Altered Expression of Imprinted Genes in Squamous Cell Carcinoma of the Head and Neck

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Abstract. *Background: Genomic imprinting is associated with many human diseases, including various types of cancers, however, no studies on gene imprinting are related to squamous cell carcinoma of the head and neck (SCCHN) directly. Materials and Methods: In this study, the expression of a panel of 15 imprinted genes in cancerous and non-cancerous tissues from 73 patients with SCCHN were investigated. Results: Altered expression of carboxypeptidase A4 (CPA4); protein phosphatase 1 regulatory subunit 9A (PPP1R9A); H19, imprinted maternally expressed transcript (non-protein coding) (H19); paternally expressed gene 3 antisense RNA 1 (PEG3-AS1); retrotransposon-like 1 (RTL1), insulin-like growth factor 2 (IGF2); solute carrier family 22 member 3 (SLC22A3); and gamma-aminobutyric acid type A receptor beta3 subunit (GABRB3) was observed. Down-regulation of PPP1R9A ($p<0.05$) and GABRB3 ($p<0.05$) was correlated with more advanced cancer stages. Down-regulation of PEG3-AS1 ($p<0.05$) and GABRB3 ($p<0.01$) was correlated with lymph node metastasis. Poor survival was related to higher expression of CPA4 ($p<0.01$) and lower expression of PEG3-AS1 ($p<0.05$) and IGF2 ($p<0.05$). Chemotherapy was also found to have an impact on the expression of imprinted genes. Conclusion: Loss of imprinting is involved in tumorigenesis of SCCHN.*

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Key Words: Squamous cell carcinoma of the head and neck (SCCHN), genomic imprinting, imprinted genes, real-time quantitative RT-PCR.

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common malignancy worldwide, affecting 600,000 new patients each year. It also had the fifth prevalence rate and was the fourth leading cause of mortality in Taiwan, 2010 (1). The survival of patients with SCCHN has remained unchanged. No matter how great the progress of surgical techniques and effective chemotherapeutic agents, patients with unresectable tumor or recurrent or metastatic cancer still have a worse prognosis and a poor overall survival (2). Recurrence and metastasis often occur following primary treatment in advanced-stage cases and are associated with significant morbidity and mortality.

Both genetic lesions (mutations, deletions, translocations *etc.*) and epigenetic aberrations can induce cancer formation (3, 4). One epigenetic phenomenon that might contribute to the development and progression of cancer in humans is genomic imprinting. Mammals are diploid organisms carrying two alleles of each autosomal gene, one inherited from the mother and the other from the father. According to classical Mendelian inheritance, the phenotype of the offspring general results from the effects of both alleles. For imprinted genes however, one of these alleles is silenced and its expression depends on either the maternal, or the paternal allele. In individuals with a paternally imprinted gene, only the allele inherited from the mother is expressed, and vice versa (5, 6). Genomic imprinting is associated with many human diseases or syndromes, such as Prader-Willi, Angelman, Beckwith-Wiedemann, Retts, and Silver-Russell syndromes, as well as various types of cancers (7). In Wilms'

tumor, hypomethylation-induced loss of imprinting (LOI) of insulin-like growth factor 2 (*IGF2*), an important autocrine growth factor, results in its pathological biallelic expression. LOI of *IGF2* is also associated with colorectal cancer (8). *IGF2* gene expression is imprinted (monoallelic), when the imprinting is lost it promotes tumor progression and metastasis (9). Thus, DNA hypomethylation leads to aberrant activation of genes and non-coding regions through a variety of mechanisms and can contribute to cancer development and progression.

The role of imprinted genes in SCCHN has not yet been investigated. Therefore in this study, we surveyed a panel of 15 human imprinted genes, namely chromosome 15 open reading frame 2 (*C15ORF2*); coatomer protein complex subunit gamma 2 (*COPG2*); carboxypeptidase A4 (*CPA4*); gamma-aminobutyric acid type A receptor beta3 subunit (*GABRB3*); H19, imprinted maternally expressed transcript (non-protein coding) (*H19*); insulin-like growth factor 2 (*IGF2*); inositol polyphosphate-5-phosphatase F (*INPP5F*); lethal (3) malignant brain tumor-like protein (*L3MBTL*); paternally-expressed gene 3 antisense RNA 1 (*PEG3-AS1*); protein phosphatase 1 regulatory subunit 9A (*PPP1R9A*); small nuclear ribonucleoprotein polypeptide N upstream reading frame (*SNURF*); retrotransposon-like 1 (*RTL1*); solute carrier family 22 member 3 (*SLC22A3*); transcription elongation factor B subunit 3C (*TCEB3C*); and zinc finger protein (*ZNF215*), in tissues of SCCHN from 73 patients using real-time quantitative reverse-transcriptase-polymerase chain reaction (qRT-PCR) to examine if the expression of imprinted genes were altered in SCCHN. The assessment of response to chemotherapy can evaluate the efficacy of chemotherapeutic agents and identify novel biomarkers. Therefore, the aims of our study were to investigate the expression of imprinted genes in tissues of human SCCHN and evaluate the impact of chemotherapy by *in vitro* studies.

Materials and Methods

Patients and samples. Samples of tumors and the adjacent non-cancerous tissues were obtained from 73 patients (70 men and three women), aged 34-82 years (mean±S.D.=55.32±10.79 years) diagnosed with SCCHN undergoing surgery at the Division of Laryngology, Department of Otolaryngology, Kaohsiung Chang Gung Memorial Hospital from 2009 throughout 2012. Clinical pathological characteristics, including age, sex, TNM staging, tumor size, and survival are listed in Table I. The specimens were obtained immediately after resection, and snap-frozen in liquid nitrogen and stored until use. Informed consent was obtained from all patients prior to tissue acquisition. This study was approved by the Institutional Review Board of the Kaohsiung Chang Gung Memorial Hospital (IRB No. 100-4455A3).

Analysis of imprinted genes by qRT-PCR. Total RNA was extracted from cancerous tissues and noncancerous tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to generate cDNA. Fifteen imprinted genes were analyzed: *COPG2, CPA4, GABRB3, H19, IGF2, INPP5F, L3MBTL, PEG3-AS, PPP1R9A, SNURF, RAS-GRF1, RTL1, SLC22A3, TCEB3C* and *ZNF215*. Sequences of the forward and reverse primers are listed in Table II. All reactions were carried out in a 10-μl final volume containing 25 ng cDNA (as total input RNA), 200 nM each primer and 10 μl 2× Power SYBR® Green PCR Master Mix (Applied Biosystems). Real-time qPCR was performed in an ABI 7500 Fast Real-Time System (Applied Biosystems) and the PCR cycling parameters were 95˚C for 10 minutes followed by 40 cycles of PCR reactions at 95˚C for 20 seconds and 60˚C for 1 minute. The expression levels of the imprinted genes were normalized to the internal control β-actin (*ACTB*) to obtain the relative threshold cycle (ΔCt) and the relative expression between cancer and noncancerous tissues was calculated by the comparative Ct (ΔΔCt) method.

Table I. *Characteristics of patients with squamous cell carcinoma of the head and neck.*

Characteristic	Value
Gender, n	
Male	70
Female	3
Median (range) age, years	55.32 (34-82)
Staging, n	
I	13
II	13
III	12
IV	35
Tumor size, n	
<3 cm	33
>3 cm	40
Neck metastasis, n	
Positive	28
Negative	45
Survival, n	
Died	22
Survived	51

SCCHN cell lines and treatment with chemotherapeutic drugs. Human SCCHN cell lines (FaDu and SCC-4) were purchased from the Food Industry Research and Development Institute, Taiwan. Cells were maintained in MEM-F15 medium (Gibco, Carlsbad, CA, USA) containing 10% HyClone fetal bovine serum (Thermo Scientific, Carlsbad, CA, USA) and grown at 37˚C with 5% CO_2. FaDu and SCC-4 cells were treated with 500 μM 5-fluorouracil (5-FU), 500 nM paclitaxel, 10 μM cisplatin, and 100 nM doxorubincin, respectively. After incubation for 24 hours, cells were harvested for RNA extraction, cDNA generation, and qRT-PCR analysis.

Statistical analysis. Paired *t*-test was used to detect the differences between two groups for expression of each imprinted gene, and the values of ΔCt were used for the statistical analysis. The test was two-sided with statistical significance set at 0.05 and all computations were carried out using SPSS for Windows Release 15.0 software (SPSS, Chicago, IL, USA).

Table II. *Oligonucleotide primers for real-time quantitative reverse transcriptase-polymerase chain reaction analysis of the 15 studied imprinted genes.*

Gene	Gene name	Location	Expressed allele	GenBank accession no.	Amplicon size (bp)	Sequence	Primer location
C15ORF2	Chromosome 15 open reading frame 2	15q11-q13	Unknown	AB527113.1	61	F: 5'-GTG ACA GCA TTG CCT CAG C-3' R: 5'-GGT CTC CTA TCT GCC TGT GC-3'	3062-3080 3103-3122
COPG2	Coatomer protein complex subunit gamma 2	7q32 AS	Paternal	NM_012133.4	67	F: 5'-TTC CAG ATG AGG ATG GGT ATG-3' R:5'-TGG TCA GAC ACA GTC ACT TCG-3'	2303-2323 2349-2369
CPA4	Carboxypeptidase A4	7q32	Maternal	NM_016352.3	85	F: 5'-GTC GGG CAC TGA GTA CCA A-3' R:5'-GTT GTC ATA CGC CCA GTC G-3'	1082-1100 1148-1166
GABRB3	Gamma-aminobutyric acid type A receptor beta3 subunit	15q11.2-q12 *AS*	Paternal	NM 000814.5	72	F: 5'-GGG TGT CCT TCT GGA TCA ATT A-3' R:5'-GTT GTC AGC ACA GTT GTG ATC C-3'	899-920 950-970
H19	H19, imprinted maternally expressed transcript (non-protein coding)	11p15.5 *AS*	Maternal	NR_002196.1	84	F: 5'-TTA CTT CCT CCA CGG AGT CG-3' R:5'-GCT GGG TAG CAC CAT TTC TT-3'	3050-3069 4570-4589
IGF2	Insulin like growth factor 2	11p15.5 *AS*	Paternal	NM_000612.4	101	F: 5'-ACA CCC TCC AGT TCG TCT GT -3' R: 5'-GAA ACA GCA CTC CTC AAC GA -3'	868-887 949-968
INPP5F	Inositol polyphosphate-5-phosphatase F	10q26.11	Paternal	NM_014937.3	87	F: 5'-TTC TTG ATA TGA AGT GGT GTT GG-3' R: 5'-GGC AGT CCA TAC AAT TAA CAC G-3'	1514-1536 1579-1600
L3MBTL	Lethal(3) malignant brain tumor-like protein	20q13.12	Paternal	NM_015478.6	72	F: 5'-AGC GCA GGG AAT ACC AGA G-3' R: 5'-TTC CTT CTT CTT GCT TCT CCA-3'	564-582 615-635
PEG3-AS1	Paternally-expressed gene 3 antisense RNA 1	19q13.4 AS	Paternal	NR_023847.2	76	F: 5'-GGG TCA AGT CCT AGG TGA AGG-3' R: 5'-CGC CAG ACA CCA GAA TAC C-3'	4802-4822 4747-4765
PPP1R9A	Protein phosphatase 1 regulatory subunit 9A	7q21.3	Maternal	NM_001166160.1	76	F: 5'-GCC CAA AAC ATC ACT GGA G-3' R: 5'-GGG ATG CTG TCA TTC CAA G-3'	4141-4159 4198-4216
RTL1	Retrotransposon-like 1	14q32.31 *AS*	Paternal	NM_001134888.2	60	F: 5'-CGC AGA GAA TTC CAC GAG TT-3' R: 5'-TCT TGG GTA GCT CTG TAA GGT CA-3'	687-706 724-746
SLC22A3	Solute carrier family 22 member 3	6q26-q27	Maternal	NM_021977.3	71	F: 5'-CCA CCA TCG TCA GCG AGT-3' R: 5'-CAG GAT GGC TTG GGT GAG-3'	460-477 513-530
SNURF	Small nuclear ribonucleoprotein polypeptide N upstream reading frame	15q12	Paternal	NM_022804.2	103	F: 5'-CTC ACT GAG CAA CCA AGA GTG T-3' R: 5'-AGC TAA ;/''GAA TGC CTG CCT CA-3'	219-240 302-321
TCEB3C	Transcription elongation factor B subunit 3C	18q21.1 *AS*	Maternal	NM_145653.3	82	F: 5'-GGC CAA GAC GCC TTA TGA T-3 R: 5'-TGG CTC CAT CTC TCC ATT TC-3'	1601-1619 1663-1682
ZNF215	Zinc finger protein 215	11p15.4	Maternal	NM_013250.2	75	F: 5'-TGT CCA AGA CAG CGA TTC C-3' R: 5'-TGC AAG TAG CTT AAG TGG CAA A-3'	220-238 273-294

F: Forward primer; R: reverse primer.

Results

Analysis of expression of imprinted genes in SCCHN using qRT-PCR. Ten pairs of cancerous and non-cancerous tissues from patients with SCCHN were used for the screening of the expression of the 15 imprinted genes using qRT-PCR. Among the 15 genes, nine (*CPA4, PPP1R9A, H19, PEG3-AS1, RTL1, IGF2, SLC22A3, GABRB3*, and *C15ORF2*) were found to be significantly altered (Figure 1A). We further used paired tissues from 73 patients to validate the expression of these nine imprinted genes and found that except for *C15ORF*, the remaining eight genes were indeed significantly altered in cancerous tissues (Figure 1B). *CPA4* was the only gene up-regulated in SCCHN ($p<0.00001$) and *PPP1R9A* was the most down-regulated gene in SCCHN (by ~40-fold $p<0.00001$) (Figure 1B).

Disease severity and expression of imprinted genes in patients with SCCHN. Disease severity depends on cancer staging. The TNM staging system was established by the American

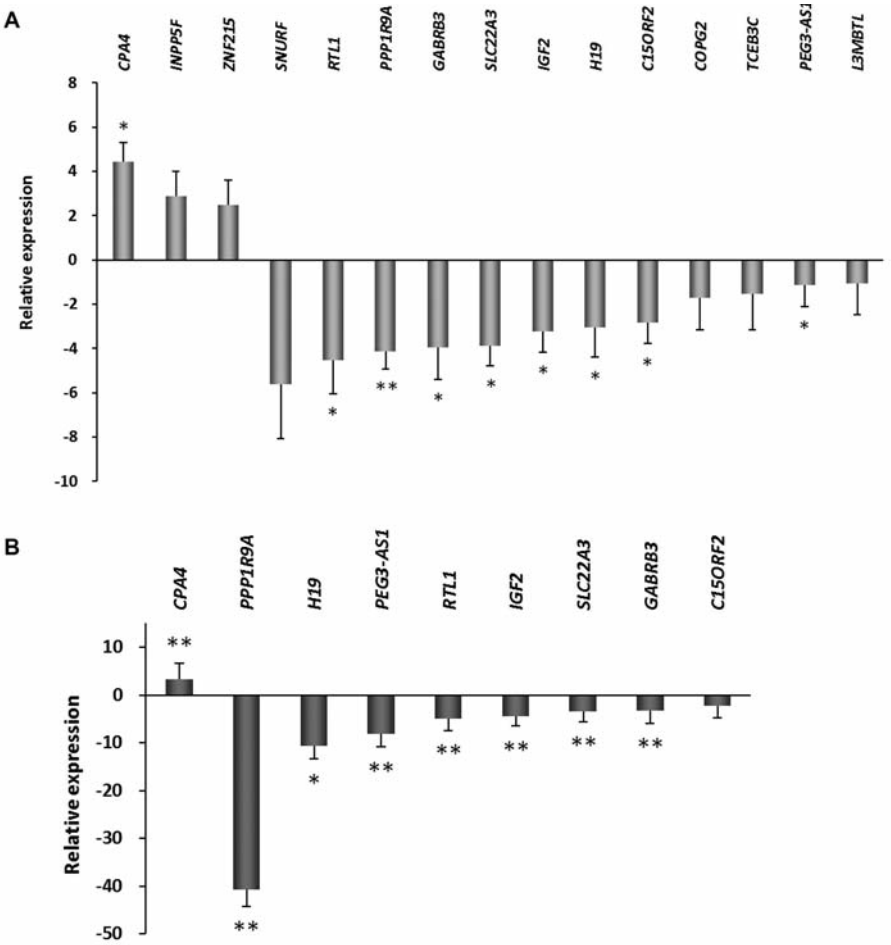

Figure 1. *Expression of imprinted genes in squamous cell carcinoma of the head and neck (SCCHN) determined by real-time quantitative reverse-transcriptase polymerase chain reaction. A: Expression of 15 imprinted genes in 10 paired cancerous and non-cancerous tissues from patients with SCCHN. Expression of nine genes was significantly altered. B: Expression of the nine imprinted genes in paired cancerous and non-cancerous tissues from 73 patients with SCCHN. After validation, expression of eight of the imprinted genes remained significantly deregulated. Expression is reported as the fold change mRNA expression level of cancerous tissues relative to non-cancerous tissue designated a value of 1. The relative expression in cancerous tissues was calculated by the comparative Ct (ΔΔCt method). Statistically significant at *p<0.05 and **p<0.01. C15ORF2: Chromosome 15 open reading frame 2; COPG2: coatomer protein complex subunit gamma 2; CPA4: carboxypeptidase A4; GABRB3: gamma-aminobutyric acid type A receptor beta 3 subunit; H19: H19, imprinted maternally expressed transcript non protein-coding; IGF2: insulin-like growth factor 2; INPP5F: inositol polyphosphate-5-phosphatase F; L3MBTL: lethal(3) malignant brain tumor-like protein; PEG3-AS1: paternally-expressed gene 3 antisense RNA 1; PPP1R9A: protein phosphatase 1 regulatory subunit 9A; SNURF: small nuclear ribonucleoprotein polypeptide N upstream reading frame; RTL1: retrotransposon-like 1; SLC22A3: solute carrier family 22 member 3; TCEB3C: transcription elongation factor B subunit 3C; and ZNF215: zinc finger protein.*

Joint Committee on Cancer includes tumor (T), neck lymph node (N), and metastasis (M) status (10). Stages from I to IV represent the general cancer status from mild to severe and are closely associated with prognosis and therapy response. We divided the patients into those with stage I and II (stage I/II) and stage III and IV (stage III/IV) groups for analysis of correlation with expression of imprinted genes. We found down-regulation of *PPP1R9A*, and *GABRB3* was correlated with more advanced cancer stages ($p<0.05$) (Figure 2A).

Tumor status and expression of imprinted genes in patients with SCCHN. To answer the question whether different tumor statuses affected expression of imprinted genes in SCCHN, we analyzed pathologic reports of the 73 patients with SCCHN and correlated them with expression levels of imprinted genes. Patients were divided into two groups according to their tumor size (< or ≥3 cm). Independent-samples *t*-test demonstrated that none of the imprinted genes displayed significant correlation with tumor size (Figure 2B).

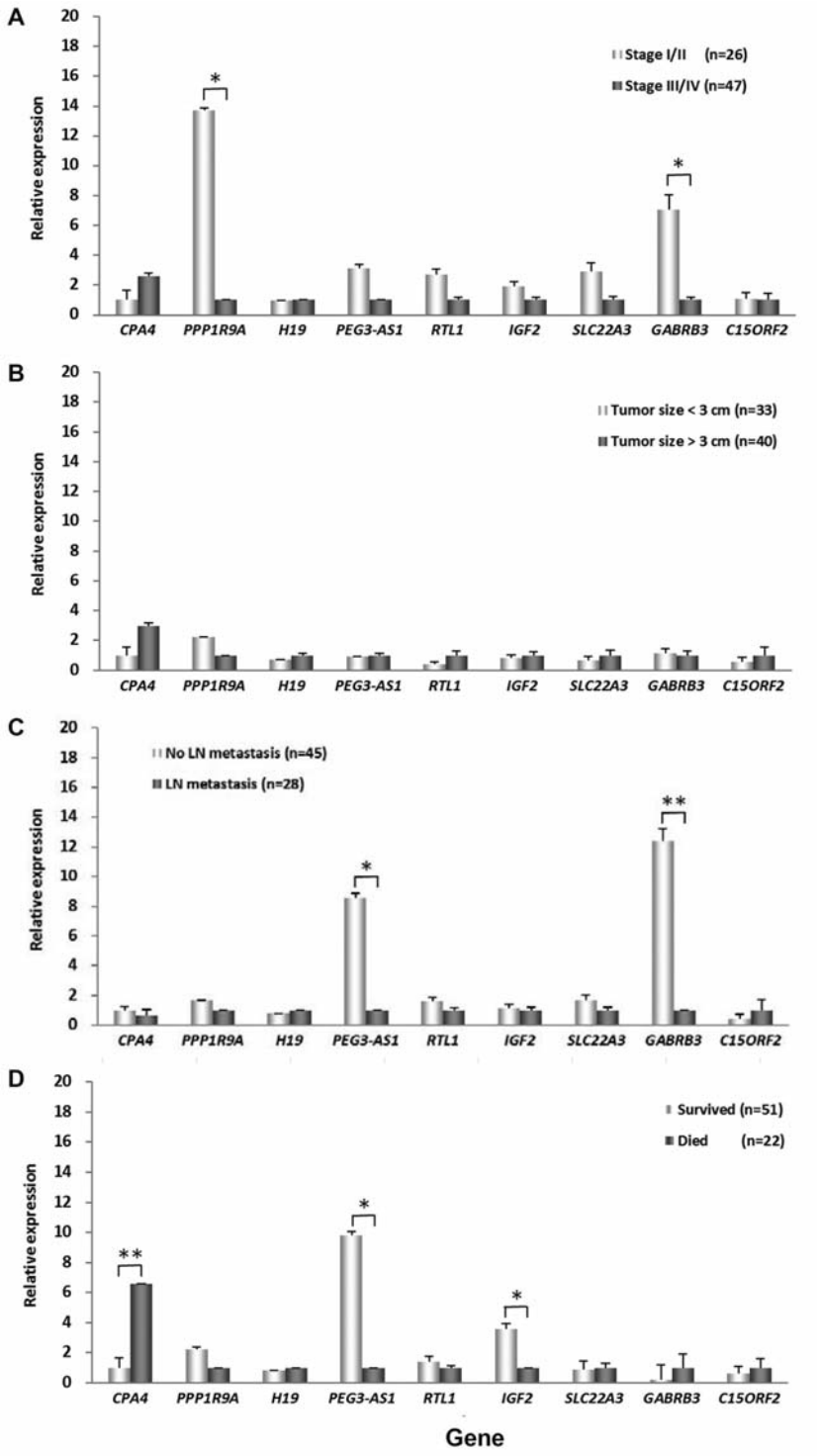

Figure 2. *Expression of imprinted genes in 73 patients with squamous cell carcinoma of the head and neck (SCCHN) according to disease severity i.e. tumor stage (A), tumor size (B), lymph-node (LN) metastasis (C) and survival (D). The relative expression in cancerous tissues was calculated by the comparative Ct (ΔΔCt method). Expression is reported as the fold-change mRNA expression level of cancerous tissues relative to mean mRNA expression in non-cancerous tissues from patients at stage III/IV, tumor size >3 cm, with LN metastases, and who did not survive, designated a value of 1. Statistically significant at *p<0.05 and **p<0.01. C15ORF2: chromosome 15 open reading frame 2; CPA4: Carboxypeptidase A4; GABRB3: gamma-aminobutyric acid type A receptor beta 3 subunit; H19: H19, imprinted maternally expressed transcript non protein-coding; IGF2: insulin-like growth factor 2; PEG3-AS1: paternally-expressed gene 3 antisense RNA 1; PPP1R9A: protein phosphatase 1 regulatory subunit 9A; RTL1: retrotransposon-like 1; SLC22A3: solute carrier family 22 member 3.*

Neck lymph-node metastasis and expression of imprinted genes in patients with SCCHN. Neck lymph-node metastasis was validated by pathology report. Among the 73 patients with SCCHN, 28 had neck lymph-node metastases and 45 did not. We analyzed the correlation between neck lymph-node metastasis in patients with SCCHN and expression of imprinted genes. Significant down-regulation of *PEG3-AS* ($p<0.05$) and *GABRB3* ($p<0.01$) was found in patients with neck lymph-node metastases. Therefore, transcripts of *PEG3-AS* and *GABRB3* displayed a neck metastasis-dependent variation in expression (Figure 2C).

Survival of patients with SCCHN and expression of imprinted genes. Patients with SCCHN were followed-up for 3-6 years after surgery. Among the 73 patients with HNSCC, 22 patients died from their disease and 51 patients survived up to the time of analysis. Correlation analysis between the survival status of patients and expression of imprinted genes demonstrated that up-regulation of *CPA4* ($p<0.01$) and down-regulation of *PEG3-AS* ($p<0.05$) and *IGF2* ($p<0.05$) were correlated with poor survival (Figure 2D).

SCCHN cells treated with chemotherapeutic drugs. To investigate the impact of chemotherapeutic drugs on imprinted genes, we treated two SCCHN cell lines, FaDu and SCC-4, with different agents. In FaDu cells, treatment with 5-FU led to a two- to three-fold increase of *PPP1R9A, PEG3-AS1, IGF2*, and *GABRB3* (Figure 3A). When treated with carboplatin, a drastic increase of *PPP1R9A* and *GABRB3* was observed (Figure 3E). In contrast, the eight imprinted genes studied were not affected by paclitaxel (Figure 3C) and doxorubicin treatment (Figure 3G). In SCC-4 cells, treatment with carboplatin led to up-regulation of *RTL1* and *SLC22A3* (Figure 3F), and treatment with doxorubicin led an 8-fold increase of *PEG3-AS1* (Figure 3H). 5-FU (Figure 3B) and with paclitaxel (Figure 3D) did not affect the expression of the eight imprinted genes in SCC-4 cells.

Discussion

Imprinted genes play significant roles in the regulation of fetal growth and development, function of the placenta, and maternal nurturing behavior in mammals (11). However, there is very limited number of studies related to cancer and imprinted genes. Genomic imprinting is one kind of epigenetic regulation whereby some genes are expressed according to parental origin and a subset of autosomal genes is monoallelically expressed (12). The exact mechanism on how imprinted genes regulate gene expression remains largely unknown, but it is generally accepted that imprinted non-coding RNAs bind to chromatin modifying complexes such as polycomb repressive complex 2, trithorax, and G9a, and generate specific silencing of genomic loci both in *cis* and *trans*. More than 100 imprinted genes have been identified and, importantly, most of them seem to coexist in clusters ranging from three to 11 genes. Each cluster has been shown to be under the control of a small stretch (2-3 kb) of a differentially methylated region, termed the 'imprinting control region' (ICR) (13, 14).

In previous studies, *IGF2* and *H19* were well-investigated for imprinting mechanism and it was found to be largely dependent upon the insulator protein CCCTC binding factor (CTCF) (15, 16). *IGF2* and *H19* genes share an enhancer region. The ICR is unmethylated on the maternal allele, permitting the insulator, CTCF, to bind and prevent interactions with a downstream enhancer acting as a boundary controller. Absence of ICR methylation allows for maternal *H19* expression. When the ICR is methylated (as on the paternal allele), *H19* expression is prevented and IGF2 expression is promoted (17, 18). Loss of imprinting at the *IGF2* and *H19* loci play a role in the oncogenesis of SCCHN (19). In our present study, we also observed altered expression of both *H19* and *IGF2*, and down-regulation of *IGF2* was correlated with poor survival.

In our study, we found the down-regulation of *GABRB3* to be associated with more advanced tumor stage (Figure 2A) and neck lymph-node metastasis (Figure 2B). The *GABRB3* gene is located at 15q11-q13 locus which encodes the gamma-aminobutyric acid A receptor β3 subunit and has been associated with both autism and absence seizures (20). We also found a lower *PPP1R9A* level to be associated with more advanced tumor stage (Figure 2A). The *PPP1R9A* gene, which encodes neurabin I, is located in a cluster of imprinted genes on human chromosome 7q21. Neurabin I protein has been shown to be a regulatory subunit of protein phosphatase I, and controls actin cytoskeleton reorganization (21). In our study, higher *CPA4* expression was also found to be associated with poor survival. *CPA4* encodes a zinc-dependent metallo-carboxypeptidase, and is located on chromosome 7q32 in a region related to prostate cancer aggressiveness (22). *CPA4* is involved in the histone hyperacetylation pathway and may modulate the function of peptides that affect the growth and regulation of prostate epithelial cells (23). Association between *CPA4* and prostate cancer has been reported (24).

Our *in vitro* study demonstrated that treatment with chemotherapeutic drugs affected the expression of imprinted genes. We also found expression of imprinted genes in FaDu cells to be sensitive to 5-FU and carboplatin, but insensitive to paclitaxel and doxorubicin. In contrast, expression of imprinted genes in SCC-4 cells was sensitive to carboplatin and doxorubicin but insensitive to 5-FU and paclitaxel. Because FaDu cells were established from hypopharyngeal tumor and SCC-4 cells from tongue squamous cell carcinoma, this raises the question of whether the expression of imprinted genes is tissue-specific. Imprinting genes can

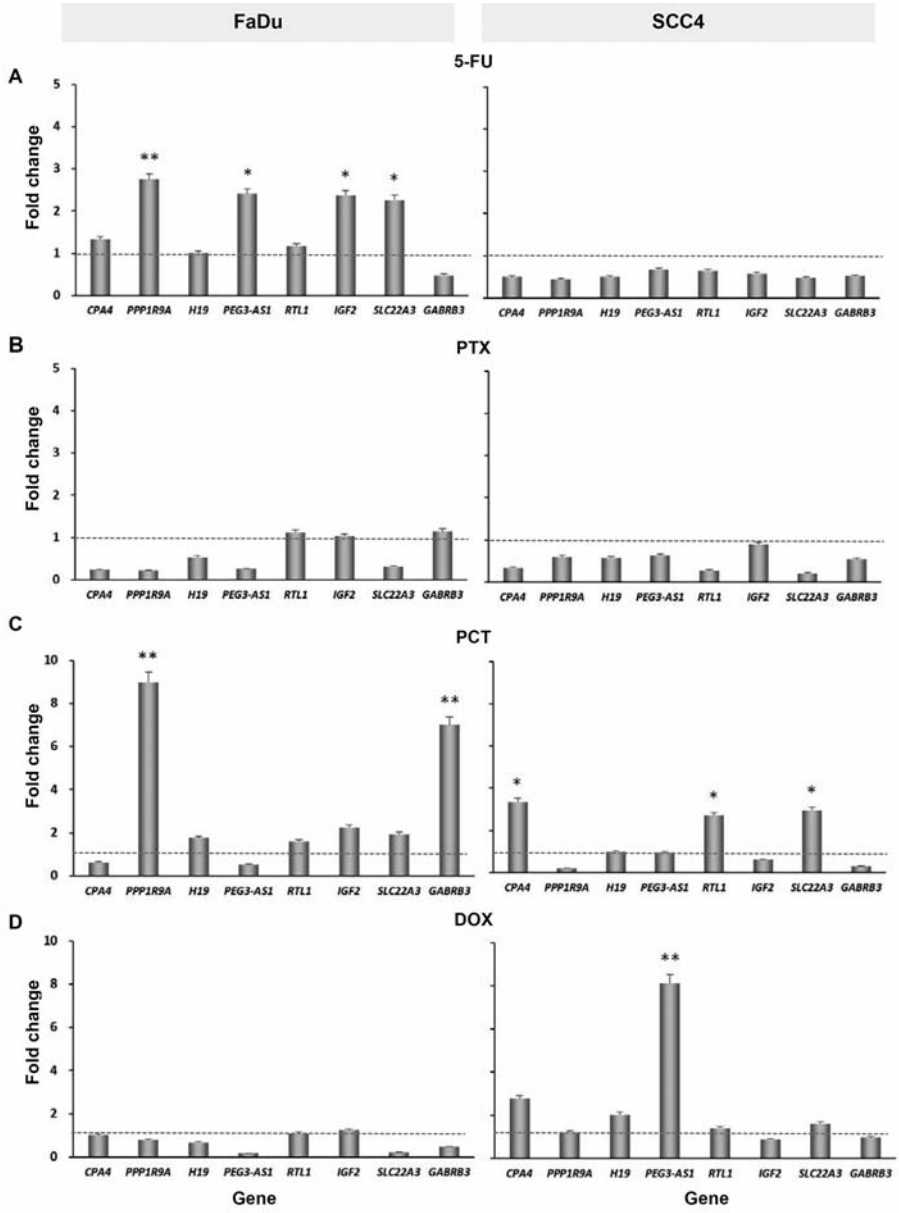

Figure 3. *Expression of imprinted genes in FaDu and SCC-4 cells treated with chemotherapeutic drugs. FaDu (left column) and SCC-4 (right column) cells were treated with 500 μM 5-fluorouracil (5-FU; A), 500 nM paclitaxel (PTX; B), 10 μM cisplatin (PCT; C), and 100 nM doxorubincin (DOX; D), respectively. After treatment for 24 h, cells were harvested for real-time quantitative reverse transcriptase-polymerase chain reaction analysis of the eight imprinted genes. Expression is reported as the fold-change in mRNA expression of cells treated with chemotherapeutic drugs relative to cells without treatment. All experiments were run in duplicates and data presented are the means of three independent experiments. *p<0.05 and **p<0.01. C15ORF2: Chromosome 15 open reading frame 2; CPA4: carboxypeptidase A4; GABRB3: gamma-aminobutyric acid type A receptor beta 3 subunit; H19: H19, imprinted maternally expressed transcript non protein-coding; IGF2: insulin-like growth factor 2; PEG3-AS1: paternally-expressed gene 3 antisense RNA 1; PPP1R9A: protein phosphatase 1 regulatory subunit 9A; RTL1: retrotransposon-like 1; SLC22A3: solute carrier family 22 member 3.*

be affected and regulated by chemotherapy, but the selection of drug will have impact on the treatment effects.

Our next challenge will be solving the limitations of our study, including the role of altered expression of imprinted genes in SCCHN and the mechanisms involved in genomic imprinting. The association of imprinted genes with disease severity and survival of patients also needs further investigation. Based on our study, focusing on *IGF2, PEG3-AS* and *CPA4* in SCCHN and study of a new pathway for HNSCC development and treatment should be a future direction.

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