RESEARCH ARTICLE

High Frequency of Codon 12 but not Codon 13 and 61 K-ras Gene Mutations in Invasive Ductal Carcinoma of Breast in a South Indian Population

C Sushma[1], Shiva Prasad[1], Rudrama Devi[1], Sudha Murthy[2], TS Rao[2], CK Naidu[3]*

Abstract

Background: *Ras* genes are thought to play an important role in human cancer since they have been found to be activated frequently in several types of tumors including breast cancer, where the overall incidence of K-RAS oncogene activation is 0-10%. Evaluation of K-RAS gene not only for mutational frequency but also for mutation types in this downstream signaling gene pathway is necessary to determine the mechanisms of action. The present study was conducted to test the hypothesis that K-RAS activation is involved in breast cancer risk of south Indian population. Materials and Methods: A total of 70 paired pathologically confirmed tumor and non-tumor tissues from the same breast cancer patients were analysed for most common K-RAS mutations of codon 12,13 and 61 by polymerase chain reaction followed by restriction digestion and direct nucleotide sequencing method. Results: We found that a high rate of homozygous and heterozygous mutations of codon 12, but not codon 13 and 61, may influence the invasive ductal carcinoma of breast risk in this study. Conclusions: Our study indicated that only codon 12 may be involved in initiating breast carcinogenesis in India.

Keywords: Breast cancer - K-ras mutations - risk - South India

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Introduction

The single most common proto-oncogenes disorder in human neoplasms is point mutation of RAS genes. About 15-30% of tumors contain RAS oncoproteins and the mutation frequencies vary in different tumors. The highest incidence is found in adenocarcinomas of the pancreas (90%), the colon (50%) and in thyroid tumors (50%). For some tumor types a relationship may exist between the presence of a ras mutation and clinical or histopathological features of the tumor (Stricker et al., 2010).

RAS has a basic role in signal transmission of growth factor receptors within cytoplasm and the activated form stimulates downstream regulators of proliferation. In inner plasma membrane these RAS family member were binds a small G proteins namely guanosine triphosphat (GTP) and guanosine diphosphate (GDP) (Stricker et al., 2010) and possess an intrinsic GTPse activity, implicates the regulation of cell proliferation activity. In the inactive form, it binds GDP, but cell Stimulation by growth factors cause, inactive (GDP-bound) form is activated to a GTP-bound state. Activated RAS stimulates RAF and mitogen-activated protein (MAP) kinase cascade to transmit growth signals to the nucleus (Chan et al., 2004; Stricker et al., 2010). The human ras family consists of three proto-oncogenes, c-Harvey (H)-ras, c-Kristen (K)-ras and N-ras, no c- prefix was added because no viral counterpart was found in N-ras. In RAS family genes K-Ras gene is important for cell proliferation and survival. When KRAS is activated by mutation, the resulting uncontrolled cell growth and division can result in cancer. K-ras gene has transforming activity after single point with in its coding sequence. Mostly, these mutations have been localized in codon 12, 13 and 61 are the most common in naturally occurring (i.e. non- experimental) (Kiaris and Spandidos, 1999) neoplasms and experimentally induced animal tumors (Mangues and Pellicer, 1992; Stanley, 1995).

The RAS protein is activated in many solid tumors and hematologic neoplasms and a wide range of variations in frequency of RAS mutations have been observed. The frequency of most common RAS mutations in breast cancer tissues are 0-10% reported. The frequency of K-RAS and breast cancer incidences are very low 0-12% (Rochlitz 1989). Breast cancer is of startlingly high incidence (approaching 1 in 9 women), but unfortunately current therapies for the disease are inadequate once it has metastasized. The disease is characterized by excessive morbidity and mortality. Normal as well as malignant growth is regulated by endocrine hormones and by local tissue factors, such as polypeptide growth factors.

*[1]Department of Genetics, Asian Institute of Gastroenterology, [2]Department of Pathology, [3]Department of Surgical Oncology, Indo-American Cancer Hospital, Hyderabad, India *For correspondence: ccknaidu@gmail.com*

Breast carcinomas seem to progress as hyperplastic ductal or lobular epithelial growth, acquiring progressive genetic changes (including those of oncogenes and tumor suppressor genes) leading to clonal outgrowths of progressively malignant cells (Dickson RB 1991).

Previous investigators have reported that the mutational activation of ras oncogenes is likely involved in the etiology or progression of human breast cancer. But these models were carried out by Chemical induction of breast adenocarcinomas in pubescent rats, it is a widely used model of carcinogenesis because the resulting tumors are histologically and behaviorally identical to human breast tumors. Notably 85% of these rat mammary carcinomas carry transforming ras mutations (Sukumar, 1988).

Additionally many investigators previously have reported over expression of the ras-encoded p21 proteins in malignant breast carcinomas (Spandidos 1984, Agnantis NJ 1986), although the role of this over expression in breast carcinogenesis has not been determined. Rochlitz et al (1989) have found the emergence of a *K-ras* mutation in codon 12 in the terminal stages of breast cancer progression, but other investigators were able to identify such mutations only in a primary tumor (Prosperi MT 1990). Using oligonucleotide hybridization analysis, Spandidos has detected point mutations at amino acid position 12 (glycine to valine) of Hras in 2/24 breast carcinomas tested (Spandidos, 1987). But these reports were 30 decades before.

Oncogenic mutations in the K- RAS signaling pathways have been instrumental in deciphering the biology of breast cancer disease pathways. Conversely, knowledge of the functional implications of oncogenic mutations has increased our understanding of human carcinogenesis, through the commonalities as well as the differences between tumor types. However, these results are addressed the mutational activation of K-RAS pathway in a single cohort of human tumor samples.

However, recent retrospective analyses (De Roock W 2010, Tejpar S 2012) have suggested that patients whose tumors harbor a specific *KRAS* exon 2 mutation, a glycine (G; single-letter amino acid code) to aspartate (D) mutation at codon 13 (G13D), may derive clinical benefit from an anti-EGFR monoclonal antibody therapy in chemorefractory settings.

Because RAS is frequently mutated in varieties of neoplasms, many attempts have been done to develop an anti-RAS drug as a therapeutic target (McCubrey et al., 2008; Mansi et al., 2011; Zhu et al., 2012). The Ras/Raf/MAP/extracellular signal-regulated kinase (ERK) pathway often contributes in sensitivity and resistance to EGFR based targeted therapies in various cancers like Cetuximab in colorectal and imatinib drug in CML.

Therefore, detection and controlling the expression of K-RAS gene pathway may helps the role in etiology and could improve chemotherapy treatment with EGFR based targeted therapies. In this study we assessed common mutations in codon 12, 13 and 61 by PCR followed by restriction Length Polymorphism and direct sequencing.

Materials and Methods

The Project was approved by the Institutional Ethics Committee of Basavatarakam Indo American Cancer Institute and Research Centre, (Hyderabad) in accordance with the Helsinki Declaration.

Enrolment of study subjects: In this study a total of 70 patients, with Breast cancer (pathologically, histologically/cytologically confirmed) between June 2011 to November 2014 were enrolled. The disease was determined clinically through FNAC, malignant cytology by Pathologists. Tumor tissue was collected from the patients after confirmed diagnosis of breast cancer and adjacent non tumor tissues were also collected from same patients for control group and informed consent was taken from all the subjects. Recording of demographic details was in a structured questionnaire carried out by direct interview and referring the medical records. The clinical information includes age, tumor type, stage of cancer, tumor size, and axillary lymph node and metastasis status. Also we have collected information of ER, PR, and HER-2 status determined immunohistochemically.

Analysis of K-ras mutations

Genomic DNA was isolated from collected tumor and healthy tissue samples of breast cancer cases on the same day. Salting out method (Miller et al., 1988) was used for DNA isolation.

For the detection of KRAS point mutations, DNA samples were analyzed by using PCR-restriction fragment length polymorphism (RFLP) for both codon 12 and 13. Primers were selected as per the literature for codon 12 (Márcia Saldanha Kubrusly et al 2002), codon 13 by Nagasaka et al. (2004). PCR was performed as per the method described by Mohammad Mahdi Kooshyar et al., 2014 with little modification in annealing temperature. For codon 12 we have used Fast digest Mva I enzyme (Fermentas)

For codon 61 exon 3 following primers were used Forward: 5'-GACTGTGTTTCTCCCTTCTC-3' and Reverse 5'-TTTCAATCCCAGCACCACCAC-3'. Mutation analysis was confirmed by direct sequencing. PCR conditions were followed as described by Atena Irani Shemirani et al in 2011.

Results

All patients were females ranging from 30-70 (mean 46.84) years. All cases enrolled in this study was invasive ductal carcinoma and found to be stage II, negative for metastasis. Demographic and clinical parameters were shown in Table 1.

There were no statistically significant differences in age, sex, breast involvement, tumor size, node involvement, grade and ER, PR status due to no mutation were found in Codon 13 and 61 in this study.

Mutations were detected both in tumor and non tumor tissues respectively. Distribution results are presented in Table 2. To enhance statistical power to detect significance and association with the risk of the disease, we compared the three variants in non tumor tissue and tumor tissue. We found that the frequency of Wild type was 33% and 14%, Homozyous mutation were 0% and 4% and Heterozygous

Table 1. Clinical Parameters of Study Subjects

Variables		No of Subjects (N=70)	Percentage (%)
Age (in years)	<40	22	31
	41-60	32	46
	>61	16	23
Breast Involved	Right	38	54
	Left	32	46
Tumor Size (in cm)	≤2.0	43	62
	>2.1-5.0	22	31
	≥5.1	5	7
LVSI*	Positive	54	77
	Negative	16	23
Grade	I	11	16
	II	43	61
	III	16	23
ER Status	Positive	48	69
	Negative	22	31
PR status	Positive	45	64
	Negative	25	36
ER and PR status	ER+ + PR+	42	60
	ER+ + PR-	8	11
	ER- + PR+	0	0
	ER- + PR-	20	29

Table 2. Distribution of Mutations in Tumor and Non Tumor Tissues

	Non Tumor Tissue (n=70)	Tumor Tissue (n=70)	OR	95%CI	p value
Wild	23 (33%)	10 (14%)	2.93	1.3-6.8	0.01
Homo	0 (0%)	03 (04%)	0.13	0.0-2.7	0.1
Hetero	47 (67%)	57 (82%)	0.46	0.2-1.0	0.05

Table 3. ??

Control Tissue	Tumor Tissue				
	Wild	Homo	Hetero	ChiSquare	pvalue
Wild	10	03	10		
Homo	00	00	00	32.7	<0.05
Hetero	00	00	47		

were 67% and 82% respectively. Statistically the Odds ratio for wild type was (OD: 2.93, p=0.01) and for Hetero (OD: 0.46, p=0.05) which was significant when compared with control tissue. Any among these two variants Gln/Gln or Arg/Gln were found to be significantly associated when compared with control group.

Even when we analyzed the individual varient by the Cochran-Armitage test between tumor and non tumor tissue samples it showed significance in breast cancer samples, where presence of homozygous mutation and heterozygous mutations showed significance when compared with wild type (Table 3). We found that presence of at least one nucleotide substitution in tumor tissue when compared with non tumor tissue e may be increases the risk of breast cancer in same patient sample (p<0.05).

For codon 13 and codon 61 We didn't observe any KRAS mutations in this study. For codon 13 we did not found any *BglI* restriction site in 70 cases. For codon 61 PCR product was sent for sequencing, after analyzing the sequence we did not found any nucleotide substitution/deletion in all sample.

Discussion

In this present study except codon 12 we did't found any mutation in codon 13 and 61. The review of literature also shown wide range various in the frequencies of RAS mutations which ranging from 0-12% in breast cancer disease (Rochlitz et al., 1989). Our results are similar with Koff et al 1993 where mutational activation of the *K-ras (codon 12)* gene may be involved in the development of a small percentage of breast tumors. A recent report also found the similar finding in breast cancer in where only 7.76% of patients showed codon 12 mutations in breast cancer and associated with Her-2 (Pereira et al., 2013). Even in Chinese population where they found only 2.5% which much lower than pancreatic and colorectal cancers (Tong et al., 2012).

We have higher frequency of mutation in this study, as this procedure was by restriction digestion method. For other two codon we did't found any mutations. This variation may be due to the nature of the disease. Because, it is a complex disease with heterogeneous clinical evolution. Several analyses have been performed to identify the risk factors for breast cancer progression. Also ethnic disparities also depends the mutation frequencies. Also frequency of the mutations may be depends up on the stage, where the frequency of the K-RAS codon 12 is about to 50% in colorectal cancers. A recent study in thai population the overall incidence of K-Ras mutations in patients was 23%. In the same reference publication authod described K-ras mutation frequencies with respect to stages (AJCC) I, II, III and IV were 6.7%, 16.1%, 23.3% and 26.6%, respectively (Welawee Chaiyapan et al 2013), metastasis (Jakovljevic K et al 2012) and hormonal status (Dickson RB et al 1991). We can correlated the low frequency of the K-ras with colorectal cancers where the differences in the KRAS mutational status between primary and metastatic tumors within lymph nodes and visceral metastases or even different portion of the primary lesion (Baldus et al., 2010), thus supporting the overall current theory of neoplastic heterogeneity. In this present study we did not found any mutation of codon 13 and 61 may be due to stage of disease as our sample consist of only stage II. Null association studies with K-RAS mutation were also reported by Mohammad et al. (2013) Chronic Myloid Leukemia, Takao Asai et al. (2014) in Gall bladder cancer.

Many investigators believe that the mutational activation of *ras* oncogenes is likely involved in the etiology or progression of human breast cancer, particularly since the *ras* p21 protein has been shown to mediate autocrine production of growth factors in transformed cells and even bypass the estrogen depedency of human breast cancer cell lines (Kasid, 1985, Sommers, 1990, but this was negatively proven by (Christoph, 1989).

Availability of K-ras mutations and cancer risk in breast disease are very few, and the data also not consistent. The reasons for this inconsistency was not convinced, it is likely that they reflect the variable sensitivities and specificities of the different techniques used. Alternatively, they may reflect heterogeneity in the patient populations in a very small number of cases. To

out knowledge this the first presentation where we found codon 12 mutation frequency is associated with breast cancer than codon 13 and 61.

In conclusion, we found that codon 12 of K-RAS gene mutation is significant with breast cancer risk. To confirm these findings, further studies are needed to examine more cases globally.

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