

Population Screening of K-ras Gene and Genetic Counselling for Patients Affected with Ampulla of Vater in Tamil Nadu

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ABSTRACT Carcinoma of the ampulla of Vater is a relatively infrequent neoplasm, approximately six percent of periampullary tumours. The aim of the study is to identify the chromosomal alterations and the K-ras mutations in the familial and sporadic carcinomas of the ampulla of Vater. A totally of 21 samples were selected which included 18 familial and 3 sporadic cases which were categorized based on their age group (group I < 50 years; group II > 50 years). Techniques such as the GTG-banding and PCR-RFLP were used to identify the genetic alterations. The result revealed a high frequency of chromosomes 1p- and 12p+ involved in the poorly differentiated (PD) tumor grade and an increased prevalence of the K-ras mutations at the codon 12 associated with > 2cm tumor size in the familial carcinomas of the ampulla of Vater. The researchers concluded that the chromosomes 1p- and 12p+ region may play a vital role for the development of a high grade tumor and the K-ras gene mutation is an early molecular event leading to an abnormal proliferation of the cells.

INTRODUCTION

Carcinoma of the ampulla of vater is a rare malignant tumor arising from the precancerous lesions (Matsubayashi et al. 1999). The prevalence is approximately 5.7 percent per million (Albores-Saavedra et al. 2009). Ampullary carcinoma is about five percent of all the gastrointes-

tinal tumors and it accounts for thirty-six percent of the pancreatic-duodenal surgical resections (Achille et al. 1998). The 5 year survival of the patients with the ampullary carcinoma is significantly better than the cancer of the common bile duct and the pancreatic carcinoma (Matory et al. 1993), because of its early clinical presentation with obstructive jaundice (Matsumoto et al. 2000). Ampullary carcinoma may occur sporadically and in the familial adenomatous polyposis (Grobmyer et al. 2008). The most precancerous lesions leading to the ampullary cancer via the adenocarcinoma sequence is generally accepted as a valid reason for the colorectal cancer (Seifert et al. 1992).

The K-ras mutation occurs at a premature stage of tumorigenesis in the gastrointestinal tract and the bile duct cancers (Kihana et al. 1991; Kobayashi et al. 1996; Matsubayashi et al. 1998). A mutation in the KRAS gene is highly dependent on the cellular circumstances, which is an important factor in the development of the carcinogenesis (Guerra et al. 2003). An activating point mutation in the K-ras codon 12 is the most common oncogene alteration in the human adeno-carcinomas. These genes are closely

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encoded with the related proteins, which are able to acquire the transforming potential when altered at one of the major position at codons 12, 13, or 61 (Bos et al. 1989). The chromosome region 12p is a common site for the allelic losses in the human cancers, and the *KRAS* gene mutation is frequently detected at position 12p12-13 regions (Takeuchi et al. 1996). Some molecular studies have reported the abnormalities of the *APC* and the *KRAS* genes are likely to represent early pathogenetic events, which progress into high grade aggressive cancers and the ampullary carcinoma is frequently associated with the *TP53*. Hence, it has been suggested that, alterations of the genetic mechanism might progress as ampullary carcinoma (Scarpa et al. 1993; Achille et al. 1996; Achille et al. 1998). *K-ras* codon 12 point mutations occur in about forty percent of the ampullary neoplasms at a relatively early stage in the tumorigenesis. The pattern of mutation in these tumors resembles that of the adenocarcinoma sequence in the colon and the rectum (Chung et al. 1996). *K-ras* point mutations are used as a genetic marker, because of the high incidence rate of these mutations in the human carcinoma (Scarpa A et al. 1994). The recent study of Cytology genotyping revealed that the majority of mutations were recognized in the *KRAS* (93%), *TP53* (72%), *SMAD4* (31%), and *GNAS* (10%) in the ampullary carcinoma and the pancreatic adenocarcinoma (Gleeson et al. 2016).

Cytogenetic studies are useful in identifying an abnormal chromosomal region. Ragnarsson et al. (1999) have reported about different types of tumors, such as the colon cancer, neuroblastoma and oligodendrogliomas have a significant association with the loss of chromosome 1p regions (Iolascon et al. 1998; Ragnarsson et al. 1999). The chromosome 1p region plays a possible role in the differentiation of the neoplastic cells, which are associated with a poor prognosis in the ampullary carcinoma (Barbara et al. 2002). Genetic counselling is mainly concerned with the provision of information about the risk for the individuals with heritable condition (Hadjipavlou et al. 2014). An important motive for a cancer genetic counsellor is to exchange the medical informations, obtain personal and family history of the cancer patients, explain about the DNA-testing, and the risk management options of the hereditary cancers (Butow et al. 2004).

Objective

The aim of the study was to identify the specific abnormal chromosomal region and observe whether the occurrence of *K-ras* mutation is a frequent feature in the carcinoma of ampulla of Vater, its correlation with the occurrence of the precursor lesions and examine the related risk in the Tamil Nadu population.

METHODOLOGY

Subject Requirement

In the present study, totally 21 paraffin embedded tissues and peripheral blood samples were obtained. Among the 21 patients, 18 were diagnosed with familial and 3 with sporadic ampulla of vater carcinoma. All the subjects were divided into two groups: < 50 years (group I) >50 years (group II). The mean age of the patients in both the groups was $n=9$ (45.22 ± 3.11) and $n=12$ (55.8 ± 2.50). The study was approved by the Ethics Committee of the participating hospital and the patients were informed and their consent form to be included in the study. All the medical and the pathological reports provided the details regarding the patients' gender, age, tumor size, location, histological grade, TNM stage, extent of the nerves and the vascular metastasis were shown in the relevant characteristic Table 1a and the supplementary Table 1b. Tumors were classified in two modes: 11 (52.4%) cases related to well differentiated, 10 (47.61%) moderately differentiated to poorly differentiated (PD). Additionally, 4 (19.04%) cases matched with TNM stage I; 8 (39.09%) cases to stage II; 5 (23.80%) cases to stage III and 4 (19.04%) cases to stage IV.

Sample Collection

In this study, 21 patients were diagnosed as having carcinoma of the ampulla of vater were recruited from the Government Stanley Medical College Hospital, Chennai, Tamil Nadu, India. Two types of formalin fixed paraffin embedded tissue samples were obtained along with 10ml of peripheral blood from each patient by the venipuncture method in two sterile tubes containing EDTA heparin and stored at 4°C.

Isolation of DNA from FFPE Tissue Samples

Formalin fixed paraffin embedded tissue blocks were treated with Xylene which is an organic solvent, helps to dissolve the paraffin. Xylene step was repeated several times to ensure complete paraffin removal. Tissue blocks were treated with ethanol for a complete removal of xylene. After ethanol rehydration the sample was treated with lysis buffer. The composition of lysis buffer was TrisHCl, EDTA, NaCl, Proteinase K was also added at the end of the reaction and the samples were incubated at 55°C in a water bath or a heating block. After the digestion of the tissue, samples were treated with buffer saturated phenol for separating the aqueous layer in which the DNA may present in the aqueous phase of the sample. This step was repeated until a pure form of DNA was obtained and centrifuged. It was then treated with phenol: chloroform: isoamyl alcohol to repurify the aqueous layer. The volume of aqueous layer was measured and sodium acetate and isopropanol were added, mixed well and kept on ice or at -20°C for overnight incubation, which was followed by a high spin microcentrifugation. The supernatant was discarded and after washing the pellets with seventy percent ethanol to remove the salts, finally the samples were air dried and suspended in the buffer of choice (Pikor et al. 2011).

Cytogenetic Analysis

The blood culture was carried out under aseptic conditions into a culture vial composed of 5.0 ml of culture medium, 1.0ml of AB serum and 0.2 ml of Phytohaemagglutinin (PHA). The cultures were incubated for a period of 72hrs. A volume of 0.05 ml of colchicine solution was used for arresting the dividing cells at the metaphase stage. This content was spun at 1000 rpm for 10 minutes. The supernatant was removed and 6ml of hypotonic solution was added to the tube. The test tubes were incubated for 7 minutes and freshly prepared fixative [Methanol: Glacial Acetic acid (3:1 v/v)] was added and centrifuged at 1000 rpm for 10 minutes. The chromosome slides were treated with 0.25 percent trypsin for 3 to 8 seconds and were stained in 3 percent Giemsa stain solution for 2 minutes. The slides were

analysed under the microscope (Moorhead et al. 1960).

Genotyping

DNA was isolated from the Formalin Fixed Paraffin embedded tissue samples. The *K-ras* genotypes were determined by PCR- RFLP. An initial denaturing step at 95°C (10 min) followed by 30 cycles of 95°C (30 s) denaturation step, 60°C (30 s) annealing step, 72°C (1 min) extension step and a final an elongation step of 72°C (10 min). The PCR products were electrophoresed on 1 percent agarose gels containing EtBr and viewed under the ultraviolet light. Further, the PCR products were digested by *Rsa-I* restriction enzyme and the reaction mix was incubated at 37°C for 8 to 12 h. The digestion products were visualized on four percent Metaphor agarose gel containing EtBr.

Statistical Analysis

The analysis was performed using IBM-SPSS software 20.0 version. Following, a descriptive analysis of the Pearson correlation coefficients were separately calculated between age dependent chromosomal alterations. Odds ratios (OR) and confidence intervals were calculated to estimate the strength of the association of polymorphism by genetic alleles in the patients. Mean and standard deviations were calculated to assess the difference between the patients and the controls and the level of significance was calculated by ANOVA.

RESULTS

A total of 21 paraffin embedded tissue samples were obtained. Out of these 21 samples, 18 were familial and 3 sporadic cases were recruited. The mean age of the patients in both the groups was $n=11(45.36\pm0.87)$ and $n=10(55.8\pm0.78)$. Present study investigated the chromosome and genetic alteration in 21 patients with carcinoma of the ampulla of vater by using karyotyping and PCR – RFLP. Table 2a and 2b display the comparison of the mean $\pm$ SD values of chromatid type aberration (CTA) and the chromosomal type (CSA) frequency of both, group I and group II carcinomas. In group I ≤ 50 years of age, CTAs and CSAs represented the frequency values of 5.22 ± 2.77 and 2.33 ± 1.93 and in

group II > 50 years of age, the frequent values were 7 ± 2.76 and 4.3 ± 1.46 , which shows a statistically high significant level for group II when compared to group I. The mean values of the total CAs in group I demonstrated 8.45 ± 3.10 and group II 11.56 ± 4.3 , which shows statistically significant results of group II when compared to group I respectively. All the subjects showed significant values at $p < 0.05$ level by ANOVA.

In the current analysis, major CA was detected in all the subjects. Losses in chromosome were observed in chromosome 9p, 18q, 6q, and 1p, and gains of chromosome were seen in chromosomes 12p, 17q and 8q. In the research, the frequent gain and loss of chromosomal alterations were noted in all the cases, the loss of chromosome 9p was frequently found in 3 cases out of 21 (14.21%), 18q found in 2 of 21 (10.2%) cases, 6q in 1 of 21 (5.6%) and most frequent loss of chromosome 1p was found in 4 out of 21 (19.04%). 3 cases (27.6%) were observed with a poorly differentiated (PD) tumor grade and 1 case (10%) revealed a well differentiated (WD) tumor grade. The gain of chromosome 12p was found in 8 of 21 (38.10%), 17q was found in 2 of 21 (10.2%) and 8q was found in 1 of the 21 patients (5.6%). A high percentage of deletion were noted as 46XY, del(1p) and gain 46XX, add (12p), but the high frequency of chromosomal aberration was observed mainly (38.10%) in chromosome 12p, as shown in Table 3.

PCR - RFLP was carried out to identify the K-ras mutation in both the familial and the sporadic carcinomas of the ampulla of vater. The activating K-ras mutations were identified in 8 of 18 (86%) of the familial carcinomas of the ampulla of vater and these were not observed in the sporadic carcinomas of the ampulla of vater. The majority of the two mutations occurred at codon 12, which is a replacement of glycine with arginine (GGT → GAT) and glycine to valine (GGT → GTT). The research revealed the nucleotide transition from G to A (Glycine to arginine) in the second nucleotide of codon 12 which was most frequent, 62.5 percent from 5 cases in the familial carcinomas of the ampulla of vater and thirty-eight percent of the 3 cases had a frequent nucleotide transition from G to T (glycine to valine), are display in Table 4. The mutation frequently observed depended upon the tumor size, 43.5 percent mutations were observed in 9 cases of tumors <2cm size and 86.6 percent K-ras

mutations detected in 6 cases of tumors >2cm size, which showed that, the K-ras mutations occurred most frequently in the tumors >2cm.

DISCUSSION

Cytogenetic studies are important in identifying specific chromosome regions involved in the tumorogenesis of the ampulla, as carcinoma of the ampulla of vater is an uncommon neoplasm and accounts for six percent of the peri-ampullary tumors. However, previously few studies have been reported about the chromosomal abnormalities, detected in ampullary carcinoma using karyotyping (Johansson et al. 1992; Bardi et al. 1993; Barbara et al. 2002). For the first time, the current study documented the major CAs, detected in all carcinomas of the ampulla of vater subjects, which is the loss of chromosome observed in 9p, 18q, 6q, and 1p, and gains of chromosome in 12p, 17q and 8q. The mean $\pm$ SD values of the total chromosomal aberrations in groups II statistically displayed significant results when compared to group I. Similarly, Johansson et al. reported on the pancreatic cancer, the nonrandom pattern of the chromosomal changes with trisomy 7 and 20, monosomy 18, gain of 1q, 3q, 8q, 11q, and 19q, and loss of 1p, 3p, 6q, 8p, 9p, 17p, and 19p (Johansson et al. 1992; Bardi et al. 1993). Solinas et al. (1996) reported the gain of chromosomes observed in 17q and 20q, and losses of chromosomes observed in 9p and 13q in the primary pancreatic cancer. Additionally, this study finds the most frequent loss of chromosome 1p with the tumor grade, which is 27.6 percent observed in a poorly developed (PD) tumor grade and 10 percent revealed in a well - developed tumor grade. The 1p region is represented in the poorly differentiated (PD) tumors in the ampullary carcinoma (Barbara et al. 2002) and other several studies identified the 1p36 region as a hot spot of LOH in different types of tumors, because the region is associated with different tumor suppressor genes (Hilgers et al. 1999; Bello et al. 2000; Mizzotto et al. 2000). Furthermore, the different tumors as blastoma, colon carcinoma and oligodendrogliomas were significantly associated with the loss of 1p and poor prognosis (Ogunbiyi et al. 1997; Iolasco et al. 1998; Ragnarsson et al. 1999; Smith et al. 2000). Similarly, the 1p36 region may play a relevant role in the progression towards a high grade of anaplasia in the

amullary adenocarcinoma (Barbara et al 2002). Therefore, the researchers suggest that, the loss of chromosome 1p is frequently associated with poor prognosis, which might be an evolution of a high tumor grade in the ampullary carcinoma.

Meanwhile, the researchers found that there is a gain of chromosome 12p, 17q and 8q and the high frequency of chromosomal aberration (38.10%) in 12p. A single case of pancreatic cancer was reported with an unpredicted high frequency gain of chromosome 12p, because of the over representation of this chromosome arm (Solinas et al. 1999). As per the knowledge of the researchers it is suggested, the gain of chromosome 12p is essential to the frequent *K-ras* mutation which occurs in ampullary carcinomas. Heterozygotic analyses have shown frequent allelic loss at 1p, 3p, 6p, 6q, 8p, 9p, 10q, 12q, 13q, 17p, 18p, 18q, 11q, 12p, 21q, and 22q were identified by a cytogenetic study, which is also associated with the tumor suppressor genes (TSG) (Seymour et al. 1994; Hahn et al. 1995; Kimura et al. 1996; Shridhar et al. 1996).

K-ras gene mutation occurs somewhere from 0 to seventy-five percent in the ampullary carcinoma (Smit et al. 1988; Stork et al. 1991; Scarp et al. 1993), further description of the *K-ras* gene mutation in the ampullary carcinoma is extremely similar to the colon cancer *K-ras* gene mutation (Finklestein et al. 1993; Vogelstein et al. 1988). *K-ras* gene mutation is observed more in the pancreatic cancer (Almoguera et al. 1988; Smit et al. 1988).

The present study found eighty-six percent of the *K-ras* gene mutation in the familial carcinomas of the ampulla of vater but it's not observed in the sporadic cases when compared to the report by Alexis et al. which stated that familial and sporadic pancreatic cancers are sharing the same molecular pathogens of the *K-ras* gene (Alexis et al. 2015). Vogelstein stated that eighty-six percent prevalence of the *K-ras* mutation at codon 12 occurred in the familial carcinomas of the ampulla of vater. Different researchers have described that sixty-eight percent of the *K-ras* mutations at either codon 12 or 13 in the adenomas, which was generally in conformity with most studies from the USA (Vogelstein et al. 1988; Ando et al. 1991; Bell et al. 1991). In the same way, in a case of 388 patients, 175 (45%) had the *K-ras* mutation, the 134 (76.5%) cases of the *K-ras* mutation at codon 12 in ampullary adenocarcinoma (Kim et al. 2016). Farr et al. (1988)

had projected in his study that, the *K-ras* mutation at codon 12 is a replacement of nucleotide from glycine to valine and glycine to aspartic acid. Likewise, in FAP adenomas the *K-ras* mutation at codon 12 is represented by a replacement of glycine to valine and glycine to arginine. High frequency of 68 percent *K-ras* mutations occur in tumors greater than 2 cm in diameter than those less than 2 cm in the non-FAP adenomas Vogelstein et al. (1988). Gallinger et al. (1995) reported the prevalence of 37 percent *K-ras* mutation in >1 cm tumor size in the periampullary adenomas from FAP, but no mutation has been found in the adenomas < 1cm in size. Howe et al. (1997) reported 27.5 percent *K-ras* mutation have been frequently occurred in <2 cm tumor size and 48.8 percent in >2cm size in the ampulla of vater. In the research, results showed 43.5 percent *K-ras* gene mutation were frequently observed in tumors <2cm size and 86.6 percent *K-ras* mutations detected in tumors >2cm size in the familial carcinomas of the ampulla of vater. Thus, these findings suggest, that the *K-ras* mutations occurred earlier in the cancer process, which was combined with the lack of correlation between the *ras* mutations and the adenoma size. Accordingly, the genetic analysis is essential and an important tool for identifying the early onset of cancer.

CONCLUSION

This is the first research regarding the genetic alterations and genetic counselling in the familial and sporadic carcinomas of the ampulla of vater. The result justifies, CAs as an intermediate end point in carcinogenesis and thus activates the point mutation in codon 12 of the *K-ras* gene in familial carcinomas of the ampulla of vater. Consequently, from these findings, the researchers are predicting that the *K-ras* gene can be used as a molecular marker for an early diagnosis event of the ampulla of Vater carcinoma. Additionally, the research suggest that the genetic counselling and research in ampulla of vater carcinoma is essential to exchange medical information regarding personal and family cancer risk, DNA-testing, and risk management. The combination of a molecular prognostic and genetic counselling is necessary to identify the early event of cancer risk and guide the management of the risk in the ampulla of Vater carcinoma patients.

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