Analysis of R213R and 13494 g → a polymorphisms of the p53 gene in individuals with esophagitis, intestinal metaplasia of the cardia and Barrett’s Esophagus compared with a control group

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Abstract  Protein p53 is the tumor suppressor involved in cell cycle control and apoptosis. There are several polymorphisms reported for p53 which can affect important regions involved in protein tumor suppressor activity. Amongst the polymorphisms described, R213R and 13949 g → a are rarely studied, with an estimate frequency not yet available for the Brazilian population. The purpose of this study was to investigate the genotype and allele frequencies and associations of these polymorphisms in a group of patients with altered esophageal tissue from South Brazil and compare with the frequency observed for a control population. A total of 35 patients for R213R and 45 for 13494 g → a polymorphisms analysis with gastro-esophageal reflux disease (GERD) symptoms diagnosed by upper digestive endoscopy and confirmed by biopsy were studied. For both groups, 100 controls were used for comparison. Loss of heterozygosity (LOH) was also analyzed for a selected group of patients where normal and affected tissue was available. There was one patient with Barrett’s Esophagus (BE) showing LOH for R213R out of two heterozygous samples analyzed and two patients (esophagitis and BE) for 13494 g → a polymorphism. We also aimed to build a haplotype for both polymorphisms collectively analyzed with R27P polymorphism, previously reported by our group. There were no significant differences in allele and genotype distribution between patients and controls. Although using esophagitis, intestinal metaplasia of the cardia and BE samples, all non-neoplastic lesions, we can conclude that these sites do not represent genetic susceptibility markers for the development and early progression of GERD to BE and esophageal cancer. Additional studies are required in order to investigate other determiners of early premalignant lesions known to predispose to esophageal cancer.

Keywords  Polymorphism · p53 · Esophagus

Abbreviations
BE    Barrett’s esophagus
IMC   intestinal metaplasia of the cardia
TP53  p53 gene
LOH   loss of heterozygosity
GERD  gastroesophageal reflux disease

Introduction
Barrett’s Esophagus (BE) is a complication of the gastro-esophageal reflux disease being an acquired premalignant disorder that predisposes to the development of adenocarcinoma of the esophagus in 10% of the patients. It is also used as a model to investigate the order of genetic events in neoplastic progression (Audrézet et al. 1996).

The progressive evolution of the normal esophageal epithelium into the columnar-type epithelium in BE is characterized by a complex sequence of events beginning...
with inflammation of the normal squamous epithelium (esophagitis), resulting from chronic reflux, followed by intestinal metaplasia of the cardia (IMC) and BE, increasing levels of dysplasia, and adenocarcinoma. This process is modulated by environmental stimuli and genetic predisposition that result in genomic instability. Cells with multiple developed genetic abnormalities result in cellular clones with acquired genetic errors. Some clones acquire proliferative advantage and may develop capacity for invasion, resulting in neoplasia (Blount et al. 1990).

Several studies have focused on the metaplasia-adenocarcinoma transition while little is still known with respect to molecular factors that contribute to the development of alterations preceding intestinal metaplasia (Reid et al. 1987; Rabinovitch et al. 1989; Reid et al. 1992). The activity of several genes has been used to pinpoint alterations in the progression that leads to dysplasia and adenocarcinoma.

Neschat et al. (1994) described the molecular alterations related to esophagus neoplasia. Gene mutations in the tumor-suppressing p53 gene were the abnormalities most commonly reported. The incidence of mutations tends to increase with the metaplasia-dysplasia-adenocarcinoma progression, ranging from rare in non-dysplastic epithelium to up to 90% in adenocarcinoma (Neschat et al. 1994; Fitzgerald 2005).

More than 90% of the mutations described for the p53 gene occur between exons 4 and 9, which encompass protein domains II-V. This region is evolutionarily highly conserved and involves the DNA-binding domain, essential for the activity of p53 (Donehower and Bradley 1993; Soussi 1996).

p53 gene mutations can damage its DNA-binding or transactivation functions, thereby inhibiting its key role in the cell cycle control. In the majority of these cases where the mutation is recessive, tumor cells often retained only the mutated allele and lose the wild type, while blood cells harbor both alleles. This loss is named loss of heterozygosity (LOH) (Baccouche et al. 2003).

Chromosomal regions with frequent LOH may point out those genes with higher susceptibility and help to understand the molecular events involved in carcinogenesis (Hu et al. 1999).

Mutations in tumor suppressor p53 gene have been detected in more than 25% of patients with Barrett’s metaplasia submitted to endoscopy and in more than 75% of Barrett’s adenocarcinomas (Aldulaimi and Jankowski 1999).

In addition to cancer-associated mutations, at least 200 polymorphisms have already been described at the p53 gene, both in coding and noncoding regions (IARC, NCI, NCBI, 2007), although most of them are located in introns outside the consensus region of splicing sites.

Studies indicate that the preferential polymorphic alleles of the gene may be involved with susceptibility and predisposition to cancer. (Soulitzis et al. 2002; Olschwang et al. 1991; Baccouche et al. 2003).

Among these, the polymorphism R213R in exon 6 and the polymorphism 13494 g → a in intron 6 are rarely studied, with an estimate frequency not yet available for the Brazilian population (IARC). The R213R polymorphism retains the arginine amino acid, the most frequent codon being CGA and the polymorphic, CGG. The polymorphism 13494 g → a shows adenine replacing guanine. No phenotypic differences were reported in both cases.

Our study aimed to estimate the frequency of R213R and 13494 g → a polymorphisms in control individuals, who represent the population of Porto Alegre, and in individuals with alterations in the esophageal tissue from the southernmost region of Brazil. In addition, we studied the potential association between the frequencies of this polymorphism in these two groups. The analysis of normal and altered tissue obtained from some of the patients allowed us to check for the existence of loss of heterozygosity in these regions of the gene. Furthermore, it also purports to build a haplotype for both polymorphisms collectively analyzed with the R27P polymorphism of exon 4 of the TP53, previously analyzed by our group (Leistner-Segal et al. 2006).

Materials and methods

Study design

This is a case control study designed to determine the relative frequency of three p53 polymorphisms in controls versus patients with GERD and complications such as BE. In addition, the study was designed to identify LOH at these loci in patients.

Patients and controls

A total of 35 patients (17 female and 18 male; ages ranging between 23 years and 82 years; average of 51.1 years) with gastroesophageal reflux disease (GERD) symptoms diagnosed by upper digestive endoscopy and confirmed by biopsy were analyzed for the R213R polymorphism, while for the 13494 g → a polymorphism analysis a total of 45 individuals (22 female and 23 male; ages ranging between 23 years and 82 years; average of 51.4 years) were used. The biopsies were analyzed according to Segal et al. 2004. For controls, 100 donor samples from Hospital de Clínicas of Porto Alegre Blood Bank were analyzed. This project was approved by the Ethic Committee of HCPA (03191).
and National Committee for Ethics and Research (7945). All procedures were performed with written informed consent of the patients.

**DNA extraction**

Ten milliliters of peripheral blood in EDTA was withdrawn from controls and patients and DNA was isolated using the salting out procedure described by Miller et al. (1988). In addition, tissue from the lesion site was obtained by biopsy at endoscopy from patients showing esophageal alterations. DNA extraction from tissue was performed freshly, carried out as previously described (Leistner-Segal et al. 2006). Because we did not perform laser microdissection in the affected tissue, it is possible that these samples may also contain cells carrying normal DNA.

**Conditions for polymerase chain reaction (PCR)**

The long PCR technique of exons 5 to 8 of the p53 gene was set as standard. Hence, exon 5 forward (5'CTGCCCTGACTTTCAACTCTG3') and exon 8 reverse (5'TGCACCCTTGTCTCCTCAC3') primers were used, according to Segal et al. 2004 (31). Amplifications were carried out using approximately 200 ng DNA, 1 U Taq DNA Polymerase (Invitrogen™), 5 l enzyme buffer (Tris-HCl 200 mM, KCl 500 mM), 5 l dNTP 0.2 mM (Invitrogen™), 1.5 l MgCl2 (50 mM), and 1.0 l of each specific primer at a concentration of 20 pmol for a final reaction volume of 50 l.

PCR conditions were: initial denaturation at 94°C for 3 min., followed by 35 cycles of 40 s, with 94°C of denaturation, 55°C of annealing and extension at 72°C, and final extension of 72°C for 10 min. The amplified fragment of 1619 pb was submitted to 1.5% agarose gel electrophoresis and visualized through ethidium bromide staining.

**Digestion with restriction enzymes**

**R213R (exon 6) polymorphism**: to identify the R213R polymorphism, digestion of the long PCR product was carried out using the *Msp*I (New England BioLabs™) enzyme to cleave the C'CGG site, according to the manufacturer’s instructions. The digestion used 10 µl of the PCR product and 2 U of the enzyme in a total final volume of 30 µl. The reaction was incubated at 37°C in dry bath. Fragments resulting from cleavage of the PCR product with the *Msp*I enzyme are 124, 356, 276, 299, 168, 276 and 120 bp in the presence of the CCG sequence, and 124, 632, 299, 168, 276 and 120 bp in the presence of the CAG sequence.

Both digestion products were subject to electrophoresis in 3% agarose gel. The band pattern found is visualized in Fig. 1.

**13494 g→a (intron 6) polymorphism**: to identify the 13494 g→a polymorphism, digestion on the long PCR product was carried out using the *Msp*I enzyme to cleave the C'CGG site, according to the manufacturer’s instructions. The digestion used 10 µl of the PCR product and 2 U of the enzyme in a total final volume of 30 µl. The reaction was incubated at 37°C in dry bath. Fragments resulting from cleavage of the PCR product with the *Msp*I enzyme are 124, 356, 276, 299, 168, 276 and 120 bp in the presence of the CCG sequence, and 124, 632, 299, 168, 276 and 120 bp in the presence of the CAG sequence.

Both digestion products were subject to electrophoresis in 3% agarose gel. The band pattern found is visualized in Fig. 1.

The statistical analysis of results was performed using a Pearson’s chi-square test in the MedCalc™ software.

**Loss of heterozygosity analysis**

For this study, the DNA extracted from blood and affected tissue of the same patient was analyzed. Only individuals who presented a heterozygous pattern in their blood were considered (n = 2 for the R213R polymorphism and n = 12 for the 13494 g→a polymorphism), respectively 8% and 33% of the sample for each polymorphism.

**Results**

**Clinical signs**

All patients showed signs of GERD. For R213R polymorphism analysis, 20% (7/35) showed esophagitis, 37.1% (13/45) showed IMC and 42.9% (15/35) showed BE. For the 13494 g→a polymorphism, 27% (12/45) showed esophagitis, 37.8% (17/45) IMC and 35.6% (16/45) BE.

**Long PCR technique standardization**

The presented methodology proved to be adequate for amplification of a relatively long segment involving four significant exons for the p53 protein function, as shown in Fig. 1.

**Identification of R213R (exon 6) polymorphism**

In both groups of patients and controls a predominance of the allele (A) was noticed, with no homozygosis for the polymorphic allele (G) being reported. This was only found in heterozygosis in two individuals in the patients group and four in the control group, as shown on Table 1. The frequencies found for this polymorphism were notably similar in both groups, without significant difference for
genotypic and allelic analysis \((P = 0.958\) and \(P = 0.957\), respectively).

The two patients presenting the allele (G) in heterozygosis showed IMC and BE.

These results were compared with literature data and no significant difference was observed among frequencies of the polymorphic allele \((P = 0.978)\) (Akar et al. 1999). When comparing with an American population, no significant difference was found, not even in comparison with Afro-American, Caucasian and Hispanic subpopulations within the same study \((P = 0.913, P = 0.726, P = 0.736\) and \(P = 0.687\), respectively) (NCI).

Figure 1 shows the results of TaqI digestion, highlighting the possible alleles.

Identification of the 13494 g → a (intron 6) polymorphism

In both groups of patients and controls predominance of the allele (G) was observed, with homozgyosis found for the polymorphic allele (A) in four individuals from the control group and none in the patient group, as shown in Table 2.

Similar to the R213R polymorphism, the frequency analysis of the 13494 g → a polymorphism did not present a significant difference when comparing the patients with the control group for both the genotypic and allelic analysis \((P = 0.607\) and \(P = 0.395\), respectively).

The patients that present the polymorphic allele (A) showed esophagitis (4/45), IMC (5/45) and BE (3/45).

When the frequencies found in our population were superimposed over the literature data, a significant difference was noted. The population analyzed in the Akar et al. 1999 study showed a frequency of 0.3 polymorphic alleles, thus differing from the findings in this study, which were 0.165 \((P = 0.008)\).

When comparing to an American population, no significant difference was found, not even in comparison with Afro-American, Caucasian, Hispanic and Pacific Coast subpopulations within the same study \((P = 0.928, P = 0.404, P = 0.467\) and \(P = 0.723\), respectively) (Shahseen and Ransohoff 2002).

Figure 1 shows the result of MspI digestion, highlighting the possible alleles.

Loss of heterozygosity

The analysis consisted in pairs of blood and tissue samples from the same patient.

Table 1 Genotype and allele frequency of the R213R polymorphism in patients and controls

| Group          | Genotype | Allele  |
|----------------|----------|---------|
|                | AA       | AG      | GG  | A  | G  |
| Controls \((n = 100)\) | 96 (0.960) | 4 (0.040) | 0 (0.00) | 196 (0.980) | 4 (0.02) |
| Patients \((n = 35)\) | 33 (0.943) | 2 (0.057) | 0 (0.00) | 68 (0.971) | 2 (0.028) |

Table 2 Genotype and allele frequency of the 13494g → a polymorphism in patients and controls

| Group          | Genotype | Allele |
|----------------|----------|--------|
|                | AA       | AG     | GG  | A   | G   |
| Controls \((n = 100)\) | 4 (0.040) | 25 (0.250) | 71 (0.710) | 33 (0.165) | 167 (0.835) |
| Patients \((n = 45)\) | 0 (0.000) | 12 (0.267) | 33 (0.733) | 12 (0.133) | 78 (0.867) |
One case of LOH (4.0%), out of two heterozygous samples analyzed, was found for the R213R polymorphism, a patient carrying BE. For the 13494 g → a polymorphism, LOH was confirmed in two patients (7.4%), a carrier of esophagitis and another one BE. The polymorphic allele present in the peripheral blood sample was not kept in the tissues of these two patients, confirming the stress in such tissues even when the wild allele was preserved.

Haplotype analysis for the R213R, 13494 g → a and R72P polymorphisms

The R213R and 13494 g → a polymorphisms were jointly analyzed with the R27P polymorphism, previously reported by us using the same group of patients (Leistner-Segal et al. 2006). Analysis consisted of 26 patients for the three polymorphisms collectively. The genotypic patterns obtained are shown in Table 3.

Table 3 Haplotype analysis involving R213R, 13494 g → a and R72P polymorphisms of TP53

| Patient | R213R | 13494 g → a | R72P | Histological type |
|---------|-------|-------------|------|------------------|
| Case 1  | AA    | GG          | PR   | BE               |
| Case 2  | AA    | AG          | RR   | BE               |
| Case 3  | AA    | AG          | RR   | BE               |
| Case 4  | AA    | GG          | PR   | BE               |
| Case 5  | AA    | AG          | PR   | IMC              |
| Case 6  | AA    | GG          | PR   | ESOPHAGITIS      |
| Case 7  | AA    | AG          | RR   | ESOPHAGITIS      |
| Case 8  | AA    | GG          | RR   | IMC              |
| Case 9  | AA    | GG          | RR   | BE               |
| Case 10 | AA    | GG          | RR   | IMC              |
| Case 11 | AA    | GG          | PR   | BE               |
| Case 12 | AA    | GG          | PR   | BE               |
| Case 13 | AA    | AG          | PR   | IMC              |
| Case 14 | AA    | GG          | RR   | ESOPHAGITIS      |
| Case 15 | AA    | AG          | PR   | IMC              |
| Case 16 | AA    | GG          | RR   | IMC              |
| Case 17 | AG    | AG          | PR   | BE               |
| Case 18 | AA    | GG          | RR   | IMC              |
| Case 19 | AG    | GG          | RR   | IMC              |
| Case 20 | AA    | AG          | PR   | IMC              |
| Case 21 | AA    | GG          | RR   | ESOPHAGITIS      |
| Case 22 | AA    | GG          | RR   | IMC              |
| Case 23 | AA    | GG          | RR   | BE               |
| Case 24 | AA    | GG          | PR   | BE               |
| Case 25 | AA    | GG          | PR   | IMC              |
| Case 26 | AA    | GG          | PR   | BE               |

IMC: intestinal metaplasia of cardia, BE: Barrett’s Esophagus

The analysis demonstrates the conserved haplotype [AA, GG, RR] for the three polymorphisms. The alleles present in the previous haplotype are the most frequent in the general population, except for the R72P polymorphism that in the previous analysis carried out by our group showed predominance of the PR genotype.

In the allele frequency analysis there were no significant difference between the three histological types (esophagitis, IMC and BE) for none of the polymorphisms, R213R, 13494 g → a and R72P ($P = 0.7600$, $P = 0.8438$ and $P = 0.8557$, respectively).

Discussion

The loss of cell cycle control and the progression to cancer in TP53 events in exons 5–8 are already well established. The functional significance and the frequency of polymorphisms in these regions still remain unknown. It is believed that they may contribute to a higher susceptibility (or resistance) to developing cancer (Vos et al. 2003).

A consistent association between these polymorphisms and several types of human neoplasias may serve as argument supporting such sites as susceptibility determiners (IARC). Furthermore, mutations in flanking regions of these exons are probably rare in spite of the biological significance they present.

The genes involved with tumor genesis are potential molecular markers associated to susceptibility to cancer. We thus investigated the relationship linking the R213R and 13494 g → a polymorphisms of the p53 gene and the premalignant esophageal alterations (esophagitis, IMC and BE). Also, countless studies indicate an association between the R72P polymorphism at exon 4 of TP53 and the increased risk of developing neoplasias (Malcolm et al. 2000; Gottschlich et al. 2000; Nakano et al. 2000; Lacerda et al. 2005). Hence, we also carried out a joint analysis of the three polymorphisms described by means of a haplotype.

In the present study the genotypic distribution of the R213R and 13494 g → a polymorphisms are in agreement with the Hardy-Weinberg equilibrium in the patient group as well as in the control group. The genotypic analysis of both polymorphisms did not present a significantly different allele distribution compared to the control samples, which leads us to conclude that there is no correlation, at these stages, between such molecular findings and the histological types, although a strong ethnic variation with regard to the prevalence of these two polymorphisms (Leistner-Segal et al. 2006, Segal et al. 2004) is known to exist.

While analyzing the R213R polymorphism, Mazars et al. 1992 obtained results similar to ours for ovarian carcinoma and blood bank donor controls with prevalence...
of 3% and 2.6% for the polymorphic allele, respectively. In the analysis of the 13494 g → a polymorphism for gastrointestinal and breast tumors, Peller et al. 1995 also found results similar to ours, with frequencies of the heterozygous AG genotype of 32% and 68% for homozygous GG genotypes, for the patient group. These results match the allele frequency described by Chumakov and Jenkins 1991, which was 31% for the AG genotype, and 69% for the GG genotype.

It was anticipated that there would be a smaller proportion of the polymorphism in the exon 6 since the coding sequence is more conserved than the intronic region, mainly because the region in which this polymorphism is located carries great importance to the activity of the p53 protein. Eventual differences detected in several studies may be explained by ethnic differences between the groups analyzed (Brenna et al. 2004). Different genetic attributes may bestow protection against or risk for developing cancer in populations of diverse ancestries (Rocha 2005). However, other reasons such as sample size, DNA source, and techniques employed should also be taken into consideration (Brenna et al. 2004).

In the combined analysis with the R72P polymorphism previously studied by our group, a similar frequency was observed in both controls and patients. According to Table 3, the most frequent haplotype was [AA, GG, RR] which occurred in 34.6% of analyzed patients. Since the most frequent haplotype is the same in patient and control groups, we may think again that the absence of association between these three polymorphisms and the esophageal alterations stems from the fact that molecular alterations can only be identified in more advanced stages of the normal tissue-metaplasia-adenocarcinoma progression.

The LOH findings are in agreement with the occurrence of genomic instability seen in various types of cancer, and serve as encouragement to the understanding of DNA repair processes in replication and recombination events (Thiagalingam et al. 2002). All tissue samples presented the wild allele indicating that cells carrying the analyzed polymorphisms were heterozygotic or that many normal cells (lacking polymorphism) were present in the tissue from which DNA was extracted (Campomenosi et al. 1996, Doak et al. 2003). This fact may have minimized the LOH findings in our study.

The allelic loss associated to esophageal carcinogenesis was found by Kawaguchi for the codon 72 polymorphism (Kawaguchi et al. 2000). Abnormalities involving the TP53 are common with esophageal adenocarcinoma and have been detected in pre-malignant tissues surrounding the esophageal cancer (Prevo et al. 1999; Reid et al. 2001). Neshat et al. 1994 reported molecular and genomic alterations identifying LOH in 94% of Barrett cancers and 88% of mutations in the TP53. Allelic loss of 17p has been detected in initial stages of high-degree metaplasia and dysplasia, leading to aneuploidy and suggesting that inactivation of the TP53 is a first step in carcinogenesis. Both cases of LOH located in the intron 6 reinforce the data in literature for these events being found in altered tissues and also highlight the presence of alterations within the non-coding region. Such alterations may alter the phenotype of the protein being coded and are of considerable value in understanding the genic expression.

According to Campomenosi et al. 1996 the association of the molecular findings with the histological type occurs upon development of the BE since the type I metaplasia (common in BE) would be more responsive to damage caused by GERD and therefore more prone to developing aneuploidies than the tissues analyzed in our study (Li et al. 2005). Without doubt, this could provide an explanation to the fact that our results do not differ from those in the control population, since the tissues assayed were in early stages of BE development.

There is increasing evidence that new mechanisms including mutation in splicing sites, introns and promoter regions are involved in the genic expression. Identification of these alterations may help select patients with a higher risk of developing cancer, allowing optimization of treatments (Lacerda et al. 2005).

Considering our data regarding the incidence of alleles, genotypes and haplotypes of the R213R, 13494 g → a and R72P polymorphisms of the p53 gene in patients and controls, we can suggest that these three sites do not represent genetic susceptibility determiners to esophagitis, intestinal metaplasia of the cardia and BE. Additional studies are required in order to investigate these three polymorphisms in advanced stages of carcinogenesis.

Acknowledgements This work received editorial support from the Research and Postgraduate Group of Hospital de Clínicas of Porto Alegre (RS, Brazil) and financial support from the Research Support Foundation of Rio Grande do Sul (FAPERGS), Coordination for the Improvement of Higher Education Personnel (CAPES) and the Research Support Fund/Hospital de Clínicas of Porto Alegre (FIPA-HCPA).

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