Expression of p53 Protein in Premenopausal Women as Risk Factors for Breast Cancer

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ABSTRACT

Mutation is known to occur before the development of various types of cancers. Mutation in p53 gene can be found in human breast cancer. The aim of the present study was to determine the correlation between p53 protein expressions with women

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in premenopausal age. A total of 111 breast cancer tissues were examined for p53 protein expression by IHC. The results showed that majority (36.9%; n=41/111) who were more than 41 yrs, overexpressed positive p53 (+) protein category and 2.7% (n=3/111) aged 41 yrs and less, showed less positive p53 (+) protein category. However, the Fishers exact test, indicated that, there was no significant correlation between participant’s age group with p53 protein category ($\chi^2 (1) = 0.78; p =0.52$) and no correlation strength was indicated (Cramer’s V coefficient = 0.08; $p =0.37$), respectively. The risk estimate showed probability of p53 (+) protein being overexpressed in the age group < 41 yrs was 0.66 times less likely compared to the age group > 41 yrs. In conclusion, with or without overexpression of p53 protein, women above 41 yrs were found to have greater risk.

Keywords: age, breast cancer, expression, p53 protein, premenopausal, women

INTRODUCTION

Normal breasts or mammary glands produce milk. In normal cell cycles, proliferation and regression are controlled by hormones and growth factors. Abnormal cellular changes progress from proliferative disease to atypical hyperplasia to carcinoma in situ to invasive breast cancer. While the majority of breast cancers are adenocarcinomas occurring in the upper outer quadrant, breast cancer is a complex and heterogeneous group of diseases with distinct morphologic and molecular features (Foxson et al. 2011; Cado et al. 2013; Watanabe et al. 2015). Over the past century there has been a dramatic increase in the pathological abnormality of the cases which is referred to as cancer. An American Cancer Society (2015) reported that cancer is best defined as diseases characterized by uncontrollable growth of abnormal cells which could spread to other parts of the body, and potentially be lethal.

In Malaysia, the incidence with regard to age suggested that the breast cancer increased steadily from the age group of 30 yrs and the peak was from 40 to 59 yrs. In contrast, in Western countries a high proportion of patients presented with an advanced-stage of cancer. A Malaysian women’s cumulative risk of breast cancer in her lifetime was in the ratio of 1:20 with the highest risk in Chinese (1:14) (National Cancer Registry Malaysia 2006; Hisham & Yip 2004). Breast cancer could be caused by a diversified modifiable and non-modifiable risk factors which include gender, age, history of benign breast or related diseases and reproductive factors such as menstrual history, first pregnancy’s age, lifestyle, hormonal, and genetic factors.

Genetic factors in the pathogenesis of breast cancer can be ascribed to be inherited susceptibility. Three genes (p53, BRCA1, and BRCA2) were identified, which may alter the formation of tumour suppressor genes. In breast cancer p53 mutation/alteration are reported in 50% of primary carcinomas (Callahan 1992; Muller & Vousden 2013; Bertheau et al. 2013).
The p53 is a tumour suppressor gene which responds to the DNA damage that induces the pathways for cell apoptosis, cell cycle arrest, and DNA repair. The function is to maintain the genetic integrity of the cells resulting p53, as one of the most studied proteins in oncology (Steele & Lane 2005). Previous study by Thompson, (2014) reported that the abnormalities observed in the p53 gene are common and could be the key events in the pathogenesis of breast cancer.

The Clinical Practice Guideline (CPG), Ministry of Health Malaysia (2003), emphasizes the management of breast cancer through reinforcing those women whom genetically related to higher propensity of deleterious mutations in BRCA1, BRCA2, and TP53 genes. They are advised for genetic counselling by geneticist; and genetic testing prior to their early detection. Currently, there is limited scientific evidence which proposes the evaluation of other genes or specific loci in common clinical setting of the Ministry of Health for genetic testing (MOH & AMM 2003). In diagnosing p53 expressions and relationship with breast cancer occurrence, an IHC analyses must be a warranted design by utilizing a statistical analysis. However, an IHC analysis cannot be ruled out as a useful indicator of prognosis in clinical applications of breast cancer (Bartley & Ross 2002; Rolland et al. 2007). Detection of p53 protein by IHC has been widely used as a surrogate marker for p53 mutations. Even though, the lacking of p53-binding domain was noted, the physiological activities were dependent on p53 in which, the binding to wild-type MDM2 was responsible for cancer pathophysiology (Inoue et al. 2016). Secondary cancer prevention is a set of intervention that uses screening tests or examinations which leads to the discovery, control of cancerous and precancerous processes. A study by Loud et al. (2002) stated that pathophysiological alteration and possible counteraction mechanism of breast cancer should be first well understood.

In the present study, the main aim was to determine the specific patterns in breast cancer (BC) risk factors by p53 status and identifying the way forward, in screening for early detection. A study by Maas et al. (2016) stated that 30 yrs women in the United States may develop invasive breast cancer at the rate of 11.3% by the age of 80 yrs. In addition, the proposed model which accounted for the risk factors depicted 4.4% - 23.5% of the average absolute risk in bottom and top deciles of the risk distribution in women. Apparently, women who fell in the lowest and highest deciles of non-modifiable risk factors accounted for 5th and 9th percentile range of the risk distribution related to modifiable factors and were recorded as 2.9% - 5.0% and 15.5% - 25.0%, respectively. Consequently, women who were at the highest decile of risk pertaining to non-modifiable risk factors, low BMI, non-alcoholic and non-smoker, and never used MHT had higher risks comparable to general population of average woman. According to Maas et al. (2016) that highlighted traditionally, the etiology and pathophysiology of breast cancer were similarly reported globally. However,
the differences in genetic materials of Asian women could possibly triggers the difference interaction mechanism with environmental exposure. This may include reproductive risk factors and dietary factors that may be involved in alteration of the susceptibility towards beneficial or deleterious effects of these exposures. Hence, it is suggested that the relevant contributing risk factors may contradict between Western and Asian populations. Biomarker data would enable in strengthening the link between risk factors and breast cancer as shown in epidemiological studies. Subdividing breast cancer cases by p53 protein expression and searching for important patterns in risk factors of breast cancer by p53 status could help to narrow the search for specific p53 mutations. Therefore, this provides the impetus for the present study to investigate the prevalence of breast cancer within epidemiological risk factor and association with p53 protein expressions in breast cancer tissue specimens in premenopausal women.

MATERIALS AND METHODS

DESIGN
Demographic and risk factor profiles data were taken from breast cancer tissue registries and paraffin embedded breast tissue samples were then detected for p53 protein expression using immunohistochemistry technique. The results of p53 protein were then compared with risk factor profiles available from patient’s registries.

BREAST CANCER TISSUE SAMPLING
A total of 111 breast carcinoma tissue samples in paraffin blocks were obtained from female patients who were diagnosed between June 2005 and April 2009. The age of the patients ranged from 27 to 82 yrs (mean age of 54 yrs). These paraffin embedded breast tissue samples were randomly picked from a listed serial number of paraffin blocks available from the computed registry of the Pathology Laboratory, Hospital Tuanku Jaafar, Seremban, Negeri Sembilan. A retrospective review of the study population was available from the patients clinical histopathology report (Form Perub. J.P. 06) which included patient’s age and tumour grade status and several other risk factor profiles.

DATA COLLECTION AND INSTRUMENTS
Demographic data and risk factors profile were taken from the patients’ histopathology laboratory reports, mammography and surgical outpatient follow-up case reports. Based on patients address they were residents of Negeri Sembilan and being referred to Hospital Tuanku Jaafar, Seremban. Patient’s age was defined as the age of cancer diagnosis.

ETHICAL APPROVAL
Ethical approval was obtained from Research Ethics Committee of both Universiti Teknologi MARA (UiTM) and also MOH Research & Ethics
Committee (MREC) of the Ministry of Health (MOH) (NMRR 8-16-1771).

**DATA ANALYSIS TECHNIQUE**  
*Immunohistochemical (IHC) Staining*

Paraffin embedded tissue sections, 5 μm thick, were mounted on silanized slides and fixed for 12 mins. The paraffin embedded slides were processed for IHC staining and evaluated for expression of p53. The histological type of breast cancer selected randomly included invasive and non-invasive carcinoma and other benign breast diseases. Grading of breast tumour was determined using modified Bloom and Richardson (1957) criteria as stated in the histopathology report.

IHC was performed using the DakoCytomation kit (EnVision + System-HRP; DakoCytomation Denmark A/S, Glostrup, Denmark). Paraffin-embedded tissue sections from all samples were stained with p53, accordingly. Tissue sections were de-paraffinized and hydrated prior to antigen retrieval by microwave, followed by inhibiting the endogenous peroxidase activity by 5 mins incubation and rinsed with distilled water. Following these steps, slides were treated for 10 mins with primary antibodies clone DO-7 (N158187, Dako) and optimum dilutions were determined according to experiments. Slides were then rinsed with Tris buffered saline solution (TBS) with 0.05% plus Tween 20 (S196630, Dako) twice for 3 mins each time. Sections were incubated with REAL™ Envision Polymer (K4065, Dako) as peroxidase labelled polymer to the primary antibody for 10 mins each time.

Antigen was visualized with DAB (3, 3’ diaminobezidine) chromogen (K4065, Dako) plus diaminobezidine substrate buffer (K4065, Dako) preparation and counter-stained with haematoxylin staining. The incubation protocols were conducted at room temperature and the tissue sections were carefully washed within the incubations series. In every experiment, in-house positive p53 tissue sections with one positive control and one negative control were included.

**Semiquantitation of p53**

For overexpression of p53 protein in samples, the staining intensity on tissues is scaled between 0 to +3 scores. Relatively, tissue section which did not exhibit brownish nuclear reactivity in tumour cells was recorded as 0. Tissue section which occasionally showed positive nuclear staining was scored as +1, provided the total percentage of positivity in tissue section was < 20%. Any tissue section which tumour cells exhibited positive nuclear staining in between 20% - 80% was given score of +2. For the +3 score, the scale was given to tissue section which depicted positive nuclear staining in > 80% of tumour cells, and the scales of +1, +2, and +3 were recorded as p53+.

Microscopic examination observed using 10X and 40X magnification lens field. IHC was confirmed positive if the reaction was visualized by Dako REALTM DAB+ (3, 3’-diaminobenzidine tetrahydrochloride in organic solvent) substrate-chromogen solution which results in a brown-colored precipitate at the antigen site of p53 protein (Figure 1).
RESULTS

The diagnostic age groups among breast cancers was elaborated in Table 1. The age at diagnosis ranged between 27 to 85 yrs. The peak age of diagnosis was between 45 and 55 yrs, with the mean age of 54 yrs (S.D 12.91) while the mode age was 46 yrs in the premenopausal age group. In Table 1, revealed that the majority of p53 (+) protein expression was found in the age groups above 41 yrs. The results indicated that majority (n=41/111) who were more than 41 yrs, had a positive p53 (+) protein category. In addition, minority (n=3/111) of the respondents were aged 41 yrs and less and also had positive p53 (+) protein category (n= 3/111). However, the Fishers exact test, indicated that, there was no statistically significant correlation between age category and p53 protein category ($\chi^2 (1) = 0.78$, p =0.52), and there was no correlation strength (Cramer's V coefficient = 0.08) and also not significant (p =0.37) (Table 1).

To strengthen the correlation findings of the p53 protein expression and the unmodifiable age as risk factor ,the estimation study was conducted. Table 2 showed the result of risk estimate of p53 protein and age group. The results reported that the risk factor was reduced by 0.66 (or 66.5%) for women aged $\leq$ 41 yrs in expressing p53 (+) protein, Risk estimate = 0.66, 95% confidence interval (CI): 0.24-1.79), compared to the women aged...
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> 41 yrs. Therefore, age group did not have any significants (p>0.05) in overexpressing p53 (+) protein. The risk estimate showed probability of p53 (+) protein being overexpressed in the age group less than 41 yrs was 0.66 times less likely compared to the age group above 41 yrs.

**DISCUSSION**

The risks pattern of breast cancer among women could be identified by the overexpression of p53 in breast cancer tissue samples which showed the presence of mutant p53. The findings indicated that this protein would not be degraded or metabolized in the tissues and continued to exert its original function. The present study reported inhibition of the apoptosis induction that further triggered the uncontrollable growth of tissues that resulted in tumour formation. Hence, becoming aggressively malignant and invasive. The highest percentage of expressing p53 (+) in breast cancer tissue was found in the age group > 41 yrs. However, the percentage of p53 (+) protein overexpression increased with the age group of this study. The majority of the respondents (36.9%) of p53 (+) overexpression were observed in the breast cancer tissue of respondents within the age group of > 41 yrs, while only (2.7%) of p53 (+) overexpression was observed within the age group < 41 yrs.

Our study reported that a total of 111 participants diagnosed with breast cancer between June 2005 and April 2009, with age groups within the range of 27 to 85 yrs and mean age of 54 yrs upon diagnosed as breast cancer. Their median age was 52 yrs and mode age of 46 yrs. Congruently supported with several studies (Lim et al. 2008; Musa et al. 2011). Another study Lim et al.

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Table 1: Participants’ age at diagnosis with p53 protein

<table>
<thead>
<tr>
<th>Age of diagnosis N=111</th>
<th>p53 (-) n (%)</th>
<th>p53 (+) n (%)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 41 yrs (n=11)</td>
<td>8 (7.2)</td>
<td>3 (2.7)</td>
<td>9.9</td>
</tr>
<tr>
<td>More than 41 yrs (n=100)</td>
<td>59 (53.2)</td>
<td>41 (36.9)</td>
<td>90.1</td>
</tr>
<tr>
<td></td>
<td>67 (60.4)</td>
<td>44 (39.6)</td>
<td>100 %</td>
</tr>
</tbody>
</table>

Summary Results: Fisher’s Exact test, $\chi^2(1) = 0.78$, $p = 0.52$, Cramer’s V coefficient = .084, $p = 0.37$. ($\chi^2(1) = 0.78$, $p = 0.52$), $\chi^2 1, n=111= 0.31$, $p = 0.57$, $\phi = 0.08$.

Table 2: Risk estimate between participant’s age and p53 protein expression

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>p-value</th>
<th>Risk estimate (95% C.I)</th>
<th>Odd Ratio (95% C.I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;41 yrs / &gt;41 yrs</td>
<td>0.52</td>
<td>1.85(0.46-7.40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53(-)</td>
<td></td>
<td>1.23(0.82-1.83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53(+)</td>
<td></td>
<td>0.66(0.24-1.79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of Valid Cases</td>
<td>111</td>
<td></td>
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</tr>
</tbody>
</table>
had similar results to the present study in which the median age at upon diagnosis was 52-53 yrs and age standardized rate (ASR) was between the age group 50-59 yrs.

The participant’s mode age with breast cancer was within 46 yrs. According to Hisham & Yip (2004) and Toh et al. (2008) similar findings were found that nearly 50 % of breast cancer cases occurred in women under the age of 50 yrs. Furthermore, Hisham and Yip (2004) showed that predominant age group between 40-49 yrs and a high proportion were in the advanced stage of the disease. In addition, Leong et al. (2010) reported a median age of 52 yrs for breast cancer occurrence showed a salient difference in peak age between 40-50 yrs in Asian countries and between 60-70 yrs in Western countries. However, a study by Rohan et al. (2006) stated that participant’s age may be a prognostic factor in determining the probability of increasing metastatic potential in breast cancer and it is supported with overexpression of p53 (+) protein that could be reflected to a more advanced stage of progression of the breast cancer.

Significantly, this study may suggest that p53 (+) overexpression could be an indicator for the likelihood of occult alterations existence in breast cancer tissues with non-expressing p53 (-) protein in a younger age group, whereby this predictor may provide a valuable indication for earlier detection of breast cancer occurrence. Earlier studies by Bertheau et al. (2008) and Agrawal et al. (2009) showed results ranging from 14-58% of p53 (+) protein overexpression in breast cancer tissues. The findings from this present study reported that overexpression of p53 (+) protein was found in 40% (44/111) of the overall breast cancer cases. Notwithstanding, there were strong similarities between the current study and earlier scientific works in terms of the overexpression of p53 signified in approximately 14-58 % of diagnosed breast cancer cases.

Adjustment of age in the present study would enable to reduce the risk factors of age group < 41 yrs to express p53 (+) protein. The risk estimation showed probability of p53 (+) protein being overexpressed in the age group less than 41 yrs was 0.66 times less likely compared to age group above 41 yrs. The participants’ age groups were related to a non-statistically significant increase in the risk of p53 protein. The increased odds (OR 1.85) of p53 (+) protein expression with age groups did not reach statistical significant as indicated, with (p > 0.05). The present study concurred with Erdem et al. (2005) who failed to demonstrate any association between p53 protein expression and age. However, this present study, indicated the correlation p53 protein and age groups did not reach a statistical significance (p > 0.05), the frequency rate, of p53 (+) protein expression among women > 41 yrs compared to < 41 yrs, was 36.9% (41/111) and 2.7% (3/111), respectively.

The finding from the present study showed that participant’s age groups can be a predictor of risk factors for breast cancer among older women. Congruently supported by previous studies Worsham et al. (2007), Etebary et al. (2002), and Dupont and Page (1989).
in P53 gene, or overexpression of p53 protein as a secondary marker in the mutation pathways would likely be considered as a potential prognostic factor or biomarker for numerous human malignant tumours, particularly breast cancer in which has been recognized with worse prognosis (Bergh et al. 1995; Kikuchi et al. 2013).

Limitation of this study could be attributed with the suboptimal methods in determination of p53 status due to the small sample size which did not allow more reliable assessment to associate with participant’s age factor and p53 protein. However, the analysis of p53 protein expression was carried out in the absence of knowledge regarding case-control status which unlikely contributed to the differential bias in the current evaluation of p53.

**CONCLUSION**

In conclusion with or without overexpression of p53 protein women above 41 yrs were at a greater risk diagnosed with breast cancer. The investigation of p53 (+) overexpression in relation to participant’s age group in the present study revealed that there was fluctuation in the frequency of the detection rate of p53 protein. This limitation could be contributed by the low statistical power of the clinical data with reference to the potential predictive value of the p53 protein which are still conflicting.

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