High frequency of p53 intronic point mutations in laryngeal squamous cell carcinoma*

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Abstract Intronic point mutations are rare and totally unknown for human laryngeal squamous cell carcinoma (LSCC). To explore the relationship of p53 gene intronic mutation to the development of human LSCC, DNA was extracted from both tumor tissues and matched normal tissues of 55 patients with LSCC in northeast of China. Polymerase chain reaction amplification-single strand conformational polymorphism (PCR-SSCP) combined with silver staining and DNA direct sequencing were used to detect mutations in exons 7~8 (p53E7 and p53E8) and introns 7~8 (p53I7 and p53I8) of p53 gene. The p53E7 mutation was detected in 17 out of 55 patients, and the p53I7 mutation in 21 patients. No mutation was found at p53E8 or p53I8 site. The difference between tumor group and paired normal group on the rates of both p53E7 and p53I7 mutations was statistically significant. The rate of p53I7 mutations in tumor tissue was higher than that of normal tissue, and so was that of p53E7. Sequence analysis revealed that most p53I7 mutations were at the nucleotides in the branch point sequence or the polypyrimidine tract in the 3′-splice acceptor site of the intron 7. The high incidence of p53 gene intronic mutation in LSCC indicates that genetic changes within the noncoding region of the p53 gene may serve as an alternative mechanism of activating the pathogenesis of human laryngeal squamous cell carcinoma. Mutations in the noncoding region of this gene should be further studied.

Keywords: laryngeal squamous cell carcinoma, p53 gene intronic mutation, PCR-SSCP.

Mutations of the p53 gene are widely detected in all kinds of human cancers. Point mutations in exons 5~8 occur frequently in many human tumors including human laryngeal squamous cell carcinoma (LSCC)[1, 2]. However, intronic point mutations are rare and totally unknown for human laryngeal squamous cell carcinoma. Intronic mutations may affect gene regulation through aberrant splicing or disruption of critical DNA-protein interactions. Any mutations in the polypyrimidine (py) tract and the branch point consensus sequence of introns will affect splicing by interfering with efficient spliceosome assembly and splicing of pre-mRNAs. In order to determine the association of p53 gene mutation with LSCC, we analyzed exons 7 and 8 (p53E7 and p53E8) as well as introns 7 and 8(p53I7 and p53I8) of the p53 gene from both laryngeal squamous cell carcinoma tissues and paired normal tissues of 55 patients in northeastern China by polymerase chain reaction amplification-single strand conformational polymorphism (PCR-SSCP) and sequencing analysis. The results demonstrated that the rate of p53I7 mutation in tumor tissue was higher than that of normal tissue, and so was that of p53E7, which indicate that genetic changes within the noncoding region of the p53 gene might also serve as an alternative mechanism of activating the pathogenesis of human LSCC.

1 Materials and methods

1.1 Samples and DNA extraction

The 55 LSCC tissues and matched normal tissues were obtained from the patients in surgery. All cases were the primary LSCC patients and examined by clinical and pathological data. They did not receive radiotherapies and chemotherapies before surgeries. The normal tissues were taken from the distal resection margins that were at least 2 cm apart from the tumor tissues. All tissues were quickly frozen at −70 ℃ until analyses.

Genomic DNA was isolated from the obtained

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tissues by standard saturated sodium chloride extraction.

1.2 Primers and PCR amplification of exons 7, 8 and introns 7, 8 of the p53 gene

Primers for p53E7, p53E8, p53I7 and p53I8 were designed using the computer program Primer 3. Details of the PCR primers are shown in Table 1.

Table 1. Primers used for PCR

Exon or intron	Primer	Product size (bp)
p53E7	Forward 5′-TGG CTC TGA CTG TAC CAC CA-3′	118
	Reverse 5′-GGC TCC TGA CCT GGA GTC TT-3′	
p53E8	Forward 5′-CTA CTG GGACGGAACAGCTT-3′	137
	Reverse 5′-TGC TTA CCT CGC TTA GTG CTC-3′	
p53I7	Forward 5′-AGG TTG GCT CTG ACT GTA CCA-3′	480
	Reverse 5′-AGC TGT TCC GTC CCA GTA GA-3′	
p53I8	Forward 5′-TTT TCC TAT CCT GAG TAG TGG TAA TC-3′	316
	Reverse 5′-GGG TGA AAT ATT CTC CAT CCA G-3′	

Genomic DNA from both tumor and normal tissues (25 ng) was subjected to PCR analysis in a total volume of 25μL using *Taq* polymerase (Promega), with 200 nmol/L dNTPs and 10 pmol of each primer (synthesized by Sangon Biotech, Shanghai, China). After 8 min of denaturation at 94 ℃, samples were subjected to 30 cycles of amplification, consisting of denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 45 s and extension at 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min, on a GeneAmp PCR system 2400 (Perkin-Elmer). The resulting PCR products were electrophoresed on a 1% agarose gel.

1.3 Single strand conformational polymorphism analysis (SSCP)

After confirming the amplification by agarose gel electrophoresis, 5μL of the amplified PCR products were denatured by heating at 98 ℃ for 10 min after adding 10μL denaturing-loading buffer and then loaded onto a 6% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide, 49∶1) containing 5% glycerol and the gel was prepared in 1x TBE buffer (pH 8.0). Electrophoresis was carried out for 4~6 h in 1x TBE buffer under the conditions of 300 V for exons regions and 600 V for introns at 15 ℃. After electrophoresis, the gel was stained in $AgNO_3$, and the Ag-dyed DNA was revealed under white light.

Each fragment amplified from the normal tissue was sequenced for confirming that no mutation existed, and regarded as control. Potential mutations were displayed by shifts in band mobility. For the samples with band shifts, the PCR-SSCP analysis was repeated. Normal controls were included in each analysis.

1.4 PCR fragments purification and DNA direct sequencing

The PCR fragments that gave abnormal band shifts were subjected to direct sequencing. The same DNA was reamplified by PCR using the same primers and the product was resolved on 1% agarose gel. The band was extracted using a quick Gel Extraction Kit (Huashun, Shanghai, China). The purified DNA fragments were sequenced using ABI Prism Bigdye Terminator and ABI Prism 3700 automated sequencer.

1.5 Statistical analysis

The difference between the tumor group and normal group on p53 gene mutations were examined using the χ^2 test. Two-sided *P*-values below 0.05 were considered to be statistically significant.

2 Results

2.1 PCR-SSCP

PCR reaction amplified the full length DNA fragments of p53 exon 7, exon 8, intron 7, and intron 8 from both normal and tumor tissues in 55 cases (shown in Fig. 1).

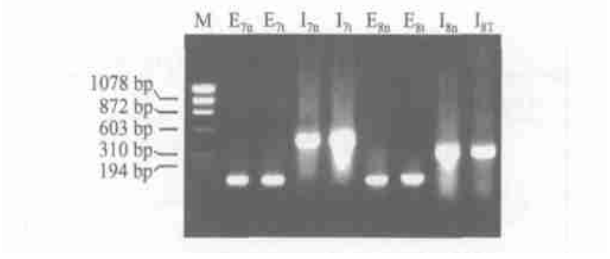


Fig. 1. PCR amplifyication of exon 7, exon 8, intron 7, and intron 8 of the p53 gene in LSCC and the controls. M, DNA marker; E_{7n}, E_{8n}, I_{7n} and I_{8n}, PCR amplified products of p53E7, p53E8, p53I7 and p53I8 from normal tissue; E_{7t}, E_{8t}, I_{7t}, I_{8t}, PCR amplified products of p53E7, p53E8, p53I7, and p53I8 from tumor tissue. The product size of p53E7, p53E8, p53I7, and p53I8 was 118, 137, 480, and 316bp respectively.

For the tumor group, band shift of p53E7 was detected in 18 out of the 55 samples (32.7%), and that of p53I7 in 21 out of the 55 samples (38.2%). No variation was observed in the electrophoretic profiles of single-strand amplified DNA of exon 8 and intron 8. In the normal group, shift in electrophoresis migration of single-strand DNA fragment of exon 7

and exon 8 was observed in 1 of the 55 samples (1.8%). No band shift was found in intron 7 or intron 8. A representative SSCP gel is shown in Fig 2.

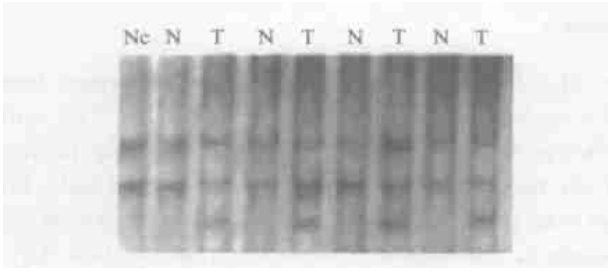


Fig. 2. A representative SSCP gel of intron 7 PCR products from 4 cases and a negative control. Nc, p53I7 of normal tissue as SSCP (−) negative system control; N, paired normal tissue, SSCP (−); T, tumor tissue, showing p53I7 band shifts, SSCP(+).

The rate of p53I7 mutations in tumor tissue was higher than that of normal tissue, and so was that of p53E7. There was a significant difference between tumor group and normal group on the rate of p53I7 mutations ($\chi^2 = 12.978$, $P < 0.01$) and that of p53E7 mutations ($\chi^2 = 8.66$, $P < 0.01$).

2.2 DNA direct sequencing

In the tumor group, exon 7 was sequenced in 10 out of 18 cases showing SSCP positive in p53E7, and intron 7 in 10 out of 21 cases. Exon 7 mutations were found in 9 of the 10 specimens and the accuracy of SSCP positive is 90%. Intron 7 mutations were found in all 10 specimens and the accuracy of SSCP positive is 100%.

In the normal group, the 2 positive SSCP samples in exon 7 and 8 respectively did not show any mutations by sequencing analysis. The difference may be caused by the false positive presence of SSCP.

In 9 cases showing exon 7 mutations, missense mutation was found in all. Simultaneously, a G insertion was found in 4 cases. No splice site mutation was found.

In 10 cases for intron 7 mutations, base pair substitutions were detected in all. The rate of transition was 51% (mainly A→G) and the rate of transversion was 49% (mainly T→G, G→T, A→T). All 10 cases had insertions and most of these changes were C insertions. Most of intron 7 mutations were located at position 14168, 14216, 14234, 14235, 14271, 14316, 14317, 14319, 14320, 14438, 14439, 14417 in the intron 7, and a C insertion was often found after position 14240 (Numbering of these variants is based on GenBank entry X54156.1 GI: 35213). 9 of the 10 samples harbored mutations at the splice acceptor site of intron 7. Details of the mutations at the splice acceptor site of the 9 cases are shown in Table 2. Fig. 3 shows a 1-bp deletion in the polypyrimidine tract of intron 7, which is located at position 14429 in the splice acceptor site of the intron 7.

Table 2. A summary of data for the 9 sequenced tumors, with splice acceptor site mutations in intron 7

Case number	Location	Sequence change	Site[a)]
1	Branch point sequence	AGGAATG→AGGAACCG	14417
2	Branch point sequence	AGGAATG→AGGAACCG	14417
3	Branch point sequence	AGGAATG→AGGAACCG	14417
4	Polypyrimidine tract	GAAGAGA→GAATGGG; GG→CC	14431-2, 4; 14438-9
5	Polypyrimidine tract	GAAGAGA→GAATGGG; GG→CC	14431-2, 4; 14438-9
6	Polypyrimidine tract	AAG→AATG; GG→CG	14430, 8
7	Polypyrimidine tract	AAG→AATG; GG→CG	14430, 8
8	Polypyrimidine tract	GAA→GA	14429
9	Polypyrimidine tract	AA→AT; GG→CC	14430; 4438-9

a) Nucleotide residue

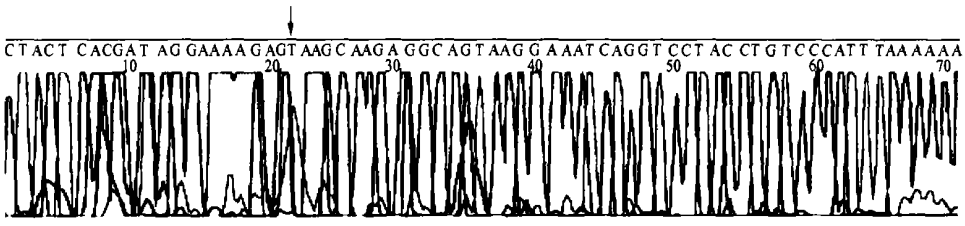


Fig. 3. Mutation in intron 7. One base was deleted at the polypyrimidine tract. The patient had an T insertion after the −21st base of intron 7.

3 Discussion

The wild-type p53 gene acts as the guardian of the genome, responding to cellular stresses such as DNA damage, ribonucleotides loss, microtubules fracture, checkpoint failure or oncogene activation by either arresting cell cycle progression to facilitate DNA repair or initiating an apoptotic pathway to remove damaged cells[1,2]. p53 protein has been demonstrated to localize in the mitotic spindles and centrosomes during mitosis and might play an important role in the regulation of the function of centrosomes[3].

Mutations in the p53 gene are the most frequently identified genetic change in human cancer and found in more than 50% of human tumors[1,4]. Loss of p53 function has been reported to result in an increased abnormality and hyperamplification of centrosomes, as well as chromosome instability (CIN) in many types of human cancers, either by loss of checkpoint control or gain of functional mutations in the presence of mitotic defects. Expression of wild-type p53 in these abnormal cells with nonfunctional p53 can significantly decrease centrosome abnormality and inhibit cell growth through G_2/M arrest[5].

Mutational inactivation of p53 has been demonstrated to initiate multiple rounds of centrosome duplication within a single cell cycle[6]. This implies that p53 regulates the centrosome duplication cycle by multiple pathways. Although the exact mechanism as how p53 is involved in centrosome regulation is not clear, evidence suggests that p53 controls the centrosome duplication cycle both in transactivation-dependent and transactivation-independent manners, and the ability to bind to unduplicated centrosomes may be important for the overall p53-mediated regulation of centrosome duplication[7]. Thus, expression of mutational p53 plays an important role in tumorigenesis through two dual ways either leading to centrosome amplification, predominantly followed by multipolar mitotic division and apoptosis, or reduced both basal and DNA damage-induced apoptosis in cells that have sustained mutant p53-related genetic abnormalities. The combination of these two ways presents a scenario in which cells are both more likely to sustain genetic aberrations and more likely to survive the presence of these aberrations, thus providing a plausible explanation for the frequency with which this p53 mutant is found in human cancers.

We examined mutations in exons 7 ~ 8 of p53 gene and found the p53E7 mutation in 17 out of 55 patients. The difference between the tumor group and paired normal group on the rate of p53E7 mutations might show that p53 mutation in exon 7 may result in the expression of mutational inactive p53, thus leading to centrosome hyperamplification and a series of mutant p53-related genetic abnormalities.

Although mutations of the p53 gene has been demonstrated to correlate with many human tumors including human laryngeal squamous cell carcinoma (LSCC) and exons 5 ~ 8 are the "hot spot", intronic point mutations are rare and totally unknown for LSCC. Now intronic mutations of the p53 gene have only been reported in a few kinds of tumors including Li-Fraumeni syndrome, chronic lymphocytic leukaemia(CML), hepatocellular carcinoma, Bloom's syndrome, multiple and malignant phaeochromocytomas, myelodysplastic syndrome, and lung cancer cell lines[8~14]. These reported mutations affected either splice donor or acceptor site, leading to aberrant splicing of p53 mRNA in all cases. To test whether intronic mutations of the p53 gene occur frequently in LSCC, we examined mutations in introns 7 ~ 8 and identified intron 7 mutations in 21 out of 55 patients. It indicates p53 mutations in intron 7 might be an important way in pathogenesis of human laryngeal squamous cell carcinoma.

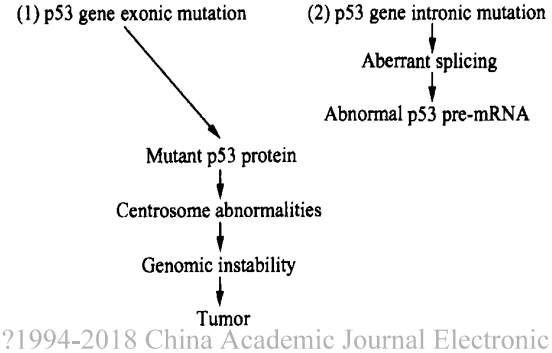
Splice site selection is mediated through the recognition of conserved sequences at the 5′- and 3′-splice sites, the branch point and the polypyrimidine tract of pre-mRNA by a number of proteins and U-rich small ribonucleoprotein particles (U snRNPs)[15]. Mutations in the branch point sequence decrease U2 binding and subsequent spliceosome formation[15]. Moreover, mutations in the branch point sequence weaken the effect of the branch-point bridging protein (BBP), defining the intron and bridging the 5′- and 3′-end of the intron prior to splicing[16]. We found 3 cases showing mutations in the branch point sequence in the sequenced 10 cases with intron 7 mutations. These mutations may lead to disordered p53 splicing and mRNA expression.

The consensus sequence polypyrimidine (py) tract downstream the branch point sequence on pre-mRNA is essential for efficient branch point utilization and 3′-splice site recognition in constitutively spliced introns. It might also play a significant role in alternative splicing. Several putative splicing factors, heterogeneous ribonucleoprotein C (hnRNP C), polypyrimi-

dine tract-binding protein (PTB), intron-binding proteins, and U2AF, a factor required for splicing of pre-mRNAs, bind the py tract. There may be specific base requirements at certain positions within the py tract. The tract length, random purine insertions, and the distance of the py tract from the branch point sequence on pre-mRNA have been shown to affect splicing[15]. The number of consecutive U(s) as well as the number of consecutive py is important for both splicing and complex formation. Specific U to purine substitutions within the py tract has a significant inhibitory effect on splicing and extensive replacement of U by C may also result in an inhibition of splicing. Thus, all mutations that could influence the polypyrimidine on pre-mRNA might reduce splicing. These mutations most likely disrupt U2 binding to the branch point, and U2AF, PTB, and hnRNP protein binding to the py tract. In the 6 cases (60% in total sequenced 10 cases) harboring mutations in the polypyrimidine tract, 5 cases had transversions from purine to pyrimidine and 1 case had transition A→G. Simultaneously, 1 case had a T insertion after 14430 and one had 1-bp deletion GAA→GA. All these mutations could abrogate the function of the py tract on pre-mRNA through transcription and subsequently lead to abnormal p53 splicing, resulting in mutant proteins without the function of wild-type p53.

It is therefore conceivable that intronic mutations may affect gene regulation through aberrant splicing or disruption of critical DNA-protein interactions. Any mutation in the polypyrimidine (py) tract and the branch point consensus sequence of introns that affected splicing did so by interferring with efficient spliceosome assembly and splicing of pre-mRNAs. Thus, p53 gene intronic mutations are associated with abnormal p53 protein, and might cause centrosome abnormalities that lead to tumorigenesis.

In summary, there may be two important pathways of p53 gene in LSCC:



High incidence of p53 gene intronic mutation was first found in LSCC. This result indicates that genetic changes within the noncoding region of the p53 gene may serve as an alternative mechanism of activating the pathogenesis of human laryngeal squamous cell carcinoma. Mutations in the noncoding region of this gene should be further studied.

References

1 Murphy, K. L. et al. A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. FASEB J., 2000, 14(14): 2291.
2 Chen, S. S. et al. Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function. EMBO J., 2002, 21(17): 4491.
3 Zhao, X. et al. Association of p53 mutation with STK15 overexpression in cancer centrosomal instability. Foreign Medical Sciences Section of Genetics (in Chinese), 2003, 26(4): 201.
4 Fang, L. et al. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. EMBO J., 2001, 20(8): 1931.
5 Ouyang, X. et al. Effect of p53 on centrosome amplification in prostate cancer cells. Biochim. Biophys. Acta., 2001, 1541(3): 212.
6 Kramer, A. et al. Centrosome replication, genomic instability and cancer. Leukemia, 2002, 16: 767.
7 Tarapore, P. et al. Difference in the centrosome duplication regulatory activity among p53 'hot spot' mutants: potential role of Ser 315 phosphorylation-dependent centrosome binding of p53. Oncogene, 2001, 20(47): 6851.
8 Lai, M. Y. et al. Splicing mutations of the p53 gene in human hepatocellular carcinoma. Cancer Res., 1993, 53(7): 1653.
9 Bromidge, T. et al. p53 intronic point mutation, aberrant splicing and telomeric associations in a case of B-chronic lymphocytic leukaemia. Br. J. Haematol., 2000, 111(1): 223.
10 Yoshimoto, T. et al. The relatively high frequency of p53 gene mutations in multiple and malignant phaeochromocytomas. J. Endocrinol., 1998, 159(2): 247.
11 Magnusson, K. P. et al. p53 splice acceptor site mutation and increased HsRAD51 protein expression in Bloom's syndrome GM1492 fibroblasts. Gene, 2000, 246(1~2): 247.
12 Wang, D. et al. Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. Cancer Res., 2000, 60(16): 4507.
13 Verselis, S. J. et al. Novel p53 splice site mutations in three families with Li-Fraumeni syndrome. Oncogene, 2000, 19(37): 4230.
14 Takahashi, T. et al. Identification of intronic point mutations as an alternative mechanism for p53 inactivation in lung cancer. J. Clin. Invest., 1990, 86(1): 363.
15 Roscigno, R. F. et al. A mutational analysis of the polypyrimidine tract of introns. Effects of sequence differences in pyrimidine tracts on splicing. J. Biol. Chem., 1993, 268(15): 11222.
16 Weaver, R. F. Post-Transcriptional Events I: Splicing. Molecular Biology. 1st ed. New York: McGraw-Hill Companies, Inc, 1999, 413~453.