

RESEARCH ARTICLE

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 PanelAditya Kurniawan¹, Erika Diana Risanti², Saihas Suhda³, Hanggoro Tri Rinonce⁴,
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Received date: Apr 9, 2019; Revised date: Jun 18, 2019; Accepted date: Jul 16, 2019

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*

Indones Biomed J. 2019; 11(3): 273-8

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

environment. One of the most common carcinogenic exposures of the environment is cigarette smoking, which reported as NPC common risk in high and low incidence population.(3)

NPC has been reported to contain more methylation than mutations, when compared with other types of tumors.(4,5) Wingless-related integration site (*WNT*) Inhibitory Factor 1 (*WIFI*) is one of the DNA segments that frequently methylated and affected by cigarette smoking. It was reported that cigarette smoking increased the risk of *WIFI* methylation by 88% in lung cancer.(6) *WIFI* 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.(7) *WIFI* methylation was reported to be found in 61.2% of biopsies and brushing samples from NPC patients collected at Dr. Sardjito Hospital in Yogyakarta. (8) Until currently, there was no report that correlates *WIFI* 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. (9) 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.(8,10)

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.(9) This was supported by an epigenome-wide association study (EWAS) of smoking conducted among twin and family, using DNA-derived peripheral blood collected as dried blood spots. The EWAS study revealed 39 CpGs differently methylated on never, former and current smokers.(11) These previous studies showed that DNA methylation could be detected from peripheral blood-derived DNA.(9,11)

Within this study, we discuss the detection of *WIFI* methylation from buffy coat of peripheral blood, and further analysed its correlation with smoking behavior comparing NPC and healthy controls. Identification of *WIFI* methylation in this study was conducted by using qualitative methylation-specific PCR (MSP) which broadly used to detect DNA methylation of known gene.

Methods

Study Sample

This was a nested case-control study, taken from the previous hospital-based case control study 2012-2014 at Dr. Sardjito General Hospital, Yogyakarta. Cases were those who newly diagnosed and histopathologically confirmed NPC. Healthy controls were those who declared healthy at the point of recruitment and never been diagnosed with any type of cancer. Data on clinical status, demographic, and 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.

WIFI Methylation Detection

DNA underwent bisulfite modification using Cells-to-CpG™ Bisulfite Conversion Kit (Applied Biosystem, California, USA). Modified DNA could be stored for a long time at -20°C. *WIFI* 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 TTG TTG TTT TTA TTT TTG 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 *WIFI* 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 ≤ 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 *WIFI* are shown as bands at 148 bp and 162 bp, respectively (Figure 1). Differences of *WIFI* methylation between cases and controls are shown in Table 2. Overall, *WIFI* 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 *WIFI* 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 *WIFI* methylation correlated with NPC risk is shown in

Table 4. Neither the smokers nor non-smokers group had significant differences in the *WIFI* methylation status based on their NPC status. *WIFI* methylation was detected among 7 (36.8%) smoker NPC patients and 9 (47.4%) smoker healthy people with Odds Ratio (OR)=0.65 and $p = 0.51$. *WIFI* 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 *WIFI* was reported to be highly detected in tissue of NPC (8,13), while the association between methylation status of *WIFI* 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 Metalloproteinase with Thrombospondin

Table 1. Subject characteristics.

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

*chi-square test; $p \leq 0.05$ considered as statistically different.

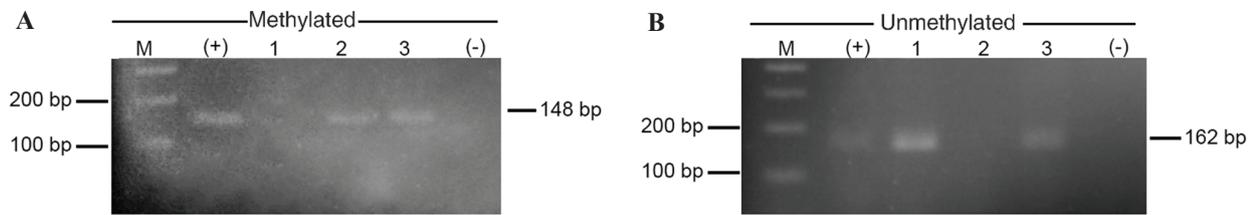


Figure 1. Methylation-specific PCR (MSP) result of *WIF1* methylation detection. A: methylated-specific primer used for methylation detection; B: unmethylated-specific primer used for unmethylation detection. Sample 1 is unmethylation sample, sample 2 is methylated sample, and sample 3 is partially methylated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

Type 1 Motif 9 (*ADAMTS9*), Interferon Regulatory Factor 4 (*IRF4*), and Forkhead Box E1 (*FOXE-1*) were higher among patients compared to healthy controls.(17) An EWAS in breast cancer, compared methylation of DNA from buffy coat of breast cancer patients and healthy people. The results showed that breast cancer patients had a higher number of methylated genes compared to healthy people. (18) Previous report from our group has shown that gene methylation could be detected in the buffycoat from NPC patient.(9) Further, both our studies may highlighted the capacity of buffy coat to be used in DNA methylation study. A study showed in the meta analysis, the superior detection of *RASFF1A* methylation for NPC diagnosis from NPC tissue compared to nasopharyngeal brushing and even less in peripheral blood.(10)

Within this study, in comparing NPC and control subjects, we did not find difference of *WIF1* methylation status by using qualitative MSP. Therefore, in this study, *WIF1* methylation status from buffy coat could not be used as a marker for the nasopharyngeal cancer. Previous study recommended a combination of five methylation marker including *WIF1*, *RASSF1A*, *p16*, Checkpoint with Forkhead

and Ring Finger Domains (*CHFR*), and Retinoblastoma-Interacting Zinc-Finger Protein 1 (*RIZ1*) to predict NPC risk.(8) In the term of sampling invasiveness, buffy coat and nasopharynx brushings, known to be less invasive, could determine *WIF1* methylation as well as biopsy sample by using multiplex MSP.(19) The *WIF1* gene was found to be methylated in the nasopharyngeal cancer cell culture (C666-1). In this cell culture, *WIF1*, nemo like kinase (*NLK*), and adenomatous polyposis coli (*APC*) genes were also methylated, resulting in the decreased expression of these three genes.(20) Other studies have shown that the methylation of *WIF1*, *APC*, O-6-Methylguanine-DNA Methyltransferase (*MGMT*) and *RASSF12A* genes from peripheral blood samples has a sensitivity of 86.5% and a specificity of 92.1% in detecting colorectal cancer.(21)

Cigarette smoke contains chemical compounds that contributes to carcinogenesis, e.g., arsenic, formaldehyde, aromatic amines, N-nitrosamines, and volatile hydrocarbon. Those carcinogens damage DNA strains and recruited DNA repair proteins, one of which is DNA methyltransferase 1 (DNMT1), which causes DNA methylation.(22) Our study showed no difference on *WIF1* methylation between non-

Table 2. *WIF1* methylation status and NPC status.

| <i>WIF1</i> Methylation Status | Control | Case | <i>p</i> * |
|--------------------------------|----------|------------|------------|
| Methylated | 20 (50%) | 15 (42.9%) | 0.3 |
| Unmethylated | 20 (50%) | 24 (61.5%) | |

*chi-square test; $p \leq 0.05$ considered as statistically different.

Table 3. *WIF1* methylation and smoking behavior.

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

*chi-square test; $p \leq 0.05$ considered as statistically different.

Table 4. Stratification analysis of *WIFI* promoter gene methylation in case-control group of smoker and non-smoker subject.

| <i>WIFI</i> Methylation Status | Smoker | | OR | <i>p</i> * | Non-smoker | | OR | <i>p</i> * |
|--------------------------------|------------|------------|------|------------|------------|------------|------|------------|
| | Case | Control | | | Case | Control | | |
| Unmethylated | 12 (63.2%) | 10 (52.6%) | 0.65 | 0.51 | 12 (60%) | 10 (47.6%) | 0.61 | 0.43 |
| Methylated | 7 (36.8%) | 9 (47.4%) | | | 8 (40%) | 11 (52.4%) | | |

*chi-square test; $p \leq 0.05$ considered as statistically different.

smokers and smokers, regardless of their NPC status. Study on non-small cell lung cancer (NSCLC) showed that *WIFI* methylation correlated with bad prognosis instead of smoking habit. *WIFI* methylation in NSCLC patients increased the risk of recurrence compared to unmethylated *WIFI*.(23) *WIFI* methylation also correlated with smoking habit in Het-1A, an esophageal epithelial cell line. *WIFI* 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, *WIFI* 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 *WIFI* methylation was found in the blood cell-derived DNA. *WIFI* methylation from the DNA buffy coat could not be used as either NPC nor smoking behavior markers.

Acknowledgements

We are grateful to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia (DIKTI) for PUPIT 2016 and Dana Masyarakat Universitas Gadjah Mada 2016 for grant funding. We also thank Sri Fatmawati and Sumartiningsih for technical assistance, as well as Dwi Hartati for taking care of the database.

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