Journal of Oncological Sciences 3 (2017) 112–116

Contents lists available at ScienceDirect

Journal of Oncological Sciences

journal homepage: https://www.elsevier.com/locate/jons

Oncogenic mutations of PIK3CA and HRAS in carcinoma of cervix in South Indian women

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ARTICLE INFO

Article history:
Received 7 June 2017
Accepted 13 October 2017
Available online 6 November 2017

Keywords:
Oncogenic mutations
Cervical cancer
Peripheral blood
PCR-CTPP method

ABSTRACT

Carcinoma of the cervix is the second most successive reason of female cancer which remains an imperative medical issue in women around the world. Mutations are normally dissected by tissue biopsy which is intrusive, costly and possibly subjective. However, detection of mutations from blood is a non-invasive procedure. However, detection of mutations from blood is a non-invasive procedure. It would offer several advantages, including greater speed and cheap at a cost such that the family members can likewise be screened. Repeated tests after surgery provide an early cautioning for the disease recurrence. The present work aimed to assess the frequency of PIK3CA and HRAS mutations in cervical cancer patients using peripheral blood. This study includes 210 cases presenting cervical cancer and 210 age and sex-matched healthy controls. Genomic DNA was isolated from peripheral blood. The PCR-CTPP method was performed for screening of exons 9 and 20 of PIK3CA and exon 34 HRAS mutations. Sequencing of PIK3CA and of HRAS genes was done for further confirmation. Out of eight mutations studied, no clear disease causing mutations were noticed in any of the cervical cancer patients in the present investigation. Cervical cancer harbors excessive rates of targetable oncogenic mutations. We couldn't find any changes in our investigation. If a connection amongst tissue and non-tissue mutations could be shown, the probability of a basic blood test to distinguish possibility for anticancer treatment comes a bit nearer. Repeated blood tests after surgery provide an early cautioning for disease recurrence. Therefore, in future evaluation of a larger data set and cases with progressive tumor (Stage III–IV) will be required to approve these findings.

1. Introduction

Cervical malignancy is our present society's most-deadliest cancer which is effectively preventable with a probability of 85% occurrence in developing countries.[1] During diagnosis of cancer in women, it occupied fourth place with a predicted 5,27,600 new cases globally. The cases pertaining to cervical cancer are assumed to increase 1.5 times by 2030 with the rising population.[2]

By adopting the method of first line screening, the increasing global implication of cervical cancer's morbidity along with mortality can be controlled. However, the high costs involved in currently available screening methods are inadequate, thus finally leading to a low participation rate. Women show their minimal interest and feel disconcerting to have Pap smear. Therefore, it is the need to develop alternative methods depending on biomarkers of blood in the tests of first line screening. Pre-determined targets

Abbreviations: PIK3CA, Phosphatidylinositol-4,5-Bisphosphate; 3-Kinase Catalytic Subunit Alpha, HRAS; Harvey rat sarcoma viral oncogene homolog, EGF; Epidermal Growth Factor, PDGF; Platelet Derived Growth Factor, GRB2; Growth factor receptor-bound protein 2, SOS; Son of seven less, MAP K; Mitogen-activated Protein Kinase, MAP KK; Mitogen-activated Protein Kinase Kinase, MAP KKK; Mitogen-activated Protein Kinase Kinase Kinase, RAF; Rapidly Accelerated Fibrosarcoma, MEK; Methyl Ethyl Ketone, RTK Receptor Tyrosine Kinase; IRS-1, Insulin receptor substrate-1; PI3K, Phosphatidylinositol-3-Kinase; PIP2, Phosphatidylinositol 4,5-Bisphosphate; AKT/ PKB, Protein kinase B; BAX, Bcl-2-associated X protein; Rheb, RAS-Homolog Enriched in Brain; mTOR, Mammalian target of rapamycin; S6K, S6 Protein Kinase; FOXO, Forkhead box O protein; SCC, Squamous cell carcinoma; ADC, Adenocarcinoma; PCR-CTPP, Polymerase chain reaction with confronting two-pair primers.

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Peer review under responsibility of Turkish Society of Medical Oncology.

https://doi.org/10.1016/j.jons.2017.10.004

can be attained by promoting active participation thereby reducing the rising rate of patients of cervical cancer globally. A screening test based on non-invasive blood sampling which is not hazardous to cancer patients is an alternative tool. In other words, it is vital for a primary screening program to distinguish the mentioned three sections: general population (average risk), high-risk group and cancer group. Keeping this in mind, the status of mutations are checked for the existence of mutations (PIK3CA and HRAS) in peripheral blood samples in cervical cancer patients, which would be a feasible alternative to a tissue biopsy.

If non-tissue mutations could be checked, the requirement for anticancer treatment for identified candidates can be done with a normal blood test. The Epidermal Growth Factor (EGF) interaction with Epidermal Growth Factor Receptor (EGFR) leads to the beginning of the main signaling pathways. The pathways get activated by mutations in RAS and/or PIK3CA is an established mechanism. These mechanisms are explained in brief.

1.1. HRAS pathway

The transformation protein p21 in humans also termed as HRAS is indeed a GTPase enzyme. HRAS gene (Fig. 1) encodes the GTPase enzyme. Binding of Receptor Tyrosine Kinase (RTK) with growth factor (e.g., EGF, HGF) activates the receptor. Proteins GRB2/SOS activate HRAS by substituting the GDP of HRAS with GTP. The active HRAS activates MAP Kinases (Mitogen-activated Protein Kinases) (MAP KKK->MAP KK/MEK->MAP K), which triggers transcription factors (TF). Once transcribed with mRNA, proteins like cyclins/CDKs etc are produced which are compulsory for cell growth and division. HRAS operates as an on/off molecular switch. If this pathway remains "ON" continuously, the normal cell will be turned in to a malignant cell, because the cell has lost control of cell division which will divide and divide, leading to cancer. So, after activation of Ras, there is also a cycle of inactivation to stop the signaling pathway. HRAS turns off when GTP converts to GDP.

1.2. PIK3CA pathway

The PIK3CA pathway is very complex (Fig. 2). IRS-1 initiates transphosphorylation from PIP2 (Phosphatidylinositol 4, 5-Bisphosphate) to create PIP3, which activates AKT/PKB, a proto-oncoprotein with many substrates and effects. The best-known effect is the prohibition of apoptosis (programmed cell death).

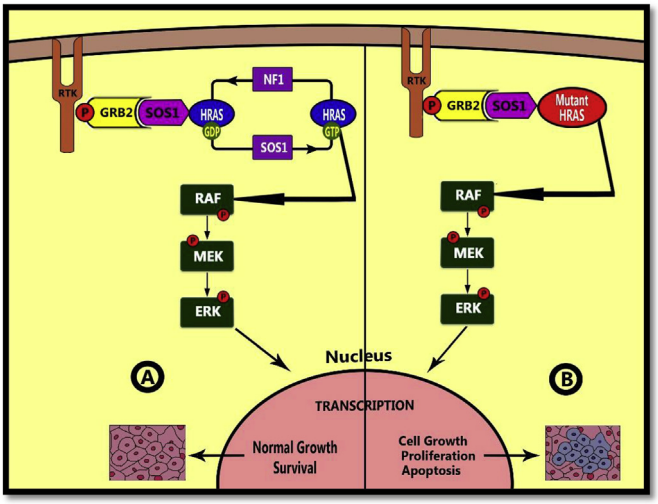


Fig. 1. Schematic representation of the HRAS pathway.

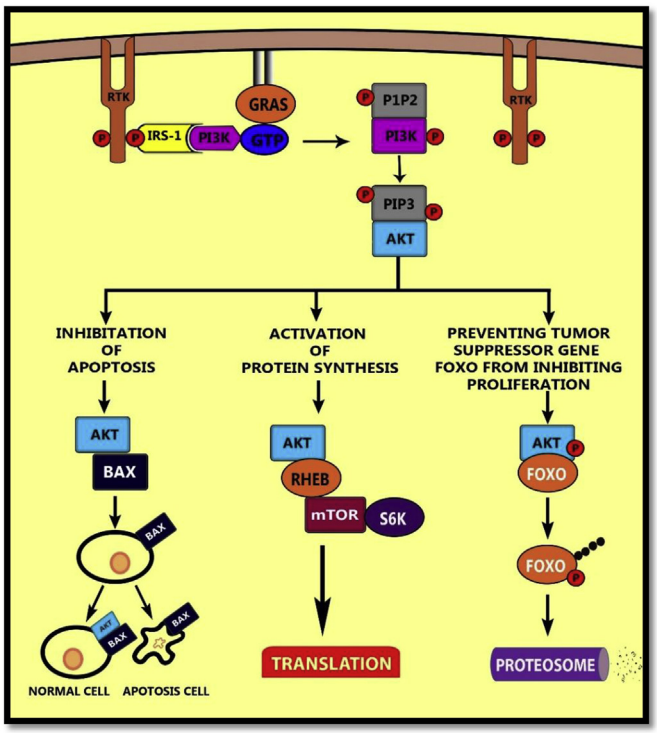


Fig. 2. A simplified overview of the PI3K–AKT–mTOR pathway.

The unavailability of AKT leads to apoptosis since AKT binds to BAX (Bcl-2-associated X protein) and hinders its capacity to form homogeny outer mitochondrial membrane. Activation of protein synthesis or translation is another important AKT effect. This effect is performed by a multi-step protein cascade Rheb (RAS-Homolog Enriched in Brain) à mTOR (mammalian Target Of rapamycin) à S6K (S6 Protein Kinase). S6K activates the transformation of mRNA to a protein which promotes growth and also the survival of cells. In addition, AKT may lower the concentration of protein FOXO protein by phosphorylating FOXO, which transfers ubiquitin peptides onto the protein. Subsequently, ubiquitin leads FOXO to store it by a complex protease, the so-called proteasome. In this way, AKT prevents the tumor suppressor protein FOXO from inhibiting proliferation.

2. Subjects and methods

2.1. Subjects

Peripheral blood samples were collected from 200 healthy females (age: 30–65 years) and 210 cervical cancer patients (stage I-II; age 30–65 years) who were diagnosed in cancer hospitals, Visakhapatnam, Andhra Pradesh. The Andhra University Institutional Ethical Committee for Research on Human Volunteers has accepted the research. The study included the participants who had given their consent in written format. Genomic DNA was isolated from the considerably modified salting-out method.[3] All the cases and controls were genotyped in a randomized and blinded fashion.

2.2. PCR-CTPP method

To detect the PIK3CA and HRAS mutations, the genotyping was done by means of PCR-CTPP.[4] Eight nucleotide mutations - 5 PIK3CA mutations (Exons 9 and 20) and 3 HRAS mutations (Exon 2) were amplified, followed by sequencing of suspicious ones and a

few samples with normal patterns. The mixture of reaction is prepared to a 15 μl final volume for 1 sample by adding all the solutions/samples in a 0.5 ml Eppendorf tube.

PCR mixture was then aliquot into the individual tubes (0.2 ml). Template DNA was then added to these tubes. Then the samples are centrifuged and kept in a LARK thermocycler and PCR was started. The PCR protocol is as follows: initial denaturation (5 min; 95 °C), followed by 30 cycles of denaturation (20 s; 95 °C), annealing (20 s; 60 °C), and extension (20 s; 72 °C). The final extension was for 5 min at 72 °C. The products were examined using 1.5% agarose gel stained with ethidium bromide.

2.3. DNA sequencing

Sequencing was done bi-directionally using Big Dye Terminator kit v3.1. The PCR conditions for the cycle sequencing are Initial denaturation at 96 °C for 10 min, denaturation at 94 °C for 10sec, annealing at 50 °C for 5sec, extension at 60 °C for 4mins for 30 cycles, using an only forward primer.

3. Results

Five nucleotide mutations of PIK3CA at codons 1633 (G>A; G>C), 1624 (G>A) and 3140 (A>T; A>G) and three nucleotide mutations of HRAS in codon 34 (G>C), 34 (G>T) and 34 (G>A) were covered (Table 1). The predicted PCR products of PIK3CA are: 128bp for wild-type (G) and 286bp for mutant (A/C) (1633G/A; G/C), 122bp for wild-type (G) and 280bp for mutant (A) (1624G/A), 230bp for wild-type (A) and 160bp for mutant (T/G) (3140 A/T; A/G). And for HRAS, 254bp for wild type and 130bp for the mutant (T/A/C) (34 G/T; G/A; G/C).

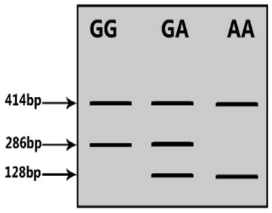
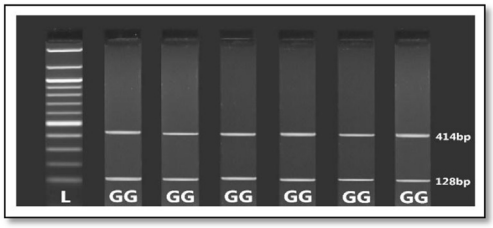
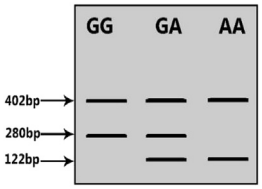
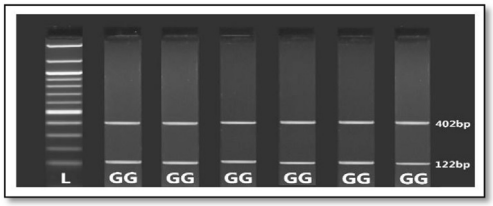
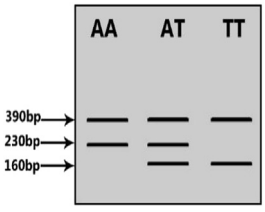
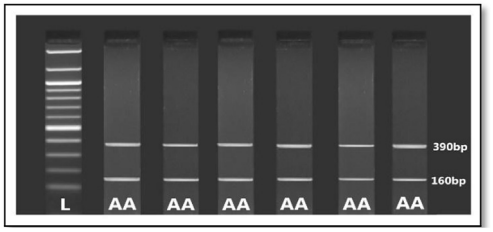
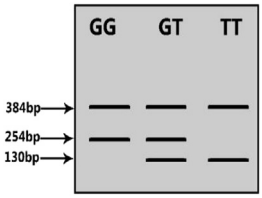
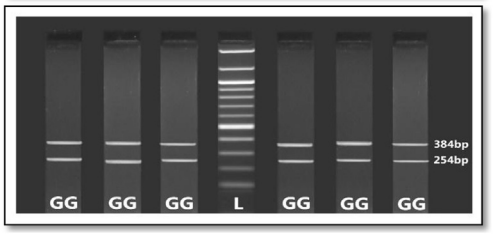
No clear mutations were found in any of the codons in the current study. The PCR products of PIK3CA were as follows: 128bp for wild type (G) for codon 1633G/A; G/C, 122bp for wild type (G) for 1624G/A, 230bp for wild type (A) for 3140 A/T; A/G and for HRAS, 254bp for wild type (G) for 34 G/T; G/A; G/C (Table 1). Sequencing of PIK3CA and of HRAS genes was done for further confirmation (Fig. 3).

4. Discussion

In cervical cancer, PIK3CA mutations play the main role. Experimental proof conveys that PIK3CA and RAS mutations generate different biologic effects and that contributing mutations enhance tumorigenic effects. PI3Ks play a major part in cellular metabolism, DNA repair, apoptosis etc.

During 1977, the occurrence of tumor DNA in plasma or serum has been reported.[5] Cell-free DNA was liberated from apoptotic

Table 1
The primer sequences, predicted and gel electrophoresis banding patterns of PIK3CA and HRAS.

Gene/Position	Primer Sequences	Predicted Band Patterns	Electrophoretic Patterns
PIK3CA_1633 (G>A; G>C)	NF: 5′ CCTGTCTCTGAAAATAAAGTCTTG3′ NR: 5′ CCATAGAAAATCTTTCTCCTGCTC3′ MF: 5′GAGATCCTCTCTCTGAAATCACTA3′ MR: 5′ GAGATCAGCCAAATTCAGTTAT3′		
PIK3CA_1624 (G>A)	NF: 5′ CCTGTCTCTGAAAATAAAGTCTTG3′ NR: 5′ TCTTTCTCCTGCTCAGTGATTTC3′ MF: 5′ TTCTACACGAGATCCTCTCTCTA 3′ MR: 5′ GAGATCAGCCAAATTCAGTTAT 3′		
PIK3CA_3140 (A>T; A>G)	NF: 5′ ATAGGTTTCAGGAGATGTGTTAC 3′ NR: 5′ TTTTGTTGTCCAGCCACCATGAT 3′ MF: 5′ATGAAACAAATGAATGATGCACG 3′ MR: 5′ CTTTGCCTGCTGAGAGTTATTAA 3′		
HRAS_34 (G>T; G>A; G>C)	NF: 5′ TAAACTTGTGGTAGTTGGAGCTGG3′ NR: 5′ AGGTACATTTCAGATAACTTAACT3′ MF: 5′ AGTGTATTAACCTTATGTGTGAC 3′ MR: 5′ CACTCTTGCCTACGCCACT3′		

Exon	Codon Position	Observed base pair change	Amino acid Change
9	1633	G to A	p.E545K
9	1633	G to C	p.E545Q
9	1624	G to A	p.E542K
20	3140	A to T	p.H1047L
20	3140	A to G	p.H1047R

Chromatogram

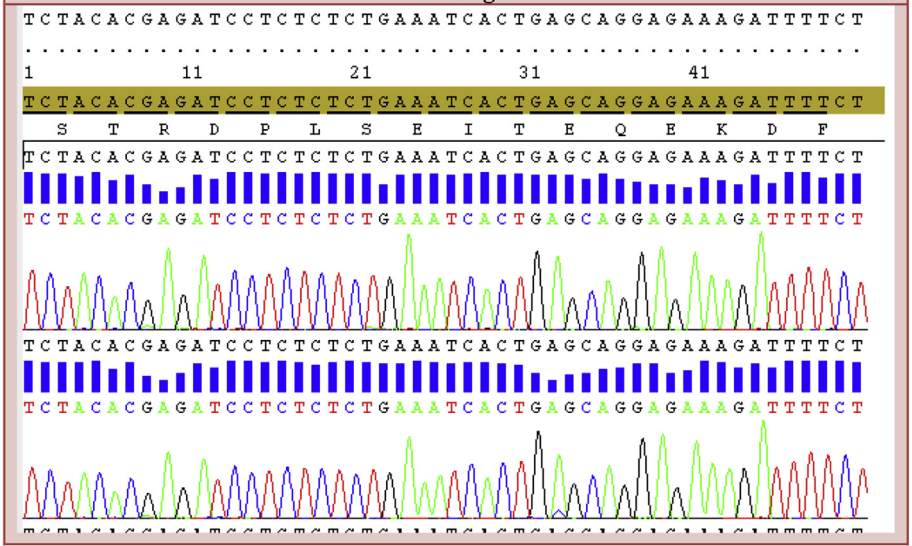


Fig. 3. Five nucleotide mutations of PIK3CA and Sequence patterns.

cancer cells as a byproduct of phagocytosis.[6,7] Initially, it received little attention, but with recent advances, it was explored extensively for the possible application to cancer detection.[8] Modern investigations used gene alterations as tumor markers which clearly advised the potential usefulness of the appearance of such gene alterations in the serum or plasma for staging, management, and detection of tumors.[9–15] This technology has been employed for somatic mutation analysis, particularly on several genes with high mutation frequency, such as K-RAS,[16–18] TP53,[19] and APC.[20] However, the spreading tumor DNA is moderately low compared with wild-type DNA.[21] Furthermore, the tumor-associated mutations are generally different to each patient,[22,23] and therefore, it is somewhat difficult to establish an inexpensive and extremely sensitive test to detect all somatic mutations for identification of various malignancies in early stages. In the detection of cervical cancer, a basic and common blood test is the alternative for Pap smear test for with an idea that a simple blood-based test will be a useful first-line screening device for all married women, we focused to explore the event of mutations in patients experiencing cervical malignancy using peripheral blood samples. But no clear mutations leading to cancer were recognized. However, the spreading tumor DNA is moderately low compared to wild-type DNA.[8–10] A positive interaction between PIK3CA mutations and malignant tissues including cervical cancer were reported with a frequency of 5–36% mutations.[24–35] Many molecular studies[36–41] on patients experiencing cervical malignancy showed HRAS mutations. But, all these studies have investigated mutations in tumor tissues.

Therefore, the fact that no genetic aberration was identified in any of the 210 patient samples suffering from cervical cancer, suggests that mutations of PIK3CA and HRAS are not probable to be recognized through blood in the patients with cancer of the cervix (with the primary tumor) are concerned.

5. Conclusion

When screened for the eight mutations, mutations were absent during amplification (PCR-CTPP method). Further, sequencing was done to confirm our results. The SCC and ADC having diverse molecular profiles suggesting distinctive clinical results may be the reason for not finding disease-causing mutations in our study since it is noticed that RAS mutations are in 30–50% of ADC, whereas our samples include only SCC resulting in no mutations. Moreover, recruiting only patients with primary tumor may be another drawback for our study for not finding mutations (circulating tumor DNA) in peripheral blood. Future works with prospective trials have to be investigated to decide the suitable method of testing for the blood mutations. Moreover, attempt to recognize and spot specific molecular biomarkers within cervical cancer provide an essential opportunity to enhance outcomes in women.

Acknowledgments

The authors were thankful to the respondents and cervical cancer patients of Srikakulam, Visakhapatnam and Vizianagaram districts, who volunteered to be the research subjects, and support for their help in data and sample collection.

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