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Low frequency of p53 mutations in cervical carcinomas among Brazilian women

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Abstract

Human papillomavirus (HPV) infections of the high-risk types are strongly linked to the development of cervical carcinoma. The HPV oncoproteins E6 and E7 are thought to play a crucial role in this process through their interactions with the p53 protein and the retinoblastoma susceptibility gene product pRb, respectively. E6 binds to p53 protein promoting its degradation. This is considered to contribute to the oncogenesis of HPV-associated anogenital cancer. On the other hand, in HPV-negative cervical carcinoma, p53 mutations are thought to have a role in the transformation process. A total of 122 HPV-positive cervical carcinoma tissue samples were evaluated for the presence of mutations in exons 5-8 of the p53 gene by single-stranded conformation polymorphism analysis and DNA sequencing. Only four missense point mutations were detected. These findings suggest that other mechanisms independent of p53 inactivation may play a role in the genesis of cervical carcinomas.

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- Suppressor gene
- HPV
- PCR
- DNA sequencing

Introduction

Carcinoma of the uterine cervix is the third most frequent of the female genital malignancies. In recent years, human papillomavirus (HPV) has been identified as the etiological agent involved in the pathogenesis of this cancer (1,2). Among more than 70 HPV types reported to date, types HPV 16 and 18 are the most prevalent and are found in more than 90% of primary cervical carcinomas in different geographic regions of the world (3,4). These high-risk HPVs encode two transforming gene products, E6 and E7, whose proteins bind to p53 and *pRb*, respectively (5-7). The high-risk HPV E6 oncoprotein targets p53 degradation through a ubiquitin-dependent proteolysis system (6,8). p53 is also functionally inactivated by interaction with SV40 TAg, and E1B of adenovirus type 5 (Ad5E1B) (9).

The mechanisms by which these viral proteins inactivate p53 are distinct. In normal primary cells, the metabolic half-life of p53 is relatively short, between 20 and 40 min in most cell types. In SV40-immortalized cells, the steady-state level of p53 is elevated and the half-life is greatly extended (10). Ad5E1B has a similar effect on p53 stability and overall concentration. On the other hand, the level of p53 in HPV-containing cervical carcinoma cell lines and HPV-immortalized keratinocytes is generally lower than the level seen in primary cells and the half-life is decreased (6).

The p53 protein is a nuclear phosphoprotein whose function is classified as a tumor suppressor (11) and has the properties of a transcriptional activator (12). The ability of p53 to bind to specific DNA sequences and to activate transcription indicates that this protein plays an important role in the regulation of cell proliferation. This gene is frequently mutated in nearly all types of human cancers (13). The majority of the studies involving the p53 gene examined only exons 5-8 in the central DNA-binding region, where the most commonly identified alterations of the p53 gene are single base pair substitutions (13,14).

Several studies have examined the status of p53 in cervical carcinomas and recent data have suggested that it may have an important role (12,15). Sequencing of p53 DNA from cervical carcinoma tissue and cell lines revealed wild-type p53 in HPV-positive tissues, whereas the mutated form was demonstrated only in HPV-negative tissues (15). Additional studies demonstrated that p53 mutations occur at higher frequencies in HPV-negative cervical carcinoma cell lines, but occur rarely in HPV-positive lines (7,16). These findings led to the suggestion that inactivation of p53 function, either by mutation or by interaction with the HPV E6 gene product, is central to carcinogenesis in the cervix (17). The p53 mutants identified in HPV-positive anogenital cancers exhibit increased resistance to HPV E6-directed degradation, suggesting that mutation of p53 may play a role in the progression of HPV-positive cervical cancer (18).

To further understand the role of this tumor suppressor gene in HPV-associated neoplasia we performed an analysis of p53 gene alterations in a large series of cervical carcinomas from Brazil.

Material and Methods

Samples, DNA extraction and HPV DNA typing

Cervical carcinoma tissues were obtained during surgery from patients admitted to the Napoleão Laureano Hospital, João Pessoa, PB, Brazil, a high-risk area for this neoplasia. The institution's Ethics Committee approved the study and all patients gave their written consent. High molecular weight DNA was extracted from the tissue samples as previously described (19). Most of these tumors were classified histologically as squamous cell carcinomas. HPV DNA sequences were evaluated by both Southern blot analysis and PCR using generic primers MY09 and MY11, which amplify a 450-bp fragment of the L1 gene from the genital HPV types, followed by dot-blot hybridization and restriction fragment length polymorphism, allowing the detection of more than 40 HPV types (20,21).

PCR-SSCP analysis of p53

Genomic DNA isolated from HPV-negative and HPV-positive cervical carcinomas was amplified by PCR for each of exon 5, 6, 7 and 8 so-called "hot spots" for p53 gene mutations. Single-stranded conformation polymorphism (SSCP) analysis of p53 mutations has been previously described (22-25).

The oligonucleotide primers employed flanked each exon and were obtained based on genomic sequences deposited in Genbank: for exon 5, sense: 5'-TACTCCCCTGCCCTCAACAAG-3' and antisense: 5'-CACCATCGCTATCTGAGCAGCG-3'; for exon 6, sense: 5'-CAGGGCTGGTTTCCCAGGGTCCCCA-3' and antisense: 5'-CAGGCGGCTCATAGGGCA-3'; for exon 7, sense: 5'-GTGTTATCTCCTAGGTTGGC-3' and antisense: 5'-CAAGTGGCTCCTGACCTGGA-3'; for exon 8, sense: 5'-AGTGGTAATCTACTGGGACGC-3' and antisense: 5'-TATCTCCATCCAGTGGTTTC-3'. These primer pairs for exons 5, 6, 7 and 8 amplify products of 184, 110, 113 and 137 bp, respectively.

DNA (200 ng) was subjected to PCR using nucleotides α^{P32}-dCTP, deoxynucle-

otide triphosphates (0.2 mM), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Cenbiot, Porto Alegre, RS, Brazil), 1 µM of each primer, and the following cycling profile: for exons 5 and 8, after heating for 5 min at 93°C, 35 cycles of 5 min at 93°C, 30 s at 58°C and 2 min at 72°C; for exons 6 and 7 heating for 5 min at 94°C, and 32 cycles of 5 min at 94°C, 1 min at 63°C and 7 min at 72°C. A 4-µl aliquot of the PCR products was diluted with a loading solution (95% formamide containing 0.05% xylene cyanol, 0.05% bromophenol blue and 20 mM EDTA), denatured at 95°C for 10 min and then applied to 5% nondenaturing polyacrylamide gel containing either 5 or 10% glycerol. Electrophoresis was performed at 3 and 6 watts for the 5 and 10% glycerol gels, respectively. The gel was dried on filter paper and exposed to X-ray film at 37°C for 12 h with an intensifying screen.

We used DNAs extracted from the following tumors, previously shown to contain mutations in the p53 gene as positive controls: a gastric carcinoma with a G→A (codon 157) change in exon 5, an HPV-negative penile carcinoma with an A→G (codon 213) change in exon 6, a penile carcinoma with two changes (C→A and C→T in codons 247 and 248, respectively) in exon 7, and C33, an HPV-negative cervical carcinoma cell line that harbors a C→T change in codon 273 of exon 8.

p53 sequencing

PCR-amplified DNA fragments with altered mobility as determined by SSCP-PCR analysis were cloned with the SureClone Ligation Kit (Pharmacia Biotech, Uppsala, Sweden) and the recombinant plasmids sequenced in an ALF Express™ DNA Sequencer (Pharmacia Biotech). Two different bacterial clones with the mutated or wild-type alleles were sequenced for each tumor sample.

Results

Analysis of the p53 gene by SSCP-PCR

HPV type distribution in these 122 cervical carcinomas was as follows: HPV 16 was the most prevalent type (79/122, 64.7%), followed by HPV 18 (6/122, 4.9%), HPV 31 (1/122, 0.81%), HPV 33 (2/122, 1.6%), and HPV 45 (2/122, 1.6%). We observed a high frequency of multiple infections: 11 tumors (9.0%) contained HPV 16 and 18, and an even larger number of samples, 21/122 (17.21%), showed multiple infections with HPV 16 and other types.

DNA extracted from the tumors was subjected to PCR-mediated amplification of exons 5-8, which cover the coding region that encompasses most of the described mutations of the p53 gene. The amplified fragments for each exon were analyzed by SSCP. A representative example is shown in Figure 1. From this analysis, we inferred p53 mutations in only 4 out of the 122 tumors analyzed.

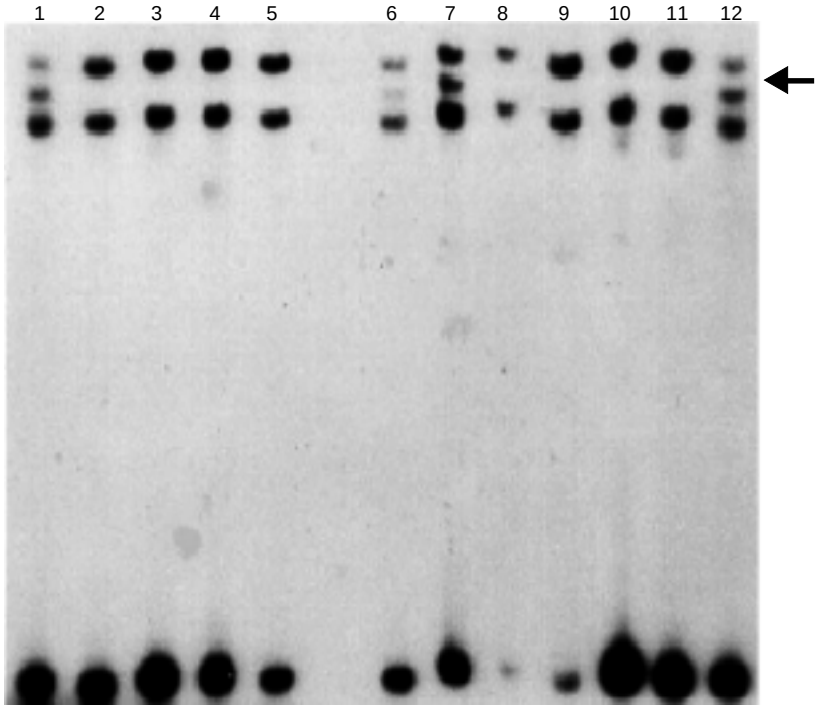

Figure 1. PCR-SSCP of p53 exon 6 in cervical carcinoma. Four samples present band shifts compatible with sequence alterations, as indicated by the arrow.

Sequencing of p53 gene exons 5-8

Sequencing was performed on both the sense and antisense strands of the cloned exon 5, 6 and 8 fragments obtained from the HPV-positive cervical carcinomas. One specimen showed a single nucleotide change at codon 175 (GC**G**→GC**A**), which does not lead to amino acid substitution. On the other hand, two samples showed mutations at codon 213 (G**A**C→G**G**C), which results in an Asp to Gly change. Another nucleotide alteration was found in codon 277 (A**C**A→A**A**A), causing a Thr-Lys substitution.

Discussion

Almost all of human cervical carcinomas harbor HPV DNA sequences, and the viral E6 and E7 oncoproteins are generally expressed within these tumors (21). Evidence that the HPV E6 oncoprotein can bind p53 protein and promote its degradation suggests one mechanism by which the HPV viruses could mediate transformation (26,27). The presence of an HPV sequence in a cell could represent the loss of the p53 function resulting from either a deletion or a mutation. If the abrogation of p53 function is really critical to cervical carcinogenesis, then either HPV infection or p53 gene mutation could fulfill this requirement. However, discrepancies are observed when comparing p53 data obtained from cell lines and clinical samples. It has been reported that HPV-negative cell lines contain p53 point mutations, whereas in HPV-positive cell lines, p53 is always of the wild type, suggesting that expression of E6 would mimic p53 mutations in the latter (6,26,28). Data from several studies employing cervical carcinoma samples have failed to substantiate this, pointing to a small percentage of p53 mutations in this neoplasm, which occur irrespective of the HPV status of the tumor (4,14, 29,30).

Interaction of E6 from high-risk HPVs and mutant forms of p53 may be rare *in vivo* and complex to study *in vitro*. Some p53 mutants may present an E6-resistant phenotype, either by reduced affinity for E6, or by being less prone to proteolysis, and may accumulate even upon HPV 16 E6 expression. This apparent contradiction may be explained by E6 and mutated p53 not being present in the same cells, which is unlikely since it has been shown that E6 expression is required for progression of HPV-related tumors. Another explanation could be that E6 expression *in vivo* is not sufficient to eliminate the p53 protein or that in these tumors p53 complexes with cellular proteins hindering E6 access to it. These data indicate that E6-p53 interaction should not be considered the single mechanism of HPV-mediated transformation.

We have screened for p53 gene mutations 122 HPV-positive cervical carcinomas by SSCP analysis and DNA sequencing of exons 5-8, which have been reported to be the most common sites of mutations in this gene (13). Codon 213 was affected in 2 of the 4 p53-mutated samples, confirming this position as a "hot spot" in tumors from different anatomical sites (13). Of the 4 point mutations detected, 3 corresponded to missense mutations that may have implications for protein conformation. Moreover, these amino acid substitutions map to a region important for p53 DNA-binding activity, implying an eventual loss of function. However, functional studies are needed to confirm this implication. In fact, it was recently reported that cervical carcinoma cell lines containing transcriptionally active HPV display normal p53 transactivating function, including cell cycle arrest at G_1 upon stimulation by genotoxic agents and irradiation.

Absences of p53 mutations in HPV-negative cervical tumors were reported by Helland et al. (31), who found no mutations in 6 HPV-negative patients. Similar results were reported by Choo et al. (32). Recently, these results were confirmed in larger series of

cervical cancers. Kim et al. (33) showed that 2 out of 136 (1.5%) tumors demonstrated SSCP band shifts. One sample (positive for HPV 18) had a nonsense mutation of codon 101 in exon 4 from AAA to TAA transversion. Another (HPV positive for the L1 consensus primer set) showed a point mutation involving codon 179 in exon 5 changing CAT to CGT transition. Three specimens negative for HPV did not contain p53 gene mutations. In another study 64 cases of primary cervix cancers were analyzed with a screening of the p53 gene mutations in exons 5 through 9 of this gene. SSCP analysis showed mobility shifts in 8 cases (6 in HPV-positive cases and 2 in HPV-negatives cases) and sequence analysis confirmed the results of SSCP (34).

Nakagawa et al. (35) analyzed mutation of the p53 gene in 45 women with cervical carcinomas. p53 mutations were analyzed by PCR-based SSCP and DNA sequencing techniques. Point mutation of the p53 gene was detected in 5 of 46 (11%) cervical carcinomas, 1 of 17 (8%) samples associated with high-risk HPVs (HPV 16 and HPV 18), and 4 of 27 samples (15%) with intermediate risk HPVs, whereas no mutation was found in 2 HPV-negative cases.

Levi et al. (36) analyzed the presence of HPV DNA in a series of 84 paraffin-embedded penile carcinomas. They also investigated the presence of p53 mutations in these tumors by immunohistochemistry, SSCP and DNA sequencing. These data indicate that subsets of penile carcinomas are etiologically related to HPV and that an overlapping subset may rise from mutational events in the p53 gene.

The p53 gene regions examined in the present study represent only a fraction of this gene. However, the vast majority of known mutations identified in different primary tumors and cell lines clustered between amino acid residues 130 and 290 (11). This is a region where the DNA sequence is highly conserved among several different species (37). Although the frequency and distribution of these mutations may differ among cancers from different tissue types, p53 mutations in cervical carcinoma are localized within this region (38). Therefore, it is relatively safe to assume that we probably would have detected some p53 mutations within exons 5-8. However, one cannot exclude that some mutations are located outside the regions of the p53 gene examined by us and others (14,32,39,40). Kurvinen et al. (40) determined the state of the p53 gene in 20 genital precancer lesions and carcinomas. Exons 5-9 of the p53 gene were analyzed by SSCP-PCR, and no mutations were detected in any of the specimens, including the 3 HPV-negative cases.

Coexistence of HPV DNA and a mutated form of p53 may suggest that these cells were mutated at the p53 locus and then became infected with HPV. Alternatively, the infection was an initial event and a point mutation on the p53 gene provided an additional growth advantage to the cells. In fact, Crook et al. (41) indicated that mutations within the p53 gene in an HPV-positive primary cancer might confer a growth advantage and contribute to the acquisition of metastatic potential in these cells.

The present results may account to the existence of other tumor suppressor genes whose inactivation or loss of function is important for cervical carcinogenesis. This is compatible with the fact that the frequency of p53 mutation reported in these tumors is low when compared with other cancers. Our results confirm that the occurrence of somatic mutations in the hot spot region of the p53 gene is indeed very low in HPV-positive cervical carcinoma.

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