

# Expression of the tumor suppressor gene hypermethylated in cancer 1 in laryngeal carcinoma

JAROSŁAW MARKOWSKI<sup>1</sup>, ALEKSANDER L. SIEROŃ<sup>2,3</sup>, KATARZYNA KASPERCZYK<sup>1</sup>,  
MONIKA CIUPIŃSKA-KAJOR<sup>4</sup>, ALEKSANDRA AUGUŚCIAK-DUMA<sup>2</sup> and WIRGINIA LIKUS<sup>5</sup>

<sup>1</sup>Department of Laryngology, School of Medicine in Katowice, Medical University of Silesia, Katowice 40-027;

<sup>2</sup>Department of Molecular Biology and Genetics, School of Medicine in Katowice, Medical University of Silesia, Katowice 40-752; <sup>3</sup>CoE Research and Teaching of Molecular Biology of Matrix and Nanotechnology, Network of CoE BioMedTech Silesia, Katowice 40-752; Departments of <sup>4</sup>Pathomorphology and <sup>5</sup>Human Anatomy, School of Medicine in Katowice, Medical University of Silesia, Katowice 40-752, Poland

Received March 31, 2014; Accepted January 22, 2015

DOI: 10.3892/ol.2015.2983

**Abstract.** Hypermethylated in cancer 1 (HIC1) is a putative suppressor gene, cooperating with TP53 in the regulation of apoptosis. The promoter site of this gene contains CpG islands susceptible to methylation. Altered methylation leads to the silencing of HIC1. Persistent loss of HIC1 function reflects the attenuation of proapoptotic characteristics of TP53 and may constitute the background for carcinogenesis. Altered methylation profiles along with diminished expression of HIC1 were documented in a number of solid neoplasms. The aim of this study was to evaluate the expression of the HIC1 gene in laryngeal carcinoma. RNA was extracted from samples of laryngeal cancer and corresponding healthy tissues of 21 patients with advanced laryngeal cancer (T3-T4). The amount of RNA (cDNA) was evaluated using reverse transcription-quantitative polymerase chain reaction with GAPDH as the reference gene. Data demonstrated that HIC1 expression was significantly reduced in laryngeal cancer tissues. The relative expression of HIC1 was found to be ~40% lower in tumor samples compared to that in healthy controls. The median tumor/normal tissue ratio for HIC1 was 0.615. These results suggest that low HIC1 expression may be associated with neoplastic transformation in the larynx.

## Introduction

Laryngeal cancer is the most common malignant neoplasm of the head and neck region. In Poland, laryngeal cancer is the seventh most common neoplasm in men (1). Despite

the advances in diagnostics and therapeutic procedures, the majority of laryngeal cancer cases are diagnosed at an advanced clinical stage. Between 1991 and 2001, a multicenter, nationwide study was conducted, which included a total of ~13,000 patients diagnosed with cancer of the larynx and hypopharynx. More than half of all the cases had a stage T3 or T4 primary tumor. Cervical lymph node metastases were identified in 47.7% of the cases (2). The 5-year survival rate for laryngeal cancer is currently ~50% (1).

Epigenetics was defined by Wu and Morris (3) as changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the DNA sequence. DNA methylation along with histone modifications and non-coding RNA dysregulation are the leading epigenetic alterations in human cancer (4).

During DNA methylation, a methyl group is added to an aromatic ring of cytosine in CpG dinucleotides. Approximately 70% of total CpG dinucleotides in the human genome are methylated. However, certain CpG islands located in the promoter region of multiple genes remain physiologically unmethylated (5). The methylation of promoter sites results in the suppression of gene expression by several mechanisms. 5-Methylcytosine may spontaneously become deaminated to thymine, leading to the formation of point mutations (5). DNA methylation directly prevents the binding of transcriptional factors to the promoter (6). The methyl-CpG-binding domain proteins recognize methylation sites. These proteins bind the methylated sequence and block the interaction between RNA polymerase and the promoter (6,7). Promoter site methylation of the suppressor gene may lead to carcinogenesis.

Environmental factors may lead to epigenetic changes in DNA. Chang *et al* (8) proved the effect of smoking combined with alcohol intake on methylation of p15 in the epithelium of the upper airways. Van Engeland *et al* (9) documented the correlation between alcohol consumption and hypermethylation of the following genes: Ras association domain-containing protein 1 (RASSF1A), O-6-methylguanine-DNA methyltransferase (O-MGMT), adenomatous polyposis coli, p16, p14 and human MutL homolog. Since alcohol abuse and smoking are the main risk factors for the development of laryngeal cancer,

---

*Correspondence to:* Professor Jarosław Markowski, Department of Laryngology, School of Medicine in Katowice, Medical University of Silesia, 20-24 Francuska Street, Katowice 40-027, Poland  
E-mail: jmarkow1@poczta.onet.pl

**Key words:** hypermethylated in cancer 1, laryngeal cancer, tumor suppressor gene

it may be hypothesized that abnormal DNA methylation is common among patients suffering from this neoplasm.

Hypermethylated in cancer 1 (HIC1) is a potential suppressor gene located on chromosome 17 (17p13.3). HIC1 is known for cooperating with TP53 in the regulation of apoptosis. The promoter region of HIC1 abounds in CpG dinucleotides and methylation of these CpG islands may lead to gene silencing (10). Hypermethylation of HIC1 was documented in the pathogenesis of several neoplasms. The aim of this study was the assessment of HIC1 expression in patients with laryngeal cancer.

## Materials and methods

**Study population and tissue samples.** The protocol of this study was approved by the Ethics Committee of the Medical University of Silesia (Katowice, Poland). The population study consisted of 21 men with histologically confirmed laryngeal squamous cell carcinoma, treated in the Laryngology Clinic of the Medical University of Silesia between 2005 and 2009. Written informed consent was obtained from all patients regarding the use of their tissue samples for the purpose of this study. All the patients were clinically at stage T3-T4, N0-N3 and M0 and underwent total laryngectomy. Paraffin-embedded 20- $\mu$ m tissue sections were collected from tumor and corresponding healthy tissues.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.** Total RNA from the formalin-fixed paraffin-embedded (FFPE) tissue samples was isolated using the AllPrep DNA/RNA FFPE kit, including DNase treatment (Qiagen, Venlo, The Netherlands). The integrity of RNA was assessed using an Agilent RNA 6000 Nano kit on Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The quantity and quality of RNA were measured spectrometrically on the NanoDrop 2000 (NanoDrop products, Wilmington, DE, USA). The total RNA concentration in each sample was 50 ng, with RNA integrity number values of  $\sim$ 2. RNA was amplified and detected in one-step RT-PCR on the LightCycler 480 (Roche, Basel, Switzerland) using a QuantiFast Probe assay (Qiagen) for the human HIC1 (Entrez target gene ID: 3090) duplexed with GAPDH\_2\_HS (as a reference gene) in combination with the QuantiFast Probe (amplicon size, 79 bp). All the samples were tested in triplicate. The results were normalized and analyzed using the  $\Delta\Delta$ cycle threshold (Ct) method (11).

**Statistical analysis.** All data are presented as the arithmetical mean  $\pm$  standard error of the mean (SEM) and the unpaired Student's t-test was used for statistical analysis. Fold change (FC) was evaluated in the target gene, or log ratio. FC was defined as the difference per gene between the averages of the group: Target gene/control.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Relative expression of HIC1 in tumor and healthy tissue samples.** A competitive RT-PCR approach with specific RNA competitive molecules for HIC1 and GAPDH was used to

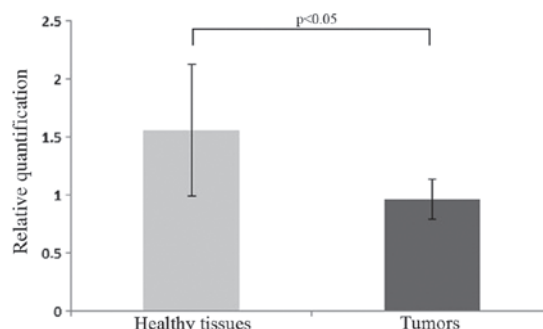


Figure 1. Relative expression of the hypermethylated in cancer 1 gene in tumor and healthy tissue samples, normalized to the GAPDH reference gene (mean  $\pm$  standard error).

evaluate the expression levels of HIC1 in samples obtained from 21 patients with laryngeal carcinoma (healthy and tumor tissues). According to the advanced relative quantification (second derivative), the mean Ct detected in tumor samples reached 0.96 (SEM = 0.57), whereas the mean Ct of healthy tissues amounted to 1.56 (SEM = 0.17). The median tumor/normal tissue ratio for HIC1 expression was 0.615. The evaluation revealed that the expression of HIC1 in tumors was 38.5% lower compared to that in healthy tissues (Fig. 1) ( $P < 0.05$ ).

The inspection of the expression profiles revealed that the differences in expression between samples and controls (comparisons between pairs of cancerous and healthy tissues in each of the 21 patients) were prominent in samples 6, 8, 9, 11, 12, 13, 14, 15, 18, 19, 20 and 21. The expression profiles of the samples are shown in Fig. 2. The FC in HIC1 was 2.54.

## Discussion

Little is known regarding the epigenetic background of carcinogenesis in the larynx. Improper methylation status of the following genes has been identified thus far in laryngeal cancer: Retinoic acid receptor  $\beta$  (RAR $\beta$ ), death-associated protein kinase (DAPK), cyclin-dependent kinase inhibitor 2A (CDKN2A), MGMT, RASSF1A, fragile histidine triad, chromodomain-helicase-DNA-binding protein 5, cellular retinol binding protein 1 and HIC1 (12-19). The majority of the studies reported certain correlations between the clinical course of the disease and the molecular changes identified. Kong *et al* (14) suggested that the hypermethylation of DAPK was associated with lymph node involvement. In addition, nodal metastases appear to correlate with the methylation profile of MGMT (15). According to Smigiel *et al* (17), CDKN2A hypermethylation is reflected by a high grade of histological differentiation of the tumor (G3) (17). Olasz *et al* (18) reported that RAR $\beta$ 2 hypermethylation is observed in patients with well-differentiated lesions.

HIC1 encodes the transcriptional factor responsible for the propagation of the biological function of p53. HIC1 contains the C-domain of five zinc-finger motifs responsible for binding with DNA at the site called the HIC1 responsive element (HiRE). The N-terminal domain BTB/POZ mediates protein-protein interactions (13). The main task of HIC1 is to suppress the expression of class III NAD-dependent histone deacetylase called sirtuin 1 (SIRT1), which causes

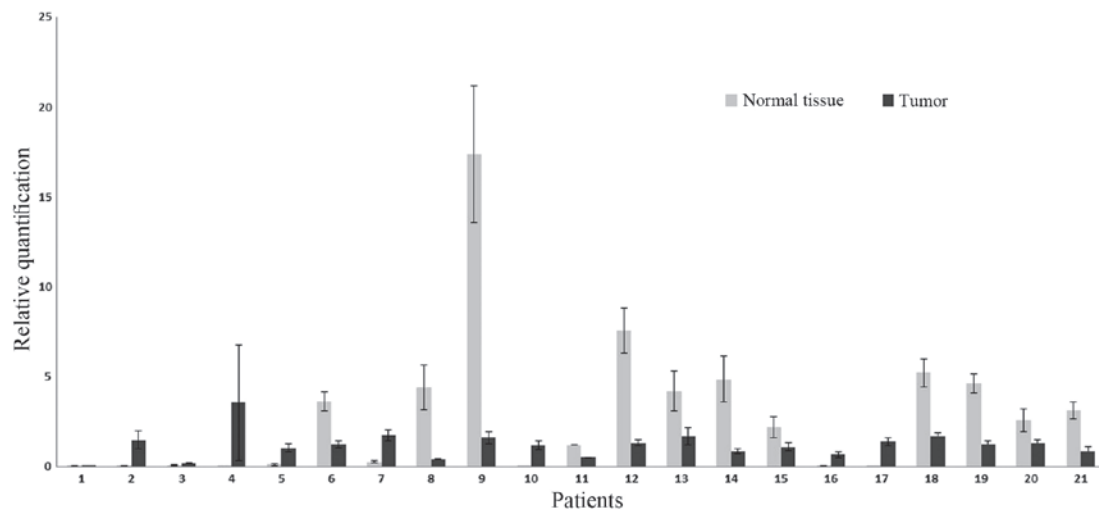


Figure 2. Profiles of expression of the hypermethylated in cancer 1 gene in pairs of cancerous and control samples in each of the investigated patients, normalized to the GAPDH reference gene (mean  $\pm$  standard error).

deacetylation of the p53 protein; this process attenuates the proapoptotic action of p53 (20). SIRT1 also affects apoptosis in pathways other than p53. Deacetylation of the Ku70 protein leads to the sequestration of B-cell lymphoma 2-associated X proteins in the cytoplasm (21). In addition, SIRT1 is involved in gene silencing during the formation of facultative heterochromatin (22).

The transcriptional activity of HIC1 is due to two structural domains: BTB/POZ enables the formation of complexes between HIC1 and SIRT1, whereas zinc finger motifs recognize and bind HiRE sequences in DNA, resulting in the suppression of HIC1 gene expression (10,23).

Under physiological conditions, there is a feedback loop between HIC1 and p53. HIC1 protects p53 from deacetylation, whereas p53 activates the transcription of HIC1 (22,23). Chronic silencing of HIC1 causes upregulation of SIRT1, persistent deacetylation of p53 and loss of the proapoptotic function of this protein. Thus, loss of expression of HIC1 may be involved in carcinogenesis (20).

The association between HIC1 and neoplastic transformation is not limited to the p53-dependent apoptosis pathway. Briones *et al* (24) recently reported that HIC1 may participate in the regulation of neoplastic angiogenesis. The presence of the HiRE sequence within the gene encoding the fibroblast growth factor-binding protein 1 (FGF-BP1) was detected. FGF-BP1 stimulates the differentiation of endothelial cells and smooth myocytes during the formation of embryonic blood vessels. FGF-BP1 also participates in neoplastic vascularisation. Increased FGF-BP1 expression was detected in the tissues of several tumors, including cancer of the colon (25).

Silencing of HIC1 may have an epigenetic background, since the promoter site of this gene contains CpG dinucleotides susceptible to methylation. Hypermethylation of HIC1 was detected in the tissues of a number of solid tumors and appears to predispose tissues to neoplastic transformation. Eguchi *et al* (26) documented the association between smoking and improper methylation at locus 17p13.3 in non-small-cell lung cancer. The alteration in methylation status occurred more frequently among smokers compared to that in non-smokers.

The incidence of hypermethylation of HIC1 may increase along with the histological progression of the tumor. Kanai *et al* (27) proved that the frequency of this epigenetic process increased from normal liver tissue, to precancerous state, to hepatocellular carcinoma. Similar conclusions may be drawn on the basis of the study conducted by Kanai *et al* (28), investigating the role of HIC1 methylation in gastric carcinogenesis.

The level of HIC1 hypermethylation may correspond to the aggressiveness of the tumor and poor prognosis. Nicoll *et al* (29) proved that the undisturbed expression of HIC1 is reflected in the promising outcome and lack of nodal involvement in breast cancer (29). Hayashi *et al* (30) suggested that low HIC1 expression may be involved in the malignant progression of non-small-cell lung cancer. Brieger *et al* (31) analyzed the demethylation of HIC1 in head and neck squamous carcinoma cell lines *in vitro* and confirmed that the demethylation of the tumor suppressor gene HIC1 increases the radiosensitivity of head and neck squamous carcinoma cells.

HIC1 hypermethylation is the process of binding methyl groups to DNA. The present study did not analyze the hypermethylation of HIC1 itself, but rather the expression of this gene, i.e. the level of the mRNA transcript. Zheng *et al* (32) analyzed the hypermethylation of HIC1 and concluded that the hypermethylation did not consistently correlate with the HIC1 expression level in breast and non-small-cell lung cancer.

The study of Stephen *et al* (33) is the only published study suggesting the effect of HIC1 methylation on laryngeal cancer. In that study, the methylation status of 38 genes was assessed in patients with laryngeal carcinoma. HIC1 was found to be methylated in 5 of a total of 79 cases. Stephen *et al* (33) revealed that the hypermethylation of HIC1 may be an independent predictor of poor survival in laryngeal carcinoma. Patients with laryngeal cancer and HIC1 methylation had a median survival time of 1.02 years, as compared to the median survival time of 4.40 years of patients without HIC1 methylation. To the best of our knowledge, the expression of HIC1 in laryngeal cancer has not been evaluated to date.

In conclusion, the present study proved that the relative expression of the HIC1 putative suppressor gene is diminished in laryngeal cancer. However, as this difference was not distinct in all the cases of laryngeal cancer, this finding requires further investigation.

### Acknowledgements

The equipment for molecular analysis was purchased using the Silesian Bio-Farma Center for Biotechnology, Bioengineering and Bioinformatics project (grant no. POIG.02.01.00-00-166/08), the Operational Programme Innovative Economy for 2007-2013, Priority Axis 2.

### References

1. The national cancer registry. <http://onkologia.org.pl/> (In Polish). Accessed March 15, 2014.
2. Bieñ S, Kamiński B, Żyłka S, Mezyk R and Piasta Z: Evolution of the epidemiology and clinical characteristics of larynx and hypopharynx carcinoma in Poland from 1991 to 2001. *Eur Arch Otorhinolaryngol* 1: S39-S46, 2008.
3. Wu CT and Morris JR: Genes, genetics and epigenetics: a correspondence. *Sciences* 293: 1103-1105, 2001.
4. Wong TS, Gao W, Li ZH, Chan JY and Ho WK: Epigenetic dysregulation in laryngeal squamous cell carcinoma. *J Oncol* 2012: 739461, 2012.
5. Jabłońska J and Jesionek-Kupnicka D: Zmiany epigenetyczne w nowotworach. *Onkol Pol* 7: 181-185, 2004 (In Polish).
6. Severin PM, Zou X, Gaub HE and Schulten K: Cytosine methylation alters DNA mechanical properties. *Nucleic Acids Res* 39: 8740-8751, 2011.
7. Baylin SB and Herman JG: DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 16: 168-174, 2000.
8. Chang HW, Ling GS, Wei WI and Yuen AP: Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma. *Cancer* 101: 125-132, 2004.
9. van Engeland M, Weijnenberg MP, Roemen GM, Brink M, de Bruine AP, Goldbohm RA, van den Brandt PA, Baylin SB, de Goeij AF and Herman JG: Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Res* 63: 3133-3137, 2003.
10. Fleuriel C, Touka M, Boulay G, Guérardel C, Rood BR and Leprince D: HIC1 (hypermethylated in cancer 1) epigenetic silencing in tumors. *Int J Biochem Cell Biol* 41: 26-33, 2009.
11. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
12. Peralta R, Valdivia A, Alvarado-Cabrero I, Gallegos F, Apresa T, Hernández D, Mendoza M, Romero P, Paniagua L, Ibáñez M, Cabrera L and Salcedo M: Correlation between expression of cellular retinol-binding protein 1 and its methylation status in larynx cancer. *J Clin Pathol* 65: 46-50, 2012.
13. Bazan V, Zanna I, Migliavacca M, Sanz-Casla MT, Maestro ML, Corsale S, Macaluso M, Dardanoni G, Restivo S, Quintela PL, Bernaldez R, Salerno S, Morello V, Tomasino RM, Gebbia N and Russo A: Prognostic significance of p16INK4a alterations and 9p21 loss of heterozygosity in locally advanced laryngeal squamous cell carcinoma. *J Cell Physiol* 192: 286-293, 2002.
14. Kong WJ, Zhang S, Guo C, Zhang S, Wang Y and Zhang D: Methylation-associated silencing of death-associated protein kinase gene in laryngeal squamous cell cancer. *Laryngoscope* 115: 1395-1401, 2005.
15. Paluszczak J, Misiak P, Wierzbicka M, Woźniak A and Baer-Dubowska W: Frequent hypermethylation of DAPK, RARbeta, MGMT, RASSF1A and FHIT in laryngeal squamous cell carcinomas and adjacent normal mucosa. *Oral Oncol* 47: 104-107, 2011.
16. Wang J, Chen H, Fu S, Xu ZM, Sun KL and Fu WN: The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. *Oral Oncol* 47: 601-608, 2011.
17. Smigiel R, Sasiadek M, Krecicki T, Ramsey D, Jagielski J and Blin N: Inactivation of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene in squamous cell carcinoma of the larynx. *Mol Carcinog* 39: 147-154, 2004.
18. Olasz J, Juhász A, Remenár E, Engi H, Bak M, Csuka O and Kásler M: RAR beta2 suppression in head and neck squamous cell carcinoma correlates with site, histology and age. *Oncol Rep* 18: 105-112, 2007.
19. Temam S, Bénard J, Dugas C, Trassard M, Gormally E, Soria JC, Faivre S, Lubinski B, Marandas P, Hainaut P, Lenoir G, Mao L and Janot F: Molecular detection of early-stage laryngopharyngeal squamous cell carcinomas. *Clin Cancer Res* 11: 2547-2551, 2005.
20. Chen WY, Wang DH, Yen RC, Luo J, Gu W and Baylin SB: Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. *Cell* 123: 437-448, 2005.
21. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R and Sinclair DA: Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305: 390-392, 2004.
22. Vaquero A, Scher M, Erdjument-Bromage H, Tempst P, Serrano L and Reinberg D: SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature* 450: 440-444, 2007.
23. Kelly KF and Daniel JM: POZ for effect-POZ-ZF transcription factors in cancer and development. *Trends Cell Biol* 16: 578-587, 2006.
24. Briones VR, Chen S, Riegel AT and Lechleider RJ: Mechanism of fibroblast growth factor-binding protein 1 repression by TGF-beta. *Biochem Biophys Res Commun* 345: 595-601, 2006.
25. Tassi E, Henke RT, Bowden ET, Swift MR, Kodack DP, Kuo AH, Maitra A and Wellstein A: Expression of a fibroblast growth factor-binding protein during the development of adenocarcinoma of the pancreas and colon. *Cancer Res* 66: 1191-1198, 2006.
26. Eguchi K, Kanai Y, Kobayashi K and Hirohashi S: DNA hypermethylation at the D17S5 locus in non-small cell lung cancers: its association with smoking history. *Cancer Res* 57: 4913-4915, 1997.
27. Kanai Y, Hui AM, Sun L, Ushijima S, Sakamoto M, Tsuda H and Hirohashi S: DNA hypermethylation at the D17S5 locus and reduced HIC-1 mRNA expression are associated with hepatocarcinogenesis. *Hepatology* 29: 703-709, 1999.
28. Kanai YI, Ushijima S, Ochiai A, Eguchi K, Hui A and Hirohashi S: DNA hypermethylation at the D17S5 locus is associated with gastric carcinogenesis. *Cancer Lett* 122: 135-141, 1998.
29. Nicoll G, Crichton DN, McDowell HE, Kernohan N, Hupp TR and Thompson AM: Expression of the hypermethylated in cancer gene (HIC-1) is associated with good outcome in human breast cancer. *Br J Cancer* 85: 1878-1882, 2001.
30. Hayashi M, Tokuchi Y, Hashimoto T, Hayashi S, Nishida K, Ishikawa Y, Nakagawa K, Tsuchiya S, Okumura S and Tsuchiya E: Reduced HIC-1 gene expression in non-small cell lung cancer and its clinical significance. *Anticancer Res* 21: 535-540, 2001.
31. Brieger J, Mann SA, Pongsapich W, Koutsimpelas D, Fruth K and Mann WJ: Pharmacological genome demethylation increases radiosensitivity of head and neck squamous carcinoma cells. *Int J Mol Med* 29: 505-509, 2012.
32. Zheng J, Wang J, Sun X, *et al*: HIC1 modulates prostate cancer progression by epigenetic modification. *Clin Cancer Res* 19: 1400-1410, 2013.
33. Stephen JK, Chen KM, Shah V, Havard S, Kapke A, Lu M, Benninger MS and Worsham MJ: DNA hypermethylation markers of poor outcome in laryngeal cancer. *Clin Epigenetics* 1: 61-69, 2010.