Infection of Human Papillomavirus Type 18 and p53 Codon 72 Polymorphism in Lung Cancer Patients From India*

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Study objectives: Infection with specific high-risk HPV types 16 and 18 and polymorphism of p53 codon 72 has been strongly associated with the genesis of various neoplasms in humans, but such study in lung cancer is limited and the results are controversial. In India, the role of these two factors has been strongly implicated in cervical and other cancers, but the occurrence of HPV or p53 codon 72 polymorphism has not been examined in lung cancer, which is the most common cause of cancer-related death in India.

Design and patients: A total of 40 tumor biopsy specimens from advanced lung cancer patients and blood samples from 40 matching control subjects were obtained for the analysis of high-risk HPV types 16 and 18 infection and p53 codon 72 polymorphism by polymerase chain reaction.

Results: Only HPV type 18 was detected in 5% (2 of 40 lung cancer patients), but no other HPV could be detected. A significantly increased frequency of Arg/Arg homozygotes was observed in patients with advanced lung cancer when compared to that of control subjects (p = 0.004; odds ratio, 5.13; 95% confidence interval, 1.59 to 17.26). However, no significant correlation could be made between p53 polymorphism and different clinical stages, except for advanced stage IV patients, who showed a higher proportion of Arg/Pro heterozygous genotype.

Conclusions: HPV detected in a small proportion of lung cancer patients in India demonstrated an exclusive prevalence of HPV type 18, and there was a significantly higher frequency of p53 Arg/Arg genotype when compared to that of control subjects. Observation of a shorter duration of symptoms (≤ 4 months) in as many as 78% (seven of nine stage IV patients) with Arg/Pro genotype may be an indication that lung cancer patients with the heterozygous p53 genotype are more susceptible to early progression.

Key words: human papillomavirus; lung cancer; p53 codon 72 polymorphism; polymerase chain reaction

Abbreviations: bp = base-pair; CI = confidence interval; HPV = human papillomavirus; OR = odds ratio; PCR = polymerase chain reaction

Lung cancer, which involves malignant proliferation of the epithelial lining of the lower respiratory tract, is one of the most common forms of malignancy leading to the major cause of cancer-related deaths around the world1 including India.2 Smoking is considered to be one of the principal causes of lung cancer; however, not all smokers acquire lung cancer, while many nonsmokers including passive smokers do acquire lung cancer.3–5 Therefore, various other etiologic factors, including genetic factors such as mutation or overexpression of oncogenes such as c-myc, erbB2, K-ras, polymorphism in P450 (CYP1A1) and glutathione transferase M1 genes, functional inactivation of tumor suppressor genes eg, Rb, p16, p53 gene including p53 codon 72 polymorphism, and infection of specific types of human papillomaviruses (HPVs), have been implicated with the development of lung cancer.6–9

Infection with HPV has been associated with the development of > 10% of human cancers,10 including cervical, oral, esophageal, laryngeal, and head and neck cancer.11 Of the > 100 types of HPV described so far, HPV types 16 and 18 are most commonly associated with malignant lesions and are...
referred to as “high-risk” types. The early oncoprotein E7 of high-risk HPV’s binds to and inactivates the cellular tumor suppressor protein Rb, while the E6 protein binds to the p53 protein and directs its ubiquitin-mediated proteolytic degradation and interferes with the cell cycle control resulting in abnormal cell proliferation and tumor growth.12–14 The reports on the involvement of HPV infection in lung cancer are not only rare but also controversial. Several authors15–18 have reported that HPV has no role to play in lung carcinogenesis, whereas others19–23 have observed a low frequency (4 to 18%) of HPV infection in lung cancer. In contrast, a moderate to a very high frequency of HPV infection (30 to 79%) has been reported by several other authors24–29 from different regions of the world. Observation of such a wide variation in HPV prevalence in lung cancer appears to be an indication of either geographic and/or ethnic variation or due to varied protocols and primers/probes used by different laboratories.

p53 protein, which exists in two polymorphic forms (p53-Pro or p53-Arg) in the general population due to single nucleotide change at codon 72 of exon 4 of the p53 gene,30,31 shows different structural and functional properties.32 It has been shown that the p53-Arg protein is more susceptible to E6-mediated proteolytic degradation than p53-Pro isoform, and women with homozygous p53 Arg/Arg genotype are at least seven times higher risk of acquiring HPV-induced cervical cancer.33 However, conflicting results have been reported for cervical and other cancers including lung cancer. While few reports34–38 supported the above findings, many others39–47 including our previous study,48 also failed to confirm the same. The association of p53 codon 72 polymorphism has been studied in lung carcinoma by several authors,22,36,49–53 but the results are contradictory. While the frequency of Pro/Pro genotype was reported to be higher in lung cancer by some authors,49,50 Papadakis and group52 observed an increased frequency of Arg/Arg genotype in advanced lung cancer cases, but Tagawa et al54 reported overrepresentation of this genotype in nonsmoking lung cancer patients. Others51–55 have observed no significant association between p53 codon 72 polymorphism and lung cancer. Furthermore, it has been demonstrated that patients with Pro/Pro genotype had a worse prognosis when compared to those with Arg/Pro genotype.36

In India, the prevalence of HPV in cervical cancer is extremely high (approximately 98%), and HPV 16 is the high-risk type exclusively prevalent not only in squamous cell carcinoma (approximately 90%),55 but in adenocarcinoma of the uterine cervix56 as well as in oral57 and esophageal cancer.58 However, no study on the prevalence of HPV as well as p53 codon 72 polymorphism in lung cancer has been reported from India. We report here an exclusive occurrence of HPV 18 infection in lung cancer patients who showed higher frequency of Arg/Arg homozygous p53 genotype, but the patients showed an aggressive progression if they harbor heterozygous Arg/Pro p53 genotype.

Materials and Methods

Tissue Specimens

A total of 40 incident lung cancer patients who reported to the Department of Medicine, Lok Nayak Hospital, New Delhi during the period 2003 to 2004 formed the study group. A detailed history relating to demographic particulars, history of smoking, tuberculosis, and family history of lung cancer or any other cancer was collected in a pretested proforma. In addition, clinical details regarding stage of the disease, histology, and degree of anaplasia were obtained from the clinical records. Tumor biopsy specimens were collected in ice-cold phosphate-buffered saline solution from the surgical operation theater, either during fiberoptic bronchoscopy or trucut needle biopsy obtained under CT guidance from lung cancer patients after obtaining their informed consent, and were stored at −70°C in a deep freezer until further analysis. Peripheral venous blood samples from 40 healthy control subjects matched for age, sex, and smoking habits were also collected for comparison. Control subjects were unrelated to the patients and were attending the outpatient department of medicine for ailments other than cancer.

DNA Extraction, Polymerase Chain Reaction, and Detection of HPV Infection

High-molecular-weight genomic DNA from biopsy and blood samples was isolated by the standard method of proteinase K digestion and phenol chloroform extraction routinely followed in our laboratory.59 Detection of HPV was carried out first by using consensus primers located within the conserved L1 region of HPV genome resulting in amplimer of 450 base-pair (bp)60 (forward primer, 5'-GGMCCAGGGWCAT AAYAATGG-3', reverse primer, 5'-CGTCCMARRGGWACTGATC-3', where M = A + C, W = A + T, Y = C + T, R = A + G) and later by type-specific primers for high risk types 16 (amplimers size, 217
method involved a 25-
mol/L of MgCl2, 12.5 of DNA, 10 mmol/L of Tris Cl (pH 8.4), 50 mmol/L of KCl, 1.5 samples. Lanes 4 and 7 represent positive samples for HPV 18. Lanes 4 to 10 are tumor samples. Lanes 4 and 7 represent positive samples for HPV 18.

Analysis of p53 Codon 72 Arg/Pro Polymorphism

The PCR amplification for the analysis p53 codon 72 arginine and proline alleles was carried out in separate reactions using the same set of primers as described by Storey et al33 (p53 Pro + 5’-GCCAGGCACTCCTCCCCC-3’, p53- 5’-CGTGAACTT-CACTTT CACCAGTTACCC-3’) with an amplimer of 268 bp served as the internal control (Fig 1). The oligonucleotide primers were synthesized in an automated DNA synthesizer (Perkin-Elmer Biosystems; Foster City, CA). The temperature profile used for amplification constituted an initial denaturation at 95°C for 5 min followed by 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, which was extended for 4 min in the final cycle. Amplification of β-globin gene (forward primer, 5’-GAAGAGCAAGGACAGGTAC-3’, reverse primer, 5’-CACTTT CATCCACGTTACCC-3’) with an amplimer of 141 bp for the analysis p53 gene (bottom lanes) alleles of codon 72 (Fig 2) on an ethidium bromide-stained 3% Nusieve agarose gel (FMC Bioproducts; Rockland, ME) under an ultraviolet transilluminator or a gel documentation system (BioRad Laboratories; Hercules, CA).

Results

Of 40 lung cancer patients, 35 patients (87.5%) were men and 5 patients (12.5%) were women (mean ± SD age, 56.8 ± 9.7 years and 62.2 ± 11.2 years, respectively). Only six patients (15%) were nonsmokers, while remaining were smokers; pack-years ranged from 6 to 150. One pack-year corresponds to a pack of 20 cigarettes smoked daily over a period of 1 year. None of the patients had any family history of lung cancer or any other cancer. Histopathologic typing revealed 22 cases (55%) of squamous cell carcinoma, 9 cases (22.5%) of adenocarcinoma, 8 cases (20%) of small cell lung carcinoma, and 1 case (2.5%) of large cell lung carcinoma.

HPV Status

HPV infection was first detected using L1 consensus primers and then typed using specific primers for high-risk HPV type 16 and HPV 18 in a total of 40 lung cancer cases. Only two cases (5%) showed HPV positivity by consensus primers, which later revealed presence of only HPV 18 DNA sequences by typespecific primers (Table 1). None of the samples showed presence of HPV type 16, which is the most prevalent HPV type in anogenital, oral, and esophageal cancer in India. Both the HPV 18-positive cases

Statistical Analysis

Means and SD were calculated for quantitative data. χ2 or Fisher Exact Tests were employed to test for significance of results.
were moderately differentiated carcinomas and belonged to TNM stage IV, which showed heterozygous p53 Arg/Pro genotypes. Since only two patients had positive findings for HPV infection, no statistical analysis could be performed either with different clinicopathologic features or p53 codon 72 polymorphism. All the 40 control subjects were also subjected for L1 consensus PCR to detect HPV infection if any, but none of them showed any HPV amplification. All the tumor and control DNA samples showed good amplification of β-globin gene, which served as an internal control.

p53 Codon 72 Arg/Pro Polymorphism

The proportion of Arg/Arg or Pro/Pro homozygosity and Arg/Pro heterozygosity in 40 lung cancer cases was found to be 47.5%, 10.0%, and 42.5%, respectively, as compared to 15.0%, 25.0%, and 60.0% in the control subjects (n = 40). The difference in the proportion of subjects revealing Arg/Arg homozygosity between the lung cancer patients and normal control subjects was found to be statistically significant (p = 0.004; odds ratio [OR], 5.13; 95% confidence interval [CI], 1.59 to 17.26). On comparing the Pro/Pro and Arg/Pro genotypes between cases and control subjects, the difference was not found to be statistically significant (p = 0.14 and p = 0.18, respectively) [Table 1].

Association of p53 Codon 72 Polymorphism With Clinicopathologic Features

Statistically, no significant association could be observed when a comparison was made between different clinicopathologic profiles and the three different p53 genotypes except for 9 of 15 stage IV patients (60%) who exhibited a heterozygous Arg/Pro genotype (Table 2). Interestingly, of these nine patients, seven patients (77.8%) showed duration of symptoms ≤ 4 months, which is a much shorter period when compared to that of other stages. The frequency of arginine homozygotes, however, showed an increasing trend with the increasing age and higher pack-years (Table 2).

Discussion

The reports on the prevalence of HPV infection in lung carcinoma worldwide are limited as well as conflicting, and the frequency ranges from zero to as high as 80% (Table 3). We have observed a very low frequency (5%) of HPV infection (HPV type 18 only), and no other HPV could be detected, including HPV type 16, which is almost exclusively prevalent in cervical, oral, and esophageal cancer in India.57–59 An exclusive occurrence of HPV 18 infection in lung cancer has also been reported by other groups20,22,23 from different regions (Table 3). The occurrence of specifically HPV type 18 in lung cancer is intriguing. It is also interesting that although the p53 Arg/Arg genotype is significantly higher in advanced lung cancer patients, the two HPV 18-positive cases that also belonged to advanced stage IV showed a heterozygous (Pro/Arg) genotype.

A high degree of difference in the prevalence of HPV infection in lung cancer around the globe has long been attributed to geographic and ethnic variation, but surprisingly two reports18,27 on lung cancer from the same location in Greece showed completely opposite results. While Papadopoulou and colleagues27 demonstrated HPV positivity as high as 69%, Gorgoulis et al18 reported the complete absence of HPV infection in lung cancer. Similarly, from Japan, HPV prevalence of 79% was reported by two groups,28,29 but Miyagi et al26 reported only 34%. In addition, Szabo et al16 found the complete absence of HPV infection in lung cancer. The above reports as well as other studies (Table 3) do indicate that there may be other factors responsible for variation in HPV prevalence. As it is generally seen in many cases of bacterial or viral infections, there could be seasonal variation of HPV infection within the same region and population. However, the major variability in results may be attributed to different methodologies, PCR protocols, sensitivity and specificity of these methods, and selection of patients including host factors.22,20,61,62 The observation of a very low frequency of HPV infection in lung cancer

Table 1—p53 Codon 72 Polymorphism and HPV Prevalence in Lung Cancer Patients and Control Subjects*

<table>
<thead>
<tr>
<th>Groups</th>
<th>p53 Polymorphism</th>
<th>HPV Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg/Arg†</td>
<td>Arg/Pro‡</td>
</tr>
<tr>
<td>Lung cancer patients (n = 40)</td>
<td>19 (47.5)</td>
<td>17 (42.5)</td>
</tr>
<tr>
<td>Control subjects (n = 40)</td>
<td>6 (15.0)</td>
<td>24 (60.0)</td>
</tr>
</tbody>
</table>

*Data are presented as No. (%).
†p = 0.004 (OR, 5.13; 95% CI, 1.59 to 17.26).
‡p = 0.18.
§p = 0.14.
by us and several other authors does not support its major role in lung carcinogenesis. However, it would be important to see the effect of HPV alone in the absence of other associated carcinogenic factors in lung cancer.

Several studies indicate a close relation between risk of cancer and p53 Arg homozygosity that confers high susceptibility to the p53 protein for degradation through HPV early protein E6-mediated ubiquitin pathways, while others failed to

<table>
<thead>
<tr>
<th>Study No.</th>
<th>HPV 16</th>
<th>HPV18</th>
<th>HPV 16 and HPV 18</th>
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<th>Total HPV</th>
<th>Region</th>
<th>Source/Year</th>
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<td>3 (9.0)</td>
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<td>6 (18.0)</td>
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<td>0</td>
<td>0</td>
<td>Kawaga, Japan</td>
</tr>
<tr>
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<td>10 (20.0)</td>
<td>5 (10.0)</td>
<td>1 (2.0)</td>
<td>0</td>
<td>16 (32.0)</td>
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<td>2 (9.3)</td>
<td>7 (32.6)</td>
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<td>9 (41.9)</td>
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<td>52</td>
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<td>11 (21.2)</td>
<td>11 (21.2)</td>
<td>25 (48.1)</td>
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<td>8</td>
<td>23</td>
<td>5 (21.7)</td>
<td>4 (17.3)</td>
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<td>15 (78.0)</td>
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<td>10</td>
<td>26</td>
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<td>2 (7.7)</td>
<td>1 (3.8)</td>
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<td>41 (33.8)</td>
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<td>2 (3.4)</td>
<td>2 (3.4)</td>
<td>5 (8.6)</td>
<td>7 (12.0)</td>
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<td>54</td>
<td>2 (3.7)</td>
<td>2 (3.7)</td>
<td>0</td>
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<td>0</td>
<td>Athens, Greece</td>
</tr>
<tr>
<td>14</td>
<td>40</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>Ankara, Turkey</td>
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<tr>
<td>15</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>New Delhi, India</td>
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</table>

*Data are presented as No. (%) unless otherwise indicated.
†HPV 6, 11, 31, 33, 35, 52, and 58.
‡Others with HPV 16 and/or HPV 18 co-infection.
§Mixed probe used 6/11,16/18, 31/33/35.
find such an association. Observations on the association between p53 codon 72 polymorphism and lung cancer have been inconsistent (Table 4). While several studies\textsuperscript{51,53} found no association between p53 codon 72 polymorphism and lung cancer, other authors\textsuperscript{36,49,50} reported an increased frequency of Pro/Pro homozygotes in lung cancer. Studies\textsuperscript{36,50} of Asian and Mexican-Americans suggested at least a twofold increase in lung cancer risk for the Pro/Pro homozygotes. Also, an increased risk was observed for African-American\textsuperscript{65} and white populations with the Pro/Pro genotype.\textsuperscript{36,50} The majority of studies that associate p53 Pro/Pro genotype with lung cancer have been reported from Southeast Asia (China, Taiwan, or Japan). In contrast, we have found an increased frequency of Arg/Arg genotypes in lung cancer patients revealing nearly a fivefold increased risk (\( p = 0.004; \) OR, 5.13; 95\% CI, 1.59 to 17.26) [Table 1]. This is in agreement with the study of Papadakis and group,\textsuperscript{22} who also observed an increased frequency of the Arg/Arg genotype in lung cancer patients compared to normal control subjects. The frequency of the Arg/Arg genotype is certainly higher in lung cancer patients and correlates positively with various clinicopathologic features. Interestingly, the frequency of Arg/Arg homozygotes also increased with the increasing severity of the disease. However, the difference was not found to be statistically significant in all stages. Several authors\textsuperscript{49,62} reported an increased frequency of Pro/Pro homozygosity in adenocarcinoma of the lung. We observed a higher frequency of the Arg/Arg allele in both squamous cell carcinoma (52.3\%) as well as adenocarcinoma (33.3\%), compared to only 15\% in healthy control subjects (Tables 1, 2). Our observation of 25\% and 10\% of the Pro/Pro allele in control subjects and cancer patients, respectively, is in good agreement with the Beckman hypothesis that with the increasing latitude, the frequency of the Pro/Pro genotype decreases, and it has been shown to vary from 63\% in African blacks to 17\% in Swedish Saamis. This is suggested to be due to natural selection through ecological adaptation to ultraviolet radiation.\textsuperscript{31}

One of the interesting observation was that of the stage IV lung cancer patients who showed a higher proportion (60\%) of Arg/Pro heterozygosity, and as high as 77.8\% of these stage IV patients showed a shorter duration of symptoms (\( \leq 4 \) months). This is indicative of the fact that the patients with the heterozygous p53 Arg/Pro genotype seem to have a higher progression rate or aggressive clinical behavior. Since the duration of symptoms is an elusive end point due to early death or other reasons, further study with a larger group of patients could establish if the prevalence of the Arg/Pro genotype can serve as a significant prognostic/susceptibility marker for advanced lung cancer patients. Although, no significant difference was found between p53 codon 72 polymorphism and different clinicopathologic features of lung cancer patients, the homozygous p53 Arg/Arg genotypes, in general, do show an increasing trend with the increasing severity of the lesions, age, and pack-years (Table 2). This suggests that a higher prevalence of arginine homozygosity could be a genetic risk factor for the development of lung cancer in India. However, due to low frequency of HPV, it is difficult to associate p53 Arg homozygosity with HPV in lung cancer.

Several studies have demonstrated contradictory results associating either p53 proline or p53 arginine homozygosity with lung cancer.\textsuperscript{22,36,49,50,54,64} The discrepancies in the results may be attributed to various reasons such as selection of patients and control subjects, variation in laboratory protocols, and geographic and ethnic background. Fan et al.,\textsuperscript{62} who demonstrated the association of p53 proline homozygosity with lung cancer, had studied only primary lung cancer stage I and II patients and excluded patients of advanced stage, whereas Papadakis and his group\textsuperscript{22} studied advanced lung cancer patients (stage III and IV) and suggested association of p53

### Table 4—Frequency of p53 Polymorphism in Lung Cancer: Worldwide Scenario*

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Sample Size</th>
<th>Arg/Arg</th>
<th>Arg/Pro</th>
<th>Pro/Pro</th>
<th>Region</th>
<th>Source/Year</th>
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<tr>
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<td>148 (45.1)</td>
<td>127 (38.7)</td>
<td>53 (16.2)</td>
<td>Saitama, Japan</td>
<td>Kawajiri et al,\textsuperscript{30} 1993</td>
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<tr>
<td>2</td>
<td>109</td>
<td>34 (31.2)</td>
<td>54 (49.5)</td>
<td>21 (19.3)</td>
<td>Texas</td>
<td>Jis et al,\textsuperscript{63} 1995</td>
</tr>
<tr>
<td>3</td>
<td>178</td>
<td>76 (42.7)</td>
<td>83 (46.6)</td>
<td>10 (10.7)</td>
<td>Chiba, Japan</td>
<td>Tagawa et al,\textsuperscript{34} 1998</td>
</tr>
<tr>
<td>4</td>
<td>114</td>
<td>39 (34.2)</td>
<td>45 (39.5)</td>
<td>30 (26.3)</td>
<td>Taichung, Taiwan</td>
<td>Wang et al,\textsuperscript{36} 1999</td>
</tr>
<tr>
<td>5</td>
<td>482</td>
<td>212 (44.0)</td>
<td>204 (42.3)</td>
<td>66 (13.7)</td>
<td>Massachusetts</td>
<td>Fan et al,\textsuperscript{62} 2000</td>
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<tr>
<td>6</td>
<td>334</td>
<td>144 (43.1)</td>
<td>138 (41.3)</td>
<td>52 (15.6)</td>
<td>Hawaii</td>
<td>Pierce et al,\textsuperscript{53} 2000</td>
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<tr>
<td>7</td>
<td>767</td>
<td>367 (48.0)</td>
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<td>Miller et al,\textsuperscript{66} 2002</td>
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<td>27 (50.0)</td>
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<td>Athens, Greece</td>
<td>Papadakis et al,\textsuperscript{22} 2002</td>
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<td>17 (42.5)</td>
<td>4 (10.0)</td>
<td>New Delhi, India</td>
<td>This study</td>
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*Data are presented as No. (%) unless otherwise indicated.*
arginine homozygosity with lung cancer. Also, in the present study, the majority (33 of 40 patients, 82.5%) belonged to stage III and IV, which showed a preponderance of p53 Arg homozygosity. However, it is highly intriguing that, although arginine homozygosity has been found to be associated with lung cancer in the present study, it is neither proline nor arginine homozygosity but Arg/Pro heterozygosity that has shown higher incidence in majority of stage IV patients who showed faster progression to advanced stage of lung cancer. A similar association of the Arg/Pro genotype with an increased risk of cervical cancer has also been reported [67, 68]. At present, it is difficult to indicate specific reason(s) or the biological basis for the increased susceptibility to progression in Arg/Pro heterozygote individuals. However, it has been observed that arginine homozygosity allows certain p53 mutants to form stable complex with p73 and block its apoptotic ability by inactivation of p73 protein [69, 70]. It indicates that the p53 Arg allele is preferentially retained in Arg/Pro germline heterozygotes. It is also possible that there might be some yet unknown mutation in the p53 gene either in the exon or in the intronic region and/or additional genetic or epigenetic changes in other gene(s) which may make Arg/Pro heterozygous patients highly susceptible for an early progression to invasive cancer. A somewhat similar situation has been observed in a Southeast Asian population, where the p53 proline homozygous genotype has been associated with lung cancer, but a low frequency of another p53 gene polymorphism at intron 3, a 16-bp duplication that offers protection against lung cancer [51], makes this population more prone to lung cancer.

In conclusion, our findings suggest no major role of HPV but a strong association of p53 Arg homozygotes with advanced lung cancer. Nevertheless, the presence of Arg/Pro heterozygotes may associate with early progression of the disease possibly due to additional genetic alteration(s). Therefore, further studies are required on a larger sample size to unravel the role of HPV and p53 codon 72 polymorphism in the genesis of lung cancer in humans.

REFERENCES


