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MAIN ARTICLE

Expression and mutational analysis of Cip/Kip family in early glottic cancer

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Abstract

Background: Genetic alteration of cyclin-dependent kinase inhibitors has been associated with carcinogenesis mechanisms in various organs.

Objective: This study aimed to evaluate the expression and mutational analysis of Cip/Kip family cyclin-dependent kinase inhibitors (p21$^{CIP1/WAF1}$, p27^{KIP1} and p57^{KIP2}) in early glottic cancer.

Methods: Expressions of Cip/Kip family and p53 were determined by quantitative reverse transcription polymerase chain reaction and densitometry. For the analysis of p21 inactivation, sequence alteration was assessed using single-strand conformational polymorphism polymerase chain reaction. Additionally, the inactivation mechanism of p27 and p57 were investigated using DNA methylation analysis.

Results: Reduced expression of p27 and p57 were detected in all samples, whereas the expression of p21 was incompletely down-regulated in 6 of 11 samples. Additionally, single-strand conformational polymorphism polymerase chain reaction analysis showed the p53 mutation at exon 6. Methylation of p27 and p57 was detected by DNA methylation assay.

Conclusion: Our results suggest that the Cip/Kip family may have a role as a molecular mechanism of carcinogenesis in early glottic cancer.

Key words: Cyclin-Dependent Kinase Inhibitor Proteins; Laryngeal Cancer; Carcinogenesis

Introduction

Laryngeal squamous cell carcinoma (SCC) represents approximately 25 per cent of all head and neck cancers, accounting for 95–98 per cent of SCCs, with drinking, smoking, viral infections, nutritional deficiencies and dietary habits being major precipitating factors.[1] Generally, laryngeal SCC has a high rate of localisation, but cervical metastasis is related to the extent of invasiveness or microscopic environment of the primary lesion.

The tumour suppressor gene p53 and the over-expression of cyclin proteins have been identified as molecular mechanisms of carcinogenesis in laryngeal SCC.[2–4] The molecular mechanisms of carcinogenesis have been studied for a long time; however, the nature of these mechanisms is only just becoming clear.

During a cell cycle, cyclin and cyclin-dependent kinase complexes drive cell proliferation and differentiation. In contrast, cyclin-dependent kinase inhibitors regulate progression by binding the cyclin and cyclin-dependent kinase complexes, and inhibiting cyclin-dependent kinase activity.[5] The cyclin-dependent kinase inhibitors are classified into two distinct families: INK4 and Cip/Kip. Members of the INK4 family, namely p16^{INK4A}, p15^{INK4B}, p18^{INK4C} and p19^{INK4D}, specifically inhibit the activity of cyclin-dependent kinases 4 and 6. In comparison, Cip/Kip members, that is p21$^{CIP1/WAF1}$, p27^{KIP1} and p57^{KIP2}, inhibit a broader spectrum of cyclin and cyclin-dependent kinase complexes.[5–7] In addition, Cip/Kip members are well characterised for their role as negative regulators of G_0 to G_1 phases, or in the G_1 to S phase transition of the cell cycle. This means that Cip/Kip members play an important role in the development of malignant cancer, because the major checkpoint of cell proliferation and differentiation occurs in the transition of the G_0 to the G_1 phase, or the G_1 to the S phase.[7]

For decades, three genes of Cip/Kip family cyclin-dependent kinase inhibitors (p21$^{CIP1/WAF1}$, p27^{KIP1} and p57^{KIP2}) have been studied in different kinds of human cancer. Reduced expression of p21$^{CIP1/WAF1}$

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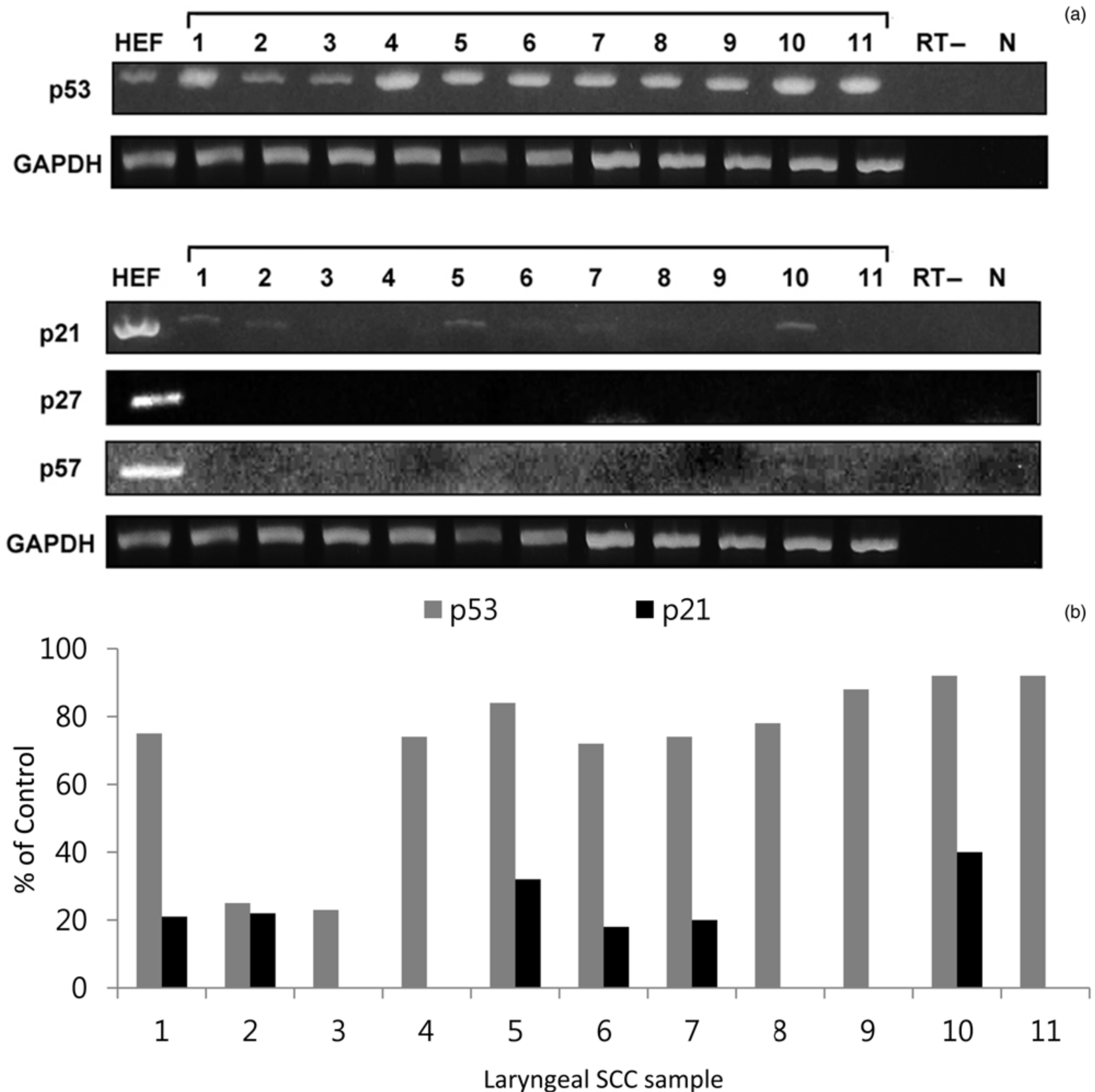

FIG. 1

Semi-quantitative reverse transcription polymerase chain reaction analysis findings for the laryngeal squamous cell carcinoma (SCC) samples (labelled 1–11). (a) Messenger RNA (mRNA) expression of p53, p21, p27 and p57 genes in laryngeal SCC specimens were analysed; glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalisation (HEF = normal human respiratory epithelium mucosa; RT− = no reverse transcriptase; N = no template). (b) Densitometry of polymerase chain reaction bands for p53 and p21 based on percentage of GAPDH control bands using ImageJ, version 1.20s (an open-source image-processing program); GAPDH mRNA was analysed as a control.

and $p27^{KIP1}$ genes are associated with increased tumour growth and risk of metastasis in several types of cancer, including lung, breast and oral cavity cancer. In addition, $p57^{KIP2}$ gene expression in certain tumours is inversely correlated with cellular proliferation, suggesting tumourigenesis.[8,9] However, to our knowledge, the role of Cip/Kip family expression in early glottic cancer has not been extensively investigated. Therefore, this study aimed to verify the expression of Cip/Kip family members and to determine the inactivation mechanism of these genes in early glottic cancer.

Materials and methods

Cancer samples

Laryngeal SCC samples were obtained during surgery from 11 patients with T_1N_0 or T_2N_0 early glottic cancer at Chuncheon Sacred Heart Hospital (Hallym University). All samples were histopathologically

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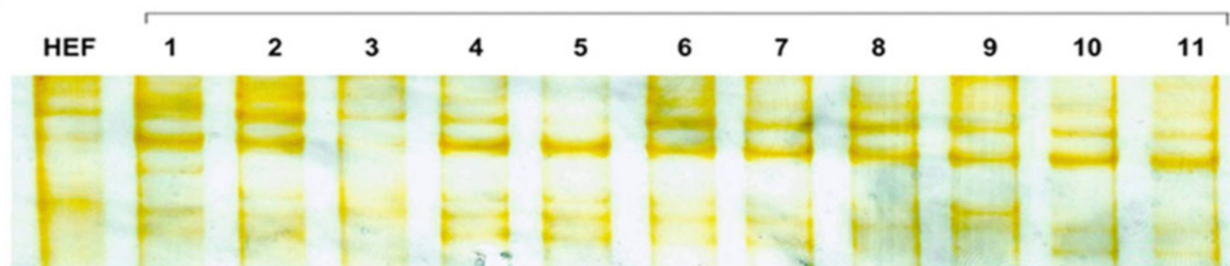

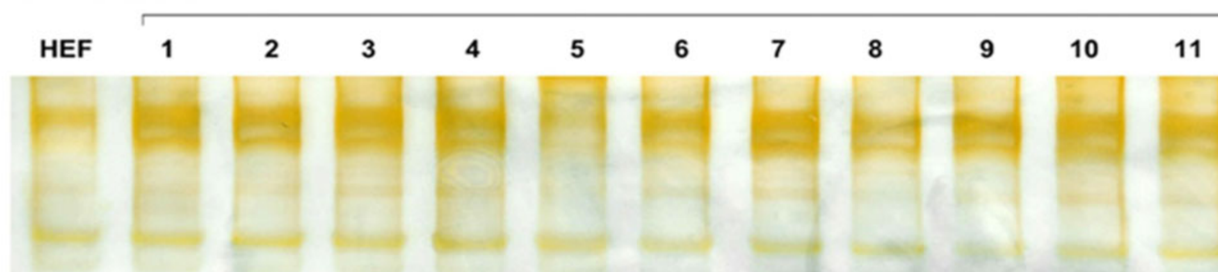

FIG. 2

Single-strand conformational polymorphism polymerase chain reaction findings for the mutation analysis of the p53 gene, for the laryngeal squamous cell carcinoma samples (labelled 1–11). HEF = normal human respiratory epithelium mucosa

confirmed as well-differentiated SCC. Patients received no radiotherapy or chemotherapy prior to surgery. Normal mucosal samples were obtained from the upper aerodigestive tract, remote from the tumour site, to act as negative controls. All samples were taken after informed consent was documented.

Reverse transcription polymerase chain reaction analysis

The RNA in tumour cells was extracted using TRIzol. TRIzol was synthesised in a reaction volume of 20 μl, containing 100 ng RNA, antisense primers (see below), 10 mM deoxynucleotide triphosphates (Boehringer Mannheim, Mannheim, Germany), 1× reaction buffer (Takara Shuzo, Otsu, Japan) and avian myeloblastosis virus reverse transcriptase (Takara Shuzo). The reaction volume was incubated at 52°C for 20 minutes.

Complementary DNA was amplified using primer sets specific for the following genes: p53 (sense, 5′-AGA GAC CTG TGG GAA GCG AA-3′; antisense, 5′-CTG AGG GTG TGA TGG GAT GG-3′); p21 (sense, 5′-CCC AAG CTT GGG CCT GCC GAA GTC AGT TC-3′; antisense, 5′-GGG AAG CTT GGG GTG ATT CTT AGC AAA TA–3′); p57 (sense, 5′-GCT GCC TAG TGT CCC GGT C-3′; antisense, 5′-GAT CAG AGG CCG GAC AGC TT-3′); and p27 (sense, 5′-TTG CCC GAG TTC TAC TAC AGA-3′; antisense, 5′-AGG GTC ATT ACC GTC GGT TGC-3′).

Polymerase chain reaction for p53 was performed as follows: 94°C for 5 minutes, 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds, followed by a final incubation at 72°C for 10 minutes. The polymerase chain reaction for p21 was performed as follows: 94°C for 5 minutes, 25 cycles at 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. The polymerase chain reaction for p57 was performed as follows: 95°C for 5 minutes, 28 cycles at 95°C for 1 minute, 65°C for 30 seconds, 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. The polymerase chain reaction for p27 was performed as follows: 95°C for 5 minutes, 28 cycles at 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes.

Polymerase chain reaction products were resolved on 2 per cent agarose gels. Human diploid fibroblast (HEP) cells were used as a normal cell control, and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a control gene; the latter shows normal expression regardless of the cell cycle phase.

Single-strand conformational polymorphism polymerase chain reaction analysis

The polymerase chain reaction of the p53 gene was performed with 100 ng of genomic DNA in 20 μl of reaction buffer, which contained 10 pmol of each primer, 0.1 mM of each of the four deoxynucleotide triphosphates, 1.5 mM magnesium chloride and 2.5 U of Taq DNA polymerase (PerkinElmer, Waltham, Massachusetts, USA).

Complementary DNA was amplified using primer sets specific for exon 6 of p53 (sense, 5′- CGG TCG

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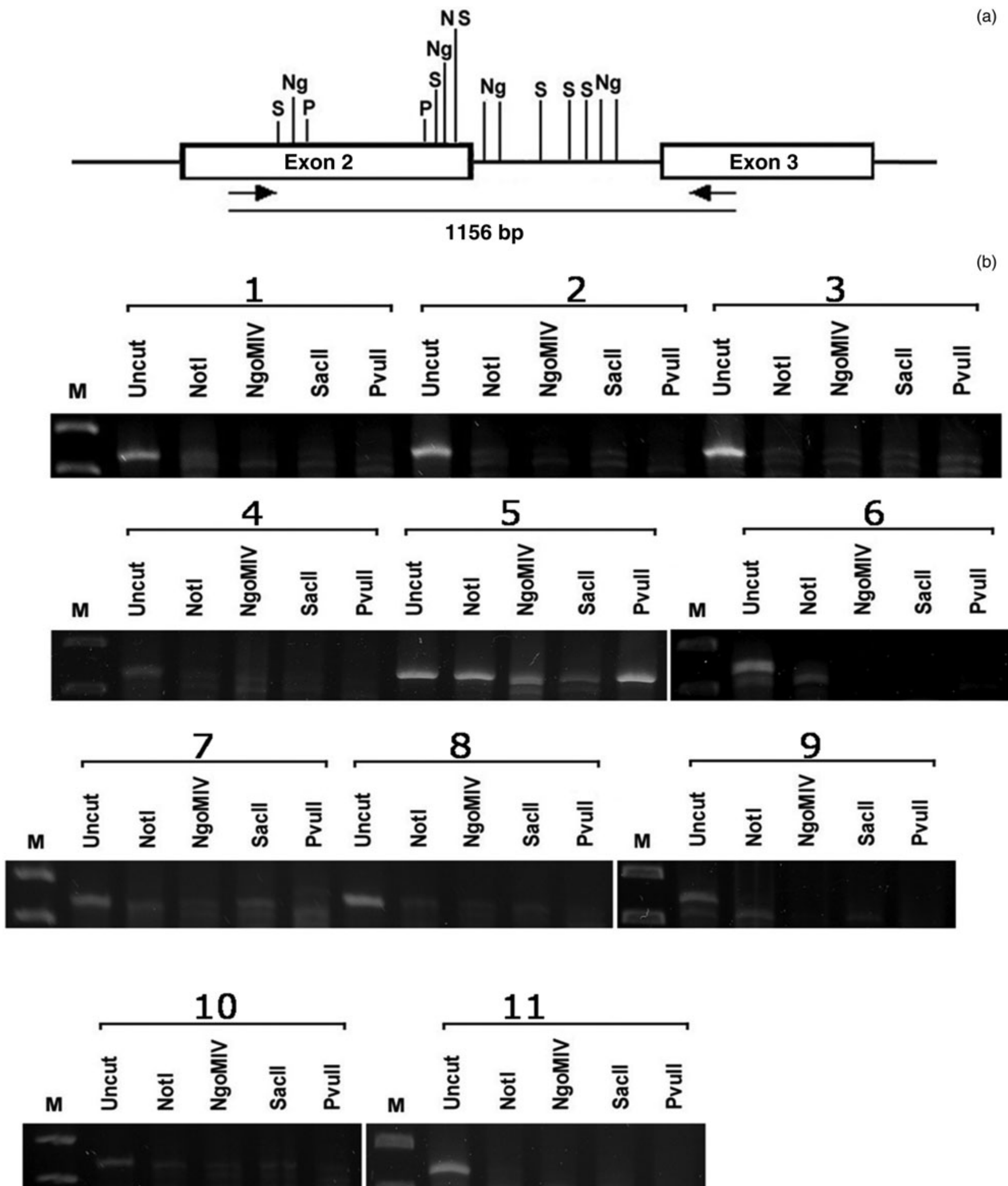

FIG. 3

Polymerase chain reaction based methylation analysis findings for the p57 gene, for the laryngeal squamous cell carcinoma samples (labelled 1–11). (a) Restriction map of the exon 2–3 region of p57 and the expected size (S = SacII; Ng = NgoMIV; P = PvuII; N = NotI; bp = base pairs). (b) Results of methylation of the p57 gene (M = molecular weight marker; Uncut = undigested DNA).

ACA GTT GCA AAC CAG A-3′; antisense, 5′-CGT CTA GAA TTC CTC ACT GAT TGC TC-3′) and exon 9 of p53 (sense, 5′-GAC TGG AAA CTT TCC ACT TGA TAA G-3′; antisense, 5′-GCC TCA GAT TCA CTT TTA TCA CC-3′).

The polymerase chain reaction conditions were as follows: 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds, followed by a final incubation at 72°C for 5 minutes. Samples were denatured at 95°C for 5 minutes and

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resolved on 8 per cent non-denaturing polyacrylamide gels containing 10 per cent glycerol. The gels were silver-stained according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

Methylation analysis

A polymerase chain reaction analysis relying on the inability of SacII, NgoMIV and NotI to cut methylated sequences was used to analyse the p57 gene. The DNA (1 µg) was digested for 5 hours using 10 units of enzyme per 1 µg of DNA. Digested DNA (100 ng) was amplified with *pfu* polymerase (Stratagene, La Jolla, California, USA) and primers flanking the restriction sites (sense, 5′-TGC CCG CGT TCT ACC GCG AGA CGG TGC AGG-3′; antisense, 5′-CGC TGA TCT CTT GCG CTT GGC GAA GAA ATC-3′) under the following conditions: 98°C for 5 minutes, 35 cycles at 98°C for 1 minute, 60°C for 30 seconds and 74°C for 1 minute, followed by a final incubation at 74°C for 10 minutes. The resulting DNA was analysed on a 1 per cent agarose gel.

Results

Figure 1a shows the messenger RNA (mRNA) expression of p53, p21, p27 and p57 in samples from patients with early glottic cancer. p53 mRNA was over-expressed in most cases (9 of 11 samples). However, the mRNA expression of Cip/Kip family members (p21, p27 and p57 genes) was markedly lower than in the control samples. The expression of p21 mRNA was considerably down-regulated in five samples and was weakly expressed in six samples (Figure 1b). The expression p27 and p57 mRNA was completely inactivated in all samples.

In order to investigate the cause of incomplete down-regulation of p21, single-strand conformational polymorphism polymerase chain reaction analysis was conducted for exons 6 and 9 of the p53 gene; this is because mutational alterations of the p53 gene have been shown to be important for p21 expression. Compared with the control samples, the p53 gene from early glottic cancer samples was most mutated at exon 6 (in 9 of 11 samples). However, no mutation was detected at exon 9 of the p53 gene (Figure 2).

Finally, to confirm the methylation of the p57 gene, we investigated polymerase chain reaction based methylation analysis by using methylation-sensitive restriction enzymes (SacII, NgoMIV and NotI) or a methylation-insensitive restriction enzyme (PvuII). Figure 3 shows no detection of bands corresponding to cleavage at the PvuII site in laryngeal SCC samples 4, 6, 7, 8, 9 and 10. However, bands corresponding to cleavage by the methylation-sensitive restriction enzymes were observed (Figure 3). It suggested that the p57 gene was methylated in six samples from patients with early glottic cancer.

Discussion

The activity of cyclin-dependent kinases is regulated by phosphorylation and the action of cyclin-dependent kinase inhibitory proteins. It has been shown that Cip/Kip family cyclin-dependent kinase inhibitors have a role in the development and progression of different kinds of human cancer, but, unlike the INK4 family, only a few genetic alterations have been found. This suggests that the mutational inactivation of Cip/Kip members is infrequent and that gene inactivation by alternative mechanisms is the general pathway.

In the present study, we found that p53 was mostly over-expressed (in 9 of 11 samples), whereas p21 was expressed more weakly than p53 in some of the samples (samples 1, 2, 5, 6, 7 and 10). In contrast, the expression of p27 and p57 was reduced in all samples. Several other studies have described different results. With regard to p53, some studies have shown that the frequency of tumour protein 53 mutations is approximately 40–60 per cent in all head and neck SCC cases.[3,10] It is likely that the discrepancy in terms of the rate of p53 anomalies may be due to differences in the stage of laryngeal SCC. Generally, previous studies investigating the correlation between the anomalous accumulation of p53 and the prognosis have included samples from patients with laryngeal carcinomas of different stages.[3,10–12] Only early stage samples were used in the present study. In addition, a few studies on p27 have shown high p27 expression in all laryngeal lesions, including the invasive carcinomas.[13,14]

To date, two known mechanisms seem to be the best candidates for inactivating the Cip/Kip members; these are gene inactivation by DNA methylation in the promoter region and changes to inactive chromatin by histone deacetylation.[15–18] The p21 gene is not DNA-methylated. Actually, the expression of p21 is controlled mainly by the inactivation of chromosomes via histone deacetylation and mutational inactivation of the p53 gene. The latter is an important transcription factor for the p21 gene and is frequently mutated in tumours.[15,19] Thus, we evaluated the mutational alterations of p53 to investigate the cause of incomplete down-regulation of p21. Our analysis of single-strand conformational polymorphism polymerase chain reaction revealed that the p53 genes were mostly mutated in exon 6, but not in exon 9. This suggests that the inactivation of p21 in early stage laryngeal SCC is due to mutation of the p53 gene, and thereby the absence of functional p53 to induce the transcription of p21. Furthermore, the inactivation mechanisms of p27 and p57 were identified by using methylation-sensitive enzymes (SacII, NgoMIV and NotI) and an insensitive enzyme, such as PvuII. No PvuII-cleaved band was observed in several of the samples, whereas the same samples showed bands for SacII, NgoMIV and NotI cleavage. These results suggest that inactivation of p27 and p57 in early stage laryngeal SCC may

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be caused by partial DNA methylation. Similar results were observed for the p27 gene analysis (data not shown).

- **Abnormal down-regulation of Cip/Kip family cyclin-dependent kinase inhibitors ($p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$) was detected in early glottic cancer samples**
- **This study showed that Cip/Kip family cyclin-dependent kinase inhibitors have a possible role as molecular mechanisms of early glottic cancer**
- **Possible mutational mechanisms include p53 mutation ($p21^{CIP1/WAF1}$) and DNA methylation ($p27^{KIP1}$ and $p57^{KIP2}$)**

This investigation has some limitations. First, this was a small study conducted for a preliminary description of the expression profiles of Cip/Kip members in early glottic cancer. Further, large-scale studies are necessary to ensure that the findings are reliable. Second, the tumour protein p53 mutation status has previously been examined on exons 5–9, but our results examined only exons 6 and 9. Thus, our study may have missed some of the many mutations. However, generally, exons 6 and 9 are known as the mutational hotspots of the tumour protein p53. Third, the RNA expression of p53 and Cip/Kip family members would be better examined with real-time polymerase chain reaction or using Western blotting to confirm their proper expression levels.

Conclusion

Our study provides a preliminary description of the expression profiles of Cip/Kip family cyclin-dependent kinase inhibitors in early glottic cancer. We demonstrated that in early glottic cancer, p21, p27 and p57 were down-regulated, with over-expression of p53. Our results also suggested that the inactivation of the p21 gene may be related to the mutation of the p53 gene. However, the relevance of the association between Cip/Kip family members and the clinical course of laryngeal SCC remains unclear; hence, further study is needed.

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