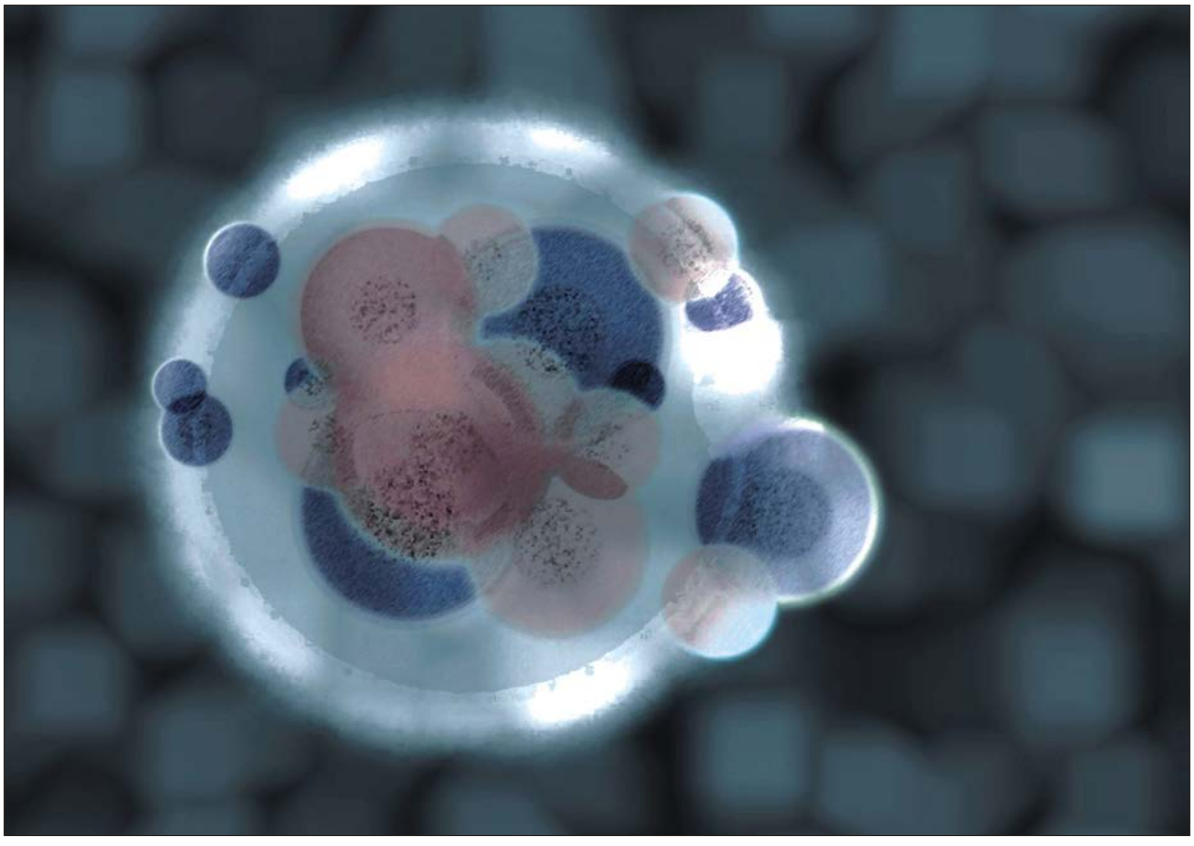

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2019.323.327

Research Article
Molecular Investigation of KRAS Gene in Breast Cancer Patients

[1,2]Rozhgar A. Khailany, [2,3]Muhammad Safdar and [2]Mehmet Ozaslan

[1]Department of Biology, College of Science, University of Salahaddin-Erbil, Iraq
[2]Department of Biology, Gaziantep University, Gaziantep, Turkey
[3]Department of Breeding and Genetics, Cholistan University of Veterinary and Animal Science, Bahawalpur, Pakistan

Abstract

Background and Objective: Breast cancer is the type of cancer that most affects women around the world. It accounts for around 30% of all cancers. KRAS gene is mutually initiated in about 20% of every single vigorous cancer. However, the further development of clinically successful KRAS coordinated malignancy treatments was usually unsuccessful and KRAS mutant malignancies are among the most resistant drugs. KRAS gene variations usually occur in many adenocarcinomas such as; lung, pancreas, breast and colon. The role of oncogene KRAS in later periods of neoplastic development that is taken after the onset is still poorly understood. The aim of this study is to investigate mutation and mRNA expression level of KRAS in breast cancer patients by using DNA sequencing and semi-quantitative reverse transcriptase techniques. **Materials and Methods:** The study included 44 paired normal and tumor samples from patients grouped based on the types of breast cancer and the patients' clinical characteristics, including age and grade of tumors. **Results:** The KRAS expression on the level mRNA was significantly increased (up-regulated) in tumor samples compared to the control samples. However, a heterozygous mutation (G>A) (GGT/GAT) was identified in two patients in the KRAS gene. **Conclusion:** The up-regulated expression of KRAS on the level mRNA can be a risk factor for breast cancer development and the changed KRAS expression level can alter individual breast cancer sensitivity.

Key words: Breasts cancer, KRAS, semi-quantitative PCR, nucleotide sequencing

Citation: Rozhgar A. Khailany, Muhammad Safdar and Mehmet Ozaslan, 2019. Molecular investigation of KRAS gene in breast cancer patients. J. Biol. Sci., 19: 323-327.

Corresponding Author: Mehmet Ozaslan, Department of Biology, Gaziantep University, Gaziantep, Turkey

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a complex of diseases that generates various pathological and metabolic changes in cellular environments[1]. It occurs through different signalling mechanisms, including cell multiplication, angiogenesis and metastasis[1,2]. It is the type of cancer that most commonly affects women worldwide[3]. It represents approximately 30% of all various cancers[3].

KRAS is initiated with mutation in about 20% of every single vigorous cancer[3]. However, the further development of malignant therapies coordinated by clinically successful KRAS was usually ineffective and mutant neoplasms are among the most resistant drugs[4,5]. KRAS changes most often occur in many adenocarcinomas such as; lung, pancreas, breast and colon and mutational activation of KRAS in these tissues are sufficient to initiate neoplasia in mice[6-7]. The role of oncogene KRAS in later periods of neoplastic development, taken after the onset, is not yet well known. Oncogenic "reliance" is a curiosity where tumors only require the supported expression and association of a distorted initial gene, despite the accumulation of numerous oncogenic lesions[8]. Clinically, this can be seen in pancreatic carcinomas (70-90%), colon (30-50%) and lung (20-30%)[9,10]. In breast cancer, however, the importance of KRAS is relatively not intensively focused due to the low frequency of mutation[10]. Molecular techniques are currently promising tools that can tackle critical problems with early recognition of breast cancer. In general, molecular markers are valuable for showing the risks and recognizing the first periods of breast tumor[11]. Recently, the look for molecular markers that can be used for the early detection of breast cancer has become an essential object of clinical research, especially since current methods are invasive and non-specific and require additional procedures for the therapeutic decision[11].

The expression and description of genes are consistent events in the progress of human cancer detection and the evaluation of a potential tumor marker[12]. These are frequent events in the progress of human cancer determination and quantification of a potential tumor[11,12]. Profiling can also change the predictions and treatment decisions in patients of breast cancer[12]. The RT-qPCR is a high-throughput advancement that uses a delicate online fluorescence reference system to estimate the expression state in disease tests[12]. RT-PCR also makes early diagnosis of cancer possible when the tumor burden is small and the disease is likely more treatable[12]. The limited clinical research is accessible on gene expression and mutation screening in KRAS-based breast cancer. The majority of the published work also relates to expression or mutation studies on patient samples.

In the current project, which studies the possible role of the KRAS gene in breast cancer, we proposed to investigate probable mutation and gene expression at the level of mRNA of KRAS gene in patients of breast cancer by following DNA sequencing and reverse transcriptase polymerase chain reaction (RT-PCR)) analysis. We investigated the role of KRAS in 44 breast tumors and 44 breast tumor-free and differentiated the results of samples in the province of Erbil in Iraq.

MATERIALS AND METHODS

Experimental site: This study was carried out at Zheen International Hospital, Erbil, Iraq, between September, 2017 and April, 2018.

Patients: The samples were collected from the Rizgary Hospital in Erbil, Iraq. A total of 88 samples were analyzed. The study included 44 paired normal and tumour samples of patients that were grouped according to the types of breast cancer and the clinical characteristics of the patients, including age and grade of tumours. The tissue samples of the breast were stored at -80°C until nucleic acid extraction. Informed consent was taken from all participants and the study approved by the local ethics committee and was conducted under the guidelines of the declaration of Helsinki.

DNA and RNA extraction: DNA and RNA samples from breast biopsied tissues were extracted using a commercial extraction kit according to the manufacturer's protocols; DNA obtained by AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea) and RNA obtained by ExiPrepTM Tissue total RNA kit (Bioneer, Korea). Quantification and qualification of DNA and total RNA concentration was performed utilizing NanoDrop (ND-1000, USA).

Complementary DNA synthesis: Complementary DNA (cDNA) synthesized by protoScript First Strand cDNA Synthesis Kit (BioLabs, England) according to the manufacturer's direction. The work area was cleaned by 70% (v/v) ethanol and filter tips were used in all steps. Thaw system components and put on ice. A variable amount of total RNA was utilized for each sample since the quality and quantity of total RNA are not equal.

Table 1: Sequence, PCR product size and annealing temperature of utilized primers

Primers	Sequence	PCR product (bp)	Annealing temperature (°C)
KRAS gene			
Exon 2			
Forward	5'-AGGCCTGCTGAAAATGACTGAA-3'	168	55.0
Reverse	5'-AAAGAATGGTCCTGCACCAG-3'		
KRAS gene			
Exp.			
Forward	5'-GCCTGCTGAAAATGACTGAAT-3'	116	55.5
Reverse	5'-TTGTGGACGAATATGATCCAACA-3'		
GAPDH gene			
Forward	5'-GGTCCACCACCCTGTTGCTGT-3'	456	59.4

Semi-quantitative RT-PCR analysis: The cDNA was amplified by semi-quantitative RT-PCR and employed the expression primers (Table 1). GAPDH gene was employed as a housekeeping gene for the normalization of KRAS gene expression data[13]. Reaction and condition of PCR were performed using Eppendorf MasterCycler Pro PCR System (Eppendorf, German). A 50 µL reaction mixture was prepared in PCR tubes containing 3 µL cDNA template, 25 µL ready master mix (Amplicon III, Danemark), 1 µL forward primer, 1 µL reverse primer and 20 µL ddH_2O. The cycling conditions comprised of initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 30 sec, annealing temperatures in Table 1 for 30 sec and extension at 72°C for 30 sec and final extension at 72°C for 5 min. Quantity of mRNA expression were discriminated employing agarose gel electrophoresis (2%) in the presence of SYBR green as a safe station. Agarose gel image was captured and quantitated expression of mRNA level by imageJ software program (version 1.46r)[13].

DNA sequencing: Mutation analysis of exon 2 in the KRAS gene was performed using genetic analyzer. Primers were obtained by Serban *et al.*[14], primer details indicated in Table 1. PCR amplification was conducted using a gradient thermal cycler device (Eppendorf, Germany), 50 µL reaction mixture was prepared in PCR tubes containing 2 µL DNA template, 25 µL ready master mix (Amplicon III, Danemark), 1 µL forward primer, 1 µL reverse primer and 21 µL ddH_2O. The cycling profile consists of an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 30 sec. At 95°C, 45 sec. At 67°C, 30 sec. At 72°C and final extension 5 min at 72°C. The PCR products were run in 2% agarose gel electrophoresis. Bands stained with SYBR green was visualized on a UV transilluminator using 100 bp ladder (Genedirex, Korea), expected size of the PCR amplicon was 168 bp.

DNA sequencing, using both forward and reverse primers, was performed separately by Applied Biosystem 3130X genetic analyzer (Singapore). The PCR fragments of the KRAS gene was excised from the agarose gel and used as a source of DNA template for sequence-specific PCR amplification.

Statistical analysis: The statistical analysis of gene expression was carried out using Wilcoxon signed rank test, significance was assumed at value of $p \leq 0.05$. The statistical tests were made by employing SPSS software (V.16).

RESULTS

KRAS mutation result: The DNA sequence of the KRAS gene was obtained from the NCBI website, to compare the resulting DNA sequence of the patient and normal samples (Query Sequence) with the reference sequence (Subject Sequence). Only two patients were detected in the study with a single nucleotide substitution mutation (G>A) (GGT/GAT) in the KRAS gene in a sample of breast cancer. Figure 1 indicates and reveals the mutated sequence.

KRAS expression result: The mRNA expression level of KRAS was measured by semi-quantitative RT-PCR and separated by 2% agarose gel electrophoresis. The expression level of KRAS gene was obtained from 44 pairs; tumor and normal samples. Various expression level of each patient was observed, the comparison between normal controls and tumors is indicated in Fig. 2. The mRNA expression level of 41 tumors according to normal controls was increased (Up-regulated). Quantity of mRNA expression of KRAS tumor samples were increased according to expression level of normal control samples, $p = 0.000$ and statistically it is significant counted on (t-test, $p<0.05$) (Fig. 3).

DISCUSSION

Most previously published researches on simultaneous KRAS mutations focused on colorectal cancer[15]. Some studies recommended that KRAS and PIK3CA mutations existed side by side within the same tumor[16]. The PIK3CA mutations coincided with the RAS mutations in colorectal cancers[17], while the previous study showed that mutation activation of the PI3K pathway was mutually particular with the RAS pathway in breast cancer[17].

In this study, we found KRAS mutations (G>A) (GGT/GAT) in two patients with breast cancer. Although a higher frequency of KRAS mutation is primarily found in colon,

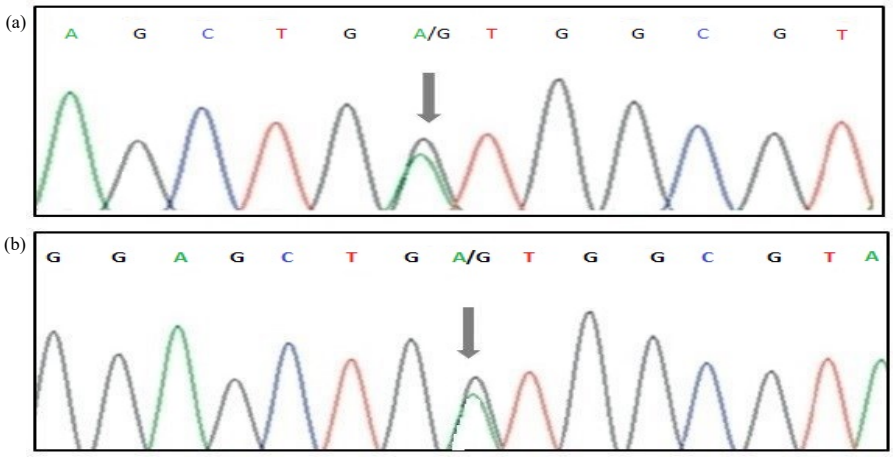

Fig. 1(a-b): DNA sequencing results is showing a single mutation in a breast cancer patient as a result of (G<A) in exon 2 of the KRAS gene, (a) Patient 1 sequence result and (b) Patient 2 sequence result

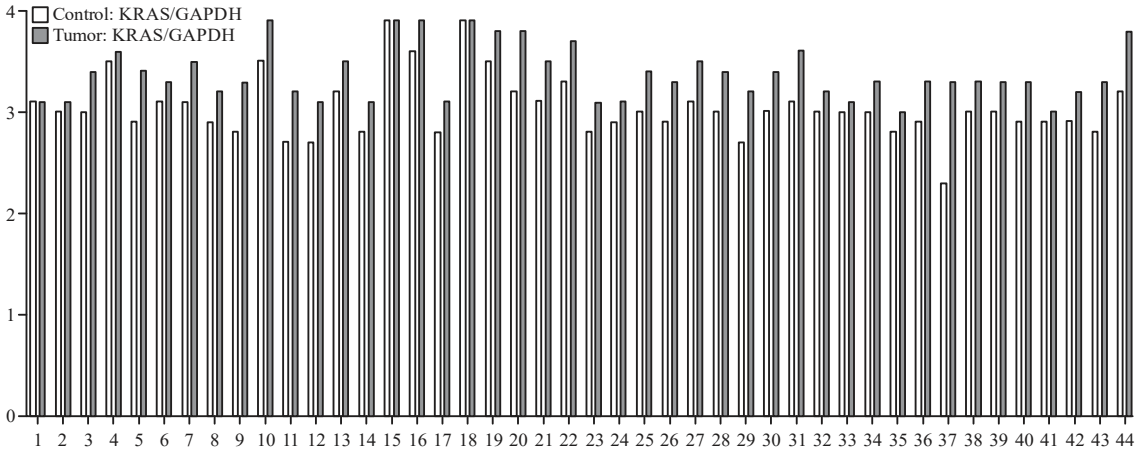

Fig. 2: The mRNA expression level of each normal control and tumor according to KRAS/GAPDH
The mRNA expression level of 41 breast cancer was up-regulated

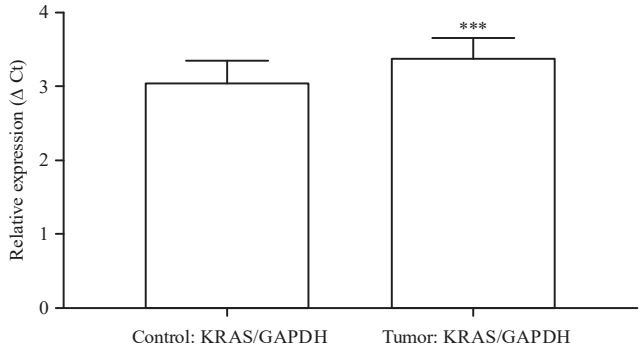

Fig. 3: Statistical results of the mRNA expression level of KRAS/GAPDH gene in both normal and breast cancer samples
***p<0.05

pancreatic and lung cancers, recent associations among KRAS hyperactivity and human breast cancer have been investigated. Mutations were found in 12.5% of cases[18], but only 5% of cases were registered in the COSMIC database of Sanger version 28 (http://www.sanger.ac.uk)/genetics/CGP/cosmic/)[18]. The lower frequency of KRAS mutations in breast cancer cell lines proposed that genetic variation is less significant in carcinogenesis of breast cancer than in other types of cancer, although at a "hotspot" in the KRAS gene mutations were found in a small subgroup of breast cancer).

An important goal in today's research is the invention of new molecular biomarkers that can identify breast tumors in initial stages[19]. Recently, molecular genetic analysis has extended the possibility of testing new potential biomarkers

because only a few markers can be recommended for practical use in the clinic[19]. Studies on gene expression analysis have been published in several new perspectives in cancer and the investigation of mRNA expression becomes a useful agent for cancer detection, classification and prediction of the disease[19]. In our study based on RT-qPCR, the KRAS gene was significantly up-regulated in patients with breast cancer. This study was similar to another observation that revealed an increase in the expression level of KRAS mRNA[19]. KRAS expression is regulated by the binding of proteins to its promoter during the initiation of transcription and as the transcriptional elongation by microRNAs that affect the stability of KRAS mRNA. Recently, it has been observed that several microRNAs (miRNAs) are involved in the KRAS signalling pathway and carcinogenesis of the breast. The specific miRNAs indirectly affected KRAS signalling by regulating the genes associated with KRAS[19].

CONCLUSION

A statistically significant association was established between the increase in KRAS expression level and the pathogenesis of breast cancer compared to normal breast tissue samples. To date, regulation of KRAS expression, downstream events of KRAS signalling and all genes that respond to KRAS remain mostly unclear. Future studies investigating the regulation of KRAS improve our understanding of the biological importance of KRAS in healthy tissues and breast cancer.

REFERENCES

1. Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, C. Smigal and M.J. Thun, 2007. Cancer statistics. CA Cancer J Clin., 57: 43-66.
2. Yezhelyev, M.V., X. Gao, Y. Xing, A. Al-Hajj, S. Nie and R.M. O'Regan, 2006. Emerging use of nanoparticles in diagnosis and treatment of breast cancer. Lancet Oncol., 7: 657-667.
3. Kravchenko, J., I. Akushevich, V.L. Seewaldt, A.P. Abernethy and H.K. Lyerly, 2011. Breast cancer as heterogeneous disease: Contributing factors and carcinogenesis mechanisms. Breast Cancer Res. Treatment, 128: 483-493.
4. Grutzmann, R., C. Pilarsky, O. Ammerpohl, J. Luttges and A. Bohme *et al*., 2004. Gene expression profiling of microdissected pancreatic ductal carcinomas using high-density DNA microarrays. Neoplasia, 6: 611-622.
5. Wagner, K.W., E.A. Punnoose, T. Januario, D.A. Lawrence and R.M. Pitti *et al*., 2007. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nature Med., 13: 1070-1077.
6. Haigis, K.M., K.R. Kendall, Y. Wang, A. Cheung and M.C. Haigis *et al*., 2008. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nature Genet., 40: 600-608.
7. Johnson, L., K. Mercer, D. Greenbaum, R.T. Bronson, D. Crowley, D.A. Tuveson and T. Jacks, 2001. Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature, 410: 1111-1116.
8. Malumbres, M. and M. Barbacid, 2003. RAS oncogenes: The first 30 years. Nature Rev. Cancer, 3: 459-465.
9. Kranenburg, O., 2005. The KRAS oncogene: Past, present and future. Biochim. Biophys. Acta, 1756: 81-82.
10. Yen, L.C., Y.H. Uen, D.C. Wu, C.Y. Lu and F.J. Yu *et al*., 2010. Activating KRAS mutations and overexpression of epidermal growth factor receptor as independent predictors in metastatic colorectal cancer patients treated with cetuximab. Ann. Surg., 251: 254-260.
11. Meola, J., L.R. Goulart, J.D. Oliveira, A.F. Neves and W.P. Oliveira, Jr. *et al*., 2006. Differential expression of the KLK2 and KLK3 genes in peripheral blood and tissues of patients with prostate cancer. Genet. Mol. Biol., 29: 193-199.
12. Burchill, S.A. and P.J. Selby, 2000. Molecular detection of low level disease in patients with cancer. J. Pathol., 190: 6-14.
13. Khailany, R.A., M. Igci, E. Bayraktar, S. Erturhan, M. Karakok and A. Arslan, 2015. VHL, PBRM1 and SETD2 genes in kidney cancer: A molecular investigation. Int. J. Med. Health Biomed. Pharm. Eng., 9: 389-392.
14. Serban, D.A., G. Weber, L. Marşavina, V.V. Silberschmidt and W. Hufenbach, 2013. Tensile properties of semi-crystalline thermoplastic polymers: Effects of temperature and strain rates. Polymer Test., 32: 413-425.
15. Patra, S., V. Young, L. Llewellyn, J.N. Senapati and J. Mathew, 2017. BRAF, KRAS and PIK3CA mutation and sensitivity to trastuzumab in breast cancer cell line model. Asian Pac. J. Cancer Prevent., 18: 2209-2213.
16. Wang, Y.L., X. Dai, Y.D. Li, R.X. Cheng, B. Deng, X.X. Geng and H.J. Zhang, 2015. Study of PIK3CA, BRAF and KRAS mutations in breast carcinomas among Chinese women in Qinghai. Genet. Mol. Res., 14: 14840-14846.
17. Ahlquist, T., I. Bottillo, S.A. Danielsen, G.I. Meling and T.O. Rognum *et al*., 2008. RAS signaling in colorectal carcinomas through alteration of RAS, RAF, NF1 and/or RASSF1A. Neoplasia, 10: 680-686.
18. Sanaei, S., M. Hashemi, E. Eskandari, S.M. Hashemi and G. Bahari, 2017. KRAS gene polymorphisms and their impact on breast cancer risk in an iranian population. Asian Pac. J. Cancer Prevent., 18: 1301-1305.
19. Mahesh, K., K.C. Kalyana, K.K. Ravi, A.V. Laxmi, M. Sudha and R. Ammi, 2012. Differences in gene expression profiles between human breast tissue and peripheral blood samples for breast cancer detection. J. Cancer Sci. Ther., 4: 379-385.