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Frequency of *Kras* Gene Mutations in Endometrioid Carcinoma of Uterus in Pakistani Population

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Abstract.- The aim of this study was to find out the frequency of *Kras* gene mutation in endometrioid carcinoma in Pakistani population. Formalin fixed paraffin embedded 70 samples of endometrioid carcinoma were collected from Shaukat Khanum Memorial Cancer Hospital and Research Centre. DNA was isolated, followed by PCR amplification using primers of exon 1 of *Kras* gene. SSCP analysis was done to detect mutation, which was confirmed by direct sequence analysis. Frequency of *Kras* gene mutation was calculated. Statistical analysis was done using SPSS 15.0 software. Frequency of *Kras* gene mutation was found to be 34%. Out of these 34% mutant samples, 62.5% patients were < 50 years of age. Well differentiated histological grade was present in 91.7% of mutant samples.

Key words: Histological grade, precursor lesion

INTRODUCTION

Endometrioid carcinoma is one of the adenocarcinoma of uterine endometrium. It has been grouped as type I adenocarcinoma. It is strongly related to high estrogen levels, whether exogenously introduced or endogenously produced. Endometrioid cancer occurs in age groups below 55 years (perimenopausal age). It is rare in postmenopausal older females (Sherman, 2000; Bokhman, 1983). Most of adenocarcinomas are type I endometrioid (80%). This type of carcinoma is less invasive and its prognosis is relatively better (Okuda *et al*., 2010; Stoian *et al*., 2010). Most of endometrioid carcinoma have low histological grade. New cases of adenocarcinoma of uterus diagnosed every year all over the world are 150,000 (Okuda *et al*., 2010). American Cancer Society had predicted that in 2012 in United States there will be 47,130 new cases of this cancer (American Cancer Society, 2012).

Global comparison was done for incidence of uterine cancer. Higher incidence regions were North America, Europe and South Australia. The regions showing low incidence included Central America, Southern Africa, South East Asia and South Central Asia (WCRF/AICR Second Expert Report 2008; Cancer Council SA, 2006).

Kras protooncogene gains function in variety of cancers. It has been reported by Okuda *et al.* (2010) that 15-30% of endometrioid cancers showed *Kras* gene mutation. Most of the *Kras* mutations have been reported in exon 1. Codon 12 and 13 are more frequently mutated. *Kras* mutation is more in endometrioid type than the rest of adenocarcinoma. Study of genes involved in carcinogenesis help in better management of the cancer. The present study was aimed at determining the frequency of *Kras* mutation in endometrioid cancer in Pakistani population.

MATERIALS AND METHODS

Seventy formalin fixed paraffin embedded (FFPE) tissue blocks of endometrioid cancer patients, between the age 35-75 years were collected from Shaukat Khanum Cancer Hospital and Research Centre in 2007. Histological sections (10µ thick) were cut from FFPE tissues. Three sections were placed in a single (1.5 ml) eppendorf and then processed for DNA isolation followed by PCR amplification. SSCP analysis was done to detect mutation, which was later confirmed by direct nucleotide sequencing.

PCR amplification of exon1 of Kras *gene*

Master pure ™DNA purification kit -cat. No

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MCD 85201 Epicentre Biotechnologies was used for extraction of DNA. The paraffin wax sections were dewaxed with 1 ml xylene followed by 100% ethanol washes. Ethanol was removed and pellets were air dried, to which later 2µl of Proteinase K @ 50µg/µl was added along with 300µl of tissue and cell lysis solution (provided in the kit).The tubes were vortexed, incubated at 65°C for 1 h and then another 150µl of tissue and cell lysis solution was added, vortexed and incubated again at 37°C overnight and then vortexed. The tubes were incubated at 65°C for 15 min. The samples were then allowed to cool down over ice for 10 min. For precipitation of DNA 175µl of MPC protein precipitation reagent (provided in the kit) was added to 300µl of above lysed sample and vortexed vigorously for 10 s. This was followed by centrifugation at 13000xg for 10 min at 4°C. Supernatant was transferred to new microcentrifuge tube. Isopropanol (500µl) was added in the supernatant. Contents were mixed by inverting tubes several times (30-40 times), and centrifuged at 13000xg for 10 min. The pellet containing DNA was rinsed with 70% ethanol twice and then air dried. The pellet was suspended in 35µl of TE buffer (provided in the kit) and used for PCR.

PCR reaction mixture (50µl), contained 1X PCR buffer, 3mM $MgCl_2$, 0.24mM dNTPs, 2.5 U Taq polymerase, 100pmole of each primer and DNA 1.5µg. Forward and reverse primers used for exon 1 of *Kras* are shown below:

KF 5'gactgaatataaacttgtgg 3'
KR 5'ctgtatcaaagaagtgtcct 3'

PCR cycling conditions included initial denaturation at 94°C for 5 min; followed by 35 cycles each comprising denaturation at 94°C for 30 s; 30 s of annealing at 54°C for exon 5, 56°C for exon 6, 58°C for exon 7, 58°C for exon 8 of P53 and 56°C for exon 1 of *Kras*; extension at 72°C for 30 s; final extension at 72°C for 7 min. The reaction was stopped by cooling mixture to 4°C. PCR products were confirmed as 163 bp band on 2% agarose gel.

Single stranded conformation polymorphism (SSCP)

For SSCP analysis 5µl of the amplified DNA and 5µl of 2X SSCP gel loading dye (95% formamide, 20mM EDTA pH 8.0, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added to microfuge tube. After gently mixing the contents of tube, it was placed in 95°C water bath for 7 min and then on ice for about 5 min.

From each of the above prepared samples, 10µl was loaded in the wells of 8% non-denaturing polyacrylamide gel 24.8ml, 10% ammonium persulphate (dH_2O) 400µl, TEMED 40µl, 100% glycerol 2.8 ml, 10X TBE 4ml, 40% acrylamide/bis acrylamide, 8ml). The gel was run at constant power of 30W for 3 h and 30 min. Running buffer used was prechilled 1X TBE. The temperature of running buffer was kept at 10°C.

After the run was complete, the gel was stained with ethidium bromide and with silver stain. The gel was photographed on UV transilluminator.

Nucleotide sequence

PCR products of mutant samples underwent direct sequencing for confirmation. DNA analyzer (Applied Biosystems) was used for sequencing. Sequencing was carried out at Center for Excellence in Molecular Biology, University of the Punjab. Nucleotide sequence was analyzed by using Chromas sequence programme.

Statistical analysis

Statistical analysis was done by using SPSS 15.0 software. The age, cancer type, stages of cancer, mutant gene type and mutant exon types were described by using frequencies and percentages. The association of cancer with age of patient and histological grade of cancer was studied. P-value $\leq$ 0.05 was considered statistically significant.

RESULTS

Figure 1 shows the amplified product of exon 1 of *Kras* gene (163bp). Total of 70 PCR reactions were carried out on samples of endometrioid carcinoma. Mutation was detected on SSCP analysis. In Figure 2 extrabands are seen in mutant samples of endometrioid carcinoma. Confirmation of this mutation was done by sequence analysis. Sequence analysis results of exon 1 of *Kras* gene is

shown in Figure 3. Point mutation has been highlighted. “G” insertion can be seen in codon 28 of exon 1 of *Kras* in reverse sequence. Due to this point mutation AAA sequence of codon 28 changes to AGA. Single nucleotide change leads to amino acid change in different codons of genes. Most common type of mutation seen was missense mutation.

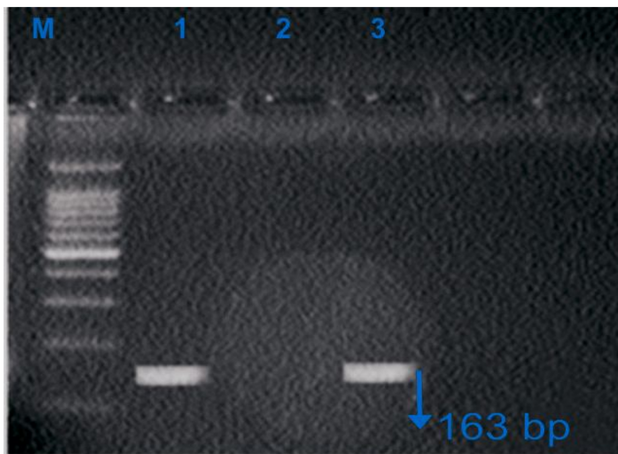

Fig. 1. Amplified product (163bp) of Exon 1 of *Kras* oncogene in 2% agarose gel. Amplified product of DNA sample from endometrioid carcinoma is seen in Lane 1 and3. M: 50bp DNA Marker.

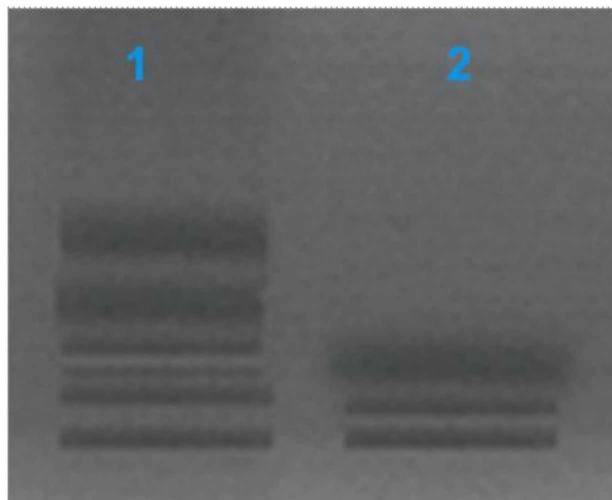

Fig. 2. SSCP analysis of amplified product of Exon 1 of *Kras* gene. Lane 1 shows multiple bands, representing mutation in this sample. Lane 2, wild type (normal). Sample used was from endometrioid carcinoma.

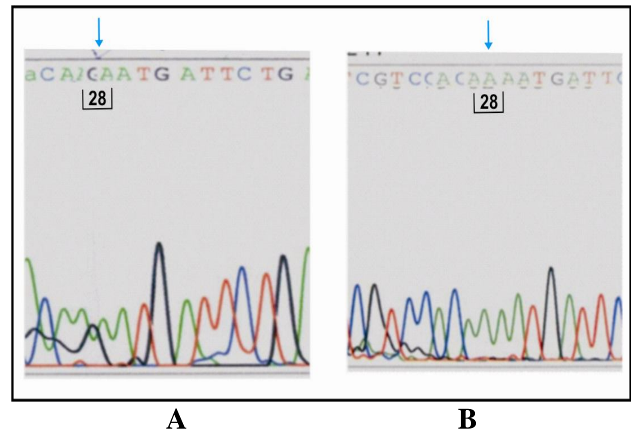

Fig. 3. A, point mutation in codon 28 of exon 1 of *Kras* gene is highlighted. In endometrioid carcinoma G insertion is seen in reverse sequence (arrow) in figure A. Figure B shows normal sequence AAA (arrow) of codon 28 of *Kras*.

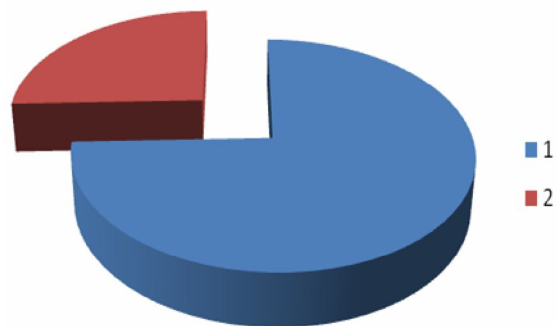

Fig. 4. Percentage of *Kras* gene mutation in endometrial cancer samples. 1, total samples; 2, mutant samples (34%).

Mutation analysis showed that out of 70 endometrial cancer samples, 34% samples showed *Kras* gene mutation in exon 1. Association of age of patient and histological grade of cancer with endometrioid cancer and gene involved was studied. It was seen that 62.5% of patients with *Kras* gene mutation were < 50 years and 37.5% patients were > 50 years.

Association of histological grade in mutant samples was studied. It was seen that 91.7% samples with *Kras* gene mutation belonged to well differentiated histological grade. 42% were moderately differentiated and 4.2% were histologically poorly differentiated.

Table I.- Association of *Kras* gene mutation with age and histological grade of endometrial cancer.

Parameters	*Kras* gene mutantation (n=24)
Age	
Less than 50 years	15 (62.5%)
More than 50 years	9 (37.5%)
Histological grade*	
Well differentiated	22 (91.7%)
Moderately differentiated	1 (4.2%)
Poorly differentiated	1 (4.2%)

*Histological grading of cancer was done according to criteria laid down by International Federation of Gynecology and Obstetrics (Creasman, 2009). According to these criteria endometrial cancers have been classified into 3 grades (G1, G2 and G3). G1 is well differentiated; G2, moderately differentiated and G, 3 poorly differentiated.

DISCUSSION

Kras gene mutation was seen in 34% samples of endometrioid carcinoma. Significant association of age of patient and histological grade of cancer was seen with *Kras* gene mutation. It was seen that *Kras* gene mutation occurs mostly in ages less than 50 years and in well differentiated cancers. In less differentiated cancers mutation in *Kras* gene was less. In a past study the frequency of *Kras* mutation in uterine endometrioid carcinoma was found to be 26%.

Kras mutations were also correlated with histological grades. In endometrioid variety 10% of mutations were present in grade 1 and grade 2 (Lax *et al*., 2000). In estrogen related endometrial carcinomas (endometrioid) the frequency of mutations in *kras* genes range from 10 to 30% (Llobet *et al*., 2009).

In a study it was reported that point mutation in *Kras* is tumorigenic and leads to endometrial cancer. Mutations reported were in codon 12 of *Kras* and these mutations changed the cell phenotype by increasing cell proliferation in normal cell. Type I endometrial cancers show greater frequency of mutation in *Kras* compared to type II. Type I cancer has association with relatively younger age group, endometrial hyperplasia and expression of estrogen and progesterone receptors. In this study it was reported that over expression of estrogen receptors is under the control of *Kras* signaling. Kras mutation upregulates the estrogen receptor transcriptional activity (Tu *et al*., 2006).

In endometriod carcinoma, mutation in *Kras* gene has been reported to be high (Engelsen *et al.,* 2009). Commonly mutations are found in exon 1 at codon 12 and 13 (Downward, 2003; Barbacid, 1990). Koul *et al.* (2002) analysed *Kras* mutations in exon 1 and 2. Point mutations were observed in codon 12 and 13. No mutation was observed in exon 2 (codon 61). Point mutations observed were transitions (G-A) and transversions (G-C:T).

Several workers have reported that *Kras* mutations form the basis of early diagnosis and better management of endometrioid carcinoma. Endometrial hyperplasia is the precursor lesion of endometrioid carcinoma and it has been seen that *Kras* mutations are present in hyperplasia of endometrium though relatively low in frequency (Doll *et al.,* 2008; Koul *et al.,* 2002). These mutations have been reported by different studies to be present in cases of atypical hyperplasia and premalignant stages. This suggests that they have main role in disease progression and early diagnosis (Sasaki *et al.,* 1993; Duggan *et al.,* 1994; Doll *et al.,* 2008; Lax *et al.,* 2000).

Recognition of important precursor lesions plays important role in cancer prevention. Therapeutic interventions can be suggested or made available before the malignancies become well developed (Mutter *et al.,* 2001).

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