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Evaluation of aberrant $p16^{INK4\alpha}$ promoter CpG methylation and its application in Vietnamese breast cancers patients

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***Abstract* - The disruption of genetic materials as the aberrant methylation at CpG islands of tumor suppressor gene's promoter, which is thought to be a driver epigenetic events of malignant cells. $p16^{INK4\alpha}$, its function is considered as an inhibitor of CDK4 and CDK6 that initiate the phosphorylation of RB (retinoblastoma tumor suppressor protein), has been identified as a tumor suppressor gene in many human cancers. In current study, methylation specific PCR (MSP) was carried out to evaluate the methylation status of $p16^{INK4\alpha}$ from Vietnamese breast patients, in order to find out the methylation signature served as biomarker applied in prognosis, early detection and diagnosis of breast cancer in Vietnamese population. The results showed that the methylation frequency of $p16^{INK4\alpha}$ promoter reached to 49.5% (47 of 95 breast cancer samples). In the case of non-cancer specimens, only 15% (3 of 20 non-cancer specimens) was methylation. According to clinical parameters, there were significant correlation between the $p16^{INK4\alpha}$ aberrant methylation and the HER(-)p53(-) combination in breast cancer ($p<0.05$). Furthermore, the $p16^{INK4\alpha}$ methylation increased the possibility to breast cancer with the high incidence via the odd ratio (OR = 5.55, $p = 0.01$). Simultaneously analyzed with hypermethylation of panel genes including *BRCA1*, *Cyclin D2*, *GSTP1* and *RASSF1A*, it showed that the coverage of diagnosis, which based on evaluation of panel genes' hypermethylation frequency, was 100% (with the methylation index (MI) ≥ 0.2). In conclusion, the hypermethylation at CpG islands of $p16^{INK4\alpha}$ was the significant characteristic of breast cancer in Vietnamese population relied on the size of samples surveyed. Notably, co-analyzed with *BRCA1*, *Cyclin D2*, *GSTP1* and *RASSF1A*, those hypermethylation characteristics could be served as significant biomarker for breast cancer.**

Keywords*: *breast cancer, methylation, MSP, $p16^{INK4\alpha}$, Vietnamese breast cancer.

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I. Introduction

Advance in understanding of the molecular mechanism of carcinogenesis and the progression from a premalignant lesion to malignancy, leading to the new information concerning about aberrant hypermethylation and its function which is considered as the regulation of tumor suppressor gene [1]. Promoter hypermethylation has been described to be consistent and early driving event in many human cancers [2]. Many recent studies indicated that hypermethylation occurs in numerous tumor suppressor genes leading to the inactivation of their function, consequently relevant to occurrence and contribute to the driving to human cancer, including breast cancer [3][19][15][20]. $p16^{INK4\alpha}$ (*CDKN2A*) gene, is located on chromosome 9p21, is important tumor suppressor gene. Its encoded protein, $p16^{INK4\alpha}$ acts through the retinoblastoma pathway to regulate the cell cycle. $p16^{INK4\alpha}$ protein acts as inhibitor of CDK (cyclin-dependent kinase) that blocks the G1/S phase of cell cycle by inhibiting cyclin-D-CDK4/6 formation via targeting at directly binding with CDK4/6, consequently, inhibits pRB phosphorylation leading to the cell arrest in G1 phase [4]. Previous studies stated that the inactivation or down-regulation of $p16^{INK4\alpha}$ by hypermethylation, could increase the incidence of carcinogenesis of various cancers, including breast cancer [3][4][5][10][21]. $p16^{INK4\alpha}$ methylation has been described in 23.9% in breast cancer, 52.8% in breast cancer and is often included in panel of markers used to access, early detect, prognosis of breast cancer [4][21][22].

In Vietnam, the incidence rate of breast cancer has increased from the crude rate of 13.8 per women in 2,000 to 28.1 per 10,000 women, it is also accessed as the common caused death of cancer and become an alarming problem in public health [11]. The early detection is necessary to be carried out for purpose of increasing the survival rate of breast cancer. The most well-known breast molecular biomarkers with prognostic and/or therapeutic value are hormone receptor, HER2 (Human epidermal growth factor receptor 2) oncogene, p53 protein, etc [6]. Actually, the amplification of HER2, a member of the epidermal growth factor, is found to be present in 15% - 30% newly diagnosis breast cancer cases and the mutation of p53 gene and/or p53 has been observed in 20% - 50% of primary breast carcinomas [14]. Therefore, the amplification of HER2/neu and p53 has well

been considered as predictive criteria and may be a good screening method for prognosis of breast cancer [8][12].

In an attempt to evaluate the role of hypermethylation of $p16^{INK4\alpha}$ in Vietnamese breast cancer population, we have evaluated the methylation frequency at CpG islands of promoter belonged to $p16^{INK4\alpha}$ in both breast cancer specimens and healthy specimens based on the MSP (Methylation Specific PCR) method. Additionally, we assessed the correlation between the methylated $p16^{INK4\alpha}$ with the clinicopathologic parameters in breast cancer. For further towards to apply promising biomarkers for prognosis and early detection in Vietnamese breast cancer patients.

II. Materials and methods

A. Sample collection

From 2010 to 2014, a total of 115 samples including 95 breast cancer specimens and 20 healthy (non-cancer) specimens were admitted to the University Medical Center Ho Chi Minh City, Vietnam. For input confirmed, all of breast cancer specimens were enrolled in evaluation of two predictive biomarkers, including HER2/neu and p53, by immunohistochemistry stained with two HER2/neu and p53 antibodies (Ventana CONFIRM anti-HER-2/neu (4B5) and Bp53-11). 20 non-cancer specimens were obtained from women underwent a biopsy of the mammary gland because of mammographic screening and for whom histology confirmed the present of only normal tissue. All tissues were obtained from the surgical specimens, then, embedded in the paraffin and stored at -20^{o}C until further used.

B. DNA extraction, bisulfite modification and MSP assay

In brief, total of genomic DNA was isolated by phenol/chloroform method from paraffin embedded breast cancer tissues and non-cancer tissues. The purity and concentration of genomic DNA was quantified by the absorbance at OD_{260} and OD_{280}. The pure preparation of DNA with OD_{260}/OD_{280} values of 1.8 to 2.0 was used to the bisulfite DNA modification assay. The bisulfite modification was carried out with approximately 2 µg genomic DNA of each sample by DNA modification Kit (Epitect Kit, Qiagen). The final precipitate was eluted in a volume of 20 µl for MSP assay.

MSP was done in a total volumn of 15 µl containing 3 µl bisulfite-modified template DNA, 0.75 unit iTaq DNA polymerase (Biorad). MSP reaction was subjected to initial incubation at 95^{o}C for 5 min, followed by 40 cycles at 95^{o}C for 30s, X^{o}C for 30s, 72^{o}C for 30s and 72^{o}C for 6 min for final incubation. (Note: X was the annealing temperature of each specific primer to $p16^{INK4\alpha}$ gene). The sequences of primers and X^{o}C for each primer annealing were noted in Table 1. Each PCR product was directly loaded onto a 2.0% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

TABLE I. METHYLATED AND UNMETHYLATED P16INK4A PRIMER SEQUENCE

Primer name	Primer sequence (5' – 3')	X^{o}C	P
p16 -M-F *p16* -M-R	TTATTAGAGGGTGGGG**CG**GAT**CGC** **G**ACCC**CG**AAC**CGCG**AC**CG**TAA	62^{o}C	150
p16 -U-F *p16* -U-R	TTATTAGAGGGTGGGG**TG**GAT**TGT** **CA**ACCC**CA**AAC**CACA**AC**CA**TAA	60^{o}C	149

*Note: CpG islands were bold and underlined; X^{o}C: primer annealing temperature. M: methylated, U: Unmethylated; F: Forward; R: Reverse; P: product size.

C. Statistical analysis

The methylation frequency of $p16^{INK4\alpha}$ was calculated. The differences in presence of methylation were determined by two sided Fisher test and Chi squared test for variables. Additionally, the correlation between methylation status and clinical parameters were examined by using the Chi-square test. Moreover, the Odd ratio (OR) and 95% confidence intervals (CIs) were also calculated. Statistical analyses were performed by using Medcalc® Version 12.7.0.0. Statistical significance was assumed at two-side p value of $p < 0.05$.

III. Results

In attempt to evaluate hypermethylation status of CpG islands belonged to promoter of $p16^{INK4\alpha}$, the MSP method was carried out for 95 tumor specimens and 20 non-cancer specimens. As the result, concerning to breast cancer specimens, the frequency of methylation was 49.5% (47 of 95 tumor specimens). Otherwise, in non-cancer specimens, methylation was observed in 3 of 20 cases, counting for 15.0%. The MSP product electrophoresis result and sequencing was shown in Fig. 1 and Fig. 2, respectively.

Figure 1. Methylated promoter of $p16^{INK4\alpha}$ gene was analyzed on some clinical samples by MSP. (*The MSP product was 150/148 bp in length. (1), (2), (3), (4): breast cancer samples; (5) (6) (7) (8): non-cancer samples*)

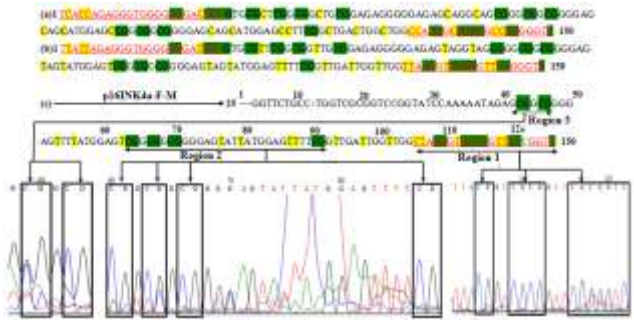

Figure 2. Sequencing profile of methylated of $p16^{INK4\alpha}$. (*CG sites were in the green highlight; Cytosine did not depend on the CpG site were in yellow. (a) DNA sequence was without bisulfite modified; (b) DNA*

sequence was bisulfite modified; (c) The $p16^{INK4\alpha}$ sequencing by using the forward methylated primer p16-M-F. Region 1: the methylated reverse primer. Region 2: four CGs in examined region. Region 3: two CGs in examined region).

According to clinical parameters, the methylation of candidate gene did not associate with patient age, tumor grade and tumor stage (Data not shown). Besides, the correlation between the methylation frequency of $p16^{INK4\alpha}$ gene and two breast cancer predictive biomarkers, including HER2/neu and p53 was also analyzed. The correlation was observed in the association between $p16^{INK4\alpha}$ and p53 immuno-staining (p = 0.03). However, regarding to HER2/neu, there was no any correlation was found out. Especially, taken these two prognosis biomarkers together, the frequent methylation of $p16^{INK4\alpha}$ in breast cancer was significant associated to HER2/neu(-)p53(-) (p = 0.04) as shown in Table 2. Moreover, the risk relative was 3.30 ($p = 0.03$) and odd ratio was found significant at 95% confidence interval with the odd ratio was 5.55 ($p = 0.01$) showing the strong association (Table 3).

TABLE II. THE ANALYSIS OF ASSOCIATION BETWEEN METHYLATION OF $P16^{INK4A}$ PROMOTER AND HER2/NEU, P53 CHARACTERISTIC

Characteristics	$p16^{INK4\alpha}$	
	U (%)	M (%)
HER2/neu		
Negative	48 (63.2)	28 (36.8)
Positive	17 (43.6)	22 (56.4)
	p = 0.07	
p53		
Negative	25 (73.5)	9 (26.5)
Positive	40 (49.4)	41 (50.6)
	p = 0.03	
HER(-)p53(-)		
No	41 (50.0)	41 (50.0)
Yes	24 (72.7)	9 (27.3)
	p = 0.04	

IV. Discussion

Results of MSP analysis was confirmed by MSP products sequencing, according to the Fig. 2, we successfully carried out the evaluation of $p16^{INK4\alpha}$ hypermethylation. By sequencing, six CG sites were observed in region 2 and 3. Moreover, we also detected five methylated CG sites in methylated reversed primer. As shown in Fig. 2c, the signal of peaks in MSP product sequencing were quite good for reading nucleotide sequencing. Additionally, making the comparison between the non-bisulfite modified (Fig. 2a) and bisulfite modified (Fig. 2b), all the unmethylated cytosine, which was indicated as yellow characteristic, were totally transfer into thymine in bisulfite modified sequence. Otherwise, the methylated cytosine were unchanged. For these reason, it was concluded that the bisulfite modification was successfully carried out.

In present study, we have analyzed the methylation status of $p16^{INK4\alpha}$ in 95 breast cancer specimens and 20 non-cancer specimens. We observed the present of the amplification band corresponding to methylated target sequence in 47 of 95 tumor specimens, counting for 49.5%, with 150-bp length. Moreover, we only observed methylation status in 3 of 20 non-cancer specimens, counting for 15.0%. The frequency of $p16^{INK4\alpha}$ promoter methylation in breast cancer specimen is significantly different from in non-cancer specimens ($p < 0.01$). So, promoter methylation of $p16^{INK4\alpha}$ gene occurs frequently in breast cancer and it is considered as a crucial epigenetic event in cell transformation from non-cancer cell into malignant cells. Compared with other reports carried out in various countries, due to geography differences, in our study, the frequency of hypermethylation was higher than the research of Hui et al., (2000) [13] as 20.0%, Zhao et al., (2010) [21] as 23.9%. Concerning to non-cancer samples, the similar to those studies was the lower hypermethylation frequency observed. Especially, in our survey on the non-cancer specimens, we remarked three cases were methylated, which we did not observe any transformation of cell into malignant growth in the moment. Therefore, it suggested that the further investigation about the whether it will transform to tumor in following years had to be considered.

Concerning to HER2/neu staining, it was clearly to consider that more 43.6% of positive cases were methylated, contract to negative HER2/neu staining in which the methylation status counting for 36.8%. Therefore, to the statistical analysis ($p > 0.05$), it could be clearly included that there was poor correlation between the HER2/neu expression and hypermethylation. According to the table 2, we found that there was a significant association between the overexpression of mutant p53 and hypermethylation of $p16^{INK4\alpha}$ ($p = 0.03$). Especially, taken p53 and HER2/neu biomarkers together, the mean of hypermethylated $p16^{INK4\alpha}$ was significant associated with the HER2/neu(-)p53(-), in which 9 of 31 cases were methylated ($p = 0.04$). It was meant that the hypermethylation of $p16^{INK4\alpha}$ gene was the specific characteristic for those trouble negative breast cancer specimens.

According to table 3, the odd ratio (OR = 5.55), it indicated that $p16^{INK4\alpha}$ methylated was highly correlated with breast cancer risk at 95% CI (p = 0.01). It could be inferred that in this model, the odds for a positive hypermethylation of $p16^{INK4\alpha}$ promoter in breast cancer was 5.55 times higher than in the case of cancer without methylation. In addition, the RR was 3.30 (p = 0.03), meant that the hypermethylation of $p16^{INK4\alpha}$ was 3.30 times higher than unmethylation in breast cancer specimens. Therefore, it was tentatively inferred that the aberrant hypermethylation of $p16^{INK4\alpha}$ promoter have an important role in driving the breast tumorgenesis. Consequently, the hypermethylation of $p16^{INK4\alpha}$ gene's promoter could be useful biomarker applied in early detection and diagnosis of breast cancer in Vietnamese population.

TABLE III. THE RESULT OF RELATIVE RISK AND ODD RATIO CALCULATION

	$p16^{INK4\alpha}$		p
	M n (%)	U n (%)	
Breast cancer Healthy sample	47 (49.5) 3 (15.0)	48 (50.5) 17 (85.0)	< 0.01
Odd ratio (OR) Relative Risk (RR)	5.55 3.30		0.01 0.03

Simultaneously analyzed with hypermethylation of panel genes in our previous study [15][16][17][18], the frequencies of methylation was 82.1% ($p < 0.001$), 62.1% ($p < 0.001$), 43.2% ($p < 0.001$) and 42.1% ($p < 0.05$) for *BRCA1*, *Cyclin D2*, *GSTP1* and *RASSF1A*, respectively. According to the methylated coverage based on results of individual methylation of five genes above carried on cancer specimens, it showed that the coverage of diagnosis was 100% (with the methylation index (MI) ≥ 0.2). It suggested that, in the case of screening on total five genes above, the ability to detect the aberrant methylation in sample was very high. Regarding to the sample with low MI value, it was the useful phenomenon for the test of the methylation drug or hypermethylation inhibitor treatment leading apoptosis of cancer cells, which was recently mentioned, such as demethylation by 5-aza-2'-deoxycytidine (5-azadC) of $p16^{INK\alpha}$ gene in human cancer treatment [7].

v. Conclusion

In our study, we highlighted that the hypermethylation frequency of p $p16^{INK4\alpha}$ gene's promoter were 49.5% and 15.0% in breast cancer specimens and non-cancer specimens, respectively. The comparative analysis of methylation frequency of $p16^{INK4\alpha}$ in cancer and non-cancer samples suggested that the OR, RR were 5.55 and 3.30. In addition, methylation of $p16^{INK4\alpha}$ were significant associated with p53 and HER2/neu(-)p53(-). Therefore, it could be inferred that the role of abnormal methylation at CpG islands of promoter belonged to $p16^{INK4\alpha}$ was a significant characteristic of Vietnamese breast cancer specimens. Interestingly, co-analyzed with other tumor suppressor genes, including *BRCA1*, *DAPK*, *Cyclin D2* and *RASSF1A*, showing the significant coverage of diagnosis, which was calculated based on the result of methylated individual genes, reached to 100%. Therefore, those hypermethylation characteristics could be served as significant biomarker for breast cancer in Vietnamese population. For the future study, the various types of cancer specimens, such as serum, biopsy, etc. should be studied in order to have an overall vision about the methylation occurs in tumor suppressor genes in Vietnamese patients.

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