Gene Mutations in Esophageal Mucosa of Chagas Disease Patients

FERNANDA DA SILVA MANOEL-CAETANO, APARECIDA F.P. SILVEIRA and ANA ELIZABETE SILVA

Department of Biology, UNESP, São Paulo State University, São José do Rio Preto, SP, Brazil

Abstract. Background: Chagas disease patients with longstanding megaesophagus have a significantly increased risk for esophageal carcinoma. Materials and Methods: PCR-SSCP analysis and DNA sequencing of esophageal mucosa from Chagas disease patients (with or without megaesophagus) in the exons of the TP53, CDKN2A and FHIT genes were performed. Results: SSCP analysis showed a mobility shift in 2/20 patients with grade IV megaesophagus (CDKN2A exon 1 and FHIT exon 7) and in 1/10 patients without megaesophagus (TP53 exons 5 and 8). However, DNA sequencing indicated a silent mutation in exon 7 of the FHIT gene (GCT → GCC) in codon 88 in only one case of megaesophagus. Conclusion: Gene mutations are rare events in the exons investigated in esophageal mucosa of Chagas disease patients and further investigations are important to clarify the possible involvement of this silent mutation in exon 7 (FHIT gene) in the advanced grades of megaesophagus and esophageal carcinogenesis.

Patients with longstanding chagasic megaesophagus (esophagus dilatation) due to achalasia in Chagas disease caused by Trypanosoma cruzi have a significantly increased risk of developing esophageal squamous cell carcinoma (ESCC), with an incidence ranging from 0.53% to 10% (1-3). This is probably due to the chronic esophagitis resulting from stasis, in addition to the action of bacteria in suspension in the lumen (4) that can induce increased proliferation of esophageal epithelial cells and subsequently dysplasia (5, 6). This could be the first step in the development of esophageal carcinoma (7).

Previous immunohistochemical studies have focused on proteins p53, p16 and p21, proliferating cell nuclear antigen (PCNA) and Ki-67 nuclear antigen, showing hyperplastic alterations and higher cellular proliferation rates in idiopathic achalasia patients, associated or not with esophageal carcinoma (5, 8-10). There is, however, a shortage of studies about genetic alterations in chagasic megaesophagus. Therefore, this paper is an attempt to establish if mutations in genes that may be related to early steps of esophageal carcinogenesis (TP53, FHIT, CDKN2A) are frequent events in the esophageal mucosa of patients with megaesophagus due to Chagas disease.

Materials and Methods

The study was performed with esophageal mucosa biopsy samples from 30 Chagas disease patients with confirmed serological and molecular tests (20 with megaesophagus alone/or associated with other clinical forms, and 10 with indeterminate/or isolated forms, but without megaesophagus). Histologically normal esophageal mucosa samples from 10 healthy individuals who had undergone endoscopic examination due to dyspeptic complaints were also obtained. All biopsies were performed on the middle and distal esophagus, at the Hospital de Base, São José do Rio Preto, SP, Brazil, from 2004 to 2006.

The Chagas disease group was composed of seventeen male and thirteen female patients (x̄±s=3.5±10.8; age range: 45-83 years). The megaesophagus cases presented different degrees of esophagus dilatation, being therefore classified as grade I (1 case), grade II (3 cases), grade III (8 cases), and grade IV (8 cases), according to the classification of Rezende et al. (11). All these patients also exhibited discrete to moderate esophagitis. The control group constituted four male and six female subjects (x̄±s=7.7±13.1; age range: 26-66 years). This work was approved by the National Research Ethics Committee (CONEP, National Health Council - Ministry of Health) for research involving human beings, and written informed consent was obtained from all patients.

DNA of all samples was extracted by the phenol-chloroform-isooamyl alcohol method, prior to precipitation with two volumes of ethanol in 3M potassium acetate (pH 6.0) (12, with modifications). After centrifugation, the pellets were resuspended in 100 μl ultrapure water, and DNA was quantitated with an ND-1000 Spectrophotometer (NanoDrop, Uniscience Inc., São Paulo, SP, Brazil).

The primers used for PCR amplification of the TP53 gene (exons 5 to 8) were: 5'-CAG TAC TCC CCT GCC TCT AA-3', 5'-CAC CAT CGC TAT CTG AGC AG-3' for exon 5; 5'-TGA CCT GGA GTC TTC CAG TG-3', 5'-TGG TCC CCA TGA GAA TAC TA-3' for exon 6; 5'-TGA CCT GGA GTC TTC CAG TG-3', 5'-TTA
CGG CTC TAA CAC TGA GG-3’ for exon 7; and 5’-CCT ATC CTG AGT AGT GGT AA-3’, 5’-GCT CCA TTC GTT CGT CCT G-3’ for exon 8. For the FHIT gene (exons 5 and 7), the primers were: 5’-GGC ATC TCT TTT GCA ACT AAG AAT C-3’, 5’-TGG CTG GTT AGG CTC AGA CTG GT-3’ (exon 7). The primers for the CDKN2A gene (exons 1 and 2) were: 5’-GAA GAA AGA GGA GGG GCT G-3’, 5’-GCC GTA CCT GAT TCC AAT TC-3’ (exon 1) and 5’-GGGA AAT TGG AAA CTG GAA GC-3’, 5’-TCT GAG CTT TGG AAG CTC T-3’ (exon 2).

The PCR reaction mixtures (45 μl) contained 60 mM Tris-SO₄ (pH 8.9), 18 mM ammonium sulphate, 1.5 mM (for TP53 exons 6 and 8, FHIT exon 7, and CDKN2A exons 1 and 2), 2.0 mM (for TP53 exons 5 and 7), or 4.0 mM (for FHIT exon 5) MgSO₄, 200 μM of each dNTP, 0.5 μM of each primer, 1 U of Platinum Taq DNA polymerase high fidelity (Invitrogen Inc., São Paulo, SP, Brazil), 5% (TP53 exons 5, 7 and 8, and CDKN2A exon 1), 7% (FHIT exon 7) or 10% (TP53 exon 6, FHIT exon 5 and CDKN2A exon 2) glycerol, and 100 ng genomic DNA. The PCR amplification conditions used were: pre-denaturation at 94°C for 3 min, followed by 35 denaturation cycles at 94°C for 45 s; annealing at 55°C for 1 min (TP53 exons 5, 7, 8), 57°C for 30 s (FHIT exon 7), or 60°C for 30 s (FHIT exon 5 and CDKN2A exon 2) or for 1 min (TP53 exon 6, CDKN2A exon 1); extension at 72°C for 2 min, and final extension at 72°C for 7 min. The amplified products were electrophoresed in 1.5% agarose gel containing 2 μg/mL ethidium bromide in TBE buffer (89.1 mM Tris base, 89.9 mM Boric acid, 2.5 mM EDTA, pH 8.3), and the gels were examined under an ultraviolet light transilluminator.

For SSCP analysis, the PCR products (4 μl) were mixed with an equal volume of gel loading solution (98% deionized formamide, 10 mM EDTA pH 8.3), and the gels were examined under an ultraviolet light transilluminator.

For SSCP analysis, the PCR products (4 μl) were mixed with an equal volume of gel loading solution (98% deionized formamide, 10 mM EDTA pH 8.3), and the gels were examined under an ultraviolet light transilluminator. The PCR products of the SSCP-positive cases were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocol, and direct sequencing was performed by the dideoxy-chain termination method, using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster, CA, USA) according to the manufacturer’s instructions. The samples were analyzed in an ABI Prism 377 Automated Sequencer (Applied Biosystems Inc.), and the sequences obtained were aligned with sequences deposited at the GenBank, using the BioEdit Sequence Alignment Editor (13).

Results

The DNA from 30 esophageal mucosa biopsy samples (20 chagasic megaesophagus (ME) and 10 chagasic without megaesophagus (CH)) were studied by PCR-SSCP to detect gene mutations involving genes TP53 (exons 5 to 8), FHIT (exons 5 and 7) and CDKN2A (exons 1 and 2). In the SSCP analysis of these DNA samples, a mobility shift (SSCP-positive) with regard to the electrophoretic mobility of each exon in normal esophageal mucosa samples was observed in three cases: two with grade IV megaesophagus (case ME13/CDKN2A exon 1, and case ME16/FHIT exon 7), and one without megaesophagus (case CH4/TP53 exons 5 and 8) (Figure 1A). DNA sequencing of these three samples showed that in cases CH4 and ME13, the DNA sequences were normal. However, in case ME16, sequencing showed a single nucleotide polymorphism (SNP) in exon 7 of the FHIT gene, consisting of a T to C transition (GCT → GCC) in the alanine-encoding codon 88 (Figure 1B), classified as a silent mutation.

Discussion

This study shows for the first time a silent mutation in codon 88 of exon 7 of the FHIT gene in a patient with advanced chagasic megaesophagus (grade IV), while exons 5 to 8 and exons 1-2 of genes TP53 and CDKN2A, respectively, were not confirmed to be altered.

Previously, Safatle-Ribeiro et al. (14) reported a mutation involving exon 6 of the TP53 gene in one out of 16 patients with advanced chagasic megaesophagus, and also mutations in exons 5 and 7 of this gene in two out of four esophageal carcinomas that developed in patients with megaesophagus due to Chagas disease. FHIT point mutations are uncommon, being observed in less than 5% of all tumors (15). In the present study, it was possible to demonstrate the presence of point mutations in 5% (1/20) of the megaesophagus cases due to Chagas disease.

The silent mutation detected in exon 7 of the FHIT gene in this study has been described as a polymorphism in the literature (16) and considered neutral (17) because no amino acid change occurs. This apparent polymorphism was also described by other investigators in esophageal cancer, ulcerative colitis, head and neck cancer cell lines, adenocarcinomas of the pancreas, and both malignant and benign breast tissues, at frequencies ranging from 12.5% to 42.8% (18-21).

Recently, synonymous polymorphisms were reported to be responsible for causing inactivation of the native splicing donor site, which results in a premature stop-codon or exon skipping, yielding a shorter mRNA (22). Some studies also showed that missense mutations and silent base substitutions in exonic regulatory sequences (ERSs) may cause exon skipping in different disease-related genes (23, 24). These ERSs play an important role in the correct splice-site identification because of their function as binding sites for specific serine/arginine-rich (SR) proteins (25). Çeçener et al. (21) believe that this silent alteration may cause a new splicing model in exon 7. Thus, splicing deregulation was suggested as an alternative mechanism for the inactivation of the tumor suppressor gene by decreasing the level of functional mRNA (21).
Reduced FHIT protein expression has been observed in approximately 33-45% of all esophageal dysplastic lesions (26) and in premalignant lesions of the esophagus, stomach, cervix and other organs, suggesting that FHIT inactivation occurs in early steps of carcinogenesis in many organs (21). Other studies suggest that silent polymorphisms can affect protein expression and the timing of co-translational folding, changing the structure of substrate and inhibitor interaction sites (27-29).

FHIT protein expression has been shown to modulate the expression of checkpoint proteins Hus1 and Chk1 at mid-S checkpoint, leading to induction of apoptosis upon DNA damage (30). Hence, frequent silent polymorphisms observed in the FHIT gene in certain tumors can allow cells to escape checkpoint control, contributing to cell proliferation, survival, increased genomic instability and tumor progression, due to failure in the FHIT-checkpoint protein interaction. Thus, this protein might be the first line of defense against the earliest stages of cancer development (30, 31).

This study is pioneering in screening TP53, FHIT and CDKN2A gene mutations in megaesophagus samples from Chagas disease patients by using PCR-SSCP analysis and DNA sequencing. Previously, aneusomies of chromosomes 7, 11 and 17 were detected in 60% of chagasic megaesophagus cases (32), reinforcing the hypothesis raised.

![Figure 1](image_url)

**Figure 1.** A) Single-strand conformation polymorphism (SSCP) analysis of genes TP53, CDKN2A and FHIT in chagasic patients without megaesophagus (CH) and with megaesophagus (ME). The arrows indicate polymorphic bands detected in cases CH4 (TP53 gene exons 5 and 8), ME13 (CDKN2A gene exon 1), and ME16 (FHIT gene exon 7), analyzed on 6% or 7.5% silver-stained polyacrylamide gels, in comparison to the wild-type bands observed in cases CH5, CH6 and ME12, and in two normal mucosa cases (C4 and C10). B) Electropherogram obtained after direct sequencing of the FHIT gene exon 7 from DNA of an esophageal sample from megaesophagus patient (case ME16), showing T to C transition (arrows).
by Duesberg (33) that aneuploidy is an early event of the initial stages of carcinogenesis, due to abnormal dosages and expression of thousands of normal genes.

In conclusion, these results indicate that TP53, FHIT and CDKN2A point mutations are not frequently occurring events in chagasic megaesophagus, at least in the exons evaluated here. However, the presence of a silent mutation in exon 7 of the FHIT gene, observed in a patient with advanced megaesophagus, is a finding whose relevance cannot be disregarded, considering the same silent alteration was also detected in two esophageal squamous cell carcinoma samples (data not published). Further studies are necessary to investigate the possible involvement of this polymorphism that can alter gene expression in the higher grades of megaesophagus and advanced stages of esophageal carcinogenesis.

Acknowledgements

We thank Dr. Paula Rahal for technical assistance with the DNA sequencing procedure. This work was sponsored by the São Paulo State Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP).

References


Received December 22, 2008
Revised January 20, 2009
Accepted February 3, 2009