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유잉육종의 p53 돌연변이

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목 적 : p53 종양억제 유전자는 사람의 암에서 변형이 가장 많이 발견되는 유전자로 유잉육종에서 p53 유전자의 변형을 관찰하고자 하였다.

재료 및 방법 : 유잉육종 환자 35례의 파라핀 블록을 사용하였으며, 유전자의 결핍과 p53 유전자의 염기서열의 변형을 관찰하였다.

결 과 : 정성적인 중합효소 연쇄반응을 이용한 p53의 4-9번까지의 유전자검사중 2례에서 동일성의 유전자 결핍이 관찰되었으며, exon 5-8의 유전자 중합효소 연쇄반응에서는 3례에서 missense 점돌연변이가 관찰되었다.

결 론 : 이상의 결과로 p53은 유전적으로 적은 부분에서 유잉육종에 관여하는 것으로 관찰되었다.

색인 단어 : 유잉육종, p53, 돌연변이

INTRODUCTION

Ewings sarcoma, an aggressive osteolytic tumor with a marked propensity for dissemination, comprises approximately 6~10% of biopsied primary malignant bone tumors and is the fourth most common primary malignancy of bone, following myeloma, osteosarcoma and chondrosarcoma[4]. Several studies have clearly documented a characteristic t(11;22)(q24;q12) chromosomal translocation in Ewing's sarcoma[17]. However, molecular genetic events, which contribute to the development or progression of Ewing's sarcoma, have been poorly characterized.

The tumor suppressor p53 plays a central role in preserving genomic integrity of the cell by arresting cell cycle progression or activating apoptosis in response to genotoxic damage[1,12]. The growth inhibitory functions of p53 are thought to result from its ability to modulate transcription of several cell cycle(or apoptosis) related genes such as $p21^{Waf1}$, Bax, or Gadd45 by binding to p53 response elements[6,16,22]. This DNA binding property and the ability to modulate gene expression is usually abrogated by the

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mutations in p53 seen in approximately 50% of human tumor cells[7]. p53 has been shown to suppress a variety of promoters including interleukin-6 or Bcl-2 through its interaction with several transcription factors such as TATA-binding protein, Sp1 or through its direct binding to a p53-dependent negative response element[13,15,21]. Mutations in p53 that occur in human cancer cells frequently affect hot spots in the region encoding the DNA-binding domain and thus inactivates its properties as a sequence-specific transcription factor, supporting the importance of transcriptional control in mediating the biological consequenses of p53 activation. These observations imply that p53 modulates transcription of multiple growth-related genes. Accumulation of p53 following treatment with DNA damage-inducing agents is associated with an arrest of the cell cycle and induction of apoptosis. p53-induced G_1 arrest of the cell cycle is believed to allow for DNA repair or apoptosis, thus preventing an accumulation of mutations[6,14].

The MDM2 gene, which maps to chromosome band 12q13, is transcriptionally induced by p53, and works as a feedback inhibitor by promoting p53 protein degradation and inhibiting p53 transcriptional activities[2]. High incidence of MDM2 amplification has been reported in soft tissues tumor, osteosarcoma and less frequently in esophageal, brain and breast carcinoma[18]. Tumors harboring MDM2 amplification or with MDM2 overexpression typically contain the wild type p53, leading to the postulation that overexpression of MDM2 may be an alternative to the mutational inactivation of p53.

It has been demonstrated that approximately one-third of the sarcomas have some type of DNA alteration at the p53 locus[1]. Mutational alterations of p53 have been frequently found in Ewing's sarcoma cell lines[8]. However, further analysis of primary Ewing's sarcoma tissues showed infrequent occurrence (5~20%) of p53 mutation, which can be identified only from patients with an advanced stage disease, indicating that mutations of p53 in Ewing's sarcoma is a rare event and might represent late genetic events related to tumor progression[5,8,9]. However, p53 mutations were 10-fold enriched in cell lines, thus indicating a selective growth advantage in vitro. It was also reported that MDM2 gene amplification occurs in approximately 10% of Ewing's sarcoma and is associated with advanced tumor stage[10]. However, data on the involvement of p53 alterations in the development and progression of Ewing's sarcoma are still limited.

To characterize the mutational status of p53 and its inhibitor gene, MDM2 in Ewing's sarcoma tissues, we have performed mutation and expression analyses of p53 and abnormal amplification of MDM2 in 35 Ewing's sarcoma tissue specimens using a quantitative PCR, PCR-SSCP, DNA sequencing analyses.

MATERIALS AND METHODS

1. Tissue Specimens

Total 35 Ewing's sarcoma tissue specimens were obtained from the Department of Pathology in the Kyung Hee University (Seoul, Korea) and the Department of Pathology, Mayo Clinic, Mayo Foundation (Rochester, MN). Two paraffin sections were prepared from the same tumor tissues and used for separately for molecular analysis of p53.

2. Extraction of Genomic DNA

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues using the method by Stanta and Schneider with a slight modification[20]. Briefly, tissue sections

(5×10㎛) were incubated with xylene for 20 minutes, washed twice with 75% ethanol, and air dried. The tissues were incubated with extraction buffer containing 1M KCl, 1M $MgCl_2$, 100% Tween 20, 100% Nonidet P-40, and 2.5㎍/㎖ proteinase K for 1 hour at 55℃. Proteinase K was inactivated by heating at 95℃ for 10 minutes and the supernatant containing genomic DNA was stored at -20℃. The concentration of extracted DNA was determined by a spectrophotometric measurement (Schimadzu Scientific Instruments, Inc., Concord, CA).

3. Quantitative Genomic PCR Analysis

For quantitative evaluation of allelic status of the p53 gene, we initially performed PCR with increasing cycle numbers (21, 24, 27, 30, 33, 36, 39, and 42 cycles) using serially diluted DNA. Each cycle was comprised of 95℃ for denaturation (1 min), 58~62℃ for annealing (45 sec), and 72℃ for extension (1 min). This showed that 200 ng of genomic DNA in 50㎕ PCR reaction undergoing 26~34 cycles was within the logarithmic phase of amplification for p53, MDM2, and a housekeeping standard GAPDH. The PCR products were resolved on 2% agarose gels, stained with ethidium bromide (0.5㎍/㎖ of 1×TBE), visualized using ultraviolet light, and photographed. The photographic negatives were soaked in distilled water until clean and air dried. Oligonucleotide primers used and its sequences primers were listed in Table 1. Specificity of PCR products was confirmed by Southern probing analysis using biotinylated internal oligonucleotides followed by chemiluminescent detection. Quantitation of gene levels was achieved by laser densitometry scanning of the ethidium bromide-stained gels. After subtraction of the background, the ratio of the area under each curve to that of GAPDH was quantitatively related to gene level. Quantitative genomic PCR was repeated at least 3 times for each specimen and the means were calculated.

4. Nonisotopic DNA/PCR-SSCP Analysis

Nonisotopic DNA/PCR-SSCP analysis was performed as previously described[3]. Briefly, the exons 4 to 10 sequences of p53 were separately amplified by PCR and 1㎕ of the PCR products was subjected to nest-PCR using intron-specific primers. The first and nest-PCRs were performed for 34~40 cycles at 95℃(1 min), 57~62℃(0.5 min), and 72℃ (1 min) using primers summarized in Table 1. Twenty ㎕ of each PCR product was mixed with 4㎕ of 0.5N NaOH, 10mM EDTA and 15㎕ of sequencing stop solution and denatured by heating at 95℃ for 5 minutes. Samples were rapidly loaded onto gels chilled to 4℃ and run simultaneously on two 8% nondenaturing polyacrylamide gels with or without 10% glycerol. These two gels were run at 18~20℃ and then repeated at 6~10℃ in a buffer-jacketed gel apparatus (DGGE-II; Aladin Enterprises, Inc., San Francisco, CA). Following a 4-hour run at 460 volts, the gels were stained with ethidium bromide and photographed under ultraviolet light.

5. DNA Sequencing Analysis of p53 PCR Products

p53 PCR products which showed abnormal SSCP patterns were subjected to DNA sequencing analysis to verify sequence substitutions. Cloning of the PCR products were performed using TA cloning kit (Invitrogen, San Diego, CA) and 30 colonies for each specimens were re-examined by PCR-SSCP. At least 3 clones showing identical SSCP abnormality were selected and sequenced. Sequencing was done in both directions to

Fig. 1. Quantitative PCR analysis of p53 in Ewing's sarcoma tissues. Genomic levels of p53 were analyzed by quantitative PCR using intron-specific primers. Ten μℓ of the PCR products were resolved in a 2% agarose gel and stained with ethidium bromide. Two human cancer cell lines, PC3 and DU145 were included as controls for one and two alleles, respectively for validation of our quantitative PCR approach. Amplification levels of an endogenous standard gene, GAPDH, was used as an internal control. Lanes 1-12: Ewing's sarcoma tissues.

confirm the findings.

RESULTS

1. Validation of Quantitative PCR Analysis

For validation of PCR approach for quantitative analysis of p53 gene level, we initially examined levels of the p53 gene in 2 human prostate carcinoma cell lines (PC3 and DU145) whose allelic status of p53 was previously characterized[3]. As shown in Fig. 1, predicted levels of p53 gene levels were detected in PC3 (0.48) and DU145 (1.12), which carry one and two alleles of p53, respectively, indicating that the genomic levels of p53 determined by our quantitative PCR are well consistent with the previously characterized allelic status of the gene. On this basis, levels less than 0.56 (one-half of DU145) were arbitrarily set as being abnormally low, which could indicate an allelic loss of the gene.

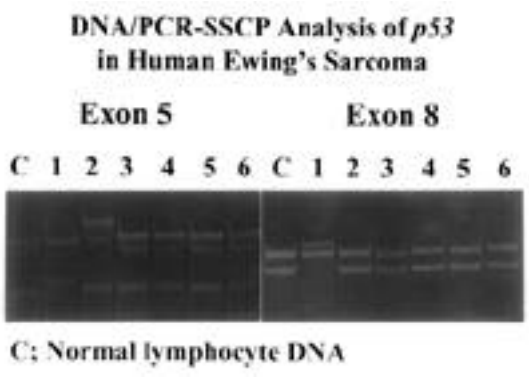

Fig. 2. Mutational analysis of p53 by PCR-SSCP. The exons 4-10 regions of the p53 gene were separately amplified with intron-specific primers using 200ng of genomic DNAs. Twenty μℓ of the PCR products mixed with 5μℓ of 0.5 N NaOH, 10 mM EDTA, 10μℓ of denaturing loading buffer, and 15 μℓ of ddH_2O. SSCP was performed using 8% nondenaturating acrylamide gels containing 10% glycerol at 4~8℃ or 18~22℃. Abnormal shifts of single-stranded DNA molecules are shown in lanes 2 and 1 in gels for exons 5 and 8, respectively. C; normal lymphocyte control, lanes 1-6: Ewing's sarcoma tissue specimens.

2. Deletion of the p53 Gene in Ewing's Sarcoma

We next examined the genomic status of the p53 gene in 35 Ewing's sarcoma tissues using quantitative PCR analysis of exon 5 region of the gene. Quantitative PCR analysis was repeated at least three times for each specimen and the mean of gene levels were obtained. As shown in Fig. 1, the p53 gene was detectable in all of the 35 specimens. However, 2 (5.7%) of these 35 showed extremely low levels of the gene (p53/GAPDH; 0.11 and 0.21, respectively), strongly suggesting that both alleles of the p53 gene might be homozygously deleted in these tumors and the amplified p53 PCR products might be derived from contaminating normal cells or infiltrating lymphocytes in

Table 1. Sequences of primers used for a quantitative PCR and PCR-SSCP analyses (All sequences are listed 5' to 3')

Gene Orientation	Primer		Sequences	
GAPDH	3		GACCATGAGAAGTATGACAACAGC	sense
	5		GAACTCAGTGCATATCCTGATCCA	antisense
p53				
4 (exon)	F		TCTGTCCCTTCCCAGAAAACC	sense
	R		TTGGGCAGTGCTCGCTTAGTGCTC	antisense
5	2064	1st	GCTGCCGTGTTCCAGTTGC	sense
	2915	1st	TCAGTGAGGAATCAGAGGCC	antisense
	SG55	Nest	TTATCTGTTCACTTGTGCCCTG	sense
	SG53	Nest	ACCCTGGGCAACCAGCCCTGTC	antisense
6	2916	1st	CTGGAGAGACGACAGGGCTG	sense
	6R	1st	TTAACCCCTCCTCCCAGAGAC	antisense
	SG65	Nest	ACGACAGGGCTGGTTGCCCAGG	sense
	SG63	Nest	CCCTCCTCCCAGAGACCCCAGTT	antisense
7	7FA	1st	CTTGCCACAGGTCTCCCCAA	sense
	2913	1st	TCAGCGGCAAGCAGAGGCTG	antisense
	SG75	Nest	CGCACTGGCCTCATCTTGGGCC	sense
	SG73	Nest	CAGTGTGCAGGGTGGCAAGTGG	antisense
8/9	2912	1st	GTTGGGAGTAGATGGAGCCTGG	sense
	SG93	1st	GGCATTTTGAGTGTTAGACT	antisense
	SG85	Nest	TCCTTACTGCCTCTTGCTTCT	sense
	SG83	Nest	TCTCCTCCACCGCTTCTTGT	antisense
	SG95	Nest	GCAGTTATGCCTCAGATTCA	sense
	P4	Nest	GGCATTTTGAGTGTTAGACT	antisense
10	F		GATTCTCTGCTCTCCTCGACGGAG	sense
	R		GCGCTGCGTAGTTGTGCTGATGTG	antisense
MDM2	F		TTACTACCACTCACCCGCAGACTC	sense
	R		TGGAGTGTATCAGTCAGCTCCCTC	antisense

tumor tissues. p53 gene levels of the other 33 tumors were found within a range of 0.73~1.32. Thus, these data demonstrate that allelic deletion of the p53 gene occur in a small fraction of human Ewing's sarcomas.

3. Mutational Alterations of p53

To investigate the mutational alterations of the p53 gene in Ewing's sarcomas, exons 4 to 10 regions of the gene, in which frequent sequence alterations have been found in a various types of human cancer, were examined by PCR-SSCP analysis. Each exons were separately amplified using intron-specific primer sets summarized in Table 1 and the PCR products were subjected to SSCP analysis. For comprehensive detection of mutations, SSCP analysis was repeated under four different gel (with or without 10% glycerol) or running (4~8℃ and 18~22℃) conditions. A representative of our nonisotopic PCR-SSCP assay is shown in Fig. 2. Among 33 tumors which showed normal levels of the p53 gene in quantitative genomic PCR, 3 (9.1%) specimens showed migration shifts of single-stranded DNA indicative of nucleotide

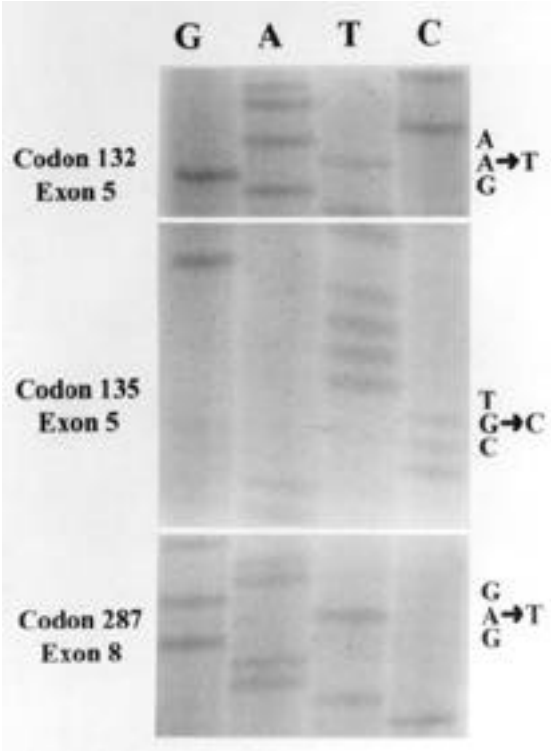

Fig. 3. Identification of p53 sequence alterations by DNA sequencing. Genomic PCR products of p53, which showed abnormal SSCP patterns, were cloned and sequenced to confirm the mutations. Missense point mutations were identified at codon 132 (AAG to ATG, lysine to methionine) and codon 135 (TGC to TCC, cystein to serine) in exon 5, and at codon 287 (GAG to GTG, glutamic acid to valine) in exon 8.

substitution. Abnormal SSCP patterns were identified in exon 5 for 2 tumors and exon 8 for one tumor. To verify sequence alterations of p53 in these specimens, we performed cloning and DNA sequencing analysis of these PCR products. As shown in Fig. 3, we detected missense mutations of p53 in these three specimens; codon 132 (exon 5; AAG to ATG, lysine to methionine), codon 135 (exon 5; TGC to TCC, cystein to serine), and codon 287 (exon 8; GAG to GTG, glutamic acid to valine). Taken together, our study demonstrate that p53 is deleted or mutated in 5 (14.3%) of 35 Ewing's sarcomas.

4. Absence of Abnormal Amplification of the MDM2 Gene

The p53 and MDM2 genes are part of a physiological pathway frequently impaired in human cancer. Overexpression of MDM2 by genomic amplification of the gene has been described to lead to function inactivation of wild-type p53. To explore the involvement of abnormal amplification of MDM2 in Ewing's sarcoma, we performed quantitative PCR analysis of the MDM2 gene in triplicate. However, compared to normal human lymphocytes included as controls, no detectable difference in levels of MDM2 in 35 tumors was recognized (data not shown). This result suggests that aberrant amplification of MDM2 might be infrequent in Ewing's sarcoma and does not play a crucial role in p53 inactivation in this type of tumor.

DISCUSSION

The role of tumor suppressor genes and oncogenes in the development of Ewing's sarcoma has not yet been fully clarified. In this study, we investigated the allelic deletion and mutation of the p53 tumor suppressor gene by quantitative genomic PCR and PCR-SSCP analyses. We demonstrated that genetic alteration of the p53 tumor suppressor gene occurs in approximately 14.3% of human Ewing's sarcoma. Thus, our results suggest that the p53 gene is genetically altered in a small fraction of Ewing's sarcoma.

The involvement of p53 alterations in the development of Ewing's sarcoma has been poorly understood. Previous study demonstrated that six of seven Ewing's sarcoma cell lines have mutations in p53 while only 2 of 14 primary tumors showed p53 alteration, which

were obtained from patients with an advanced stage disease, indicating that mutations of p53 in Ewing's sarcoma is a rare event and might represent late genetic events related to tumor progression. Kovar et al[9] examined 37 primary tumors and cell lines from 19 patients and only 5% of the primary tumor specimens were found to carry an alteration within this gene. They also showed the absence of MDM2 amplification in Ewing's tumors. However, p53 mutations were 10-fold enriched in cell lines, thus indicating a selective growth advantage in vitro. Strikingly, they found that 5 out of 9 p53 alterations detected were missense mutations within codon 273, which were reported to impair only partially the normal p53 function. Another study also found mutational alteration of p53 in only one of 24 tumor tissues[19]. They also observed that MDM2 was overexpressed in five of these 24 cases and mutations of the Ras gene were detected, suggesting that compared with other highly malignant mesenchymal pediatric tumors such as osteosarcomas, mutations of p53 and Ras in Ewing's sarcomas are an extraordinarily rare event. Recently, Abudu et al[1]. detected the overexpression of p53 protein in 14% of Ewing's sarcomas but no relationship between expression of p53 and site of tumors was recognized. Interestingly, however, they demonstrated that patients who overexpressed p53 protein appeared to have more advanced diseases at diagnosis and poorer response to chemotherapy than those without p53 overexpression. In addition, the 5-year relapse-free survival and overall survival in patients without metastases at the time of diagnosis were 66% and 71%, respectively, in p53 protein-negative patients compared with 20% relapse-free and overall survival in those with p53 protein overexpression, suggesting that overexpression of p53 protein is an independent poor prognostic factor in Ewing's sarcoma of bone. In this work, we detected genomic deletion or missense point mutations of p53 in 5 (14.3%) of 35 Ewing's sarcomas, which is consistent with the previous finding that mutational alteration of p53 is not a frequent event in tumorigenesis of human Ewing's sarcoma.

Amplification of the MDM2 gene, which maps to chromosome band 12q13 and encodes a p53-binding protein, may result in functional inactivation of p53 and has been observed in various bone and soft tissue sarcomas. Published studies have included few cases of Ewing's sarcoma or peripheral neuroectodermal tumor, a tumor group in which alterations of the p53 pathway have so far not been extensively studied[10,19]. Thus, we examined the same Ewing's sarcoma tumor tissues used for p53 analysis for abnormal MDM2 gene amplification. Our data showed that all of the 35 specimens contain normal levels of MDM2, supporting the reports that MDM2 revealed neither gene amplification in the primary tumors and cell lines nor significant overexpression in any of the cell lines[9,19]. Thus, our study excludes the possibility that abnormal amplification of MDM2 play a role in negative regulation of p53 function in Ewing's sarcoma.

In conclusion, we demonstrate p53 mutation. Further studies will be required to identify and characterize factor(s) that modulate the conformation and/or subcellular localization of p53 protein and in the deveopment or progression of human Ewing's sarcoma. Also more studies are needed for the correlation with clinical outcome with p53 mutation.

CONCLUSION

In this study, to explore the implication of p53 alteration in Ewing's sarcoma, we ana-

lyzed the deletion and sequence alterations of p53. Quantitative genomic PCR analysis showed that 2 of 35 tumors have extremely low levels of the p53 gene, indicating a homozygous deletion of the gene. Mutational analysis of exons 4 to 9 of p53 by PCR-SSCP revealed that 3 of 35 tumors carry sequence alterations in exons 5 or 8, and DNA sequencing analysis identified missense point mutations at codon 132 (AAG to ATG, lysine to methionine) and codon 135 (TGC to TCC, cystein to serine) in exon 5, and codon 287 (GAG to GTG, glutamic acid to valine) in exon 8 from these tumors. Taken together, our data demonstrate that p53 is genetically altered in a small fraction of Ewing's sarcoma.

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Abstract

p53 Mutations in Ewing's Sarcoma

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Purpose : The p53 tumor suppressor gene is one of the most frequently altered genes in human malignancies. We try to explore the implication of p53 alteration in Ewing's sarcoma.

Materials and Methods : We analyzed 35 paraffin blocks to explore the deletion and sequence alterations of p53.

Results : Quantitative PCR analysis showed that 2 tumors showed a homozygous deletion of the gene. Mutational analysis of exons 4 to 9 of p53 by PCR-SSCP revealed that 3 tumors carry sequence alterations in exons 5 or 8, and DNA sequencing analysis identified missense point mutations.

Conclusion : Taken together, our data demonstrate that p53 is genetically altered in a small fraction of Ewing's sarcoma.

Key Words : Ewing's sarcoma, p53, Mutation

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