical target for novel treatments (Marin-Ramos et al., 2019). Members of the RAS family are low molecular weight monomeric GTP-binding proteins that play important roles as core components of cellular networks controlling various signaling pathways: regulation of growth, proliferation, survival, differentiation, adhesion, cytoskeletal rearrangements, motility, and cell survival (Ferrer et al., 2018; Murugan et al., 2019). Previous researches has improved the understanding of the structure, processing, and signaling pathways of RAS in cancer cells and has opened new ways to inhibit RAS function. Abnormally activated RAS proteins regulate the function of key signaling pathways involved in the initiation and progression of one-third of human cancers (Li et al., 2018; Lindsay et al., 2018). The proteins of RAS act as cellular switch that is turned on by extracellular stimuli leading to the transient formation of an active, GTP-bound form of RAS which activates various signaling pathways to regulate fundamental cellular processes (Pązik et al., 2021).

Irreversible changes in the genetic material of a cell are key components of carcinogenesis because they can modulate gene expression and function of proteins

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involved in regulating cell growth and differentiation (Ferrer et al., 2018). Since RAS genes are one of the first mutated genes in various cancers, understanding these mutation patterns can not only assist to understand how cancer starts, but also the factors influencing the event affecting cancer prevention and treatment (Li et al., 2018). In our study, the relationship between common K-RAS gene mutations and K-RAS mRNA expression with clinicopathological findings and five-year overall survival (5y OS) of breast cancer patients has been investigated.

Materials and Methods

Patients and samples

The cases in this study were patients who underwent breast surgery and their paraffin tissue samples were available in the pathology department of Imam Hossein Hospital in Tehran. All the cases had filled informed consent at admission in hospital and before any diagnostic work up or treatment. They filled the part in that form that their innominate data provided in medical files such as demographic, pathologic reports, and treatment data could be used for future results. This study was a case-control research which used medical data and pathologic samples and no procedure was done on patients except surgery as a treatment modality. All the molecular studies were perform after pathologic report and when there was no need for pathologic consultations. The study was confirmed by local committee of medical ethics, Shahid Beheshti University of Medical Science (approval code: IR.SBMU.MSP.REC.1399. 270).

Patient demographic and clinical information such as age, sex, family history of cancer and staging, estrogen receptor (ER), progesterone receptor (PR) and Her-2 (Human epidermal growth factor receptor 2) were obtained by reviewing patients' medical files and pathologic reports. We followed 5-year survival of cases by phone calls or records in their files.

DNA/RNA extraction

Breast cancer tissue samples were obtained as paraffin-embedded tissue blocks from the pathology department and kept at room temperature until the tests were performed. After deparaffinization of tissues, extraction of RNA and DNA was performed using Exgene FFPE Tissue DNA/RNA kit produced by GeneAll Company (South Korea).

K-RAS mutation evaluation

Evaluation of common mutations (2 mutations) in codons 12 and 13 of K-RAS gene was performed using conventional PCR (polymerase chain reaction) method and gel electrophoresis. Thus, after performing the PCR reaction on the samples, the results were revealed using agarose gel. Primers which used in this study for K-RAS mutation have showed in Table 1.

K-RAS mRNA expression analysis

Specific primers and probes for miR-181a-5p and miR-181b-5p genes designed using Gene runner software (Version 3.05), BLAST, which is available on the Internet (www.ncbi.nlm.nih.gov/BLAST), was used to avoid binding primers and probes designed to other sequences similar to the desired gene sequences. Real-time PCR method was used to evaluate the expression of miR-181a-5p and miR-181b-5p genes. Probes are labeled using the FAM reporter at the ′5. Finally, before analyzing the data, the melting curves obtained from each PCR reaction were checked to confirm the correctness of the peak related to the desired gene and the lack of primer dimer. To analyze the data, first, Ct Δ of the gene in each sample was calculated from the difference of Ct of the corresponding gene and U6 Ct in somatic genes as a reference.

Statistical analysis

The results were analyzed using SPSS version 20 software. The information was reported in two descriptive and analytical sections the significance level was considered $P<0.05$.

Results

In this study, tissue sample of breast tumor in 100 female patients were examined. The average age of the patients was 54.19 ± 1.36 years with the range of 24 to 77 years. Forty cases (40%) had a family history of cancer (breast or other cancer in first and second relatives).

The results showed that 14 patients (14%) were positive and 86 patients (86%) for codon 12 mutation. 10 patients (10%) were positive and 90 patients (90%) for codon 13 mutation. Figure 1 shows the electrophoresis of K-RAS mutation PCR. The patients were divided into two groups based on mutations in K-RAS gene in codon 12 and 13 (case and control). If these mutations were detected they were named case group and patients who did not have mutation were control group. The tumor grade, stage of disease based on the TNM system (tumor, node, and metastasis) and hormonal status of receptors based on immunohistochemistry tests (IHC) in each group are shown in Tables 2. Also, the results showed that 5 year overall survival was 8% in case group and 69% in control group which was significant (P-Value =0.01).

Based on this comparison, K-RAS gene expression in patients who had T3-T4 tumors was significantly higher

Table 1. K-RAS Primers

Primer Name		Primer Sequences	PCR Product size
K-RAS codon 12	Forward	GTTGTGGTAGTTGGAGCTGTTGGCGTAGGCAAGAATGCC	111
	Reverse	GGCACTCTTGCCTACGCCAACAGCTCCAACTACCACAAG	
K-RAS codon 13	Forward	GGTAGTTGGAGCTGGT GACGTAGGCAAGAGTGCC	111
	Reverse	GGCACTCTTGCCTACGTCACCAGCTCCAACTACC	

Table 2. Relation of K-RAS Mutation with Clinicopathological Parameters

Variable	Variable (subgroup)	group		P Value
		case (N%)	control (N%)	
T stage	T1-T2	9	61	0.01
	T3-T4	15	15	
N stage	N0	9	31	0.1
	N1	15	15	
	N2-3	13	17	
Grade	G1	8	26	0.3
	G2	7	40	
	G3	9	10	
ER	Positive	11	45	0.6
	Negative	13	31	
PR	Positive	7	59	0.01
	Negative	17	17	
HER-2	Positive	6	17	0.1
	Negative	18	59	
Age (years)	18-49	7	28	0.1
	50-70	12	45	
	>70	5	3	

Case, patients who had mutations in codon 12 and 13; Control, patients who did not have these mutations; T stage, Tumor size; N, Lymph node involvement; ER, Estrogen receptor; PR, Progesterone receptor; Her-2: Human epidermal growth factor receptor 2; P-value <0.05 considered significant

than low T stage tumors (P-Value =0.01). The same significant difference was observed in PR positive tumors

Table 3. Relation of K-RAS mRNA Expression with Clinicopathological Parameters

Parameter	Status	Number (%)	Fold change	P.Value
T stage	T1-T2	70	1.8	0.01
	T3-T4	30		
N stage	N0-N1	70	2.3	0.01
	N2-N3	30		
Age (years)	18-49	35	1.1	0.1
	50-70	65		

(P-Value =0.01) . There was not a significant relationship between K-RAS mutations with N stage, ER, HER-2, age or grade. These results are summarized in table 2. Also, it was revealed that K-RAS gene expression was significantly lower in cases who survived more than 5 years from diagnosis (P-Value =0.01).

The analysis of K-RAS gene expression results in both groups is shown in figure 2. As it shown, K-RAS gene expression in case group was higher than control group with a fold-change of 1.5 and this increase in expression was statistically significant (P-Value =0.001).

It was revealed that K-RAS mRNA expression was significantly more common in advanced T stage (T3-T4) compared to T1-T2 cases with the fold change of 1.8 (P-Value =0.01). The same results were observed in lymph node involvement which K-RAS m RNA expression was significantly more in N2-N3 compared with N0-N1 with the fold change of 2.3 (P-Value =0.01). But, there was not a significant different with the age of patients in both group. These results are summarized with details

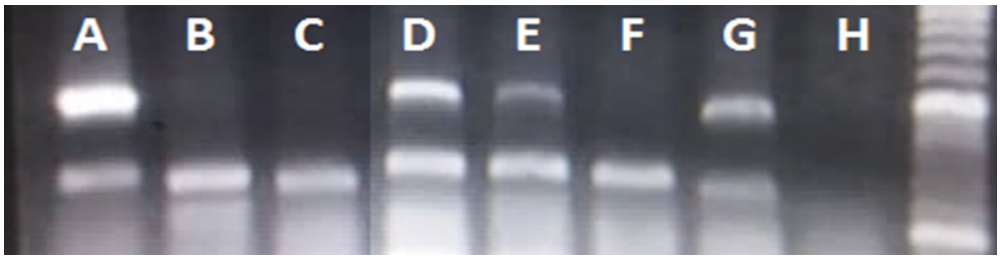

Figure 1. 2% Agarose Gel Electrophoresis of K-RAS Mutation PCR. A, D, E and G are positive for mutation and B, C, F are negative for mutation. H is negative control and we used ladder 50bp.

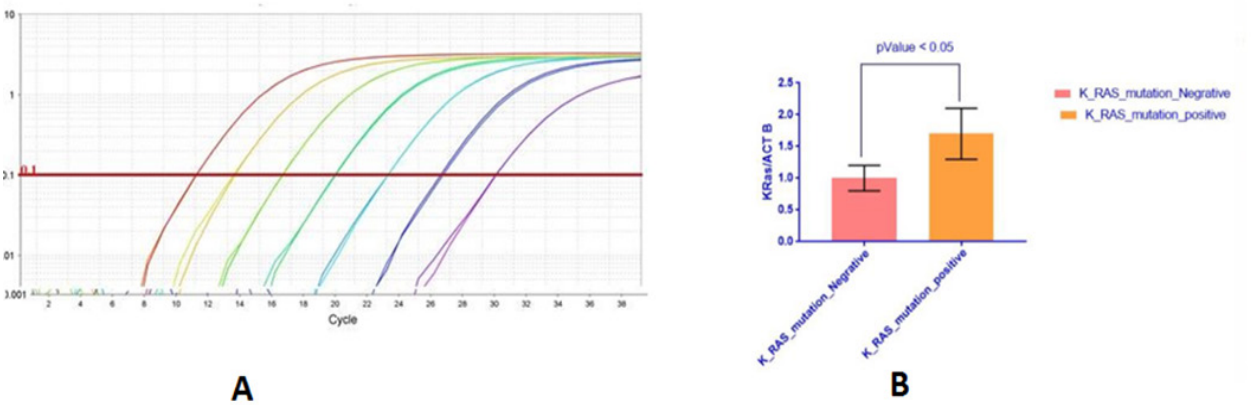

Figure 2. A, K-RAS gene amplification plot in tumor tissue compared to U6 gene; B, K-RAS gene expression in K-RAS mutation positive and negative patients.

in Table 3. Also, the analysis showed that the 5y OS in patients who had K-RAS mRNA expression was 77 % which was significant. (P-Value =0.01).

Discussion

Mammary cell lines have served as tumor models for many studies demonstrating the tumorigenic potential of RAS oncogenes. The studies have shown that oncogenic RAS mutations substantially increase the interaction of mammary cells with the basement membrane, alter three-dimensional collagen gel growth, ligand-independent phenotype, invasiveness, tumorigenic potential, TGF-β (Transforming growth factor beta) and IGF-1 (Insulin-like growth factor 1) secretion, EGFR (epidermal growth factor receptor) activation induces mitogen-activated protein kinase (MAPK) and estrogen sensitivity (Albini et al., 1986; Martinez-Lacaci et al. 2000). Mutant KRAS gene cooperates with mutant PIK3CA (Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) to induce tumor transformation in human epithelial cells Wang et al., 2013). Conditional expression of K-RAS G12V in mammary cells induces estrogen receptor alpha (ERα)-positive adenocarcinoma in mice; while HRAS Q61 stimulates breast adenomyoepitheliomas (Geyer et al., 2018).

Several pathways and downstream factors have been identified that mediate the tumorigenic phenotype caused by RAS mutations in mammary cells. Active N-RAS oncogene and its homologue N-RAS proto-oncogene act through a pathway for tumorigenesis in vivo. Oncogenic RAS mutations support cancer progression and metastatic dissemination through modulation of ΔNp63, a truncated amino-terminal isoform of p63, a member of the p53 family of transcription factors (Hu et al., 2017). Oncogenic RAS mutations promote TFG-β-induced epithelial-mesenchymal transition through activation of leukotriene B4 receptor-2-related cascade. Mutant RAS is associated with the induction of cyclooxygenase-2 (COX-2) expression in human breast cancer cell lines (Gilhooly and Rose, 1999).

The present study was conducted with the aim of investigating common K-RAS mutations and K-RAS gene expression and relation of these mutations with prognosis and overall survival. In our study, 100 patients with breast cancer were investigated. The average age of the study subjects was 54.19 ± 1.36 years and most of the patients were older than 50 years old, and this result is consistent with the average age of breast cancer worldwide.

In a study by Banys-Paluchowski et al., (2019) 198 patients were examined for the expression of RAS family genes. The results of their study showed that increased H-RAS levels were significantly higher in patients who had larger tumors and ER-positive tumors, while high K-RAS levels were associated with involvement of lymph node and HER2 positive tumors. After a median follow-up of 183 months, patients with high N-RAS expression had significantly better 5 year OS compared to patients with lower N-RAS expression. In another study, the relationship between K-RAS mRNA and OS and breast cancer specific survival (BCSS) was determined. A group of patients with higher K-RAS mRNA expression had higher OS and BCSS than the group with lower K-RAS mRNA expression, and the prognostic effect of K-RAS mRNA expression was observed only in luminal A tumors (Hwang et al., 2019). In another study on 88 samples including 44 pairs of healthy and tumor tissue samples which were sequenced for K-RAS mutation. The expression of K-RAS mRNA was measured by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) method. The level of K-RAS mRNA expression in cancer tissue was significantly higher compared to healthy tissue (Safdar et al., 2020).

In our study, K-RAS mRNA expression was significantly more common in advanced T stage (T3-T4) and N2-N3 tumors. Also, 5y OS in patients who had K-RAS mutations (case group) was 8% whereas in control group it was 69%. Additionally, 5y OS in patients who had K-RAS mRNA expression was 77 % and this was significant too.

In Conclusion, the results of the present study showed that 14% and 10% of patients had K-RAS mutations in codons 12 and 13, respectively. There was a significant relationship between K-RAS mutations with T staging and PR positivity in tumors. There was not a significant relationship between K-RAS mutations with N stage, ER, HER-2, age or grade. Five years overall survival was 8% in case group compare to control group who had 69% 5y OS. Furthermore, K-RAS mRNA expression had a significant relationship with T and N staging and 5 year survival, but there was no significant relationship with the age. In conclusion, it seems that two molecular markers of mutation and K-RAS gene expression may be used simultaneously to estimate the prognosis of breast cancer.

It is suggested that this study be conducted with a larger number of patients in order to achieve results with higher validity and accuracy. It is also suggested to use non-cancerous breast tissue samples as a control sample in order to investigate the difference in K-RAS gene expression.

Author Contribution Statement

SD and HA reviewed the medical reports of cases and wrote the initial manuscript; SK and HA reviewed and revised the initial manuscript; HA and FV worked on molecular testing in their laboratory; SK and HA and SD had the original plan for the study and managed all the process; SD reviewed the manuscript draft and finalized the draft and is the corresponding author; FV analyzed the data.

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This study was approved in the ethics committee in the school of medicine, Shahid Beheshti University of Medical Sciences with research ethics approval code: IR.SBMU.MSP.REC.1399. 270. All patients signed an informed consent form before surgery and agreed to their tissue samples being used in the research project. These forms are kept in their medical files in the hospital. All ethical requirements were observed during sample collection.

The datasets generated and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they had not any conflict of interest.

References

Albini A, Graf J, Kitten GT, et al (1986). 17 beta-estradiol regulates and v-Ha-ras transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane. *Proc Natl Acad Sci U S A*, **83**, 8182-6.

Banys-Paluchowski M, Milde-Langosch K, Fehm T, et al (2020). Clinical relevance of H-RAS, K-RAS, and N-RAS mRNA expression in primary breast cancer patients. *Breast Cancer Res Treat*, **179**, 403-14.

Bertucci F, Lagarde A, Ferrari A, et al (2012). 8q24 Cancer risk allele associated with major metastatic risk in inflammatory breast cancer. *PLoS One*, **7**, e37943.

Ferrer I, Zugazagoitia J, Herbertz S, et al (2018). KRAS-Mutant non-small cell lung cancer: From biology to therapy. *Lung Cancer*, **124**, 53-64.

Geyer FC, Li A, Papanastasiou AD, et al (2018). Recurrent hotspot mutations in HRAS Q61 and PI3K-AKT pathway genes as drivers of breast adenomyoepitheliomas. *Nat Commun*, **9**, 1816.

Gilhooly EM, Rose DP (1999). The association between a mutated ras gene and cyclooxygenase-2 expression in human breast cancer cell lines. *Int J Oncol*, **15**, 267-70.

Hu L, Liang S, Chen H, et al (2017). ΔNp63α is a common inhibitory target in oncogenic PI3K/Ras/Her2-induced cell motility and tumor metastasis. *Proc Natl Acad Sci U S A*, **114**, 3964-73.

Hwang KT, Kim BH, Oh S, et al (2019). Prognostic Role of KRAS mRNA Expression in Breast Cancer. *J Breast Cancer*, **22**, 548-61.

Khan AQ, Kuttikrishnan S, Siveen KS, et al (2019). RAS-mediated oncogenic signaling pathways in human malignancies. *Semin Cancer Biol*, **54**, 1-13.

Li S, Balmain A, Counter CM (2018). A model for RAS mutation patterns in cancers: finding the sweet spot. *Nat Rev Cancer*, **18**, 767-77.

Lindsay CR, Jamal-Hanjani M, Forster M, Blackhall F (2018). KRAS: Reasons for optimism in lung cancer. *Eur J Cancer*, **99**, 20-7.

Marín-Ramos NI, Ortega-Gutiérrez S, López-Rodríguez ML (2019). Blocking Ras inhibition as an antitumor strategy. *Semin Cancer Biol*, **54**, 91-100.

Mariotto AB, Etzioni R, Hurlbert M, Penberthy L, Mayer M (2017). Estimation of the Number of Women Living with Metastatic Breast Cancer in the United States. *Cancer Epidemiol Biomarkers Prev*, **26**, 809-15.

Martínez-Lacaci I, Kannan S, De Santis M, et al (2000). RAS transformation causes sustained activation of epidermal growth factor receptor and elevation of mitogen-activated protein kinase in human mammary epithelial cells. *Int J Cancer*, **88**, 44-52.

Murugan AK, Grieco M, Tsuchida N (2019). RAS mutations in human cancers: Roles in precision medicine. *Semin Cancer Biol*, **59**, 23-35.

Pązik M, Michalska K, Żebrowska-Nawrocka M, et al (2021). Clinical significance of HRAS and KRAS genes expression in patients with non–small-cell lung cancer - preliminary findings. *BMC Cancer*, **21**, 130.

Safdar M, Zaheer S, Khailany RA, et al (2020). The Relevance of SNPs at 3'UTR Region of CASP7 and miR-371b-5p Associated Diseases: A Computational Analysis. *Cell Biochem Biophys*, **78**, 541-57.

Tahergorabi Z, Moodi M, Mesbahzadeh B (2014). Breast Cancer: A preventable disease. *J Birjand University Med Sci*, **21**,126-41.

Wang GM, Wong HY, Konishi H, et al (2013). Single copies of mutant KRAS and mutant PIK3CA cooperate in immortalized human epithelial cells to induce tumor formation. *Cancer Res*, **73**, 3248-61.