Wnt Inhibitory Factor 1 (WIF1) Qualitative-Methylation from Peripheral Blood Could Not Be Used as Biomarker for The Risk of Nasopharyngeal Carcinoma or Smoking Behavior in Yogyakarta Panel

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Abstract

BACKGROUND: Tobacco smoking plays an important role in nasopharyngeal carcinoma (NPC) risk. The Wingless-related integration site (WNT) Inhibitory Factor 1 (WIF1) as one of the tumor suppressor gene (TSG), functions to control cell proliferation through suppression of WNT signaling pathway. WIF1 methylation is one of epigenetic mechanisms which causes overactivated of WNT pathway in NPC development. WIF1 methylation from buffy coat related to smoking status and NPC risk is not known yet.

METHODS: This was a nested case-control study involving 39 newly diagnosed NPC patients and 40 healthy controls. All subjects were enrolled at Dr. Sardjito Hospital, Yogyakarta. Subjects were male, with known smoking status, duration, and quantity of cigarette consumed. Parallel DNA isolated from buffy coat was used for WIF1 DNA methylation analysis, by using methylation-specific PCR (MSP) method. Chi-square analysis was used to determine distribution of differences among groups. Two tailed p-value≤0.05 was considered as statistically significant.

RESULTS: No significant difference between WIF1 methylation of cases and controls (p=0.30), nor smoking habit among smokers (p=0.51) and non-smokers (p=0.43).

CONCLUSION: WIF1 methylation from buffy coat could not be used as an NPC marker nor as a smoking behavior marker.

KEYWORDS: buffy coat, cigarette smoking, methylation, nasopharyngeal carcinoma, WIF1

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Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial cell malignancy located on the surface of the nasopharynx located after the nostrils and connects them to the throat. (1) The global NPC incidence is 1.7/100,000 per year. The estimated incidence of NPC in Indonesia is 8.3/100,000 per year, which makes it the fifth most common carcinoma among men in Indonesia. NPC is more common in men than in women with a ratio of 2.5:1.(2) Etiology of NPC is generally divided into three causes: Epstein-Barr virus (EBV) infection, carcinogenic exposure and genetic susceptibility. NPC will not occur if a person has only one factor, so there is no dominant factor of vulnerability. Carcinogenic exposures can be obtained from the
NPC has been reported to contain more methylation than mutations, when compared with other types of tumors. WINGLESS-RELATED INTEGRATION SITE (WNT) Inhibitory Factor 1 (WIF1) is one of the DNA segments that frequently methylated and affected by cigarette smoking. It was reported that cigarette smoking increased the risk of WIF1 methylation by 88% in lung cancer. WIF1 is one of the tumor suppressor genes (TSGs) that controls cell proliferation by suppressing WNT gene expression (oncogene). WNT controls β-catenin expression. Overexpression of β-catenin leads to nasopharyngeal cancer progression. WIF1 methylation was reported to be found in 61.2% of biopsies and brushing samples from NPC patients collected at Dr. Sardjito Hospital in Yogyakarta. Until currently, there was no report that correlates WIF1 methylation, smoking behavior and nasopharyngeal carcinoma.

Tumor tissue is considered as the best source of information, due to tumor clonality. Particularly for NPC, the small size of tissue biopsy and the impact of preserving the tissue in formalin may limit the use of formalin-fixed paraffin embedded (FFPE) tissue for epigenetic studies. The use of other type of samples such as nasopharynx brushings and peripheral blood for methylation studies were suggested as well due to the less invasiveness procedures.

Previous report from our group showed that Ras Association Domain Family Member 1A (RASFF1A) and Cyclin Dependent Kinase Inhibitor 2A (CDKN2A) DNA methylation could be observed in buffy coat of NPC peripheral blood samples. This was supported by an epigenome-wide association study (EWAS) of smoking behavior including smoking status (smoker and not smoker), smoking duration and smoking quantity were collected in database. Peripheral blood samples, including separated buffycoat were collected from subjects and stored at -80°C until used.

The inclusion criteria for this study was male, age conformity between case and control (±5 years) and had smoking status data (quantity and duration of smoking). Sample with poor quality and quantity of DNA were excluded. Further, this study could include 40 controls and 39 cases. Smoking status was determined as those who actively smoking until diagnosed, whole non smoker were those who never smoked regularly for at least a year.

The DNA used in this study were isolated from buffycoat, purified by using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), and further, kept in -20°C until used.

This study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with reference number KE/FK/0078/EC/2018.

WIF1 Methylation Detection
DNA underwent bisulfite modification using Cells-to-CpG™ Bisulfite Conversion Kit (Applied Biosoym, California, USA). Modified DNA could be stored for a long time at -20°C. WIF1 methylation detection was analyzed using MSP methods, with methylation forward primer: 5’- CGT TTT ATT GGG CGT ATC GT -3’, methylation reverse primer: 5’ - ACT AAC GCG AAC GAA ATA CGA -3’ (12); unmethylated forward primer: 5’- TTT TGG TGG TTT TTA TTT TGG TTT GT -3’, and unmethylated reverse primer: 5’- TCC CAT TTA AAC AAC TAA ACA CA -3’. (13) MSP for methylation used Amplitaq Gold 360 (Thermofisher, California, USA). MSP for unmethylation used Invitrogen Platinum Taq Polymerase (Thermofisher). MSP process was conducted by using Applied Biosystems
ProFlex PCR System (Thermofisher). Unmethylated and methylated WIF1 were observed in 2% agarose gel (in TAE buffer) with 148 bp and 162 bp DNA band, respectively.

**Statistical Analysis**
The proportions of methylation between case-control/smoking status groups were observed using Chi-Square analysis. A two tailed \( p \) value of \( \leq 0.05 \) was considered as statistically significant. Statistical analyses used IBM SPSS statistics v23.

**Results**

Characteristic of subjects are shown in Table 1. No difference was found between mean of age and smoking status between cases and controls \( (p>0.05) \). The average duration of smoking and average cigarette consumption were also similar between cases and controls \( (p>0.05) \).

Unmethylated and methylated WIF1 are shown as bands at 148 bp and 162 bp, respectively (Figure 1). Differences of WIF1 methylation between cases and controls are shown in Table 2. Overall, WIF1 methylation was observed in 20 controls (50%) and 15 cases (42.90%) \( (p=0.30) \) as shown in Table 2. Table 3 shows a comparison of WIF1 methylation between smokers and non-smokers, regardless of their NPC status. Overall, no significant difference was observed between both groups \( (p=0.79) \).

Stratified analysis of smoking behavior related to WIF1 methylation correlated with NPC risk is shown in Table 4. Neither the smokers nor non-smokers group had significant differences in the WIF1 methylation status based on their NPC status. WIF1 methylation was detected among 7 (36.8%) smoker NPC patients and 9 (47.4%) smoker healthy people with Odds Ratio (OR)=0.65 and \( p=0.51 \). WIF1 methylation was detected among 8 (40%) non-smoker NPC patients and 11 (52.4%) non-smoker healthy people with OR=0.606 and \( p=0.43 \).

**Discussion**

NPC is a malignancy reported to contain more DNA methylation than mutations, compared to other types of cancer.(4,5) Similar to many cancers, tobacco smoking has been reported as a risk factor of NPC in low and high incidence populations.(3,12) Methylation of WIF1 was reported to be highly detected in tissue of NPC (8,13), while the association between methylation status of WIF1 in peripheral blood and smoking behavior was reported only among lung cancer patients.(6)

The use of peripheral blood as an alternative to tumor tissues has been reported in different diseases, including cancers (14), diabetes (15) and cardiovascular disease (16). A study on colorectal cancer reported that methylation from whole blood could be used as a biological marker for diagnostic purpose. They reported that methylation of Aldehyde Oxidase 1 (AOX-1), Retinoic Acid Receptor-b2 (RARB2), Ras Like Estrogen Regulated Growth Inhibitor (RERG), Adam Metallopeptidase with Thrombospondin

### Table 1. Subject characteristics.

| Variable              | Control (n = 40) | Case (n = 39) | \( p \) * |
|-----------------------|------------------|---------------|-----------|
| **Age**               |                  |               |           |
| Mean ± SD (year)      | 49.58 ± 2.56     | 50.58 ± 2.38  | 0.79      |
| **Smoking status**    |                  |               |           |
| Smoker                | 19               | 19            | 0.91      |
| Non-smoker            | 21               | 20            |           |
| **Smoking duration**  |                  |               |           |
| Mean ± SD (year)      | 28.89 ± 13.07    | 33.21 ± 2.99  | 0.47      |
| **Cigarette/day**     |                  |               |           |
| Mean ± SD (year)      | 8.95 ± 1.30      | 12.89 ± 1.93  | 0.21      |
| **WHO type**          |                  |               |           |
| Type 2                | 0                | 1             |           |
| Type 3                | 0                | 38            |           |

*chi-square test; \( p<0.05 \) considered as statistically different.
A B

Figure 1. Methylation-specific PCR (MSP) result of WIF1 methylation detection. A: methylated-specific primer used for methylation detection; B: unmethylated-specific primer used for unmethylation detection. Sample 1 is unmethylation sample, sample 2 is methylated sample, and sample 3 is partially methylated sample and considered as methylated sample.

WIF1 Methylation Status  Control Case  p *

|                | Methylated | Unmethylated |  |
|----------------|------------|--------------|---|
| Methylated     | 20 (50%)   | 15 (42.9%)   | 0.3 |
| Unmethylated   | 20 (50%)   | 24 (61.5%)   |   |

*chi-square test; p≤0.05 considered as statistically different.

Table 3. WIF1 methylation and smoking behavior.

Smoking Status | WIF1 Methylation Status | p * |
|---------------|-------------------------|-----|
|               | Methylated | Unmethylated |   |
| Smoker        | 16 (45.7%) | 22 (38.5%)  | 0.79 |
| Non-smoker    | 19 (54.3%) | 22 (61.5%)  |   |

*chi-square test; p≤0.05 considered as statistically different.
smokers and smokers, regardless of their NPC status. Study on non-small cell lung cancer (NSCLC) showed that WIF1 methylation correlated with bad prognosis instead of smoking habit. WIF1 methylation in NSCLC patients increased the risk of recurrence compared to unmethylated WIF1.(23) WIF1 methylation also correlated with smoking habit in Het-1A, an esophageal epithelial cell line. WIF1 expression became downregulated and correlated to methylation in Het-1A treated with mainstream smoke extract (MSA) and side-stream smoke extract (SSE) for 6 months.(24) Our prior study on RASSF1A and CDKN2A methylation status in peripheral blood showed no association with smoking behavior.(9) Similarly, in our study, WIF1 methylation of peripheral blood cell-derived DNA could not act as a smoking behavior marker. Regarding the suggested pattern by Lee and Pausova, correlation of smoking behavior and DNA methylation still needs to be observed in NPC by using other target genes in the TSG and oncogenes pathways.(22)

DNA methylation using qualitative approach might contributed to the result of the study, as it only assessed the presence and the absence of MSP product. Previous study showed that compared to bisulfite sequencing as gold standard for DNA methylation detection, qualitative MSP found less agreement than quantitative high resolution mass-spectrometry based approach (mass-ARRAY). This protocol yielded significant difference between high degree of Inhibitor of DNA Binding 4 (ID4) gene methylation of acute myeloid leukemia (AML) patients and low degree of ID4 gene methylation or unmethylation of healthy controls. (25) For further study, we propose the use of quantitative approach or at the minimum semi-quantitative assay such as high resolution melting (HRM), to further determine the role of smoking habit to the pathogenesis of NPC related to methylation of regulating genes.

Smoking behavior is one of many potential environmental factors that correlate with NPC. There are other environmental factors that may generate DNA methylation, such as diet, physical activity, drug consumption, and occupational exposure.(26,27) In future studies, it is suggested to do further analysis that discuss the association of methylation status quantitatively and potential environmental factors in a larger set of populations.

### Conclusion

We concluded that WIF1 methylation was found in the blood cell-derived DNA. WIF1 methylation from the DNA buffy coat could not be used as either NPC nor smoking behavior markers.

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### References

1. Brennan B. Nasopharyngeal carcinoma. Orphanet J Rare Dis. 2006; 1: 23. doi: 10.1186/1750-1172-1-23.
2. WHO [Internet]. Globocan 2012: Estimated Cancer Incidence Mortality and Prevalence Worldwide in 2012. World Health Organization. 2012 [cited 2018 Apr 5]. Available from: http://globocan.iarc.fr/Pages/fact_sheets_population.aspx.
3. Tsao SW, Yip YL, Tsang CM, Pang PS, Lau VMY, Zhang G, et al. Etiological factors of nasopharyngeal carcinoma. Oral Oncol. 2014; 50: 330-8.
4. Dai W, Cheung AKL, Ko JMY, Cheng Y, Zheng H, Ngnk RKC, et al. Comparative methylome analysis in solid tumors reveals aberrant methylation at chromosome 6p in nasopharyngeal carcinoma. Cancer Med. 2015; 4: 1079-90.
5. Tsao SW, Tsang CM, Lo KW. 2017. Epstein-Barr virus infection and nasopharyngeal carcinoma. Philos Trans R Soc Lond B Biol Sci. 2017; 372: pii: 20160270. doi: 10.1098/rstb.2016.0270.
6. Zheng Y, Li X, Jiang Y, Xu Y, Song B, Zhou Q, et al. Promoter hypermethylation of Wnt inhibitory factor-1 in patients with lung cancer. Medicine (Baltimore). 2016; 95: e5433.

7. Tulalamba W, Janvilisri T. Nasopharyngeal carcinoma signaling pathway: an update on molecular biomarkers. Int J Cell Biol. 2012; 2012: 594681. doi: 10.1155/2012/594681.

8. Hutajulu SH, Indrasari SR, Indrawati LP, Harijadi A, Duin S, Haryana SM, et al. Epigenetic markers for early detection of nasopharyngeal carcinoma in a high risk population. Mol Cancer. 2011; 10: 48. doi: 10.1186/1476-4598-10-48.

9. Risanti ED, Kurniawan A, Wahyuningsih L, Dwianingsih EK, Rinonce HT, Fachiroh J. Association of peripheral blood RASSF1A and CDKN2A methylation status with smoking behaviour in nasopharyngeal carcinoma. Indones Biomed J. 2018; 10: 123-7.

10. Ye M, Huang T, Ni C, Yang P, Chen S. Diagnostic capacity of RASFF1A promoter methylation as a biomarker in tissue, brushing, and blood samples of nasopharyngeal carcinoma. EBioMedicine. 2017; 18: 32-40

11. Li S, Wong EM, Bui M, Nguyen TL, Joo JHE, Stone J, et al. Causal effect of smoking on DNA methylation in peripheral blood: A twin and family study. Clin Epigenetics. 2018; 10: 1-12.

12. Xu FH, Xiong D, Xu YF, Cao SM, Xue WQ, Qin HD, et al. An epidemiological and molecular study of the relationship between smoking, risk of nasopharyngeal carcinoma, and Epstein-Barr Virus activation. J Natl Cancer Inst. 2012; 104: 1396-410.

13. Dai W, Zheng H, Cheung AKL, Lung ML. Genetic and epigenetic landscape of nasopharyngeal carcinoma. Chinese Clin Oncol. 2016; 5: 16.

14. Li L, Choi JY, Lee KM, Sung H, Park SK, Oze I, et al. DNA methylation in peripheral blood: a potential biomarker for cancer molecular epidemiology. J Epidemiol. 2012; 22: 384-94.

15. Otterdijk SD Van, Binder AM, Szarc K, Schwald J, Michels KB. DNA methylation of candidate genes in peripheral blood from patients with type 2 diabetes or the metabolic syndrome. PLoS One. 2017; 12: e0180955. doi: 10.1371/journal.pone.0180955.

16. Nakatochi M, Ichihara S, Yamamoto K, Naruse K, Yokota S, Asano H, et al. Epigenome-wide association of myocardial infarction with DNA methylation sites at loci related to cardiovascular disease. Clin Epigenetics. 2017; 9: 54. doi: 10.1186/s13148-017-0353-3.

17. Luo X, Huang R, Sun H, Liu Y, Bi H, Li J, et al. Methylation of a panel of genes in peripheral blood leukocytes is associated with colorectal cancer. Sci Rep. 2016; 6: 29922. doi: 10.1038/srep29922.

18. Severi G, Southey MC, English DR, Jung C, Lonie A, McLean C, et al. Epigenome-wide methylation in DNA from peripheral blood as a marker of risk for breast cancer. Breast Cancer Res Treat. 2014; 148: 665-73.

19. Zhang Z, Sun D, Hutajulu SH, Nawaz I, Van DN, Huang G, et al. Development of a non-invasive method, multiplex methylation specific PCR (MMSP), for early diagnosis of nasopharyngeal carcinoma. PLoS ONE. 2011; 7: e45908. doi: 10.1371/journal.pone.0045908.

20. Zhao Z, Liu W, Liu J, Wang J, Luo B. The effect of EBV on WIF1, NLK, and APC gene methylation and expression in gastric carcinoma and nasopharyngeal cancer. J Med Virol. 2017; 89: 1844-51.

21. Lee BB, Lee EJ, Jung EH, Chun HK, Chang DK, Song SY, et al. Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clin Cancer Res. 2009; 15: 6185-91.

22. Lee KWK, Pausova Z. Cigarette smoking and DNA methylation. Front Genet. 2013; 4: 132. doi: 10.3389/fgene.2013.00132.

23. Yoshino M, Suzuki M, Lei T, Yasumitsu M, Hidehisa H, Tatsuro O, et al. Promoter hypermethylation of the p16 and Wif-1 genes as an independent prognostic marker in stage IA non-small cell lung cancers. Int J Oncol. 2009; 35: 4-11.

24. Huang Y, Chang X, Lee J, Cho YG, Zhong X, Park IS, et al. Cigarette smoke induces promoter methylation of single-stranded DNA-binding protein 2 in human esophageal squamous cell carcinoma. Int J Cancer. 2011; 128: 2261-73.

25. Claus R, Wilop S, Hielscher T, Sonnet M, Dahl E, Galm O, et al. A systematic comparison of quantitative high-resolution DNA methylation analysis and methylation-specific PCR. Epigenetics. 2012; 7: 772-80.

26. Lim U, Song M-A. Dietary and lifestyle factors of DNA methylation. In: Dumitrescu RG, Verma M, editors. Cancer Epigenetics: Methods and Protocols. Totowa, NJ: Humana Press; 2012; p.359-76.

27. Pacchierotti F, Spanò M. Environmental impact on DNA methylation in the germline: state of the art and gaps of knowledge. Biomed Res Int. 2015; 2015:123484. doi: 10.1155/2015/123484.