Biomarkers, 2012; 17(6): 552–556
© 2012 Informa UK, Ltd.
ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.699556

informa
healthcare

RESEARCH ARTICLE

A novel p16^{INK4A} mutation associated with esophageal squamous cell carcinoma in a high risk population

Meenu Asaas Qureshi[1], Nishawar Jan[1], Nazir Ahmed Dar[2], Mahboobul Hussain[1], and Khurshid Iqbal Andrabi[1]

[1]*Department of Biotechnology, University of Kashmir, Hazratbal, Srinagar, India and*
[2]*Department of Biochemistry, University of Kashmir, Hazratbal, Srinagar, India*

Abstract
This study describes identification of p16^{INK4A} sequence variants and their potential association with esophageal squamous cell carcinoma (ESCC) in a high risk population from Kashmir, India. We report a novel 7 base pair exon 2 deletion in 22 out of 106 (~20%) surgically resected tumor samples. The deletion beginning at the second base of codon 103, results in a frame shift causing premature termination of the protein at codon 142, with structural and functional consequences predicted by insilico analysis. The described mutation is a previously unreported variant of p16^{INK4A}, perhaps representing a founder mutation unique to the population.

Keywords: Sequence variations, heteroduplex analysis, deletion, Kashmiri population

Biomarkers Downloaded from informahealthcare.com by Changhua Christian Hospital on 11/14/12
For personal use only.

Copyright © 2012 Informa UK Limited
Not to be sold or commercially distributed
Unauthorized distribution prohibited. Authorised users can print a single copy for personal use

Introduction

P16 is an important component of cell cycle regulatory system. It accomplishes its regulatory role by binding to and inhibiting the function of cyclin dependant kinases (CDKs), to bring about cell-cycle arrest at G1 (Serrano et al. 1993; Kamb et al. 1994a). Loss of P16 function, rendering the protein unable to bind or inhibit CDKs, can result in uncontrolled cell proliferation leading to tumorogenesis. Homozygous deletions of p16^{INK4A} gene and other loss of function mutations have been reported in a variety of tumors including esophageal squamous cell carcinoma (ESCC; Holland et al. 1994; Cairns et al. 1994; Caldas et al. 1994; Kamb et al. 1994b; Mori et al. 1994; Nobori et al. 1994; Hussussian et al. 1994; Lukas et al. 1995; Koh et al. 1995; Ranade et al. 1995). ESCC however reveals a variable spectrum of p16^{INK4A} mutational pattern in different populations, attributed to distinct geographical and environmental factors, a population is exposed to (Muzeau et al. 1997). This is validated by the observation that the differences between incidences of esophageal cancer in distinct geographical areas are more extreme than observed for any other cancer (Landis et al. 1999; Pickens & Orringer 2003). Regions of high incidence in Asia, characterized as the "Esophageal Cancer Belt," also exhibit striking variations within, presumably due to local dietary and environmental factors (Rosenberg et al. 1985; Khuroo et al. 1992).

Kashmir valley, in North India, has been reported as a high-incidence area for ESCC (Mattoo et al. 1974; Khuroo et al. 1992). ESCC accounts for 42.9% of all types of cancers in this region (Dhar et al. 1993). Kashmiri population distinguishes itself in pure ethinicity, unique geographical locale, intra community marriages, traditional dietary habits and unique cooking recipes. The high incidence has been associated with potential exposure to nitroso compounds, amines and nitrates present in the local food stuffs, such as dried and smoked fish, red meat, dried pickled vegetables and traditional hot salted green tea (Nun Chai) (Siddiqi et al. 1988, 1991, 1992; Siddiqi & Preussmann, 1989; Kumar et al. 1990). The present study was therefore undertaken to investigate the potential basis for high risk in these patients by examining sequence variations in tumor suppressor p16^{INK4A}. A novel 7 base pair (bp)

Address for Correspondence: Khurshid Iqbal Andrabi, Department of Biotechnology, Ground Floor, New Science Block, University of Kashmir, Hazratbal, Srinagar, 190006, Jammu & Kashmir, India. Tel: +91–194-210–2154. Fax: +91–194-242–8723. E-mail: andrabik@uok.edu.in
(Received 25 Apr 2012; revised 24 May 2012; accepted 30 May 2012)

RIGHTSLINK

deletion in 22 out of 106 (~20%) samples was observed in $p16^{INK4A}$ exon 2.

Methods

Patients presenting themselves for treatment of ESCC for the first time at the Departments of Cardiovascular and Thoracic Surgery and Gasteroenterology of the Sher-I-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir were recruited for the study, with prior informed consent. Patients underwent endoscopic, radiologic and histopathologic examinations to establish the clinical profile (Mir et al. 2005). Blood and surgically resected samples from 106 patients diagnosed with ESCC were collected along with matched normal esophageal tissue from a distant site. Samples were snap-frozen at –70°C until analyzed. A pretested, semistructured questionnaire was used to collect the information on clinicopathologic parameters including age, gender, site, histopathologic grade, socioeconomic factors, personal habits, and dietary history of ESCC patients.

Genomic DNA isolation from tissue samples was carried out by standard procedure (Blin & Stafford 1976). DNA from cancerous tissue, normal counterpart and blood was used to amplify all three exons using following primers: Exon 1 forward primer 5′ CTGCGGAGAGGGGGAGAGC 3′ and reverse primer 5′GCGCTACCTGATTCCAATT 3′; Exon 2 (N-terminal) forward 5′GATAGAGAACTCAAGAAGG 3′, and reverse primer 5′ TCGGGTGAGAGTGGCGG 3′; Exon 2 (C-terminal) forward 5′ ACTCTCACCCGACCCGT 3′ and reverse primer 5′ GGAAGGTCCCTCAGGTG 3′; Exon 3 forward primer 5′ ATGTGCCACACATCTTTGACC 3′ and reverse primer 5′ CAGTTGTGGCCCTGTAGGA 3′. PCR amplifications (35 cycles) were performed in a reaction volume of 50 µL containing 50–100 ng of genomic DNA, 200 µM of each dNTP (Fermentas, Germany), 0.4 pm of each primer (Fermentas, Germany), one unit of Taq Polymerase (Sigma, Ronkonkoma, USA) in 1X Taq Buffer containing 1.5 mM $MgCl_2$ (Fermentas, Germany) For amplification, PCR program used was: denaturation at 95°C for 1 min, annealing at 58°C (Exon 1)/59°C (Exon 2 N terminal)/56°C (Exon 2 C terminal)/60°C (Exon 3) for 1 min and extension at 72°C for 1 min. Amplification products were analyzed on 1.5% agarose gels and purified wherever required using glass milk as matrix (Vogelstein & Gillespie 1979).

Amplicons were subjected to heteroduplex assay for which heteroduplex formation was accomplished by denaturing the PCR product at 95°C followed by random annealing at 68°C. The heteroduplexes were analyzed on a 10% acrylamide gel using standard conformation sensitive gel electrophoresis (CSGE) protocol (Ganguly et al. 1993). The gel was silver stained according to procedure by Bassam and Gresshoff (2007). Purification of heteroduplex bands from acrylamide gel was carried out using "Crush and Soak" method (Sambrook et al. 1989).

Allele separation was accomplished on a 3% agarose gel, bands were excised and purified (Vogelstein & Gillespie 1979) for cloning using TA cloning kit (InsTAclone™, Fermentas, Germany). Plasmids recovered after cloning were sequenced using sequence specific or end primers. All sequencing data were obtained through commercial sequencing services (Macrogen Inc., Seoul, Korea).

The mutant version of P16 protein was modeled using I-TASSER Server (Zhang 2007, 2008) and the quality assessment of the structure was performed using Ramachandran Plot generated by iMolTalk web resource (Diemand & Scheib 2004). DOT 1.0 β (ClusPro Server) (Comeau et al. 2004) was used to generate the docked conformations of two proteins viz, mutant P16 protein and cyclin dependent kinase 6. The total energy of the crystallized wild type P16-CDK6 complex (PDB entry 1bi7) and mutant P16-CDK6 complex was computed using "Compute Energy" option in SWISS PDB Viewer.

Results

Individual exons amplified as per primer details given above yielded 279 bp and 150 bp amplicons for exon 1 and 3 respectively. Heteroduplex analysis was not suggestive of any heterozygous sequence variation. Half of the samples, representing both cancerous and normal were however subjected to direct sequence analysis to establish the reliability of heteroduplex screen and also to inspect for any homozygous sequence changes that might have remained undetectable during heteroduplex assays. No known or novel sequence changes were observed over the entire coding length of the two exons, i.e. exon 1 and exon 3. Exon 2 was PCR amplified as a 254 bp N-terminal fragment (data not shown) and a 232 bp C-terminal fragment (Figure 1a). Preferential amplification of the exon as smaller fragments was undertaken on purpose to facilitate effective and reliable heteroduplex analysis by Conformation Sensitive Gel Electrophoresis (CSGE; Körkkö et al. 1998). While the N-terminal portion of the exon did not reveal any abnormal band patterns (data not shown), prominent heteroduplex bands (Figure 1b) were observed in 22 out of 106 cancer tissue samples when the C-terminal 232 bp PCR fragment was analyzed. Excised and purified heteroduplex bands from the acrylamide gel yielded a 232 bp fragment upon reamplification to verify their authenticity of having originated from 232 bp PCR product. All the samples that showed heteroduplex bands were sequenced alongside the ones derived from their respective normal tissues. The sequencing chromatograms revealed overlapping peaks beginning at nucleotide 308 and at nucleotide 314 (reverse), to suggest a possible 7 bp deletion, observed in all the samples that showed heteroduplex formation whereas normal sequencing pattern was observed for all corresponding normal samples (Figure 1). The distribution of the mutation however did not seem to correlate with tumor stage or other tumor characteristics. The extent of deletion raised the prospect of physical separation of the two alleles on a 3% agarose gel (Figure 2a). Although we were able to separate the deletion allele, attempts to sequence it without contamination of the other allele were successful only after they were cloned. Sequencing of the

Biomarkers Downloaded from informahealthcare.com by Changhua Christian Hospital on 11/14/12
For personal use only.

© 2012 Informa UK, Ltd.

RIGHTSLINK

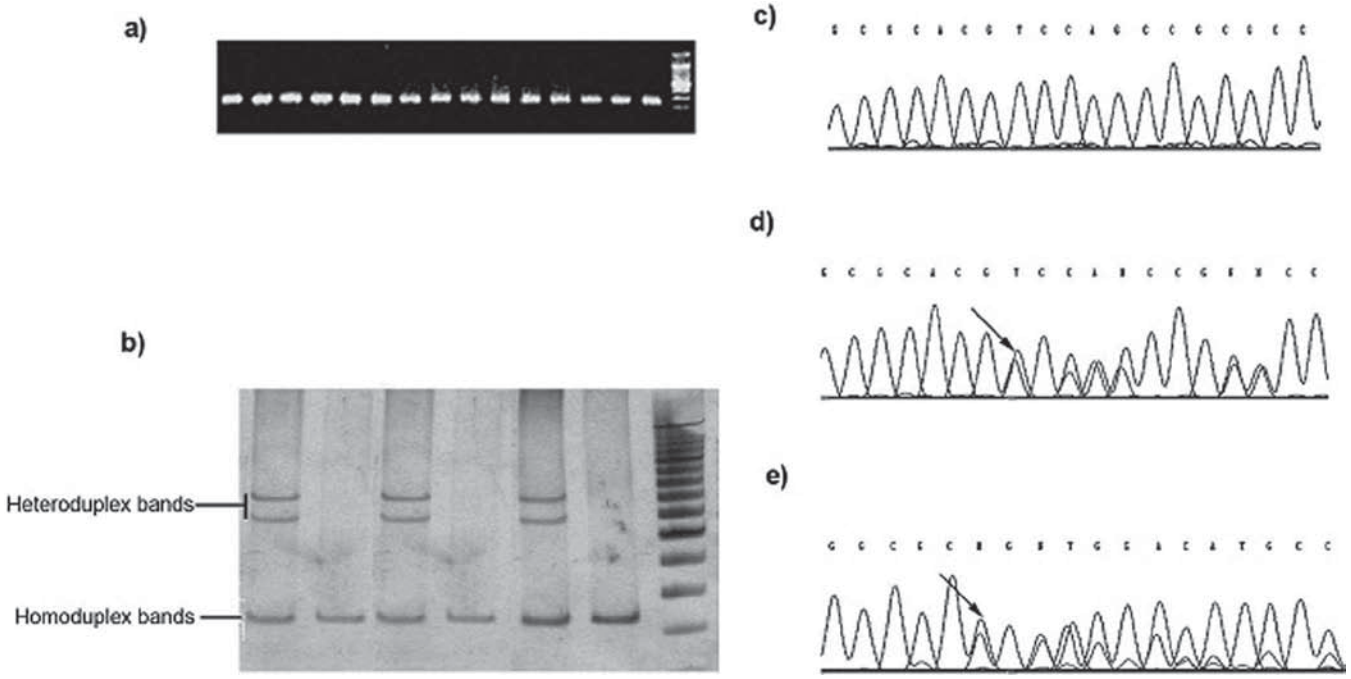

Figure 1. p16^{INK4A} mutational analysis in human ESCC. (a) Amplification pattern of 232 bp C-terminal Exon 2 fragment from different ESCC samples with 100 bp ladder on the side lane. (b) Heteroduplex analysis by conformation sensitive gel electrophoresis. (c) Sequencing chromatogram of normal sample using reverse primer, and that of diseased sample from the same patient, using (d) reverse primer (e) forward primer. Solid arrows indicate beginning of potential deletion.

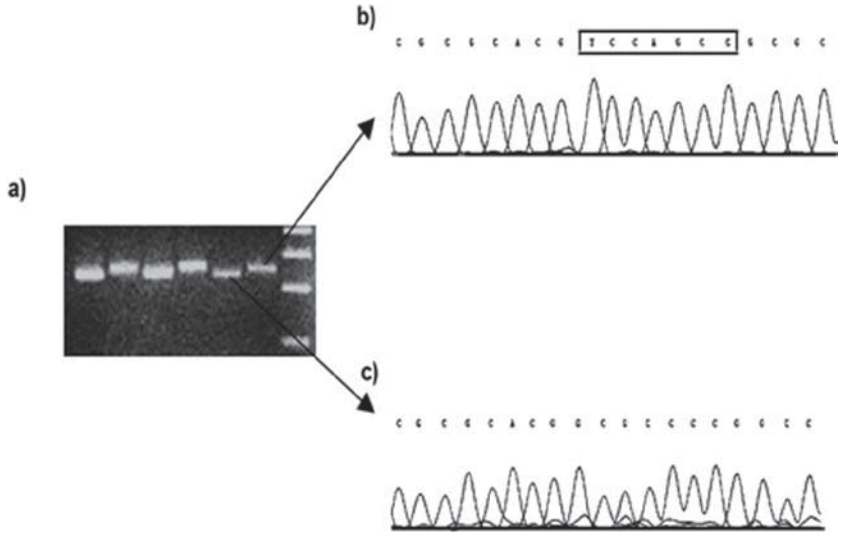

Figure 2. Separation of Alleles and their Sequencing. (a) Alleles separated on 3% agarose gel. Sequencing chromatograms of purified alleles; (b) wild type and (c) mutant obtained after cloning. Note: Sequencing chromatogram showing 7 bp sequence TCCAGCC (boxed), deleted in the mutant allele.

cloned alleles confirmed a 7 bp deletion (Figure 2b and 2c) beginning at the second nucleotide of codon 103 that brings about a frame shift resulting in substitution of 40 amino acids and premature termination to generate a polypeptide, 14 amino acids short of the wild type.

Insilico study was undertaken to predict the effect of this 7 bp deletion on structural and functional attributes of P16, by predictive molecular modeling and molecular docking. The 3 D model of mutant P16 obtained from I-TASSER, on visual inspection by PyMOL showed a prominent change in the last two helices (ankyrin repeat 4; Figure 3). In addition, the total energy values of –12632.216 kJ/mol for crystallized wild type P16-CDK6 complex (PDB entry 1bi7) compared to –11788.572 kJ/mol for mutant P16-CDK6 complex, indicated the latter being unstable than the former.

Discussion

We have described a novel p16^{INK4A} mutation in a group of ESCC patients from high risk ethnic Kashmiri

Biomarkers Downloaded from informahealthcare.com by Changhua Christian Hospital on 11/14/12
For personal use only.

RIGHTSLINK

Biomarkers Downloaded from informahealthcare.com by Changhua Christian Hospital on 11/14/12
For personal use only.

population. The analysis of blood and normal tissue revealed that the mutation was tumor specific. The mutation at the start of loop 3 that joins 3rd and 4th ankyrin repeat domains of P16 has the potential to dramatically distort the structural integrity of the protein. The change of frame and the substitution of the remaining amino acids is likely to undermine the contribution of the loop 3 in the overall structure of the protein and the loss of ankyrin repeat 4 would pronounce the distortion even further (Byeon et al. 1998). This is in conformity with the observation that ankyrin repeat 3 and 4 fall within a region of 9–133 amino acids, considered critical for P16 activity (Lilischkis et al. 1996). The intactness of the ankyrin repeats has also been shown to be important in constituting the minimal folding unit of ankyrin repeat proteins in general and P16 in particular (Zhang & Peng 2000). Furthermore ankyrin repeat 4 has also been implicated to contribute in P16-CDK interaction (Lilischkis et al. 1996).

The total energy of the mutant P16-CDK6 complex was found to be higher than that of the crystallized wild type P16-CDK6 complex. This structural instability in the mutant protein with 7 bp deletion can be envisaged to be the cause of its functional inefficiency, though functional assays remain the proper assessment tool. It is imperative, therefore, to envisage the loss of P16 function with the observed mutation, given the fact that tumor associated point mutations reported in this region viz G101W, P114L, and H123Q have all been found to be devoid of any CDK inhibitory activity (Byeon et al. 1998). Interestingly, none of the reported mutations were observed in this cohort of population, suggesting that the observed mutation might represent a unique genetic or ethnic marker for ESCC in high risk Kashmiri population.

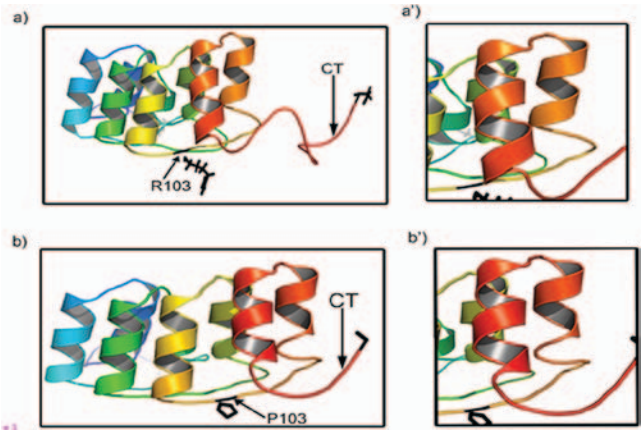

Figure 3. Three dimensional model of wildtype P16 and mutant P16. (a) Three dimensional structure of wildtype P16 showing arginine at 103, the frameshift due to the mutation changes it to proline in addition to the premature termination at codon 142. (b) The helices 7 and 8 (a') are both shortened considerably in (b') along with an elongated loop 3 and a truncated carboxy terminal (CT) domain.

Conclusion

This study describes the identification of a novel p16^{INK4A} deletion variant specific for ESCC in a pure ethnic population. The mutation defines atleast one of the basis in a compendium of factors that may contribute to ESCC in this high risk cohort. Structural distortions together with changed energy dynamics envisaged by insilico analysis are strong indicators of functional consequences associated with the mutation.

Acknowledgments

Research Associate fellowship in favor of MAQ from Department of Biotechnology (DBT), India is duly acknowledged.

Declaration of interest

The financial support for this project by Department of Science and Technology (DST), India under No. SR/SO/BB-21/2009 is gratefully acknowledged. Infrastructural Grant under FIST program and Special Assistance grant under SAP program, from DST India and UGC India is duly acknowledged.

References

Bassam BJ, Gresshoff PM. (2007). Silver staining DNA in polyacrylamide gels. *Nat Protoc* 2:2649–2654.

Blin N, Stafford DW. (1976). A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res* 3:2303–2308.

Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ, O'Maille P, Tsai MD. (1998). Tumor suppressor p16INK4A: Determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. *Mol Cell* 1:421–431.

Cairns P, Mao L, Merlo A, Lee DJ, Schwab D, Eby Y, Tokino K, van der Riet P, Blaugrund JE, Sidransky D. (1994). Rates of p16 (MTS1) mutations in primary tumors with 9p loss. *Science* 265:415–417.

Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE. (1994). Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* 8:27–32.

Comeau SR, Gatchell DW, Vajda S, Camacho CJ. (2004). ClusPro: An automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics* 20:45–50.

Dhar GM, Shah GN, Naheed B, Hafiza. (1993). Epidemiological trend in the distribution of cancer in Kashmir Valley. *J Epidemiol Community Health* 47:290–292.

Diemand AV, Scheib H. (2004). iMolTalk: An interactive, internet-based protein structure analysis server. *Nucleic Acids Res* 32:W512–W516.

Ganguly A, Rock MJ, Prockop DJ. (1993). Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: Evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90:10325–10329.

Holland EA, Beaton SC, Edwards BG, Kefford RF, Mann GJ. (1994). Loss of heterozygosity and homozygous deletions on 9p21-22 in melanoma. *Oncogene* 9:1361–1365.

Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA, Dracopoli NC. (1994). Germline p16 mutations in familial melanoma. *Nat Genet* 8:15–21.

© 2012 Informa UK, Ltd.

RIGHTSLINK

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. (1994a). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 264:436–440.

Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, Hussey C, Tran T, Miki Y, Weaver-Feldhaus J. (1994b). Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 8:23–26.

Khuroo MS, Zargar SA, Mahajan R, Banday MA. (1992). High incidence of oesophageal and gastric cancer in Kashmir in a population with special personal and dietary habits. *Gut* 33:11–15.

Koh J, Enders GH, Dynlacht BD, Harlow E. (1995). Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* 375:506–510.

Körkkö J, Annunen S, Pihlajamaa T, Prockop DJ, Ala-Kokko L. (1998). Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: Comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. *Proc Natl Acad Sci USA* 95:1681–1685.

Kumar R, Mende P, Tricker AR, Siddiqi M, Preussmann R. (1990). N-nitroso compounds and their precursors in Brassica oleracea. *Cancer Lett* 54:61–65.

Landis SH, Murray T, Bolden S, Wingo PA. (1999). Cancer statistics, 1999. *CA Cancer J Clin* 49:8–31, 1.

Lilischkis R, Sarcevic B, Kennedy C, Warlters A, Sutherland RL. (1996). Cancer-associated mis-sense and deletion mutations impair p16INK4 CDK inhibitory activity. *Int J Cancer* 66:249–254.

Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, Peters G, Bartek J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* 375:503–506.

Mattoo AR, Kaul HK. (1974). Incidence of malignant neoplasms in Kashmir. *J Indian Med Assoc* 62:309–311.

Mir MM, Dar NA, Gochhait S, Zargar SA, Ahangar AG, Bamezai RN. (2005). p53 mutation profile of squamous cell carcinomas of the esophagus in Kashmir (India): A high-incidence area. *Int J Cancer* 116:62–68.

Mori T, Miura K, Aoki T, Nishihira T, Mori S, Nakamura Y. (1994). Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res* 54:3396–3397.

Muzeau F, Fléjou JF, Thomas G, Hamelin R. (1997). Loss of heterozygosity on chromosome 9 and p16 (MTS1, CDKN2) gene mutations in esophageal cancers. *Int J Cancer* 72:27–30.

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 368:753–756.

Pickens A, Orringer MB. (2003). Geographical distribution and racial disparity in esophageal cancer. *Ann Thorac Surg* 76:S1367–S1369.

PyMol Molecular Viewer. Available at: http://www.pymol.org/

Ranade K, Hussussian CJ, Sikorski RS, Varmus HE, Goldstein AM, Tucker MA, Serrano M, Hannon GJ, Beach D, Dracopoli NC. (1995). Mutations associated with familial melanoma impair p16INK4 function. *Nat Genet* 10:114–116.

Rosenberg JC, Budev H, Edwards RC, Singal S, Steiger Z, Sundareson AS. (1985). Analysis of adenocarcinoma in Barrett's esophagus utilizing a staging system. *Cancer* 55:1353–1360.

Sambrook J, Fritsch EF, Maniatis T. (1989). *Molecular cloning: Laboratory manuals.* New York: Cold Spring Harbor Laboratory Press.

Serrano M, Hannon GJ, Beach D. (1993). A new regulatory motif in cell-cycle control specific inhibition of cyclin D/CDK4. *Nature* 366:704–707.

Siddiqi M, Tricker AR, Preussmann R. (1988). The occurrence of preformed N-nitroso compounds in food samples from a high risk area of esophageal cancer in Kashmir, India. *Cancer Lett* 39:37–43.

Siddiqi M, Preussmann R. (1989). Esophageal cancer in Kashmir–an assessment. *J Cancer Res Clin Oncol* 115:111–117.

Siddiqi MA, Tricker AR, Kumar R, Fazili Z, Preussmann R. (1991). Dietary sources of N-nitrosamines in a high-risk area for oesophageal cancer–Kashmir, India. *IARC Sci Publ* 105:210–213.

Siddiqi M, Kumar R, Fazili Z, Spiegelhalder B, Preussmann R. (1992). Increased exposure to dietary amines and nitrate in a population at high risk of esophageal and gastric cancer in Kashmir (India). *Carcinogenesis* 13:1331–1335.

Vogelstein B, Gillespie D. (1979). Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* 76:615–619.

Zhang B, Peng Z. (2000). A minimum folding unit in the ankyrin repeat protein p16(INK4). *J Mol Biol* 299:1121–1132.

Zhang Y. (2007). Template-based modeling and free modeling by I-TASSER in CASP7. *Proteins* 69 Suppl 8:108–117.

Zhang Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40.

Biomarkers Downloaded from informahealthcare.com by Changhua Christian Hospital on 11/14/12
For personal use only.

RIGHTSLINK