RESEARCH COMMUNICATION

Lack of Germ Line Changes in KISS1 and KAI1 Genes in Sporadic Head and Neck Cancer Patients of Pakistani Origin

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Abstract

Background: Head and neck cancer is included among the top five most commonly prevailing cancers worldwide. Abnormalities of either genetic or epigenetic factors are found responsible for the development and progression of head and neck cancer. Metastasis is the leading cause of death in patients with head and neck cancer. Down regulation of metastasis suppressor genes (MSGs) expression have been frequently observed in advanced tumours. Methodology: The present study was designed to screen two of the most frequently down-regulated MSGs (KISS1and KAI1) for mutations in 120 diagnosed head and neck cancer affected Pakistani patients. The questionnaire was filled for basic information about age, gender, smoking habits and area of cancer affected and other relevant details. Primers for both genes were designed using "Primer 3" software in such a way that both intron exon boundaries were included in this region. DNA isolation and estimation was done by using organic method and agarose gel electrophoresis. Single Strand conformational polymorphism technique was used after amplification of the respective genes. Mobility patterns were analyzed using BioDoc Analyzer. Results: Data of patients were analyzed on the basis of age, sex and type of cancer as variables. The mean age of patients and controls was 44 years. There were 53% females and 47% males in this group of study, 63% nonsmokers and 37% smokers and larynx cancer was found to be most frequent type of cancer with a percentage of 64. Lack of germ line mutation was observed in the entire coding region in both coding regions as well as splice sites of the respective genes. Conclusion: Germ line mutations in KISS1 and KAI1 are thus considered to be a less frequent event in head and neck cancer patients. However, two polymorphisms in intronic region of exon 3 and exon 9 of KAI1 gene were observed in 1% of patients. In non coding region downstream of exon 3 (KAI1), there was a C 29166 T substitution and in intronic region upstream exon 9 of KAI1 gene, a C 52840 A substitution was observed. Both patients were females with ages 47 and 50 years respectively. A detailed analysis of regulatory mechanism is required to explore the genetic basis of down regulation of these MSGs for a better understanding of head and neck cancer progression.

Key words: PTEN - breast cancer - germline mutations - PCR - SSCPS

Asian Pacific J Cancer Prev, **12**, 2767-2772

Introduction

Head and neck cancer include tumours of the upper aerodigestive tract, parapharyngeal space, paranasal sinuses, thyroid gland, parathyroid gland as well as major and minor salivary glands. Besides these, tumours of the bone and skin present in the head and neck region are also included in head and neck cancer (Stadler et al., 2006). Head and neck cancer (HNNC) is the fifth most common cancer affecting across the globe (Marcu and Yeoh, 2009). Its different types of head and neck cancer like oral cavity, laryngeal and pharyngeal cancers generally share a similar morphology, epidemiology, risk factors and control measures in Pakistani population (Bhurgi et al., 2006). Genetic alterations associated with head and neck cancer are numerous and include a variety of different pathways. Altered expressions in signaling pathways may sometimes be random, but more commonly, they are due to a lifetime of environmental exposure, such as tobacco and alcohol addictions (Ha et al., 2009). Metastasis suppressor genes also play an important role in a way that if these genes are altered, the process of metastasis speeds up and leads to more devastating form of cancer.

Metastasis is a multistep process involving complex interactions between cancer and host cells. To metastasize,

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tumour cells must evade from primary site, dissociate from the primary tumour, extravastion in circulation and movement in circulation, be transported to nearby or distant secondary site (Jeong-Hyung et al., 1996). The whole cascade of events is controlled by altered expression of several genes. There are certain genes which suppress metastasis at certain steps of metastatic cascade without affecting the primary tumour growth termed as metastasis suppressor genes (Steeg et al., 1998). The two most promising genes in this family of MSGs are KISS1 and KAI1 (Jeong-Hyung et al., 1996; Ichikawa et al., 1991).

KISS1 is a metastasis suppressor gene, identified and cloned by Lee (1996). KISS1 was originally identified as a metastasis suppressor by microcell-mediated transfer in melanoma lines, responsible for tumour cell invasive and migratory properties without affecting their tumourigenicity (Lee and Welch, 1997). It has been found in many human malignant tumours including breast (Stark et al., 2005), esophageal (Ikeguchi et al., 2004) and gastric cancers (Yu et al., 2006). KISS1 has been shown to act as a potent anti-metastatic agent either by treatment using synthesized KISS1 peptide or exogenous expression in metastasized cells (Lee and Welch, 1997; Ohtaki et al., 2001; Masui et al., 2004). Although, KISS1 an important metastasis suppressor gene (Muir et al., 2001) however, germline variants of the coding region of KISS1 has not been explored in relation to head and neck cancer in Pakistani population. KAI1 gene (kangai1), located on chromosome 11p11.2, was identified based on its functions as a metastasis suppressor gene in prostate cancer. It was first described in 1991 as a 267 amino acid, tetraspanin protein, thought to be involved in inhibiting cancer cell migration and invasion (Ichikawa et al., 1991). The role of KAI1 in the metastatic process of prostatic carcinoma has already been generally accepted (Dong et al., 1996). Involvement of KAI1 has not only been limitsized to prostate cancer progression but is also observed in several other types of cancers. KAI1 role in the progression of human pancreatic cancer, non-small cell lung cancer, bladder, breast and esophageal cancer has also been explored (Guo et al., 1996; Yu et al., 1997; Yang et al., 1997; Higashiyama et al., 1998; Miyazaki et al., 2000).

So far KISS1 and KAI1 have not been screened for mutations in head and neck cancer in Pakistani population. The current study was designed to screen the KISS1 and KAI1 genes for germline mutations in head and neck cancer. Aim of this study was to explore KISS1 and KAI1 genes for any deletion, insertion or frame shift mutations in head and neck cancer patients.

Materials and Methods

In this study, 120 patients of head and neck cancer along with normal individuals as controls were included. Blood samples were collected with approval of Ethical Committees of the participating institutes and hospitals named as Allied Hospital Faisalabad, Combined Military Hospital Rawalpindi (CMH) and Nuclear Oncology and Radiotherapy Institute (NORI) Islamabad. Normal individuals with similar age group to disease patients and no prior history of any other disease in general or any type of cancer in particular were included in this study. Samples were collected after an informed consent and diagnosis of histopathologist.

Sample collection and storage

Blood samples from each patient were collected in EDTA vacutainers. Recommended guidelines for storage, transportation and preservation of these samples were followed (Anderson et al., 1998).

DNA isolation and dilution

For DNA extraction phenol chloroform method was adopted, as described by Comey et al. (1994). Isolated DNA was confirmed by agarose gel electrophoresis and quantified using a spectrophotometer for the polymerase chain reaction. Stock DNA samples were stored at -20°C and working DNA samples were kept at 4°C for further usage.

Amplification and mutation screening

Primer 3 software was used to design primers. Amplification size along with primer sequences for respective exons is shown in Table 1. Intron exon

Table 1. Primer Sequences for KISS1 and KAI1 Gene

Exon	Product	Product size (bp)
KISS1		
F1	TCTTCAGGAGGGTCTGAGGAG	371
R1	GGCTGGTAAACAGGAAAGATCA	
F2	CTCTACCAGGAGCCTCCAAAG	395
	TAGATTTCCACCAAATGCAATG	
F3	AGGTCAAGGAAGGAAAAGAAGG	675
	GCACTGACCTTAATGACACCAG	
KAI1		
F1	GTTGGGGTACGGCCATAGTG	367
	CCTGTCACTAGTTCCGAGGAAAG	
F2	GGGAGCCTGGATTTAAAGTGA	331
	AATGCTGTAGGAGCCAGAGAAG	
F3	ACAGGGTTAGTACCCACCTCCT	294
	CTCAGTCCCTACCCACCAAAT	
F4	GACTTGGGTTCCAGGGACAG	261
	AAAAGCAACAGGCATTGAAGC	
F5	CAATCCTGAGAAGCCTTACGAA	492
	ATGCTCTCCACCCGATGTTAC	
F6	CTGCCCATTTCCTCTCATCC	381
	CCCCATTTGATCACAAGGAATA	
F7	GGGGGAGGCAGTTTAAGTAGG	278
	CAGATGCCAGTCCCAGACAT	
F8	GTGGCATATCTCAGTCTCTGTCC	478
	GCTCTGCCTGTTCCTTGAATAA	
F9	CAGCAATTCCTTCCTGCATTTA	373
	CACCTCCCCAAGGAGATCAG	
F10	TCAAGTTGAGGATCCACTTAATC	397
	GAGCAGAGGAACCACAGAAC	

junctions were also included in the amplification so that any splice site variability may also be identified. BLAST using NCBI server was also carried out to confirm the primer binding sites specificity. After optimization, amplification conditions were 95°C for 4 min; 95°C for 30 sec, annealing temperature for 30 sec, 72°C for 1 min for 30 cycles with a final extension of 72°C for 45 min. Amplified products were resolved on ethidium bromide stained 2% agarose gels. For mutation detection, SSCP technique was used and samples were screened for any mobility shift in their banding pattern (Lallas and Buller, 1998). DNA sequencing of the suspected samples was carried out by Macrogen Korea. Bio Edit software was used to compare normal and suspected samples.

Statistical Analysis

G-power Analysis: Significant number of controls for 120 patients' samples was determined by using G-POWER 2 software. The power at α 0.05 was 0.90 which is >0.84. Therefore, the no. of controls i.e. 50 was significant to demonstrate association of normal and diseased individuals.

SPSS Analysis: SPSS software was used to determine significant or non-significant association between different variables e.g. age, gender, cancer site and smoking habit of different individuals.

Results

Age of head and neck cancer patients ranged from 7 to 80 years with a mean age of 44 years. Frequency of head and neck cancer was significantly higher in age group of 40-60 years. There was no significant difference between percentage of males and females ($p>0.05$). It was observed that non smokers were 63.34% of total number of patients as compared to smokers which were 37%. Most common cancer site in head and neck cancer patients was found to be larynx with a percentage of 64 and second most common site was oral cavity which accounts for 24% of total number of patients. Pharyngeal cancer was 12% and thus least common cancer site. It was observed that larynx cancer is more common in females and oral cavity cancer is more common in males.

There is a significant difference in smoking habits of males and females ($p<0.05$). Smoking was more common in males than in females. Pharynx cancer was more common in smokers than in non smokers while larynx and oral cavity cancer were more common in nonsmokers.

All the exons of KAI1 and KISS1 were selected to be screened for mutational analysis. Fifteen variants were found using SSCP. Polymorphism was confirmed by using sequencing analyzer (3730L DNA analyzer (AB, USA) by Macrogen, Korea. Any mutation in exonic sequences was not found but two polymorphisms in intronic region of exon 3 and exon 9 of KAI1 gene were observed. In non coding region downstream exon

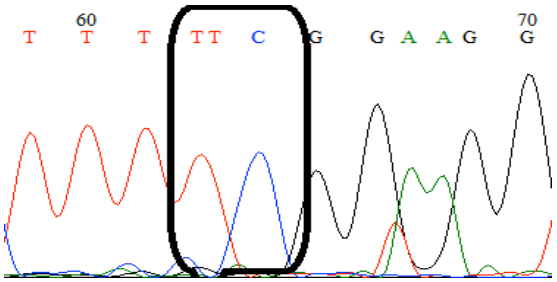

Figure 1. Sequencing Results Obtained with Sample C87, Exon 3 of KAI1 Gene. At position 63, there is a C 29166 T substitution downstream intronic region.

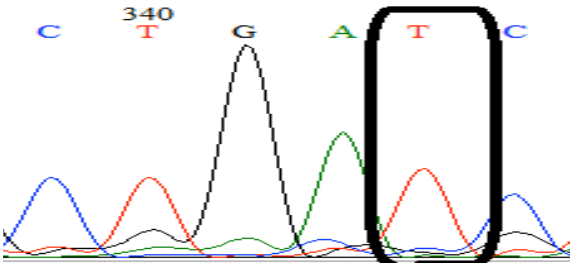

Figure 2. Sequencing Results Obtained with Sample C413, Exon 9 of KAI1 Gene. There is a C 52840 T substitution upstream the intronic sequence.

3 in KAI1 gene at position 29166, there was a C to T substitution. In intronic region upstream exon 9 of KAI1 gene at position 52840, a C to A substitution was observed as shown in Figures 1 and 2.

The finding that there was almost no germ line mutation in exonic regions of these genes in sporadic head and neck cancer patients highlights the following features:

- Germ line mutations of both KAI1 and KISS1 are less frequent in sporadic head and neck cancer patients.
- Coding regions of these genes are conserved among the patients with head and neck cancer and the controls.
- Almost complete absence of any germ line mutations for these metastasis suppressor genes may also indicate that involvement of some other regulatory mechanisms can also be responsible for down regulation of the genes in disease progression.

Thus, metastasis suppression mechanism can be attributed more towards these regulatory factors rather than the impaired coding genome. Contribution of germ line mutation of these MSGs is less likely responsible for their down-regulation in cancer.

Discussion

Data of patients were analyzed on basis of cancer site, gender, age and smoking patterns. Larynx was found to be the most common cancer site and oral cavity was second most common cancer site. Larynx cancer rate is significantly higher ($P<0.05$) as compared to other cancers (pharynx, oral cavity and nasal cavity cancers). Similar results were found in a study on Pakistani population by Akhtar et al. (2007) that laryngeal cancer

is most common malignant tumour. Larynx cancer was found to be most common in Finland (Makitie et al., 1999). Frequency of head and neck cancer is significantly higher in age group of 41-60 years. This is in accordance with other studies carried out in Scotland, Nigerian and Pakistani populations (Bhurgri et al., 2006; Macfarlane et al., 1999; Bukola et al., 2008). Generally, frequency of head and neck cancer is much more commonly diagnosed in males than females with a quite late disease onset in developed countries among those aged 60 years and older.

In Pakistani population number of male and female patients was almost same. There was no significant difference in occurrence of head and neck cancer on gender basis ($P>0.05$). In US, head and neck cancer was more common in males than in females. In the 1950s, the male to female ratio in patients with HNC was 15:1 in US. Increase in the number of cases per year has been reasonably steady during the past decades in both sexes (Ferlito et al., 2001). This number had changed to 5:1 by the year 2000, and the proportion of women afflicted by the disease is projected to increase in years to come (Titcomb et al., 2001). Now in our population, this ratio has become almost same as shown in this study. These changes are likely a reflection of shifts in smoking patterns, with women smoking more in recent years. Association of different factors including rapid and rational industrialization, industrial wastes, inadequate nutrition and exposure to pesticides were also observed. Larynx cancer was more common in females while pharynx in males. Pharynx cancer was more common in smokers than in non smokers observed.

The salient objective of this study was to assess the mutational spectrum of KISS1 and KAI1 genes in both head and neck cancer and normal patients. So far germline variants of the coding region of both these genes have not been explored in relation to head and neck cancer. In breast cancer, mutational analysis of both these genes was carried out but no mutation was found after screening of all exons of both genes (KISS1 and KAI1) (Malik et al., 2008).

Expression of KAI1 in a highly malignant prostate cancer cell line resulted in a significant suppression of lung metastases (Yang et al., 1997). The role of KAI1 in the metastatic process of prostatic carcinoma, human pancreatic cancer, non-small cell lung cancer, bladder cancer, breast cancer, and esophageal cancer is established (Dong et al., 1996; Guo et al., 1996; Yu et al., 1997; Higashiyama et al., 1998; Miyazaki et al., 2000). A similar trend of KAI1 protein down regulation in both normal and cancer cells cell lines of a variety of tissues had also been observed (White et al., 1998). The role of KAI1 expression in gastric cancer seems to be controversial. One study showed that KAI1 was unchanged in metastatic and non-metastatic gastric cancers whereas a more recent study demonstrated that KAI1 expression was decreased in high-grade gastric cancer (Hinoda et al., 1998). Low expression of KISS1 and KAI1 was associated with gallbladder associated cancer. KISS1 and KAI1 were also suggested as target genes to inhibit metastasis of gallbladder carcinoma (Zhiqiang et al., 2009). Screening for KAI1 gene mutations in breast and prostate cancer was carried out. In breast cancer, no mutation was found after screening of all exons of KAI1 gene (Malik et al., 2008).

In the present study, screening of KISS1 and KAI1 gene mutations in 120 head and neck cancer patients was carried out. Coding regions as well as intronic exonic junctions were included in this screening to identify the presence of any splice site variations as well. All the exons of KAI1 and KISS1 were selected to be screened for mutational analysis. Any mutation in exonic sequences was not found but two polymorphisms in intronic region of exon 3 and exon 9 of KAI1 gene were observed. In non coding region downstream exon 3 in KAI1 gene at position 29166, there was a C to T substitution. In intronic region upstream exon 9 of KAI1 gene at position 52840, a C to A substitution was observed. Similar result has also been found in earlier studies as well (Uzawa et al., 2002). Coding regions of KISS1 and KAI genes were screened for germline mutations in breast cancer patients of Pakistani population (Malik et al., 2008). An extensive screening for the whole coding region for both KISS1 and KAI1 was done. No mutation was found in coding regions of KISS1 and KAI1 genes in the breast cancer samples. The study thus strongly suggested that frequent down regulation of these proteins in breast cancer progression was unlikely due to any germline mutations.Although the coding sequence of a gene is entirely in the exons, however, if a mutation occurs at an exon-intron junction, then aberrant splicing may occur and the protein would be affected. There are also examples of intron mutations influencing variable splicing, thus affecting the final protein. Nucleotide signals at the spliced sites guide the enzymatic machinery. If a mutation alters one of these signals, then the intron is not removed and remains as a part of the final RNA molecule. The translation of its sequence alters the sequence of the protein product. So mutations found in intronic region are also important.

In conclusion, there is a need to further explore and analyze these genes at expression level. It is possible that polymorphisms in exonic region are present in low frequency. So studies should be carried out on larger set of data to find conclusive results regarding germline as well as somatic alterations responsible for disease progression.

Acknowledgements

All authors would like to acknowledge COMSATS Institute of Information and Technology, Islamabad and Higher Education Commission of Pakistan for assisting this research. We would also express deep regards to all the individuals and institutes (NORI, CMH & Allied Hospital) who participated in this study.

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