RESEARCH ARTICLE

Methylation Status and Immunohistochemistry of BRCA1 in Epithelial Ovarian Cancer

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Abstract

Background: Cancer initiation and progression are controlled by genetic and epigenetic events. One epigenetic process which is widely known is DNA methylation, a cause of gene silencing. If a gene is silenced the protein which it encodes will not expressed. Objectives: 1. Identify the methylation status of *BRCA1* in patients with epithelial ovarian cancer (EOC)and assess *BRCA1* protein expression in tumor tissue. 2. Examine whether BRCA1 gene methylation and BRCA1 protein are associated with survival of epithelial ovarian cancer patients. Methods: The study design was a prospective-cohort study, conducted at Sardjito hospital, Yogyakarta, Indonesia. Results: A total of 69 cases were analyzed in this study. The data showed that the methylation status of BRCA1 in EOC was positive in 89.9%, with clear protein expression of BRCA1 in 31.9%. Methylation status and expression of BRCA1 were not prognosticators of EOC patients. Menarche, CA125 level, clinical stage and residual tumor were independent factors for prognosis.

Keywords: EOC - methylation - *BRCA1* - prognosis factors - survival

Asian Pac J Cancer Prev, **15 (21)**, 9479-9485

Introduction

Cancer develops through a multistep process in which the genomes of the new cancer cells undergo mutations in some groups of the specific genes such as protooncogen, tumor suppressor genes and other genes that directly or indirectly control a cell proliferation. Cancer cells also have a genetic instability that allows cells to get other changes all the time. One of the characteristics of malignant tumors is its heterogeneity. Cancers are caused by the accumulation of mutations of several categories of genes, the initiation and the progression of cancer are controlled by the genetic and epigenetic events.

The most known epigenetic process is a DNA methylation. Methylation is adding four atoms on cytosine, one of four DNA nucleotides. This additional atom blocks the protein that transcribes genes. DNA methylation is an epigenetic mechanism that becomes very clear in the recent years that there is a synergy between the genetic and epigenetic changes.

The expression of BRCA1 protein is ubiquitous in humans, located in the nucleus, whereas the highest levels are obtained in the ovarian, testis and thymus. It is a tumor suppressor and the reduced expression is associated with the transformation procedures and the etiology of sporadic breast cancer and ovarian cancer. The reduction of expression is said to be regulated by the transcriptional implications of methylation of CpG nucleotide promoter. Esteller et al. research in the methylation of some suppressor genes in various types of cancer showed that the methylation of BRCA1 in sporadic ovarian cancer was 19% (11/58) (Esteller et al., 2001), in Vietnam found the methylation status of BRCA1 in women with ovarian cancer patients was 11/59 (18.6%) (Lan et al., 2013). The examination of the profile of BRCA1 methylation in ovarian cancer is very crucial to understand the molecular pathology of ovarian cancer, which in the end it will be very useful in the clinical management, due to methylation is a reversible procces.

As it was known that the use of azacitidine (AZA), an inhibitor of DNA methylation, has been approved by the FDA for the treatment of myelodysplastic syndrome (MDS), a precancerous condition of acute myeloid leukemia (Issa et al., 2005). Different combinations of genetic changes are found in genomes of more than 100 different types of human cancers. Thus, each cancer may be unique and the spectrum of genetic changes that initiates the incidence of cancer may have many variations. There is no single rule that underlies the occurrence of all cancer and cancer is a phenomenology of infinite complexity. Hahn and Weinberg made an alternative view that the pathogenesis of cancer in humans was governed

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by a number of genetic and biochemical rules applied in most types of human cancers (Hahn and Weinberg, 2002).

This study was conducted to determine the methylation status of BRCA1 gene in epithelial ovarian cancer and the expression of BRCA1 protein in the tumor whether the proportion of expression appropriate with the proportion of methylation. Does the proportion of methylation status of BRCA1 genes and the proportion of expression of BRCA1 protein influence survival of patients with epithelial ovarian cancer?

Materials and Methods

The study design was a prospective cohort, it was conducted at Sardjito Hospital, Yogyakarta, Indonesia in which patients with ovarian tumors when the results of frozen section showed malignant ovarian neoplasm, then some of the tumors were stored at -70 C, then the tissues were used as the sample of the study to examine the methylation status by *methylation specific PCR* (MSP). While the other tumor tissues were examined at pathology anatomy to determine the type of histopathology, the degree of differentiation and examined the expression of BRCA1 in the epithelial ovarian cancer.

BRCA1 *methylation status examination*

DNA tissue extraction was performed with *QIAamp*^{R} *DNA Mini Kit (Qiagen, Germany)* as appropriate with the manual kit. The conversian procedure of DNA bisulphite used protocol of *MethylEasy Xceed* Rapid DNA Bisulphite modification Kit. For PCR reaction, 1µl DNA which was converted with sodium bisulphide add 18µl PCR mix which contain 1×PCR buffer, optimum concentration Mg2+ for each primer, 0.4µl primer and 0.1µl Taq polymerase. The primer length which were used for BRCA1_M75 bp, BRCA1_U76 bp, with a nukleotid base sequence as follows: BRCA1_***MF***: 5'-TCG TGG TAA CGG AAA AGC GC-3', BRCA1_***MR***: 5'-AAA TCT CAA CGA ACT CAC GCC G-3', BRCA1_***UF***: 5'-TTG GTT TTT GTG GTA ATG GAA AAG TGT-3', BRCA1_***UR*** : 5'-AAA AAA TCT CAA CAA ACT CAC ACC A-3',

Immunohistochemistry (IHC) for BRCA1 *expression and scoring*.

Paraffin block section 3-5μm thick of ovarian tumor tissue on glass slide was examined for IHC. The IHC assay for BRCA1 expression used the MS110 clone monoclonal antibody (Biocare Medical, LLC, 4040, Pike Lane Concord, CA 94520, LA, USA) that reacts with N-terminal portion of the BRCA1 protein. The possitive BRCA1 expression of breast cancer tissue were used for positive control, and negative control used the same tissue and the staining without BRCA1 antibody. The percentage of staining was determined by an independent pathologist he was blinded to the identity and clinical outcome of the samples. The ascribes score was based on the number of cells with nuclear staining. The score was classified as Thrall et al. used: slides was score as 0 if there was no staining, 1 if there was scattered staining (<10%). 2 if 10-50% of the cells were stained, 3 if 50-90% of cells were stained and 4 if nearly all cells (>90%) were stained (Figure 2). Tumor were catagorised as having aberrant BRCA1 expression for very low to no staining (<10%); 0 or 1 score) and normal BRCA1 expression for >10% BRCA1 staining (2-4 score). (Thrall et al., 2006; Lesnock et al., 2013).

Results

The cases of ovarian tumors had been collected found 69 cases were malignant epithelial ovarian cancer, which were then used for the study of this manuscript. The patient's survival was followed up and the longest follow-up was 54 months and the shortest was 12 months. Several clinicopathological characteristic which were associated with risk factors of ovarian tumors such as patients age, menarche, parity, menopausal age, nutritional status (BMI), the CA125 level before operation, residual tumor during operation, histopathological type and grade of differentiation were recorded and analyzed. Those clinicopathological characteristic of the patients were clasified in two or three group. Table 1 showed the results of the methylation of BRCA1 genes in the tissue of EOC was 62/69 cases (89.9%) without methylation 7/69 cases (10.1%). IHC staining of BRCA1 normal (positive) was 22/69 cases (31.9%) and aberrant (negative) was 47/69 (68.1%). The agreement between methylation status of the BRCA1 gene and the expression of BRCA1 protein in the tumor was -0.019 its mean that there is no agreement between methylation and expression of BRCA1. This result can be proved either in the electrophoresis seen that although some cancer cells the BRCA1 gene was found methylated some cells found unmethylated either and these cells still express BRCA1 protein (Figure. 1). Distribution of methylation status and IHC BRCA1 staining correlated with clinicopathologial factors were not statistically significant different with p>0.05 (Table

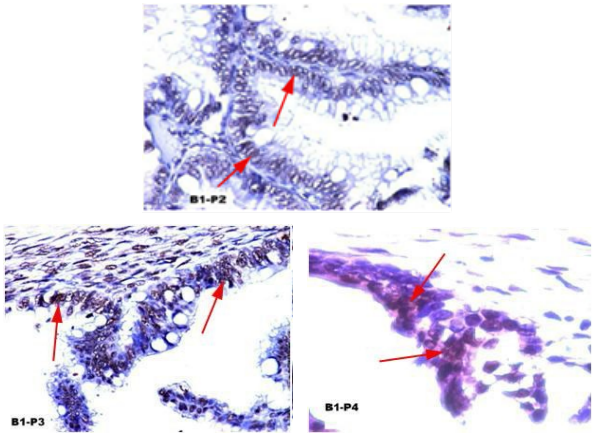

Figure 2. IHC Examination of BRCA1: (B1-P2) Show Positive 2 Staining, (B1-P3) Show Positive 3, (B1-P4) Show Positive 4

Table 1. Kappa Statistic of the Methylation Status of BRCA1 Gene and Expression of BRCA1 Protein

Methylation Status	I H C		p value	Kappa
	Aberrant<10%(-)	Normal≥10%(+)		
Methylated (+)	42 (60.9%)	20 (29.0%)	0.84	-0.019
Unmethylated (-)	5 (7.2%)	2 (2.9%)		
Total	47 (68.1%)	22 (31.9%)		

2). In the survival analysis by bivariable analysis found that menarche, CA125 level, stage of disease and residual tumor during operation were significantly influence the survival of the EOC patients with HR 2.55 (p=0.03), 4.42 (p=0.01), 3.77 (p=0.01), 2,4 (p=0.04) respectively, but in multivariable analysis there were no factors influence the survival of the patients, even methylation status of BRCA1 gene and expression of BRCA1 protein as well (Table 3).

From the results of the present research, its were found that the methylation levels of BRCA1 was 89.9% compared with the results of other studies elsewhere was high. Methylation of BRCA1 in sporadic ovarian cancer was 19% (11/58) (Esteller et al., 2001), 10% of 49 patients ovarian cancer (Rathi et al., 2001), 31% (Wang et al., 2004), Lan et al (2013) in Vietnamese women suffer from ovarian cancer was 18,6%. It was considered whether there was a possibility of technical error, for checking and proving the absence of errors in the examination of this research, it had carried out an internal validation with positive and negative controls using positive and negative controls on each of the electrophoresis examination (Figure 1). We also conducted an external validation in the way of re-examining randomly samples of the study. We also did the examination to some samples of other normal

Table 2. Characteristic of Epithelial Ovarian Cancer and Comparibility of the Methylation Status of *BRCA1* Gene and IHC Result of BRCA1 Protein

Variable		Methylation Status BRCA1			IHC BRCA1 protein		
		Methylated (%)	Unmethylated (%)	p value	Aberrant/- (%)	Normal/+ (%)	p value
Age of patients:	< 40 years	17 (24.6)	1 (1.4)	0.45	12 (17.4)	6 (8.7)	0.87
	≥ 40 years	45 (65.2)	6 (8.7)		35 (50.7)	16 (23.2)	
Age of menarche	< 15 years	42 (60.9)	5 (7.2)	0.84	32 (46.4)	15 (21.7)	0.99
	≥ 15 years	20 (29.0)	2 (2.9)		15 (21.7)	7 (10.1)	
Parity:	< 2	30 (43.5)	3 (4.3)	0.78	23 (33.3)	10 (14.5)	0.78
	≥ 2	32 (46.4)	4 (5.8)		24 (34.8)	12 (17.4)	
Menopauze:	Not menopauze	33 (47.8)	2 (2.9)	0.21	22 (31.9)	13 (18.8)	0.34
	Menopauze	29 (42.0)	5 (7.2)		25 (36.2)	9 (13.0)	
B M I:	< 25 kg/m^2	47 (68.1)	5 (7.2)	0.79	35 (50.7)	17 (24.6)	0.8
	≥ 25 kg/m^2	15 (21.7)	2 (2.9)		12 (17.4)	5 (7.2)	
CA 125 level	≤ 70 IU/ml	21 (30.4)	3 (4.3)	0.63	17 (24.6)	7 (10.1)	0.72
	> 70 IU/ml	41 (59.4)	4 (5.8)		30 (43.5)	15 (21.7)	
Clinical Stage:	Early Stage	20 (29.0)	3 (4.3)	0.57	18 (26.1)	5 (7.2)	0.2
	Late Stage	42 (60.9)	4 (5.8)		29 (42.0)	17 (24.6)	
Histophatological type:	Serous	18 (26.1)	1 (1.4)	0.5	10 (14.5)	9 (13.0)	0.23
	Musinous Ca.	30 (43.5)	5 (7.2)		26 (37.7)	9 (13.0)	
	Others	14 (20.3)	1 (1.4)		11 (15.8)	4 (5.8)	
Grade of Differentiation	Well differentiated	30 (43.5)	5 (7.2)	0.3	24 (34.8)	11 (15.9)	0.69
	Moderate	15 (21.7)	0 (0.0)		9 (13.0)	6 (8.7)	
	Poor differentiated	17 (24.6)	2 (2.9)		14 (20.3)	5 (7.2)	
Residual tumor:	Optimal operation	36 (52.2)	4 (5.8)	0.96	29 (42.0)	11 (15.9)	0.35
	Not optimal	26 (37.7)	3 (4.3)		18 (26.1)	11 (15.9)	

Table 3. Survival Analysis for Bivariable and Multivariable Analysis with Cox's Regression

Variable		Bivariable analysis		Multivariable analysis	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Age of patients:	< 40 years	Ref		Ref.	
	≥ 40 years	1.54 (0.52-4.59)	0.43	1.38 (0.35-5.33)	0.63
Menarche	< 15 years	Ref.		Ref.	
	≥ 15 years	2.55 (1.07-6.03)	0.03	2.64 (0.93-7.48)	0.06
Parity:	< 2	Ref.		Ref.	
	≥ 2	2.00 (0.80-4.96)	0.13	2.28 (0.91-7.79)	0.22
Menopauze:	Not menopauze	Ref.		Ref.	
	Menopauze	1.07 (0.45-2.53)	0.86	0.74 (0.26-2.15)	0.59
B M I:	< 25 kg/m^2	Ref.		Ref.	
	≥ 25 kg/m^2	0.67 (0.22-2.01)	0.48	0.41 (0.11-1.49)	0.17
CA 125	≤ 70 IU/ml	Ref.		Ref.	
	>70 IU/ml	4.42 (1.29-15.10)	0.01	3.01 (0.74-12.19)	0.12
Clinical Stage:	Early Stage	Ref.		Ref.	
	Late Stage	3.77 (1.11-128.6)	0.01	3.15 (0.58-17.15)	0.18
Histophatological type:	Serous	0.84 (0.25-2.78)	0.77	0.78 (0.20-2.98)	0.71
	Musinous Ca.	0.82 (0.30-2.27)	0.71	1.75 (0.35-7.99)	0.46
	Others (Clear cell+ Endometrioid)	Ref.		Ref.	
Grade of Differentiation	Well differentiated	Ref.		Ref.	
	Moderate	1.98 (0.66-5.92)	0.22	2.25 (0.52-9.63)	0.27
	Poor differentiated	2.29 (0.82-6.36)	0.11	3.83 (0.71-20.69)	0.11
Residual tumor:	Optimal operation	Ref.		Ref.	
	Not optimal	2.45 (1.02-5.89)	0.04	0.81 (0.24-2.73)	0.74
Methylation status BRCA1	Methylated	Ref.		Ref.	
	Unmethylated	1.02 (0.23-4.41)	0.97	2.11 (0.38-11.71)	0.39
IHC BRCA1 Protein	Aberrant	Ref.		Ref.	
	Normal	0.83 (0.32-2.15)	0.7	0.39 (0.11-1.36)	0.14

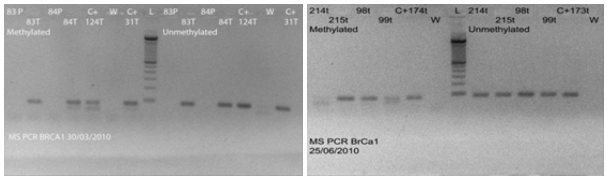

Figure 1. The Result Electrophoresis of BRCA1 Methylation of Some Sample of the Epithelial Ovarian Cancer

cells, namely leukocytes cells of the normal people and the result were same. The above validation checks proved that the possibility of technical errors in the examination could be ruled out or the results of the examination in the study were reliable. The results of this study might show characteristics of ovarian cancer patients in Indonesia, particularly in areas of southern Central Java.

As it was known that the process of carcinogenesis was caused by various factors as described in the literature. Likewise, promoter methylations of suppressor genes could be caused by various factors, namely, environment, nutrition, chemicals, infections, and so on. For Indonesians, these factors were different to those in Western countries or the other more developed countries. So, it would greatly affect the methylation status of the genes in these individuals in our population.

Discussion

The data showed that frequency methylation status of the patients were 89.9% and the expression of BRCA1 were 31.9%. If all of the tumor cells underwent methylations in the BRCA1 genes, certainly, the tumor cells would not express the BRCA1 protein but on the examination it obtained 22/69 cases (31.9%) that still showed the expression of BRCA1 protein and 47/69 case (68.1%) showed no expression of BRCA1. It meant that not all ovarian tumor cells in a patient who on the examination had positive methylation status had cells without methylation either. This was confirmed on the electrophoresis examination of any sample even it gained a band that showed the positive methylation genes but on the other hand there was also a band which showed the presence of the genes not methylated (Figure 1). there was almost no agreement between methylation status of BRCA1 genes and expression of BRCA1 protein in EOC of this study as the result of Kappa statistic=0.01 (Table 1). The methylation of BRCA1 promoter is important in silencing BRCA1 in sporadic EOC. The loss of expression of tumor suppressor genes such as BRCA1 is known occur through biallelic inactivation. The sugestion is that somatic mutation of BRCA1 is rare in sporadic EOC, thus, the loss of BRCA1 expression is considered to be due to a combination of allelic loss and methylation (Esteller et al., 2001). Other authors suggested that the silent or inactive BRCA1 gene on the tumor development were not only because of the epigenetic or genetic processes (gene mutation), but also because of a variety of other mechanisms that might occur together, as the role of micro RNA (miRNA) where miRNA was an RNA with a short nucleotide chain length less than 22 bases of nucleotide and would pause the mRNA translation process if the protein that was resulted from the translation of gene was no longer needed by binding to the mRNA so mRNA would be silent or undergo degradation. The protein expression of BRCA1 and others were regulated by various molecular devices within the cell. The setting was at the level of transcription, translation and post translation. In the result of the latest research, it was reported that the causes and the early incidence of EOC were governed by a group of genes including microRNA genes, which could be modulated at four levels, namely the level of genomic, transcriptional regulation, post-translation modification and regulatory processes of miRNA (Li et al., 2011). The position of miRNA genes had been mapped to be scattered in chromosome 1 to 22, and the X and Y sex chromosomes. Apparently, around 16% miRNA was regulated at the level of post-translational. Changes in the pair of A-T base became the pair of A-I base (inosine that acts on RNA editing) caused a conversion of miRNA biogenesis, the changes of miRNA product and the targeting changes of specific miRNA (Kawahara et al., 2009).

It had also been reported that lin28, a repressor let7 miRNA when a change of the base pairs occurred, caused a Dicer enzyme that did not function and the degradation of pre-miRNA; consequently, the target protein of mRNA had over-expression (Heo et al., 2008). This might explain why there was a difference of expression profile of BRCA1 and they had methylations, but the expression of the proteins in the tissue was high. As mentioned by Hilton et al. that the universal inactivation of BRCA1 had multiple mechanisms that were not only because of mutation and hypermethylation but there were still other factors or mechanisms (Hilton et al., 2002). These mechanisms could include: first, the mutations of BRCA1 promoter, so failing to identify the correct CpG island of BRCA1 promoter; second, the loss of the function of gene products required for the transcription of BRCA1, as well as no loss of heterozygosity (LOH) as the third cause (Hilton et al., 2002). Similarly, not all cancer cells on these examination showed the negative expression of BRCA1 although the results of the examination of the methylation of BRCA1 gene were positive.

There were no correlation among BRCA1 methylation and BRCA1 expression with several clinocopathological variables such as; age, menarche, parity, menopauze status, BMI, CA125 level, clinical stage of disease, histopathological type, grade and residual tumor during surgery as seen in the distribution of methylation status of BRCA1 gene and expression of BRCA1 protein in EOC tissue statistically were not different with Chi square test $p>0.05$ (Table 2). Lan et al (2013) found that association between methylation status of BRCA1, RASSF1A and ER genes with the clinical and pathological parameters of women suffering from ovarian cancer in Vietnam. Other studied find expression of BRCA1 protein in EOC was 40% and no association between BRCA1 expression with tumor grade, stage and overall survival of EOC patients (Shawky et al., 2014). As already were mentioned that methylation of promoter gene or aberrant methylation was influenced by several environmental agents. Aberrant DNA methylation currently is recognized as a common

molecular abnormality in cancer and its become potential molecular marker for diagnosis, prognosis and treatment (Laird, 2003). Previous studies regarding the association between BRCA1 gene methylation and patient survival remain controversial. Yang et al. and Montovan et al. indicated that BRCA1 methylation was not assosiated with patient survival (Yang et al., 2011; Montovan et al., 2012). Chiang et al. demonstrated survival disavantage in patients whose neoplasm were methylated at BRCA1 (Chiang et al., 2006). However, reported significant association between BRCA1 gene methylation and improved survival rate in patients with advanced stage (stage III-IV) (Bai et al., 2014). The inconsistency of these results may be due to the varying population and different environtment condition that were involved in the studies.

In the present study demonstrated that there was no significant association between methylation status of BRCA1 with survival of the patients. Even though, the data from this studied show methylation status of BRCA1 at EOC was high (89.1%) in this population. By acquiring a fact that the proportion of methylation BRCA1 gene was high, the population of patients with epithelial ovarian cancer in Indonesia especially in Central Java would be used as the basic rationale for the presence of a strong indication of the use of gene targeted therapy with inhibitors of DNA methylation such as DMT inhibitors (azacitidine) and HDAC inhibitors in the therapy of ovarian cancer in the future in Indonesia when the use of this therapy is already approved by authorized institutions for being used in ovarian cancer. As it was known that the use of azacitidine (AZA), an inhibitor of DNA methylation, has been approved by FDA since May 2004 for the therapy of myelodysplastic syndrome (MDS), a precancerous condition of acute myeloid leukemia. This was an example of drug pioneer in which the target was "epigenetic gene silencing", a mechanism that occurred in cancer cells to inhibit the expression of genes where the effect inhibited a malignancy phenotype (Issa et al., 2005). Because ovarian cancer was a highly heterogeneous cancer in which it has many types of the histopathology and malignancy with a progression ranging from slow to very fast and the patient could die quickly. Thus, using the molecular profiles of ovarian cancer to reduce the heterogeneity will be important for the patient selection in determining the therapy. It also happened in the MDS (Myelo Dysplastic Syndrome), a heterogeneous disease group in which the outcomes differed greatly depending on the profile of clinical pathology from chronic and slow to aggressive with a short survival. The results of this study got that the high frequency of BRCA1 methylation was a new fact for us in Indonesia. Hypermethylation in BRCA1 gene in the epithelial ovarian cancer had largely various levels of variation and it might likely be very different in each country because of differences in environmental factors, nutrition, chemical exposures and polution factors, as these greatly affected the occurrence of promoter methylation of suppressor genes and other genes.

Without the BRCA1 protein expressed in ovarian tumors it showed the presence of dysfunction of BRCA1 gene due to either genetic or epigenetic changes that could lead to the occurrence of the transformation of cells into cancer. BRCA1 was a tumor suppressor that reduced the expression associated with the transformation process and the etiology of breast cancer and sporadic ovarian cancers. Reduced expression of BRCA1 was quite possible related to the presence of gene methylation. The present study of the examination of BRCA1 gene methylation obtained as much as 89.9%. Thus, 68.1% of epithelial ovarian tumors without the BRCA1 protein expressed was very likely because of methylation while the remaining 31.9% expressed with the positive levels (≥10%) or normal. Therefore, it was very likely that the lack of BRCA1 protein expression was because of the presence of down regulation due to the methylation process.

It was said that ovarian cancers caused by the germline mutation had frequency of 10-15% while the rest were the sporadic very likely due to the role of lost BRCA1 gene because of the presence of methylation on the promoter to be inactive. Thrall et al (2006) found 84% of cases of EOC were still expressed positives, statistically there was a significant correlation ($p < 0.001$) between the expression of BRCA1 in the tumor tissue and the tumor stage, where the expression seemed in all stage I and stage II, completely negative in 16% of tumor in stage III, or 65% of tumors in stage III had minimal expression until completely negative (0-<10%) compared with 22% in stage I and 14% in stage II. So, overall there was a significant decrease in BRCA1 protein expression with increasing stages of epithelial ovarian cancer (Bast et al., 2000). The data studied found that early stage (stage I) compare to late stage (stage II - IV) no significance different between the two group p=0.20. Expression of BRCA1 protein in tumor cells seem to have advatages to the survival of EOC patients independently or adjusted even though it was not statistically significance, where with expression were positive HR=0.83 (p=0.70) and HR 0.39 (p=0.14) respectively.

The data of this studied also found that menarche, CA125 level, clinical stage, residual tumor during surgery were independently as prognostic factors for survival of EOC with p=0.03, p=0.01, p=0.01, p=0.04 respectively. However, after adjusted for other variables they were not significant as a prognostic factors ($p>0.05$). As far we researched no study mentioned menarche corellated with prognosis of EOC. Age of menarche usually corellated with risk of ovarian cancer pathogenesis. However, in this study menarche was independently as prognostic factor for survival. The levels of CA125 in this studied was classified into two groups with the cut off point of 70 U/ml due to the level of CA125 was still normal is <35U/ml. There are several conditions of non neoplastic conditions CA125 level are increase, nearly 6% of women without ovarian cancer had CA125 levels more than 35mlU/ml (Bast et al., 2000; Urban, 2003), then in this study of malignancy condition espescially EOC the level of CA125 twice of normal limit used as the cut of high level. The data showed that CA125 was as independent prognostic factor of the survival of EOC of all stage with HR 4.42 and p=0.01, after adjusted to other clinicopathological factors HR 3.01 (95% CI 0.74-12.18) and p=0.12. Other studied found that CA125 were as prognostic factor for EOC stage I (Nagele et al., 1995; Paramasivam et al.,

2005; Petri et al., 2006). A review of 15 studies showed that CA125 levels increased in 50% of patients with stage I of the disease, 90% in stage II, 92% in stage III, 94% in the stage IV of disease (Jacob and Bast, 1989). It was also reported a positive correlation between the increased serum levels of CA125 and the expression levels of CA125 in the epithelial ovarian cancer tissues. However, in the epithelial ovarian cancer of serous type the expression was significantly more positive than the other types of epithelial ovarian cancer. It was also found significantly shorter survival in the patients with ovarian cancer of stage III and IV without the expression of CA125 compared with the patient of ovarian cancer in stage III and IV with the expression of CA125 in the tumor (Hogdall et al., 2007). Clinical stage is the most important prognostic factor of the cancer. Relative five years survival of ovarian cancer in all stages was 53%, for stage III and IV were 31 % and for stage I and II were 95% (Landis et al., 1998). The present study found that clinical stage independently as prognostic factor of EOC in which the late stage (stage II-IV) had HR 3.79 (p=0.01) compare with early stage eventhough in the adjusted analysis had HR 3.15 (p=0.18). Residual tumor was demonstrated to be a prognostic factor to determine survival in patients with EOC stage IV (Bristow et al., 2002; Winter et al., 2008). Elstrand et al (2012) reported that among patients with EOC stage IV who underwent at least one surgical prosedure residual disease was an important prognostasicator for overall survival. While in this study found residual tumor was as prognostic factor independently for survival of EOC patients. Grade of differentiation mainly in the early stages of the disease is an important prognostic factor that affects treatment planning (Morgan et al., 2011 cited Hoffman et al., 2012), This study found that well differentiated, moderate differentiated and poor differentiated were clinically significant and likely to be a prognostic factor of EOC which the Hazard Ratio (HR) were 1.22 and 3.83 respectively even though statistically they were not significant. Then menarche, CA125 level, clinical stage, residual tumor during surgery were independently prognostic factors for survival of EOC, however, in the multivariable analysis showed to be insignificant as prognostic factors of survival, its seems that those results are due to the power of study being low.

Age of EOC patients most commonly occured in age ≥40 years 73.9% and 26.1% aged <40 years, others studied reported in Lahore India that the median age of EOC was 47 years old (Saeed and Akram, 2012) and in Sweden median age of ovarian cancer was 75 years old (Segelman et al., 2010). The studies on the prognostic implication of age in ovarian cancer are inconclusive. Although most reports have shown that younger women with ovarian cancer have an improved outcome compared to older women due to they have lower stage and well differentiation tumors (Rodriguez et al., 1994: Chan et al., 2006), others researchers have found that age was not an independent prognostic factor (Massi et al., 1996: Duska et al., 1999). However, population-based studied found that across all stages of EOC very young women (<30 years) had significant survival advantage over young (30-60 years) and older (>60 years) group with 5-years survival estimates at 78.8% *vs* 58.8% and 35.3% respectively (p<0.001), even after adjusted for race, stage, grade and surgical treatment the difference between the age group persist (Chan et al,. 2006). In this studied show that older women ≥ 40 years had lower survival than younger women <40 years, but statistically not significant with HR 1.54 (p=0.43) and after adjusted for other variables HR 0.93 (p=0.83). The present study show that parity and more than one child had less chances of survival compared to patients with no or one child as seen independently as well as adjusted analysis HR more than twice though statistically not significant, Other factor in this study were histopathological type, menopauze status apparently were not influential to the survival of EOC patients independently as well as on adjusted analysis.

In conclussions, methylation status and expression of BRCA1 were not to be prognosticator of EOC patients and they were not correlated to clinicopathological characteristics of the patients such as; age, menarche, parity, menopauze status, BMI, CA125 level, clinical stage, histopathological type, grade, residual tumor. The study found that menarche, CA125 level, clinical stage and residual tumor were independently as prognosticator of EOC patients, eventhough in the multivariable analysis statistically were not significant its due to the power of the study was low.

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