The Potential of *SIRT6* and *SIRT7* as Circulating Markers for Head and Neck Squamous Cell Carcinoma

CHENG-TUNG LU[1], CHENG-MING HSU[1,2], PAI-MEI LIN[3], CHI-CHIH LAI[1], HSIN-CHING LIN[1], CHAO-HUI YANG[1,2], HUI-HUA HSIAO[4,5], YI-CHANG LIU[4,5], HUGO YOU-HSIEN LIN[6,7], SHENG-FUNG LIN[4,5] and MING-YU YANG[2]

[1]*Department of Otolaryngology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung City, Taiwan, R.O.C.;*
[2]*Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan, R.O.C.;*
[3]*Department of Nursing, I-Shou University, Kaohsiung City, Taiwan, R.O.C.;*
Divisions of [4]*Hematology-Oncology and* [6]*Nephrolog, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung City, Taiwan, R.O.C.;*
[5]*Faculty of Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan, R.O.C.;*
[7]*Department of Internal Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University, Kaohsiung City, Taiwan, R.O.C.*

Abstract. *Background: We have previously demonstrated altered SIRT gene family in head and neck squamous cell carcinoma (HNSCC). In the present study we aimed to investigate whether the SIRT gene family was also altered in peripheral blood (PB) of patients with HNSCC and the possibility to be used as circulating biomarkers. Patients and Methods: The expression profiles of the 7 SIRT genes of PB leukocytes from 34 patients with HNSCC before and after surgery and 31 healthy individuals were investigated. Results: In the cancer group, the expression level of SIRT1 was down-regulated ($p<0.05$); in contrast, SIRT6 and SIRT7 were significantly up-regulated ($p<0.001$). Patients with advanced-stage HNSCC had lower expression of SIRT1 and SIRT3. Recovery of SIRT6 and SIRT7 was observed in postoperative patients ($p<0.005$). Conclusion: SIRT genes were altered in PB leukocytes of HNSCC patients and SIRT6 and SIRT7 are potential circulating prognostic markers for HNSCC.*

*These Authors contributed equally to this study.

Correspondence to: Sheng-Fung Lin, Division of Hematology-Oncology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung City, Taiwan. Tel: +886 73121101 (ext. 7071), e-mail: shlin@cc.kmu.edu.tw and Ming-Yu Yang, Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan. Tel: +886 77317123 (ext. 8865), e-mail: yangmy@mail.cgu.edu.tw

Key Words: HNSCC, sirtuin genes, *SIRT6, SIRT7*, peripheral blood leukocytes.

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common malignancy worldwide affecting 600,000 new patients each year. It also had the fifth prevalence rate and is the fourth leading cause of mortality rate in Taiwan in 2010 (1). These malignancies are strongly associated with certain environmental and life-style risk factors such as tobacco, excessive alcohol consumption (2, 3), endemic betel quid chewing (especially in the Asia-Pacific region) (4, 5) and viruses (principally human papillomavirus and Epstein-Barr virus) (6). HNSCC consists of several distinct structures (*e.g.* lip, tongue, oropharynx, larynx, hypopharynx, pyriform sinus) with distinct microscopic features and lymphatic and venous drainage in anatomy. Thus, treatment approaches and outcomes vary significantly according to the different involved area (7). In spite of the advances in cancer treatment, HNSCC is often reported as having high rates of recurrence and poor disease-free survival and overall survival rates (7). Since most of the mortality begins with an undetectable recurrence of the postoperative cases, the challenge would be how to detect early and monitor the progression of HNSCC. Therefore, studies in discovering new molecular pathways that regulate cancer cell biology are crucial for the development of biomarkers for early screening and better approaches for cancer prevention and treatment.

Sirtuins, silent information regulator (SIR), are a family of nicotine adenine dinucleotide (NAD^+)-dependent protein deacetylases or adenosine diphosphate-ribosyltransferases (8). Each of the seven human sirtuin genes (*SIRT1-7*) identified has its own unique characteristics, functions and

localization. Re-localization of some sirtuins has been shown to be involved in function of cell or tissue type, developmental stage, metabolic status and certain stress conditions, suggesting that localization is important for regulating their function (9). They are heavily implicated in cellular and tissue functions through chromatin regulation, cell survival under stress, metabolic homeostasis regulation and developmental and cell differentiation (8). For the clinicians, sirtuins have been linked to aging (9-11), diabetes (12), cardiovascular diseases (13) and neurodegenerative diseases (14). The connection between sirtuins and circadian rhythms has been suggested by the role of SIRT1 in linking cellular metabolism to the circadian core clockwork machinery (15). Moreover, the sirtuin family has also be linked to cancers but with contradictory roles, as its members may act as tumor suppressors in some types of cancers and as tumor promoters in others depending on the cell- and tumor-type and the presence of different stress or cell death stimuli (16).

According to our previous studies, the expression levels of *SIRT1, SIRT2, SIRT3, SIRT5, SIRT6* and *SIRT7* were significantly down-regulated in HNSCC tissues compared to their adjacent non-cancerous tissues (17). We had further discussed about if the expression of the sirtuin family can be detected in patients with HNSCC before surgery or management. Therefore, the aim of this study was to investigate the expression of circulating *SIRT* gene expression in patients with HNSCC and the possibilities of using their differential expression levels for early detection and prognosis. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze the expression levels of the *SIRT* genes in peripheral blood (PB) leukocytes of patients with HNSCC and healthy volunteers.

Materials and Methods

Patients, healthy subjects and samples. The study enrolled 31 healthy volunteer individuals (22 men and 9 women), aged 25 to 71 years (mean±SD, 47.7±13.3) and 34 patients (33 men and 1 woman), aged 42 to 69 years (57.6±8.7) from September 2009 through June 2012 who have undergone wide resection of head and neck proven tumor at the Department of Otolaryngology, Kaohsiung Chang Gung Memorial Hospital. PB samples of patients were collected between 8:00 AM and 9:00 AM on the day of surgery and two months after surgery. Clinical and pathologic data including patients' sex, tumor (T), neck lymph node (N) and metastasis (M) staging, tumor size, depth of invasion and survival status are listed in Table I. All of the PB samples were processed within 2 hours of collection using ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) to deplete red blood cells from PB. Informed consent was obtained from all patients and healthy volunteers prior to PB collection. This study was approved by the Institutional Review Board of the Kaohsiung Chang Gung Memorial Hospital.

Table I. *Characteristics of HNSCC patients*.

Characteristic	Number
Gender	
Male	33
Female	1
Median age year (range)	59.0 (42-69)
Staging	
CIS	1
I	3
II	3
III	9
IV	18
Site	
Tongue	5
Buccal mucosa	6
Gingival	7
Tonsil	1
Tongue base	4
Palate	1
Larynx	2
Hypopharynx	8
Tumor size	
<3 cm	12
>3 cm	22
N stage	
N0	19
N1	7
N2a	1
N2b	4
N2c	3
N3	0
Survival	
Expired	11
Survived	23

qRT-PCR analysis of SIRT1-7 genes. Total RNA was extracted and purified first from PB leukocytes by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The 2-μg RNA input for cDNA synthesis was determined by spectrophotometric OD_{260}/OD_{280} measurement and cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocols. The expression of *SIRT* genes were analyzed using the TaqMan® system and all the TaqMan® Gene Expression Assays were purchased from Applied Biosystems as previously described (17). Expression of human housekeeping genes, *ACTB* (β-actin), was used for normalizing *SIRT1-7* gene expression in qRT-PCR. All reactions were carried out in a 20-μl final volume containing 50 ng cDNA (as total input RNA), 1 μl 20× TaqMan® Gene Expression Assay and 10 μl 2× TaqMan® Universal PCR Master Mix (Applied Biosystems). Real-time quantitative PCR was performed in an ABI 7500 Fast Real-Time System (Applied Biosystems) and the PCR cycling parameters were set as follows: 95˚C for 10 minutes followed by 40 cycles of PCR reactions at 95˚C for 20 seconds and 60˚C for 1 minute. Relative expression levels were calculated by the comparative threshold cycle Ct (ΔΔCt) method. The Ct of *SIRT1-7* genes was first normalized to Ct of *ACTB* to obtain the relative threshold cycle (ΔCt) of *SIRT1-7* genes. The $2^{-\Delta\Delta Ct}$ was then used to calculate the relative expression-fold between HNSCC patients and healthy individuals.

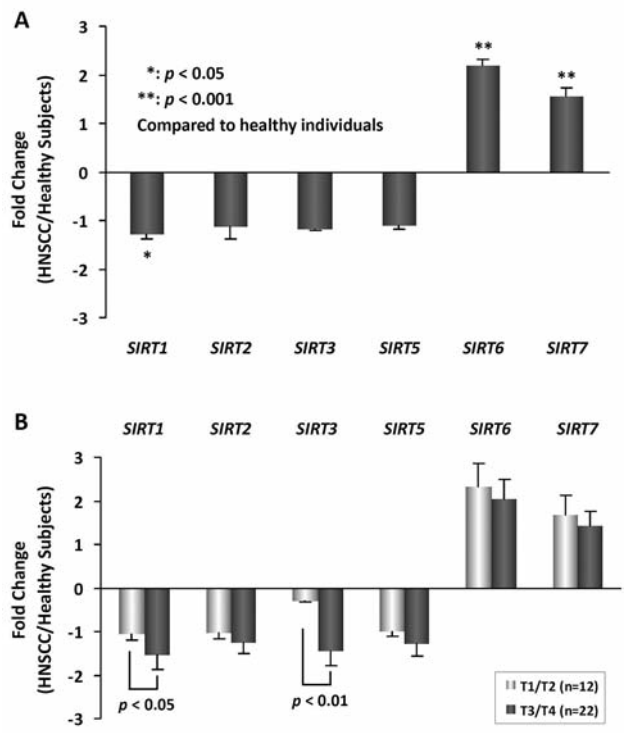

Figure 1. *Expression of the SIRT gene family in peripheral blood (PB) leukocytes of patients with head and neck squamous cell carcinoma (HNSCC) determined by real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). (A) Expression of the SIRT genes in PB leukocytes from 34 patients with HNSCC and 31 healthy individuals. Compared to the PB leukocytes of healthy individuals, the expression of SIRT1 was significantly down-regulated ($p<0.05$), while SIRT6 and SIRT 7 were significantly up-regulated (both $p<0.001$) in HNSCC patients. The y-axis represents the relative mRNA expression level. The relative expression in patients with HNSCC is calculated by the comparative CT ($\Delta\Delta$CT method). The value of mRNA expression in healthy individuals is designated 1, whereas the level of mRNA expression in patients with HNSCC is calibrated to obtain the fold change in patients with HNSCC. ** and * indicate the statistical significance $p<0.001$ and $p<0.05$, respectively. (B) Shows the expression of SIRT genes in patients with early- and advance-stage HNSCC. The 34 patients with HNSCC were divided into two groups by their disease stages, T1/T2 and T3/T4, for correlation analysis with expression of SIRT genes. The y-axis represents the relative mRNA expression level. The relative expression in patients with HNSCC is calculated by $\Delta\Delta$CT. The value of mRNA expression in healthy individuals is designated 1 and the level of mRNA expression in the T1/T2 or T3/T4 group is calibrated to obtain the fold change in patients with HNSCC. The p-value indicates the statistical significance evaluated between the T1/T2 and T3/T4 group.*

Immunocytochemical (ICC) staining of SIRT proteins. ICC staining was performed on PB total leukocyte samples with HNSCC patients and healthy individuals. For cytospin preparations, 5×10^5 cells were cytocentrifuged onto glass slides and fixed in 1% formaldehyde/phosphate-buffered saline (PBS) and blocked for nonspecific binding with 10% bovine serum albumin/PBS. Polyclonal antibodies against SIRT6 and SIRT7 (Epitomics, Burlingame, CA, USA) were used as the primary antibodies. Samples were incubated with primary antibodies (1:50 dilutions) for 30 min then incubated with ImmPRESS™ universal

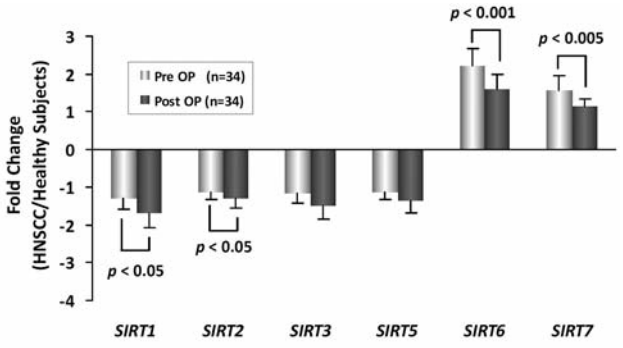

Figure 2. *Comparison of SIRT family expression in peripheral blood (PB) leukocytes of paired preoperative (Pre OP) and postoperative (Post OP) samples from patients with head and neck squamous cell carcinoma (HNSCC). The expression of SIRT1 and SIRT3 was up-regulated in the 1-month Post OP PB leukocyte samples as compared with the levels in the Pre OP group (both $p<0.05$). In contrast, the expression of SIRT5 and SIRT6 was significantly down-regulated in the 1-month Post OP PB leukocyte samples as compared with the levels in the Pre OP group ($p<0.001$ and 0.005, respectively). The p-value indicates the statistical significance evaluated between Pre OP and Post OP groups. The y axis represents the relative mRNA expression level. The value of mRNA expression in PB leukocytes of healthy individuals is designated as 1, while the level of mRNA expression in patients' Pre OP or Post OP sample is calibrated to obtain the fold change.*

reagent anti-mouse/rabbit Ig (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. The specific binding of the secondary antibodies to the primary antibodies was visualized using an ImmPACT™ DAB peroxidase substrate kit (Vector Laboratories). After staining, the cells were mounted, cover-slipped and examined using a Zeiss microscope (Zeiss, Gottingen, Germany).

Statistical analysis. All statistical analyses were computed with SPSS for Windows Release 15.0 (SPSS, Chicago, IL, USA). The paired *t*-test was used to detect the differences between patients with HNSCC and healthy subjects in each *SIRT* gene expression of leukocytes. The values of ΔCt were used for all the statistical analyses. A *p*-value of <0.05 was considered statistically significant.

Results

Analysis of SIRT gene expression in PB leukocytes using qRT-PCR. PB samples from 34 patients with HNSCC and 31 healthy individuals were examined for the expression of the 7 *SIRT* genes (*SIRT1-7*) using qRT-PCR to elucidate whether the expression levels of *SIRT* genes were altered in patients with HNSCC. Our data demonstrated that the expression level of SIRT1 was down-regulated in the PB of patients with HNSCC ($p<0.05$), in contrast, the expression levels of *SIRT6* and SIRT7 were significantly up-regulated (both $p<0.001$) when compared to those in healthy individuals (Figure 1A). *SIRT4* expression was undetectable in the PB leukocytes of patients with HNSCC or healthy individuals; therefore, *SIRT4* was not included in the subsequent analyses.

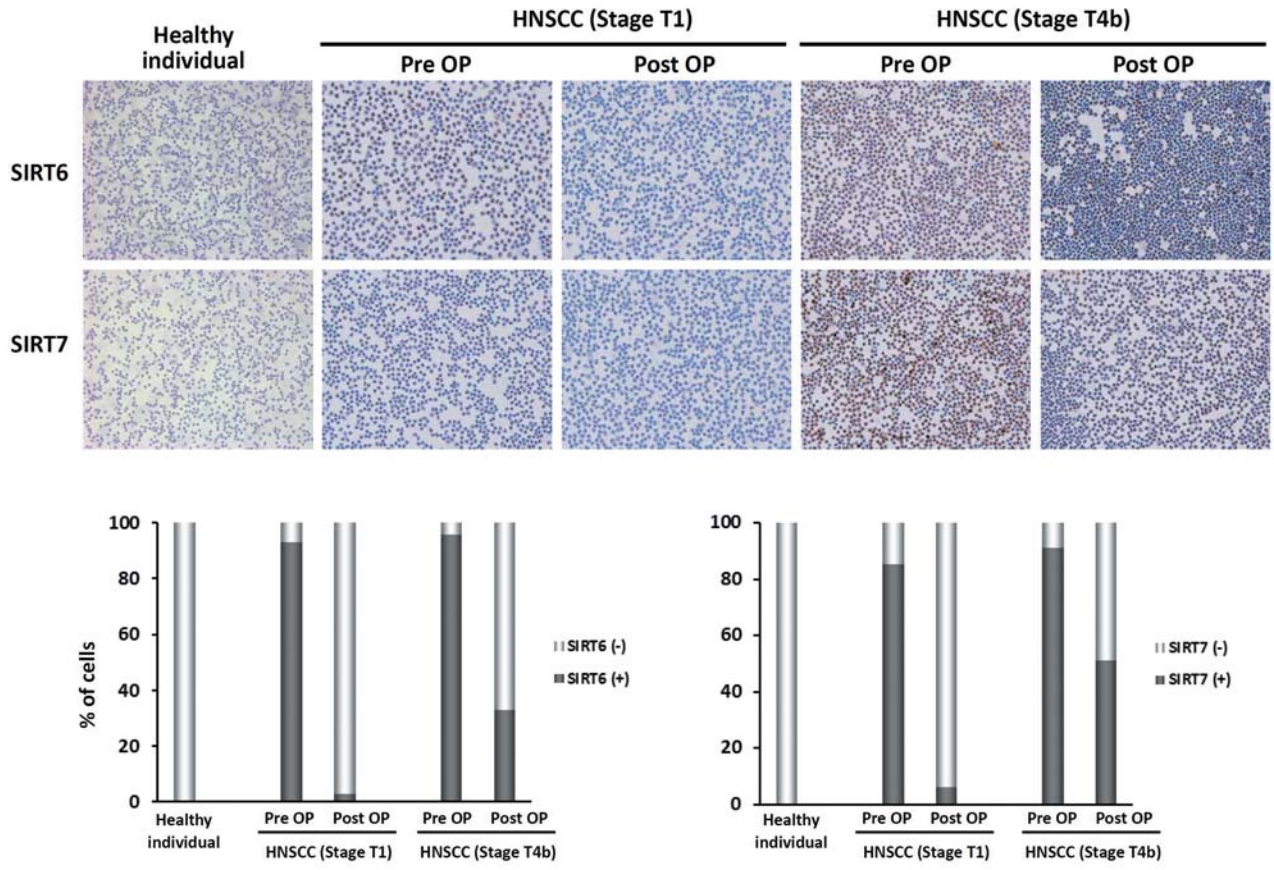

Figure 3. *Immunocytochemical staining for SIRT6 and SIRT7. In peripheral blood (PB) leukocytes from healthy individuals only a few cells are positively stained (brown) for SIRT6 and SIRT7 antibodies; most cells of the preoperative (Pre OP) patients with head and neck squamous cell carcinoma (HNSCC) were stained. In the cells from post-operative (Post OP) HNSCC patients, a significant reduction of SIRT6 or SIRT7 positively stained cells was observed and the reduction was more remarkable in patient with stage T1 HNSCC than in the patient with T4b HNSCC. Percentages of cells positively stained for SIRT6 and SIRT7 antibodies are also shown. Antibody staining was detected using peroxidase with diaminobenzidine (DAB) substrate. Cells were counterstained with hematoxylin-eosin. (Original magnifications ×200.)*

Correlations between tumor staging and expression of SIRT genes in PB leukocytes of patients with HNSCC. We defined the clinical and pathological tumor staging by the TNM staging system, which includes tumor, neck lymph node and metastasis according to the American Joint Committee on Cancer, seventh edition. We categorized our patients into early- (stage I/II) and advanced-stage (stage III/IV) groups to survey the correlation with expression of *SIRT* genes in PB leukocytes. We found that, in advanced stages, the expression levels of *SIRT1* and *SIRT3* were more down-regulated than those in early stages and their correlation with tumor stage were statistically significant ($p<0.05$ and $p<0.01$, respectively) (Figure 1B).

SIRT gene expression in pre-operative and post-operative PB leukocytes in patients with HNSCC. To investigate whether the altered *SIRT* gene expression in PB leukocyte of patients with HNSCC recovers after surgery, we analyzed 34 paired preoperative and one-month postoperative PB samples from patients who underwent head and neck cancer surgery. Among the 34 patients, 10 patients died within 5 year postoperatively and the other 24 patients survived for more than 2 years postoperatively. In patients who survived for more than one year after surgery, the expression of *SIRT1* ($p<0.05$) and *SIRT2* ($p<0.05$) was significantly down-regulated, whereas *SIRT6* ($p<0.001$) and *SIRT7* ($p<0.005$) levels were significantly up-regulated in postoperative as compared to the levels in preoperative samples (Figure 2).

Immunostaining of sirtuin proteins. We conducted an ICC analysis of PB leukocytes to validate the protein expression of the two genes that were most up-regulated in preoperative samples among the 7 genes, *SIRT6* and *SIRT7*. As shown in Figure 3, only few cells of healthy individuals were positively stained by the SIRT6 and SIRT7 antibodies. In contrast, most cells of HNSCC preoperative samples were positively stained.

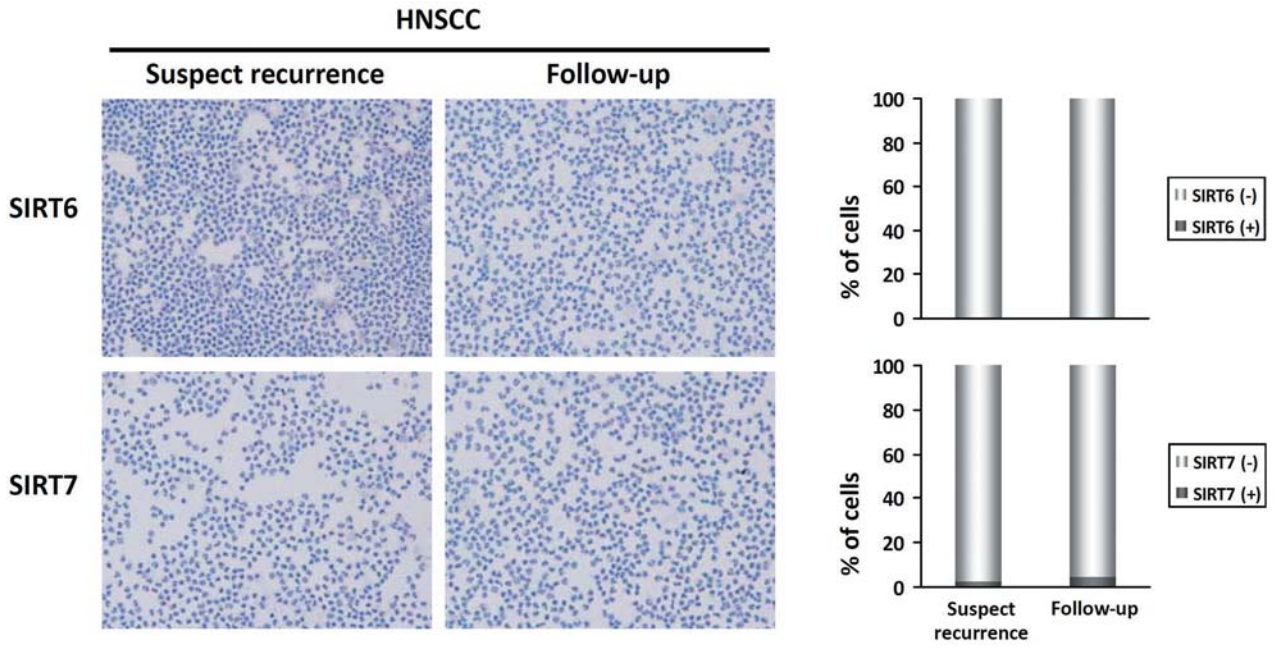

Figure 4. *SIRT6 and SIRT7 immunocytochemical (ICC) staining of peripheral blood (PB) leukocytes from a head and neck squamous cell carcinoma (HNSCC) patient with suspected recurrence. In this representative case, only a few cells are positively stained (brown) for SIRT6 and SIRT7 antibodies in the postoperative PB leukocytes of this patient. After a six-month follow-up, this patient was proven to be not recurrent and the ICC staining of this patient's PB leukocytes for SIRT6 and SIRT7 demonstrated that the numbers of positively-stained cells remained low. Percentages of cells positively stained for SIRT6 and SIRT7 antibodies are also shown. Antibody staining was detected using peroxidase with diaminobenzidine (DAB) substrate. Cells were counterstained with hematoxylin-eosin. (Original magnifications ×200.)*

We also examined the SIRT6 and SIRT7 protein expression in preoperative and postoperative patients with HNSCC. In Figure 3, two representative patients with stage T1 or stage T4b HNSCC are shown. In both patients, more cells of the preoperative samples were positively stained by SIRT6 and SIRT7 antibodies than those of the postoperative samples. Interestingly, we also found that the cells of the patient with T4b HNSCC were more strongly stained than that of the patient with T1 HNSCC (Figure 3). These ICC results are consistent with our observation obtained from qRT-PCR analysis of preoperative and postoperative samples.

Recovery of SIRT6 and SIRT7 in postoperative patient. We also surveyed a postoperative case that was, by image study, a suspected recurrence. However, the result of this patient's CT-guided biopsy was negative and this patient was proved to be non-recurrent after follow-up for six months. We performed ICC to examine the SIRT6 and SIRT7 expression of PB leukocytes of this patient during the time of suspected recurrence and follow-up. The ICC results revealed that the numbers of cells positively stained for SIRT6 and SIRT7 antibodies remained low (Figure 4). This ICC result implies that SIRT6 and SIRT7 expression may have the potential to be a candidate predictor for recurrence.

Discussion

This is the first study to investigate the altered expression of circulating *SIRT* genes in patients with HNSCC. *SIRT* genes are involved in the preservation of genomic stability, linkage of DNA repair and metabolism, as well as prolongation of lifespan (7), though many of their target interactions remain unknown. Cancer cells alter normal cellular machineries to promote persistent cell proliferation and maximize their lifespan, but excessive proliferative signaling can trigger cell senescence and lead to the shortened lifespan of the host organism (18). Therefore, functional loss of *SIRT* genes (particularly *SIRT1*), which is involved in maintaining genome integrity and DNA repair, will promote tumorigenesis. However, cancer cells also need sirtuins for survival, proliferation and repairing the catastrophic genomic events (19). In our previous study, we found down-regulation of *SIRT* genes (*SIRT1, SIRT2, SIRT3, SIRT5* and *SIRT7*) in tumor tissues from patients with HNSCC and suggested that loss of *SIRT* genes may contribute to development of cancer and drive the cancer cells to more advanced stages (17). Based on our previous observation in HNSCC tissues, we think *SIRT* genes might have the potential to be prognostic biomarkers for HNSCC. If the alteration of *SIRT* genes in PB

reflects the alteration in HNSCC tissues, this would make PB *SIRT* gene expression an even more useful and accessible diagnostic tool. Therefore, in the present study, we analyzed the expression of the *SIRT* genes in PB leukocytes from patients with HNSCC and healthy individuals and we found that SIRT1 was dowregulated; in contrast, *SIRT6* and *SIRT7* were significantly up-regulated in the PB of patients with HNSCC when compared to those in healthy individuals. These results revealed that the alteration of *SIRT* genes in PB might not be compatible to the alteration in HNSCC tissues.

Even though the circulating SIRT6 and SIRT7 did not reflect the levels in HNSCC tissues, another interesting finding of this study is the recovery of circulating SIRT6 and SIRT7 expression in patients with HNSCC after surgery (Figure 3). We also found that the cells of a patient with higher grade (T4b) HNSCC were more deeply stained than the cells of the patient with lower grade (T1) HNSCC (Figure 3). In the post-near-total laryngectomy case we have surveyed, although suspected recurrence by CT, the low circulating SIRT6- and SIRT7-positve cells at the times of suspected recurrence and follow-up for six months suggested this patient was non-recurrent. This implied that we might predict no recurrence by circulating SIRT6 and SIRT7 in some equivocal cases without positron emission tomography (PET) or surgical biopsy.

In the last two decades, more and more bodily secretions or plasma biomarkers were proposed to early screen, diagnose and evaluate tumorgenesis of HNSCC (20). Park *et al*. (21) and Zimmermann *et al*. (22) had proposed that the mRNA extracted from the saliva may harbor a broad spectrum of biological information for clinical diagnostic applications. Metalloproteinase (MMP) families are proteolytic enzymes that cause degradation of extracellular matrix allowing for tumor cell migration, which play the role of differentiation, proliferation and apoptosis. It has been reported that members of the MMP family could be biomarkers but this requires more study to evaluate their specificity and clinical significance (23-25). IL-8 in saliva and IL-6 in serum have also been indicated to hold promise as biomarkers for oral cavity and oropharyngeal squamous cell carcinoma (OSCC) (26). Annexin A1 (*ANXA1*) was recently identified in PB by real-time PCR and has been proposed as a potential diagnostic biomarker for OSCC (27). In our previous study, we have demonstrated that detection of circulating miR-21 and miR-26b pre- and postoperatively might provide a novel tumor marker for HNSCC (28). We have also demonstrated altered circadian clock genes in PB leukocytes of patients with HNSCC and PER1 and CLOCK are potential circulating prognostic markers for HNSCC (29).

In this study we further demonstrated expression of *SIRT6* and *SIRT7* in PB leukocytes that may be possibly account as potential diagnostic biomarkers for HNSCC. *SIRT6* and *SIRT7* are both mainly located over the nucleus. *SIRT6* is an important promoter of fat utilization and key regulator of glucose metabolism, and lack of this chromatin factor leads to a phenotype that is reminiscent of the Warburg effect (30). Indeed, *SIRT6* has been recently described as a tumor suppressor that regulates cancer metabolism. Mechanistically, *SIRT6* suppresses aerobic glycolysis and Myc-dependent ribosome biosynthesis, and lack of this *SIRT6* leads to robust metabolic reprogramming, sufficient to promote tumorigenesis (31). In consistence with this, *SIRT6* expression is down-regulated in human pancreatic and colorectal cancers, while conditional deletion of *SIRT6* promotes intestinal tumorigenesis *in vivo* (31). Consistent with a tumor-suppressive function, it has recently been shown that over-expression of SIRT6 induces apoptosis in cancer cell lines (32). However, the mechanism and physiological relevance of this observation remain as yet unexplored. A recent study has demonstrated that *SIRT7* functions as an H3K18 deacetylase that could repress transcription of multiple genes involved in anchorage-independent growth and contact inhibition (33). *SIRT7* depletion markedly reduced tumorigenicity of cancer cells, suggesting that *SIRT7* may play a critical role in maintaining oncogenic transformation (33).

Our next challenge will be to solve the limitations of our study, including the detection range and detection specificities of circulating SIRTs and the evaluation of some confounding factors of the patients' clinical condition, inflammation and homeostatic status. Metabolic diseases (such as diabetes mellitus or liver problem) will effect the result of the *SIRT* family expression also need further investigation. More studies will be needed to discover the mechanism of action of surtuins in relation to genomic, proteomic and metabolic abnormalities associated withcancer. Based on our study, focusing on PB examination and development of recurrence predictors for HNSCC prevention and treatment should be a future direction.

Acknowledgements

This study was partly supported by grants from the Ministry of Science and Technology of Taiwan (MOST 101-2314-B-182A-051, MOST 102-2314-182A-083, MOST 102-2314-B-037-066-MY2, MOST 103-2320-B-182-023 and MOST 103-2314-B-182A-063), Chang Gung Memorial Hospital (CMRPG8B0361, CMRPD8B0661 and CMRPD8C0911) and Kaohsiung Medical University Hospital (KMUH101-1R11, KMUH102-2R11, KMUH102-2T03 and 102-20).

References

1 Department of Health ET, R.O.C.: Cancer registry annual report in Taiwan area, 2010. 2013.

2 Pinto FR, Matos LL, Gumz Segundo W, Vanni CM, Rosa DS and Kanda JL: Tobacco and alcohol use after head and neck cancer treatment: influence of the type of oncological treatment employed. Rev Assoc Med Bras *57(2)*: 171-176, 2011.

3 Hashibe M, Hunt J, Wei M, Buys S, Gren L and Lee YC: Tobacco, alcohol, body mass index, physical activity, and the risk of head and neck cancer in the prostate, lung, colorectal, and ovarian (PLCO) cohort. Head Neck *35(7)*: 914-922, 2013.
4 Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, Lu YC, Li HF and Cheng AJ: Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. Cancer Sci *99(8)*: 1507-1514, 2008.
5 Chang MC, Chiang CP, Lin CL, Lee JJ, Hahn LJ and Jeng JH: Cell-mediated immunity and head and neck cancer: with special empha-sis on betel quid chewing habit. Oral Oncol *41(8)*: 757-775, 2005.
6 Smith EM, Rubenstein LM, Haugen TH, Pawlita M and Turek LP: Complex etiology underlies risk and survival in head and neck cancer human papillomavirus, tobacco, and alcohol: a case for multifactor disease. J Oncol *2012*: 571862, 2012.
7 Mao L, Hong WK and Papadimitrakopoulou VA: Focus on head and neck cancer. Cancer Cell *5(4)*: 311-316, 2004.
8 Imai SI and Guarente L: NAD^+ and sirtuins in aging and disease. Trends Cell Biol 24(8): 464-471, 2014.
9 Hallows WC, Albaugh BN and Denu JM: Where in the cell is SIRT3?—functional localization of an NAD^+-dependent protein deacetylase. Biochem J *411(2)*: e11-13, 2008.
10 Lafontaine-Lacasse M, Richard D and Picard F: Effects of age and gender on Sirt 1 mRNA expressions in the hypothalamus of the mouse. Neurosci Lett *480(1)*: 1-3, 2010.
11 Suwa M and Sakuma K: The potential role of sirtuins regarding the effects of exercise on aging- related diseases. Curr Aging Sci *6(2)*: 178-188, 2013.
12 Pulla VK, Battu MB, Alvala M, Sriram D and Yogeeswari P: Can targeting SIRT-1 to treat type 2 diabetes be a good strategy? A review. Expert Opin Ther Targets *16(8)*: 819-832, 2012.
13 Donato AJ, Magerko KA, Lawson BR, Durrant JR, Lesniewski LA and Seals DR: SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans. J Physiol *589*: 4545-554, 2011.
14 Suzuki K and Koike T: Mammalian Sir2-related protein (SIRT) 2-mediated modulation of resistance to axonal degeneration in slow Wallerian degeneration mice: a crucial role of tubulin deacetylation. Neuroscience *147(3)*: 599-612, 2007.
15 Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW and Schibler U: SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell *134(2)*: 317-328, 2008.
16 Bosch-Presegue L and Vaquero A: The dual role of sirtuins in cancer. Genes Cancer 2(6): 648-662, 2011.
17 Lai CC, Lin PM, Lin SF, Hsu CH, Lin HC, Hu ML, Hsu CM and Yang MY: Altered expression of SIRT gene family in head and neck squamous cell carcinoma. Tumour Biol *34(3)*: 1847-1854, 2013.
18 Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell *144(5)*: 646-674, 2011.
19 Roth M and Chen WY: Sorting out functions of sirtuins in cancer. Oncogene *33(13)*: 1609-1620, 2014.
20 Lee KD, Lee HS and Jeon CH: Body fluid biomarkers for early detection of head and neck squamous cell carcinomas. Anticancer Res *31(4)*: 1161-1167, 2011.
21. Park NJ, Zhou X, Yu T, Brinkman BM, Zimmermann BG, Palanisamy V and Wong DT: Characterization of salivary RNA by cDNA library analysis. Arch Oral Biol *52(1)*: 30-35, 2007.
22 Zimmermann BG, Park NJ and Wong DT: Genomic targets in saliva. Ann NY Acad Sci *1098*: 184-191, 2007.
23 Kuropkat C, Plehn S, Herz U, Dunne AA, Renz H and Werner JA: Tumor marker potential of serum matrix metalloproteinases in patients with head and neck cancer. Anticancer Res *22(4)*: 2221-2227, 2002.
24 Somiari SB, Shriver CD, Heckman C, Olsen C, Hu H, Jordan R, Arciero C, Russell S, Garguilo G, Hooke J and Somiari RI: Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer. Cancer Lett *233(1)*: 98-107, 2006.
25 Yen CY, Chen CH, Chang CH, Tseng HF, Liu SY, Chuang LY, Wen CH and Chang HW: Matrix metalloproteinases (MMP) 1 and MMP10 but not MMP12 are potential oral cancer markers. Biomarkers *14(4)*: 244-249, 2009.
26 St John MA, Li Y, Zhou X, Denny P, Ho CM, Montemagno C, Shi W, Qi F, Wu B, Sinha U, Jordan R, Wolinsky L, Park NH, Liu H, Abemayor E and Wong DT: Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg *130(8)*: 929-935, 2004.
27 Faria PC, Sena AA, Nascimento R, Carvalho WJ, Loyola AM, Silva SJ, Durighetto AF, Oliveira AD, Oliani SM and Goulart LR: Expression of annexin A1 mRNA in peripheral blood from oral squamous cell carcinoma patients. Oral Oncol *46(1)*: 25-30, 2010.
28 Hsu CM, Lin PM, Wang YM, Chen ZJ, Lin SF and Yang MY: Circulating miRNA is a novel marker for head and neck squamous cell carcinoma. Tumour Biol *33(6)*: 1933-1942, 2012.
29 Hsu CM, Lin PM, Lai CC, Lin HC, Lin SF and Yang MY: PER1 and CLOCK are potential circulating biomarkers for head and neck squamous cell carcinoma. Head Neck *36(7)*: 1018-1026, 2014.
30 Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, Guimaraes A, Marinelli B, Wikstrom JD, Nir T, Clish CB, Vaitheesvaran B, Iliopoulos O, Kurland I, Dor Y, Weissleder R, Shirihai OS, Ellisen LW, Espinosa JM and Mostoslavsky R: The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell *140(2)*: 280-293, 2010.
31 Sebastián C, Zwaans BM, Silberman DM, Gymrek M, Goren A, Zhong L, Ram O, Truelove J, Guimaraes AR, Toiber D, Cosentino C, Greenson JK, MacDonald AI, McGlynn L, Maxwell F, Edwards J, Giacosa S, Guccione E, Weissleder R, Bernstein BE, Regev A, Shiels PG, Lombard DB and Mostoslavsky R: The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell *151(6)*: 1185-1199, 2012.
32 Van Meter M, Mao Z, Gorbunova V and Seluanov A: SIRT6 overexpression induces massive apoptosis in cancer cells but not in normal cells. Cell Cycle *10(18)*: 3153-3158, 2011.
33 Barber MF(1), Michishita-Kioi E, Xi Y, Tasselli L, Kioi M, Moqtaderi Z, Tennen RI, Paredes S, Young NL, Chen K, Struhl K, Garcia BA, Gozani O, Li W and Chua KF: SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. Nature *487(7405)*: 114-118, 2012.

Received August 13, 2014
Revised September 16, 2014
Accepted September 23, 2014