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# Allele-Specific Expression Analysis of *PEG1/MEST* in Head and Neck Squamous Cell Carcinomas

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**Genomic imprinting is an epigenetic feature that plays a significant role in carcinogenesis. In this study, we examined the expression status of an imprinted gene, paternally expressed gene 1/mesoderm-specific transcript (*PEG1/MEST*), in 38 cases of head and neck squamous cell carcinomas (HNSCCs) and in 17 oral squamous cancer cell lines. Loss of imprinting (LOI) of *PEG1/MEST* was found in 8 of 10 (80%) in tumor specimens, and 6 of 10 (60%) informative cases even in the extracted normal tissue specimens. As for the oral squamous cancer cell lines, LOI was detected in 5 of 8 (62.5%) informative cases in *PEG1/MEST*. Thus, these data showed that abnormal expression of *PEG1/MEST* was found at a high frequency in the tumor, the extracted normal tissue specimens and the oral squamous cancer cell lines. *PEG1/MEST* LOI in extracted normal tissue specimens may have a potential individual cancer risk for HNSCC.**

**Key words:** head and neck squamous cell carcinoma; loss of imprinting; *PEG1/MEST*

Head and neck cancers are almost always squamous cell types arising in the oral cavity, tongue, pharynx and larynx. Understanding the molecular mechanisms involved in tumor development and progression has enabled the design of new biological approaches. This study focuses on molecular studies on the roles of genomic imprinting. Almost all imprinted genes identified to date can be classified as regulators of embryonic growth, placental growth or adult metabolism (Morison et al., 2005). Monoallelic expression of either the maternal or paternal copy occurs while the other copy is silenced in normal somatic tissue specimens. Imprinted genes are often clustered in chromosomal domains and are thought to be coordinately regulated by imprinting control centers. More than 60 types of imprinted genes have been identified in humans (Delaval and Feil, 2004).

Mutations that affect the epigenetic states of imprinted domains underlie a number of human diseases (Walter and Paulsen, 2003). Loss of imprinting (LOI) is one of the most frequent alterations

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HNSCC, head and neck squamous cell carcinoma; LOI, loss of imprinting; *PEG1/MEST*, paternally expressed gene 1/mesoderm-specific transcript; RFLP, restriction fragment length polymorphism; RT, reverse transcriptase; SNP, single nucleotide polymorphism

found in cancers and involves abnormal activation of a normally silent allele. Jaenisch and colleagues have demonstrated that global LOI leads to tumor formation in chimeric mice. All the tumors in the mice were derived from non-imprinted cells. Most importantly, tumors were not seen in the offspring of the chimaeras, as imprinting is reset during gametogenesis (Goymer, 2005).

Imprinting is therefore an epigenetic tumor-suppressing phenomenon. When imprinting is lost, cells are immortalized through the inappropriate regulation of both tumor suppressors and oncogenes (Holm et al., 2005). The genes exhibiting LOI in human cancer leading to biallelic expression has been reported in a variety of tumors including head and neck squamous cell carcinomas (HNSCCs) (Schofield et al., 2001; Feinberg and Tycko, 2004). Previous studies regarding imprinted genes in HNSCC suggested that LOI of *IGF2*, *H19* and $p57^{KIP2}$ loci play an important role in oncogenesis (el-Naggar et al., 1999; Lai et al., 2000; Rainho et al., 2001). However, the involvement of other imprinted genes is still poorly understood in HNSCC.

Paternally expressed gene 1/mesoderm-specific transcript (*PEG1/MEST*) is a paternally expressed gene located on human chromosome 7q32 (Kobayashi et al., 1997). *PEG1/MEST* LOI has been reported in lung cancers (Suda et al., 2003; Nakanishi et al., 2004), breast cancers (Pedersen et al., 1999, 2002) and colorectal cancers (Nishihara et al., 2000). In these previous studies, it was postulated that the disruption of *PEG1/MEST* might be implicated in the etiology of these cancers. In this study, we examined the imprinting status of *PEG1/MEST* in HNSCC specimens and oral squamous cancer cell lines with special attention to the role of *PEG1/MEST* LOI on oncogenesis in HNSCC tissue specimens and several oral squamous cancer cell lines.

## Materials and Methods

### Tissue samples

HNSCCs, diagnosed by histopathologic examination and matched with extracted normal tissue specimens from 38 patients, were analyzed in this study. They consisted of 16 oral cancers, 4 oropharyngeal cancers, 5 hypopharyngeal cancers and 13 laryngeal cancers. All tissue specimens were obtained during surgery at the Clinical Department of Otolaryngology, Head and Neck Surgery, Tottori University Hospital. Each resected tumor was carefully trimmed to remove normal tissue. Then extracted normal tissue was selected as far from the cancerous areas as possible. We usually picked extracted normal tissue from the muscle, which was removed when neck dissections were performed. Informed consent to participate in this study was obtained from each patient. The institutional review board of Tottori University approved this study (approval number 746). All tissue specimens were stored at –80°C until analysis.

### Cell lines

Seventeen oral squamous cancer cell lines were analyzed. HSC-2, HSC-3 and HSC-4 were established and provided by Uzawa and associates (Uzawa et al., 1995). HO-1-N-1, HO-1-u-1, KON, KOSC-2 cl3-43, OSC-19, OSC-20, SCC-4 and SKN-3 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). SAS were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Eight oral squamous cancer cell lines (HSC-2, HSC-3, HSC-4, HSC-5, HSC-6, HSC-7, KON and SCCKN) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Two (HO-1-N-1 and HO-1-u-1) were grown in DMEM-F12 medium (1:1 mix) with 10% fetal bovine serum. Two (OSC-19 and OSC-20) were grown in DMEM-F12 medium (1:1 mix) without serum. SCC-4 was grown in DMEM-F12 medium (1:1 mix) with 10% fetal bovine serum and 0.4 μg/mL hydrocortisone. Four (KOSC-2 cl3-43, SAT, SAS and SKN-3) were grown in RPMI 1640 medium with 10% fetal bovine serum. These cells were cultured and growing cells with 70% confluence were harvested for analyses.

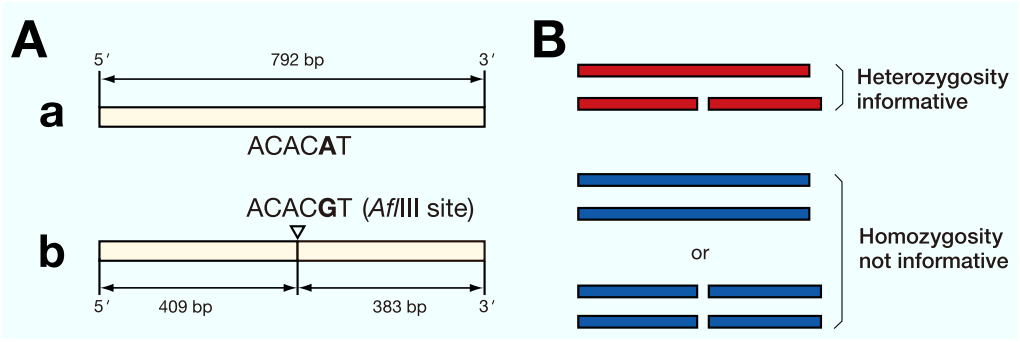


**Fig. 1.**
**A:** A schematic of the *Afl*III polymorphism of human *PEG1/MEST* is shown. The arrow indicates the site of the polymorphism. PCR products contain this polymorphic site. **A-a** represents the undigested product (792 bp) and **A-b** represents product digested with *Afl*III (383 and 409 bp). *PEG1/MEST,* paternally expressed gene 1/mesoderm-specific transcript.
**B:** Heterozygosity called informative is necessary for allele-specific analysis.

### *DNA and RNA sample preparation*

Genomic DNA was isolated using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Total RNA was isolated using an RNeasy Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase I (Takara Bio, Shiga, Japan) to remove any DNA contamination. First-strand cDNA synthesis was carried out with or without SuperScript III Reverse Transcriptase (RT+ or –) (Invitrogen, Carlsbad, CA) by using an oligo $(dT)_{15}$ primer (Roche, Basel, Switzerland). The sequences of the primers were 5-CACTGATGCAGAAAGACGTTC-3 and 5-CAGCACCATTTGCTCATAGG-3.

### *Identification of polymorphisms*

Imprinting analysis for *PEG1/MEST* was carried out by single nucleotide polymorphism (SNP) analysis for informative heterozygotes at the G/A (exon 12; rs10863) polymorphism. An informative DNA polymorphism at an *Afl*III site was identified as the *Afl*III-digestible (ACACGT) or indigestible (ACACAT) sequence. PCR was performed for 30 cycles including denaturation at 95˚C for 30 s, annealing at 62˚C for 30 s and extension at 72˚C for 30 s.

### *Allele-specific expression analysis*

Allele-specific expression analysis with RT-PCR and digestion by restriction enzymes was performed under the same conditions as those used to detect genomic polymorphisms. To confirm the results of restriction fragment length polymorphism (RFLP), we also performed a method for allele-specific expression analysis of imprinted genes by real-time PCR with Cycling Probe Technology (Takara Bio). In this method, one end of the probe is labeled with a fluorescent substance and the other end is labeled with a quencher, which quenches the fluorescence emitted from the substance. RNaseH specificially cuts the RNA region of this probe, resulting in emission of strong fluorescence, but it does not cut the RNA probe region including any mismatches.

## Results

We analyzed 38 primary HNSCCs for the LOI in *PEG1/MEST.* First of all we identified genomic polymorphisms using RFLP analysis. Informative cases were then further analyzed to determine their allele-specific expression. As shown in Figs. 1 and 2, we distinguished the 2 alleles and determined whether a case is informative or not. This polymorphism (G/A; rs10863) results in the presence

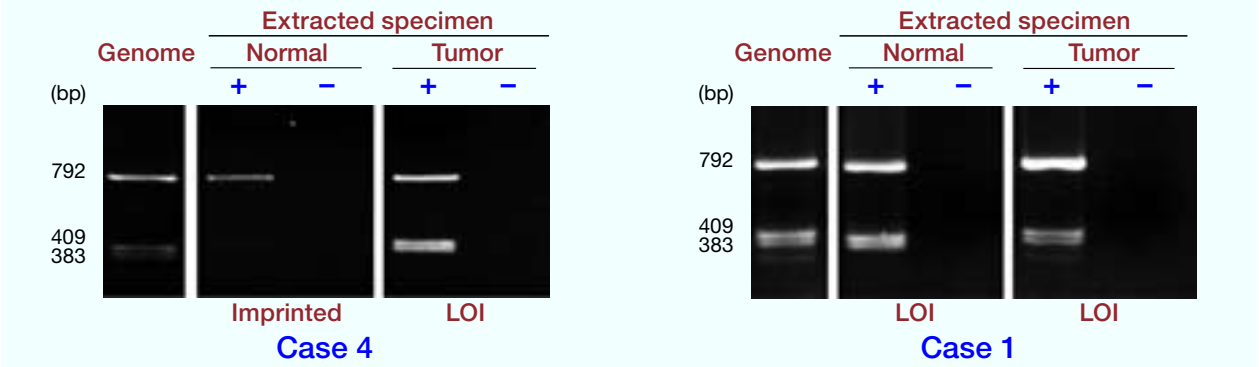


**Fig. 2.** Allele-specific expression analysis of *PEG1/MEST* in head and neck squamous cell carcinoma tissue specimens. The allele-specific expressions of *PEG1/MEST* were assessed by restriction fragment length polymorphism analysis. Representative results of *PEG1/MEST* are shown. +, presence of reverse transcriptase (RT); –, absence of RT; LOI, loss of imprinting; *PEG1/MEST,* paternally expressed gene 1/mesoderm-specific transcript.

or absence of an *Afl*III site. The 3 different bands (792, 409 and 383 bp) after *Afl*III digestion of the genomic PCR fragment indicated an informative heterozygous case. Next, to determine the expression status of *PEG1/MEST*, the RT-PCR products are digested with *Afl*III to determine whether one or both copies are expressed. The presence of 3 bands indicates biallelic expression, that is, *PEG1/MEST* LOI. On the other hand, a single band of 792 bp or 2 bands of 409 bp and 383 bp showed monoallelic expression, indicating the normal imprinting status of *PEG1/MEST*. Among the cases screened, we observed 10 informative cases of *PEG1/MEST*. *PEG1/MEST* LOI was found in 8 informative cases (80%), and in 6 cases (60%) it was detected even in the normal tissue specimens (Table 1), regardless of tumor location or histology. These data suggest that *PEG1/MEST* LOI occurs in HNSCC specimens, suggesting its fundamental role in HNSCC oncogenesis.

Next, we analyzed the imprinting status of *PEG1/MEST* in 17 oral squamous cancer cell lines to substantiate the results from the tissue specimens. The results for the histopathologic types of these oral squamous cancer cell lines are summarized in Table 2. *PEG1/MEST* LOI was observed in 5 of 8 informative cases (62.5%). The results from the cell lines are summarized in Table 2, which were consistent with the high frequency of LOI at the *PEG1/MEST* loci detected in the HNSCC tissue specimens. Thus, abnormal expression of *PEG1/MEST* was found at a high frequency in the cancer tissue specimens, the extracted normal tissue specimens and the oral squamous cancer cell lines.

**Table 1. Summary of *PEG1/MEST* gene expression profiles in informative cases of tissue specimens of head and neck squamous cell carcinoma**

| Case number | Type of cancer | Histological classification | *PEG1/MEST* | |
|---|---|---|---|---|
| | | | Normal | Tumor |
| 1 | Tongue cancer | Moderately differentiated | LOI | LOI |
| 2 | Laryngeal cancer | Moderately differentiated | LOI | LOI |
| 3 | Laryngeal cancer | Well differentiated | LOI | LOI |
| 4 | Hypopharyngeal cancer | Poorly differentiated | Imprinted | LOI |
| 5 | Tongue cancer | Well differentiated | Imprinted | Imprinted |
| 6 | Tongue cancer | Moderately differentiated | Imprinted | LOI |
| 7 | Tongue cancer | Moderately differentiated | Imprinted | LOI |
| 8 | Laryngeal cancer | Moderately differentiated | LOI | LOI |
| 9 | Oropharyngeal cancer | Moderately differentiated | LOI | LOI |
| 10 | Laryngeal cancer | Poorly differentiated | LOI | Imprinted |

LOI, loss of imprinting; *PEG1/MEST,* paternally expressed gene 1/mesoderm-specific transcript.

## Discussion

Several lines of evidence suggest that the disruption of imprinting mechanisms play a critical role in oncogenesis including HNSCC, but none have examined *PEG1/MEST* in HNSCC. The data from mice studies of *Mest* have suggested a possible role of *Mest* as a regulator of embryonic growth. *Mest* paternal knockouts have been shown to result in an imprinted phenotype characterized by fetal and placental growth retardation (Lefebvre et al., 1998). In our report, we provide the first evidence regarding the presence of biallelic expression of *PEG1/MEST* in HNSCC. *PEG1/MEST* LOI was identified 8 out of 10 cases in HNSCC tissue specimens and 5 out of 8 cases in oral cancer cell lines. Interestingly, *PEG1/MEST* LOI was observed in the extracted normal tissue surrounding the tumor specimens.

*IGF2* and *PEG1/MEST* are known as imprinted genes important in fetal growth. The correlation between *IGF2* LOI in normal cells and colorectal cancer has been well documented (Cui et al., 2003; Nakano et al., 2006). *IGF2* LOI has been found in 10% of normal individuals (Sakatani et al., 2001) and it appears to be 5 times more common in patients with a family history of colon carcinoma, and 21 times more common in patients with a personal history of colorectal neoplasia (Cui et al., 2003). Moreover, a mouse model with *Igf2* LOI suggests that mice with abnormal imprinting acquired twice as many intestinal adenomas as those whose imprinting was normal (Sakatani et al., 2005). These studies indicated that *IGF2* LOI might be a valuable predictive marker of an individual's risk for colorectal cancer.

*PEG1/MEST* LOI in both extracted normal and tumor tissue specimens suggests that the detection of *PEG1/MEST* LOI in extracted normal tissue specimens may also pose a potential cancer risk in individuals for HNSCC. *IGF2* LOI has been found in 10% of normal individuals. However, there are no reports indicating the incidence of *PEG1/MEST* LOI in normal individuals. It will be necessary to clarify the incidence of *PEG1/MEST* LOI in normal individuals in order to elucidate its predictive value for an individual's cancer risk for HNSCC.

**Table 2. Summary of allele-specific expression of *PEG1/MEST* in 17 oral squamous cancer cell lines**

| Cell line | | Type of cancer | *PEG1/MEST* |
|---|---|---|---|
| Number | Name | | |
| 1 | HSC-2 | Oral cavity | Imprinted |
| 2 | HSC-3 | Tongue | Imprinted |
| 3 | HSC-4 | Tongue | * |
| 4 | HSC-5 | Poorly differentiated | * |
| 5 | HSC-6 | Tongue | * |
| 6 | HSC-7 | Tongue | LOI |
| 7 | HO-1-N-1 | Bucchal mucosa | LOI |
| 8 | HO-1-u-1 | Mouth floor | * |
| 9 | KON | Oral floor | * |
| 10 | KOSC-2 cl3-43 | Oral floor | Imprinted |
| 11 | OSC-19 | Tongue | LOI |
| 12 | OSC-20 | Tongue | * |
| 13 | SAS | Tongue | LOI |
| 14 | SAT | Oral cavity | * |
| 15 | SCCKN | Oral cavity | * |
| 16 | SCC-2 | Tongue | * |
| 17 | SKN-3 | Oral cavity | LOI |
| Frequency of LOI | | 5/8 (62.5%) | |

* Non-informative case.
LOI, loss of imprinting; *PEG1/MEST,* paternally expressed gene 1/mesoderm-specific transcript.

## References


1 Cui H, Cruz-Correa M, Giardiello FM, Hutcheon DF, Kafonek DR, Brandenburg S, et al. Loss of *IGF2* imprinting: a potential marker of colorectal cancer risk. Science 2003; 299:1753–1755.

2 Delaval K, Feil R. Epigenetic regulation of mammalian genomic imprinting. Curr Opin Genet Dev 2004;14: 188–195.

3 el-Naggar AK, Lai S, Tucker SA, Clayman GL, Goepfert H, Hong WK, et al. Frequent loss of imprinting at the *IGF2* and *H19* genes in head and neck squamous carcinoma. Oncogene 1999;18:7063–7069.

4 Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004;4:143–153.

5 Goymer P. Cancer epigenetics: dangerous unmarked genes. Nature Reviews Genetics 2005; 6:878-878.

6 Holm TM, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout WM 3rd, Jaenisch R. Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell 2005; 8:275-285.

7 Kobayashi S, Kohda T, Miyoshi N, Kuroiwa Y, Aisaka K, Tsutsumi O, et al. Human *PEG1/MEST*, an imprinted gene on chromosome 7. Hum Mol Genet 1997;6:781–786.
8 Lai S, Goepfert H, Gillenwater AM, Luna MA, El-Naggar AK. Loss of imprinting and genetic alterations of the cyclin-dependent kinase inhibitor *p57$^{KIP2}$* gene in head and neck squamous cell carcinoma. Clin Cancer Res 2000;6:3172–3176.
9 Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene *Mest*. Nat Genet 1998;20:163–169.
10 Morison IM, Ramsay JP, Spencer HG. A census of mammalian imprinting. Trends Genet 2005; 21:457–465.
11 Nakanishi H, Suda T, Katoh M, Watanabe A, Igishi T, Kodani M, et al. Loss of imprinting of *PEG1/MEST* in lung cancer cell lines. Oncol Rep 2004;12:1273–1278.
12 Nakano S, Murakami K, Meguro M, Soejima H, Higashimoto K, Urano T, et al. Expression profile of LIT1/KCNQ1OT1 and epigenetic status at the KvDMR1 in colorectal cancers. Cancer Sci 2006;97:1147–1154.
13 Nishihara S, Hayashida T, Mitsuya K, Schulz TC, Ikeguchi M, Kaibara N, et al. Multipoint imprinting analysis in sporadic colorectal cancers with and without microsatellite instability. Int J Oncol 2000;17:317–322.
14 Pedersen IS, Dervan PA, Broderick D, Harrison M, Miller N, Delany E, et al. Frequent loss of imprinting of *PEG1/MEST* in invasive breast cancer. Cancer Res 1999;59:5449–5451.
15 Pedersen IS, Dervan P, McGoldrick A, Harrison M, Ponchel F, Speirs V, et al. Promoter switch: a novel mechanism causing biallelic *PEG1/MEST* expression in invasive breast cancer. Hum Mol Genet 2002;11:1449–1453.
16 Rainho CA, Kowalski LP, Rogatto SR. Loss of imprinting and loss of heterozygosity on 11p15.5 in head and neck squamous cell carcinomas. Head Neck 2001;23: 851–859.
17 Sakatani T, Kaneda A, Iacobuzio-Donahue CA, Carter MG, de Boom Witzel S, Okano H, et al. Loss of imprinting of *Igf2* alters intestinal maturation and tumorigenesis in mice. Science 2005;307:1976–1978.
18 Sakatani T, Wei M, Katoh M, Okita C, Wada D, Mitsuya K, et al. Epigenetic heterogeneity at imprinted loci in normal populations. Biochem Biophys Res Commun 2001;283:1124–1130.
19 Schofield PN, Joyce JA, Lam WK, Grandjean V, Ferguson-Smith A, Reik W, et al. Genomic imprinting and cancer: new paradigms in the genetics of neoplasia. Toxicol Lett 2001;120:151–160.
20 Suda T, Katoh M, Hiratsuka M, Fujiwara M, Irizawa Y, Oshimura M. Use of real-time RT-PCR for the detection of allelic expression of an imprinted gene. Int J Mol Med 2003;12:243–246.
21 Uzawa N, Yoshida MA, Oshimura M, Ikeuchi T. Suppression of tumorigenicity in three different cell lines of human oral squamous cell carcinoma by introduction of chromosome 3p via microcell-mediated chromosome transfer. Oncogene 1995;11:1997–2004.
22 Walter J, Paulsen M. Imprinting and disease. Semin Cell Dev Biol 2003;14:101–110.

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