Loss of Imprinting and Genetic Alterations of the Cyclin-dependent Kinase Inhibitor \( p57^{KIP2} \) Gene in Head and Neck Squamous Cell Carcinoma\(^1\)

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**ABSTRACT**

The \( p57^{KIP2} \) gene is a maternally expressed and paternally imprinted cyclin-dependent kinase inhibitor located on chromosome 11p15.5. Because of its location, biochemical functions, and imprinting status, \( p57^{KIP2} \) has been considered a candidate tumor suppressor gene. To determine, for the first time, the involvement of this gene in the development of head and neck squamous carcinoma (HNSC), we analyzed the imprinting and expression status and loss of heterozygosity (LOH) within the \( p57^{KIP2} \) gene flanking loci on the 11p15.5 region in 64 primary untreated tumors. Of the 30 (47%) informative cases for this gene, loss of imprinting and LOH were noted in 4 (13%) and 10 tumors (33%), respectively. Analysis of the microsatellite markers flanking the \( p57^{KIP2} \) gene on chromosome 11p showed infrequent alterations at these loci. \( p57^{KIP2} \) was expressed in all tumors with LOH within and around the gene. Quantitative reverse transcription-PCR analysis showed elevated \( p57 \) mRNA expression in tumor with loss of imprinting. Sequencing analysis of exons 1 and 2 of the \( p57^{KIP2} \) gene failed to detect any mutations. Our data indicate: (a) infrequent genomic abnormalities at the \( p57^{KIP2} \) gene in HNSC; (b) leaky or incomplete imprinting of the paternal allele is associated with increased expression of this gene in a subset of tumors; and (c) minimal evidence for suppressor function for this gene in HNSC.

**INTRODUCTION**

Genomic imprinting is the epigenetic marking of a gene, based on parental origin, that results in monoallelic expression (1–4). This phenomenon differs from the classical sequence-based qualitative changes in that gene expression and effective gene dosage are controlled by epigenetic dysregulation of parental alleles of an imprinted gene. Imprinting dysregulation may contribute to tumorigenesis either by activating a transcriptionally repressed allele resulting in gene activation, or by inactivating an expressed allele of an imprinted tumor suppressor gene, leading to loss of function (5). Evidence implicating this process in tumorigenesis is based on the finding of selective parental LOH\(^3\) and LOI at certain imprinted domains in several pediatric tumors (6–13).

The chromosome 11p15.5 region contains imprinted domains in which \( H19, IGF2 \), and the recently identified \( p57^{KIP2} \) genes reside (14–25). Several studies have shown that imprinting abnormalities of the \( H19 \) and/or \( IGF2 \) genes play a role in tumorigenesis of both embryonal and adult neoplasms (14, 18–21, 26–36). \( p57^{KIP1} \), a maternally expressed gene located ~400–500 kb centromeric to \( H19 \) and \( IGF2 \) genes, is a member of INK’s CDK inhibitor family, which includes \( p21^{CIP1} \) and \( p27^{KIP1} \) genes (13–15, 22). A tumor suppressor role for the \( p57^{KIP2} \) has been suggested in studies of different tumors based on its association with cell cycle control and imprinting status. Therefore, the dysregulation of \( p57^{KIP2} \) by imprinting of the maternally expressed allele, or by relaxation of this process in the paternal allele, results in uncontrolled proliferation and tumor development (3, 9, 17, 18, 21, 22, 36, 37).

We investigated, for the first time, the incidence of imprinting and genetic alterations at the \( p57 \) gene and its flanking loci on chromosome arm 11p15.5 region in 64 primary oral squamous carcinoma to determine their association with head and neck squamous tumorigenesis.

**MATERIALS AND METHODS**

**Specimens.** The materials for this study consisted of matched pairs of fresh normal squamous mucosa and tumor specimens, which were surgically removed from 64 patients with primary oral squamous cell carcinoma between 1993 and 1996 at the Department of Pathology, The University of Texas M. D. Anderson Cancer Center (Houston, TX). All specimens were received by one pathologist (A. E. N.) and were subjected to frozen section evaluation and stored at \( -80^\circ \) C until used. Normal mucosae from the same patients were obtained from the farthest margin of resection after frozen section verification.

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\(^3\)The abbreviations used are: LOH, loss of heterozygosity; LOI, loss of imprinting; CDK, cyclin-dependent kinase; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; QC-RT-PCR, quantitative competitive RT-PCR; HNSC, head and neck squamous carcinoma.
DNA and RNA Extraction. DNA and RNA isolation were performed according to previously published protocol (38).

Characterization of p57 and Chromosome 11p Polymorphism. Primers C and D (Table 1) were used to PCR amplify normal and tumor genomic DNA to determine the heterozygosity of p57 (21). The reaction mixture was composed of 1× PCR buffer (Promega Corp., Madison, WI) with 1 μm of primers, 2000 μm of deoxynucleotide triphosphate, 2.5 units of Taq polymerase, and 100 ng of template DNA. PCR conditions were 98°C for 5 min, 35 cycles of 95°C for 1 min, 65°C for 30 s, and 72°C for 30 s, followed by a 72°C 5-min extension.

Two microsatellite markers, THO1 and D11S2359 (Research Genetics, Inc., Huntsville, AL), flanking p57 loci on 11p15.5 were also used to detect the heterozygosity of 11p. PCR products were separated in 10% acrylamide gel and stained using a color silver staining kit (Pierce Chemical Co., Rockford, IL).

RT-PCR. For allele-specific expression, 1 μg of total RNA from normal mucosa and tumor specimens was reverse-transcribed for the first strand of cDNA using the Gene Amp RT-PCR system (Perkin-Elmer Cetus, Branchburg, NJ) in a 20-μl reaction. The reaction mixture was added to 80 μl of 100 μM deoxynucleotide triphosphate and 2 mM MgCl₂, 10% glycerol, and 2.5 units of Taq polymerase in 1× PCR buffer. PCR was carried out with 0.4 μm of primer E₁ and D (Table 1). The PCR condition for primers E₁ and D was similar to those of primers C and D, except that annealing was performed at 60°C for 1 min and extension was performed at 72°C for 30 s. This resulted in a 547-bp p57 cDNA band, whereas primers E₁ and D yielded a 668-bp DNA fragment (data not shown) and served as a size reference (21) on 2% agarose gel.

QC-RT-PCR was performed with the PCR mimic construction kit (Clontech Industries Inc., Palo Alto, CA). Primers 11 and 12 consisted of the flanking nucleotide sequence of p57 cDNA (Table 1). PCR amplification of the v-erb fragment as a competitor was developed using the same primer (11 and 12) sequences in addition to fragments A and B of the v-erb gene. Thus, primers 11 and 12 were used to coamplify the 1:10 dilution of the competitor and p57 cDNA under the same PCR conditions. Products were run on 2% agarose gel stained with ethidium bromide, and band density was calibrated by a densitometer (Molecular Dynamics, Sunnyvale, CA). The level of p57 mRNA was determined by comparing the intensity ratio of competitor:target bands.

Imprinting Analysis. In heterozygous cases for the p57 gene, primers C and D for the nested PCR product from the 547-bp template obtained by primers E₁ and D. Cases 42 and 44 yielded one allele. Cases 45 and 62 had biallelic (LOI) expression (103- and 91-bp bands), indicating LOI. G, heterozygous genomic DNA; C, nested cDNA PCR product; N, normal; T, tumor.

SSCP Analysis. Mutation screening of exons 1 and 2 was carried out by SSCP analysis. Exon 1 was amplified as one fragment size (bp) for PCR. Normal and tumor genomic DNA PCR products were also run in parallel with normal and tumor genomic DNA PCR product by primers C and D.

Table 1 Oligonucleotide primers used for genomic and RT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>CCACCCCGCCCGAGTC</td>
<td>103 or 91</td>
</tr>
<tr>
<td>D</td>
<td>GGGGCGAGGACCGGCCAC</td>
<td>668 (genomic PCR)</td>
</tr>
<tr>
<td>E₁</td>
<td>GACGCCAGAGGAGTCCACC</td>
<td>547 (RT-PCR)</td>
</tr>
<tr>
<td>D</td>
<td>GGGGCGAGGACCGGCCAC</td>
<td>596 (v-erb DNA PCR)</td>
</tr>
<tr>
<td>11 + A</td>
<td>ACGGCGAGGAGGAGGTTCACTTGCAGAATCTCRTTCGG</td>
<td>205 (RT-PCR) or 596 (competitor)</td>
</tr>
<tr>
<td>12 + B</td>
<td>AGTGCGTTACCCAGGCTGTTCTGAGTCACATTGAGGAGCGTTT</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>ACCGACCCGCGACAGAGTCCCA</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>AGTGGGTTAATCGCCCTCCGCCCCGAGGAGGCAGTGGATTCCTTTCGG</td>
<td></td>
</tr>
<tr>
<td>1U</td>
<td>GGCGCCAGGCTAGGCCG</td>
<td></td>
</tr>
<tr>
<td>1D</td>
<td>CCAGGGACGCGCCGCTGC</td>
<td></td>
</tr>
<tr>
<td>2AU</td>
<td>CACATCCAGATGAGCGCG</td>
<td></td>
</tr>
<tr>
<td>2AD</td>
<td>AGGGCCCGAGGCCTCACCCACC</td>
<td></td>
</tr>
<tr>
<td>2BU</td>
<td>CTGCCGCGGCAGGGGC</td>
<td>281</td>
</tr>
<tr>
<td>2BD</td>
<td>GGGGCCAGGACCGGCCGCC</td>
<td></td>
</tr>
<tr>
<td>2CU</td>
<td>GGCAGGGCCAGGGCCGGCTCC</td>
<td>232</td>
</tr>
<tr>
<td>2CD</td>
<td>CGGAGATCCAGAGGCGCGCGGA</td>
<td>280</td>
</tr>
</tbody>
</table>

Table 2 Genetic alterations at the p57 gene and its flanking loci on chromosome 11p15.5 in HNSC

<table>
<thead>
<tr>
<th>Genetic locus</th>
<th>Het (%)</th>
<th>LOI (%)</th>
<th>LOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p57</td>
<td>30 (46.9) 103.3 (13.3)</td>
<td>10 (33.3)</td>
<td></td>
</tr>
<tr>
<td>D11S2359</td>
<td>45 (70.3) N/A</td>
<td>6 (13.3)</td>
<td></td>
</tr>
<tr>
<td>THO1</td>
<td>51 (79.7) N/A</td>
<td>7 (14.0)</td>
<td></td>
</tr>
</tbody>
</table>

Het, heterozygous; N/A, not applicable.

Fig. 1 Imprinting status in p57 heterozygous HNSC cases using primers C and D for the nested PCR product from the 547-bp template obtained by primers E₁ and D. Cases 42 and 44 yielded one allele. Cases 45 and 62 had biallelic (LOI) expression (103- and 91-bp bands), indicating LOI. G, heterozygous genomic DNA; C, nested cDNA PCR product; N, normal; T, tumor.
fragment with primers 1U and 1D, whereas exon 2 was split into three fragments with primers 2AU and 2AD, 2BU and 2BD, and 2CU and 2CD for amplification (Table 1). Five μl of PCR products were denatured by heating at 95°C for 5 min in 5 μl of sequencing stop solution (United States Biochemical Corp., Cleveland, OH). The reaction mixture was applied to 8% non-denaturing acrylamide gel at 150 V overnight at 4°C. The gel was subjected to silver staining using a color silver stain kit (Pierce Chemical Co.).

RESULTS

Of the 64 tumors analyzed, 30 (47%) were heterozygous for the p57 gene (Table 2), with two bands of 103- and 91-bp PCR products identified in normal specimen (data not shown). These were considered informative for further analysis. Five μl of PCR products were denatured by heating at 95°C for 5 min in 5 μl of sequencing stop solution (United States Biochemical Corp., Cleveland, OH). The reaction mixture was applied to 8% non-denaturing acrylamide gel at 150 V overnight at 4°C. The gel was subjected to silver staining using a color silver stain kit (Pierce Chemical Co.).

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Table 3  Correlation between p57 gene alterations and LOH at its flanking microsatellite markers in HNSC

<table>
<thead>
<tr>
<th>11p15.5 loci</th>
<th>Yes</th>
<th>No</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH</td>
<td>2</td>
<td>2</td>
<td>0.61</td>
</tr>
<tr>
<td>Het*</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

* Het, heterozygous.

Fig. 2  QC-RT-PCR analysis of the p57 mRNA level in a case (45) with LOI. Serial dilutions of the competitor (v-erb) were coamplified with either normal or tumor cDNA samples. The gel photograph represents competitor (C) PCR products with normal (N; top) and tumor (T; bottom) cDNA samples. The intensity ratio of competitor:normal as well as competitor:tumor p57 mRNA was plotted in relation to the log scale of competitor concentration. At the ratio of 1, tumor concentration is higher than that of the normal specimen.

Fig. 3  Photomicrograph of LOH at p57KIP2 polymorphic sites. Loss of one allele in tumor samples of cases 47 and 61 are demonstrated.

Fig. 4  Comparative DNA and cDNA for preferential loss of a parental allele: Variable parental allelic loss in DNA and cDNA from cases 47 and 61; Table 2). Analysis of the microsatellite markers flanking the p57KIP2 locus on chromosome 11p15.5 showed LOH in six tumors (13%) of the 45 informative cases at marker D11S2359 and in seven tumors (14%) of the informative cases at marker THO1 (data not shown). One tumor specimen showed instability at the THO1 marker. Of the 27 (45%) informative cases for both the p57KIP2 polymorphic locus and markers D11S2359 and THO1, two tumors (7%) showed simultaneous LOH at the p57 locus and either one of the microsatellite markers, and one tumor manifested LOH at the p57 locus and instability of the THO1 locus. None of the tumors manifested concurrent LOI and LOH.

LOH was scored if one band showed a >50% intensity reduction in tumor specimen. LOH at the p57 polymorphic locus on exon 2 was noted in 10 (33.3%) tumors (Fig. 3, cases 47 and 61; Table 2). Analysis of the microsatellite markers flanking the p57KIP2 locus on chromosome 11p15.5 showed LOH in six tumors (13%) of the 45 informative cases at marker D11S2359 and in seven tumors (14%) of the informative cases at marker THO1 (data not shown). One tumor specimen showed instability at the THO1 marker. Of the 27 (45%) informative cases for both the p57 polymorphic locus and markers D11S2359 and THO1, two tumors (7%) showed simultaneous LOH at the p57 locus and either one of the microsatellite markers, and one tumor manifested LOH at the p57 locus and instability of the THO1 locus. None of the tumors manifested concurrent LOI and LOH.

No correlation between LOI and LOH at the p57 gene or the flanking microsatellite markers was noted (P = 0.61; Table 3).

Our mutational screening of the p576 exons by SSCP analysis failed to yield any mutations in exons 1 and 2. However, a polymorphism representing a 12-bp deletion in one allele was observed. To determine preferential parental allelic loss, comparison of the banding pattern between cDNA of normal mucosa and normal and tumor DNA from the 10 cases with LOH at the p57KIP2 gene were performed. Loss of maternal allele was considered if the same DNA band that appeared in normal cDNA was lost (assuming that the maternal allele is expressed in normal cDNA). We observed equal allelic loss at both alleles of the p57 gene, indicating lack of specific paternal allelic loss of this gene (Fig. 4, cases 18, 39, 47, and 61).
**DISCUSSION**

Genomic imprinting is a unique epigenetic feature that may play a significant role in tumorigenesis (1–3, 39). The human chromosome 11p15.5 region contains imprinted domains that include H19, IGF2, and p57kip2 genes (5, 6, 15, 16, 19–22). Imprinting dysregulation of these genes has frequently been reported in several embryonal and adult solid malignancies, suggesting an association with their development (8, 9, 11, 15–18, 22–29, 31, 32). p57kip2, a CDK inhibitor, is a newly described maternally expressed cell cycle gene (13, 15, 22). Alterations of the maternal allele of p57kip2 by LOH or mutation without changes in the imprinted paternal allele have been observed in both embryonic and adult solid malignancies (3, 6, 7, 14, 17, 37, 39). The association of G1 cell arrest with overexpression (9, 14, 44–47) may involve genome imprinting. Proc. Natl. Acad. Sci. USA, 86: 7480–7484, 1989.

Our results show infrequent alterations of the p57kip2 gene and detectable expression in all cases with LOH within and at the flanking 11p15.5 loci of this gene in HNSC. We also observed biallelic expression and elevation of mRNA content in tumors with LOI. These results, together with the lack of sequence alterations and the consistent expression, argue against a tumor suppressor role for this gene in HNSC (3, 9, 11, 17, 22, 36, 37, 39–41). Our results, however, are in agreement with previous studies of embryonal and lung carcinomas (10, 16, 42, 43) and are supported by in vitro experiments negating an association between p57kip2 and tumor suppression (12). Para-adoxically, a tumor suppressor function is evinced by the detection of nonsense mutation in its CDK inhibitory domain, development of Beckwith-Wiedemann syndrome phenotype in knockout mice, lack of expression in certain tumors, and association of G1 cell arrest with overexpression (9, 14, 44–47). The differences between these studies could be attributed to organ- or tumor-specific modifications of this gene.

Our findings of expression in cases with LOH at the p57 gene in our case are similar to those previously reported in lung carcinoma (39). Contrary to the lung carcinoma study (39), no selective parental LOH of the p57kip2 in our cases was observed. These results suggest that variations in the imprinting level account for the p57 expression level in tumors. Quantitative studies to precisely determine the effect of imprinting status on the expression level of this gene are needed. Taken together, the lack of preferential parental loss, infrequent imprinting dysregulation, and rare genetic alterations indicate a minimal role for the p57 gene in HNSC. However, the biallelic and elevated mRNA expression in some of our cases and in other tumor subtypes (22, 45) indicate either a leaky/incomplete imprinting or paternal LOI and suggest a possible oncogenic role for this gene in a subset of these tumors. Further studies are needed to determine the biological effect of the biallelic expression of this gene in tumorigenesis.

**REFERENCES**

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