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Reduced expression of *AURKA* in peripheral blood of breast cancer patients

LUCKY POH WAH GOH[1], EDWIN UN HEAN SEE[2], KEK HENG CHUA[3], PING-CHIN LEE[1]*

[1] Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia
[2] Department of Surgery, Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia
[3] Department of Biomedical Science, University of Malaya, Kuala Lumpur, Malaysia

Abstract

AURKA, *CENPW*, and *TIMP1* are reported to be genes involved in the regulation of the cell cycle and are highly associated with various cancers, including breast cancer (BC). These genes appear to be overexpressed in cancerous tissues, but unknown in peripheral blood. This study uses quantitative real-time PCR (qPCR) to assess the differential expression of *AURKA*, *CENPW*, and *TIMP1* genes in the peripheral blood of BC patients and correlates expression levels with clinicopathological characteristics and etiological factors. We found that *AURKA* expression was significantly reduced in BC patients, as well as: 1) those without any family cancer history, 2) nonsmokers, and 3) those who engage in frequent consumption of red meat in the peripheral blood. However, all three genes in this study were not significantly correlated with clinicopathological characteristics of BC. Our study reveals that the expression of *AURKA* is significantly downregulated in the peripheral blood of BC patients.

Key words: Aurora kinase A, breast neoplasm, gene expression, peripheral blood

Introduction

Breast cancer (BC) has the highest incidence and mortality among all cancer types in women globally (Siegel et al., 2013). Previous studies have reported the use of biopsy-based gene expression to determine BC tumorgenesis, prognosis, and treatment (Lu et al., 2011; Mihály and Győrffy, 2013; Song et al., 2015). BC is, however, highly heterogeneous because it is composed of different cell types, which often results in a misclassification of the BC subtypes and the consequent mistreatment of BC(Kim et al., 2005). Peripheral blood that shares more than 80% of the transcriptome with other tissues and elicits specific changes in the whole blood environment, represents an interesting non-invasive sample for the study of gene expression in various diseases (van der Velden et al., 2004). Moreover, peripheral blood was reported to contain circulating tumor cells and DNA that indicated early and late stages of cancer malignancies (Bettegowda et al., 2014; Sarioglu et al., 2015). Analyses of gene expression in blood have already been used in the diagnosis of various cancer types such as colorectal cancer (Xu et al., 2014), lung cancer (Zander et al., 2011), and BC (Aaroe et al., 2010).

A cell cycle consists mainly of two consecutive processes, a DNA replication and a segregation of replicated chromosomes into two separate cells. These processes are tightly regulated to ensure a correct cell division process. In cancer, genetic mutations in cell cycle-associated genes cause uncontrolled cell proliferation, which leads to the rise of cancer. Aurora kinase A (*AURKA*) is a gene that encodes a protein involved in the centrosome formation during the chromosomal segregation of cell division, and is widely known to be implicated in carcinogenesis. The overexpression of *AURKA* in cells confers the ability to inhibit apoptosis in human cancer cells and has been associated with BC tumor develop-

* Corresponding author: Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia;
e-mail: leepc@ums.edu.my

ment (Rouquier et al., 2014; Huang et al., 2008). These findings indicate the importance of *AURKA* in carcinogenesis.

Centromere protein W (*CENPW*) was originally identified as a cancer-up-regulated gene 2 (*CUG2*) that caused *in vitro* and *in vivo* transformations of the normal NIH3T3 mouse fibroblast cell line into highly tumorigenic cells (Lee et al., 2007). An overexpression of *CENPW* has been associated with aneuploidy, BC, and colon cancer (Yuen et al., 2005; Lee et al., 2007; Lau et al., 2014). A subsequent functional analysis revealed that *CENPW* encodes a kinetochore protein required for the segregation of chromosomes during cell division (Kim et al., 2009). These studies showed the importance of the *CENPW* expression in the tumorgenesis process.

The expression of another gene, the tissue inhibitor of metalloproteinase 1 (*TIMP1*), was reported to be significantly elevated in colon cancer. *TIMP1* is known as an inhibitor of *MMP*s (metalloproteinases), which inhibit the proteolytic activity of cleaving the components of the extracellular matrix (Folgueras et al., 2004). *TIMP1* also regulates cell growth (Brew et al., 2000; Taube et al., 2006) and demonstrates anti-apoptosis activities (Liu et al., 2003) in BC. The overexpression of *TIMP1* was reported to confer resistance toward chemotherapy treatment; this finding highlighted the importance of this gene in cell cycle regulation (Zhu et al., 2012). *AURKA*, *CENPW*, and *TIMP1* are cell cycle-associated genes that play important roles in BC development, progression, and metastasis. A limited report on the expression of these genes in BC has drawn us to investigate the expression of these genes in BC.

The peripheral blood gene expression profiling of BC was previously reported using a limited set of genes (Aaroe et al., 2010). The up-regulation of genes that were involved in the translation, response, and defense mechanisms was reported (Aaroe et al., 2010). However, the study did not report any involvement of the cell cycle-associated genes such as *AURKA*, *CENPW*, and *TIMP1*. Hence, we report here the differential expressions of *AURKA*, *CENPW*, and *TIMP1*, the cell cycle-associated genes in the peripheral blood of BC patients as compared to those of healthy volunteers. We also include the clinicopathological characteristics and other etiological factors such as age, family cancer history, smoking, and dietary habits.

Materials and methods

Subjects and sample collection

In all, 14 healthy females (mean age ± SD = 66.1 ± 22.0 years) were randomly selected from blood donation campaigns as healthy controls, together with 14 females, clinically diagnosed BC patients (mean age ± SD = 57.5 ± 11.0 years), who were admitted to the Queen Elizabeth Hospital, Kota Kinabalu. They were recruited for this study in 2014–2015. Blood samples were collected from all of the subjects in Tempus™ Blood RNA Tubes (Applied Biosystems, USA) with the consent of the patients. The sampling for the BC patients was performed prior to any clinical treatments such as chemotherapy, radiotherapy, or major resections of tumors. Several etiologies (i.e., age, family cancer history, smoking status, fast-food consumption, and red meat intake) were recorded for all of the tested subjects.

The clinicopathological characteristics of breast tumors from each BC patient were recorded according to The American Joint Committee on Cancer (AJCC). The AJCC TNM cancer stage grouping incorporates the tumor size and spread (T), the spread to lymph nodes (N), and the metastasis to distant organs (M). TNM staging describes the least advanced stage (Stage I) to the most advanced stage (Stage IV) of BC (Edge et al., 2010). The clinical data of three patients were unavailable because of the misfiling of case notes; consequently, these patients were excluded from the statistical analysis of the clinicopathological characteristics. This study was approved by the university's ethical committee with Ref. JKEtika 1/15(8).

Total RNA isolation and quality determination

Total RNA was isolated using Tempus™ Spin RNA Isolation Kit with the DNase treatment (Applied Biosystems, USA) according to the manufacturer's recommendations. The purity (A260/A280) and the RNA integrity number (RIN) of the total RNA were assessed using Nanophotometer® (Implen, Germany) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The purity RNA (A260/A280) ranged from 1.89 to 2.55 for the control group and 1.63 to 2.39 for the BC samples, respectively. The RINs ranged from 7.60 to 9.20 and 7.90 to 9.40 for the control and the BC samples, respectively.

cDNA synthesis and quantitative real-time PCR (qPCR)

cDNA was synthesized using 1 μg of total RNA with high-capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's protocol. qPCR was performed on the StepOnePlus™ Real-Time PCR (Applied Biosystems, USA) instrument using a final volume of 20 μl containing 10 ng of cDNA, 1 × TaqMan® Fast Advance Master Mix (Applied Biosystems, USA), and 1 × custom/pre-designed TaqMan® gene expression assays (Applied Biosystems, USA). The primers for the target genes of the TaqMan® gene expression assays were as follows: *AURKA* (forward primer: 5′-CTGCCCC CTGAAATGATT-3′; reverse primer: 5′-GGCTCCAGAGA TCCACCTTCT-3′; TaqMan® Probe: 5′-AAGGTCGGA TGCATGAT-3′), *CENPW* (Lau et al., 2014), and *TIMP1* (Lau et al., 2014); the reference genes were actin beta (*ACTB*) (Assay ID: Hs01060665_g1), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Assay ID: Hs0275 8991_g1), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (Assay ID: Hs02800695_m1). GeNorm (Vandesompele et al., 2002) and NormFinder (Anderson et al., 2004) were used to determine the two most stable reference genes among *ACTB*, *GAPDH*, and *HPRT1*. The PCR conditions were as follows: denaturation at 95 °C for 20 s, followed by 40 cycles of 1) denaturation at 95 °C for 1 s, and 2) annealing and extension at 60 °C for 20 s. The qPCR fluorescence signals were captured at the end of the annealing and extension step. The efficiency of each TaqMan® gene expression assay was experimentally determined to be 90–110%.

Gene expression level analysis

The relative gene expression levels of *AURKA*, *CENPW*, and *TIMP1* were normalized by the two most stable reference genes (*GAPDH* and *HPRT1*). By using healthy controls as the reference sample in the overall gene expression levels, we calculated the relative fold change ($2^{-\Delta\Delta Cq}$) values by using the following formulas (Livak et al., 2001):

$$\text{Cq (Target gene)} - \text{Cq (Reference genes)} = \Delta\text{Cq}$$
$$\Delta\text{Cq (Sample)} - \Delta\text{Cq (Reference sample)} = \Delta\Delta\text{Cq}$$
$$\text{Relative fold change} = 2^{-\Delta\Delta Cq}$$

The statistical analysis of the Mann – Whitney *U*-tests was performed with the SPSS software v17.0 (IBM SPSS, USA) to determine the significant difference in the gene expression between BC patients and healthy controls. A $P < 0.05$ was considered statistically significant. Bonferroni's correction of the significant level was applied in the multiple testing of the etiologies (Dunn, 1961).

Results and discussion

BC has been associated with the infiltration of the neutrophils and macrophages of peripheral blood (Laoui et al., 2011; Fridlender et al., 2012), suggesting that the study of the peripheral blood gene expression in BC patients may reflect the clinicopathological characterization of the tumor and disease progression. In this study, we used the qPCR technology to assess the expression levels of cell cycle–associated genes such as *AURKA*, *CENPW*, and *TIMP1* in the peripheral blood of patients with BC and compared the results with the blood analysis of healthy volunteers.

Table 1. Changes in the *AURKA*, *CENPW*, and *TIMP1* expression levels in the BC group as compared to the healthy control as the reference sample; fold change ($2^{-\Delta\Delta Cq}$) and *P*-values are given

Gene	Fold change ($2^{-\Delta\Delta Cq}$)	*P*-value
AURKA	0.66	**0.017**[a]
CENPW	0.81	0.874
TIMP1	0.86	0.164

[a]Significant at $P < 0.05$ when compared to the healthy control

AURKA is highly expressed at the end of the S phase and accumulates until the G1 phase or the late mitosis of the cell cycle regulation (Giet et al., 1999). Our results revealed that the overall expression of *AURKA* ($P = 0.017$) was significantly down-regulated by 0.66-fold in BC patients as compared to the healthy controls (Table 1). The reduced expression of *AURKA* was previously reported to be associated with the progression of BC from *in situ* breast carcinoma to ductal invasive breast carcinoma (Hogue et al., 2003). Our findings further confirmed the association of the reduced expression of *AURKA* with the invasiveness of BC. Besides, *AURKA* phosphorylation activated the microtubule-associated oncogene known as the targeting protein for xenopus kinesin-like protein 2 (*TPX2*), which contributes to the migration and invasion of cancer cells through the Akt and focal adhesion kinase signaling

Table 2. The relative fold change ($2^{-\Delta\Delta Cq}$) and *P*-value of the *AURKA*, *CENPW*, and *TIMP1* genes of BC patients as compared to their respective reference sample with clinicopathological characteristics

Clinicopathological characteristics	Overall percentage of BC patients % (*N*)	*AURKA* (fold change)	*P*-value	*CENPW* (fold change)	*P*-value	*TIMP1* (fold change)	*P*-value
Lesion site							
Left	63.6 (7)	1.00	Ref	1.00	Ref	1.00	Ref
Right	36.4 (4)	0.54	0.315	1.35	0.164	1.09	0.412
Tumor diameter (cm)							
Small (≤ 5)	54.5 (6)	1.00	Ref	1.00	Ref	1.00	Ref
Big (> 5)	45.5 (5)	0.74	0.792	1.29	0.177	0.89	0.429
Differentiation							
Poor	54.5 (6)	1.00	Ref	1.00	Ref	1.00	Ref
Moderate	45.5 (5)	1.66	0.177	1.15	0.537	0.80	0.126
TNM stage[a]							
Early (1 & 2)	36.4 (4)	1.00	Ref	1.00	Ref	1.00	Ref
Late (3 & 4)	63.7 (7)	0.95	0.788	1.06	0.788	1.01	0.927
Depth of invasion[a]							
T1	18.2 (2)	1.00	Ref	1.00	Ref	1.00	Ref
T2	36.4 (4)	0.98	1.000	1.14	0.800	1.37	0.267
T3	27.3 (4)	1.17	0.400	1.41	0.400	1.31	0.200
T4	18.2 (2)	0.36	0.333	1.40	0.667	1.35	0.333
Lymph node metastasis[a]							
N0	36.4 (4)	1.00	Ref	1.00	Ref	1.00	Ref
N1	27.3 (3)	0.92	0.857	1.21	0.400	1.01	0.857
N2	9.1 (1)	1.74	1.000	1.02	1.000	0.75	0.800
N3	27.3 (3)	1.92	0.400	0.94	0.857	1.09	0.857
Distant metastasis[a]							
M0	54.5 (6)	1.00	Ref	1.00	Ref	1.00	Ref
M1	27.3 (3)	1.19	0.381	1.32	0.167	1.02	0.905
M2	18.2 (2)	0.40	0.286	1.76	0.071	1.12	0.429
HER2 status							
Negative	9.1 (1)	1.00	Ref	1.00	Ref	1.00	Ref
1+	27.3 (3)	1.73	0.500	1.01	1.000	0.88	1.000
2++	27.3 (3)	2.14	0.500	1.22	0.500	0.80	1.000
3+++	36.4 (4)	1.30	0.800	1.46	0.400	0.95	1.000

[a]According to American Joint Committee on Cancer (2010); Ref: Reference sample

pathway (Yang et al., 2015; Wu et al., 2016). As shown in this research, the reduced expression of *AURKA* was detectable in the peripheral blood of cancer patients.

In contrast to the above, an elevated expression of *AURKA* was reported to be involved in the transition of BC from stage I to stage III and the corresponding possible effects on the progression of the disease (Miyoshi et al., 2001; Rouquier et al., 2014). Our study found no significant ($P > 0.05$) differences in the *AURKA* gene expression in both the peripheral blood of the controls

Table 3. The relative fold change ($2^{-\Delta\Delta Cq}$) and *P*-value of the *AURKA*, *CENPW*, and *TIMP1* genes of BC patients as compared to the healthy controls with the inclusion of dietary and lifestyle etiological factors

Etiology factors	Number of patients	*AURKA* (fold change)	*P*-value	*CENPW* (fold change)	*P*-value	*TIMP1* (fold change)	*P*-value
Age							
<50	3	0.30	0.071	0.70	1.000	0.89	0.250
≥50	11	0.71	0.025	0.94	0.710	0.89	0.370
Family cancer history							
No	9	0.66	**0.013**[b]	0.81	0.905	0.82	0.182
Yes	3	0.60	0.400	0.67	0.400	0.93	0.629
Unsure	2	–	–	–	–	–	–
Smoking habit							
No	12	0.52	**0.002**[a]	0.79	0.843	0.89	0.378
Yes	2	1.18	0.667	0.95	1.000	0.72	0.333
Fast-food intake							
No	2	0.04	0.267	0.78	0.533	0.87	0.800
Yes	12	0.61	0.036	0.83	0.722	0.84	0.123
Red meat intake							
No	6	0.58	0.643	0.57	1.000	0.86	0.429
Yes	8	0.54	**0.020**[a]	0.83	0.734	0.87	0.384

[a] Significant at $P < 0.025$ when compared to the healthy controls (Bonferroni corrected); [b] Significant at $P < 0.017$ when compared to the healthy controls (Bonferroni corrected)

and that of the BC patients (Table 2). We did not observe a significant ($P > 0.05$) difference in the *AURKA* expression at the lesion site nor its dependence on the tumor diameter, differentiation, TNM stage, depth of invasion, lymph metastasis, distant metastasis, or HER2 status when compared to the respective reference. Our findings implied that *AURKA* was associated with the BC invasion but not BC progression.

Similarly, no difference in the expression of *CENPW* and *TIMP1* in the BC patients was detected when compared to the healthy controls with a relative fold change of 0.81 and 0.86, respectively (Table 1). As shown in Table 2, we observed elevated but not statistically significant ($P > 0.05$) changes in the *CENPW* and *TIMP1* expression levels at the lesion site nor any dependence on the tumor diameter, differentiation, TNM stage, depth of invasion, lymph metastasis, distant metastasis, and HER2 status when compared to the respective reference. Although previous studies reported elevated expression levels of *CENPW* (Park et al., 2010) and *TIMP1* (Nakopoulou et al., 2002) in BC tumors, our results indicated that the expression difference was not detectable using peripheral blood. Peripheral blood is not a homogenous cell population but contains numerous subpopulations of erythrocytes, thrombocytes, and leucocytes. Consequently, gene expression studies using peripheral blood are challenging as the subpopulation-specific gene expression can be masked by other cell populations that reduce the ability to detect significant *CENPW* and *TIMP1* expression changes.

The age of patients remains one of the associative factors of BC incidence and gene expression. Women older than 50 years made up roughly 80% of all the BC cases as the risk of BC development increased with age (Smigal et al., 2006). We observed higher levels of *AURKA* and *CENPW* expressions in women ≥ 50 years old, but no differential *TIMP1* expression was observed (Table 3). A higher level of the *AURKA* transcript was reported to be associated with a poorer prognosis, which contributed to a higher mortality rate in women over 50 years old (Weier et al., 2013). The overexpression of *CENPW* and *TIMP1* was reported previously, but its association with different age groups has not yet been proven (Park et al., 2010; Nakopoulou et al., 2002). Younger BC pa-

tients with a positive family cancer history were reported to have more aggressive BC with a distinctive gene expression than their older counterparts and therefore, a poorer prognosis (Anders et al., 2008).

In addition, the dietary intake of fast food, red meat, and smoking status associated with the increased risk of BC development, cellular proliferation and the expression of growth factors, which eventually influenced the expression of genes in the peripheral blood (Luo et al., 2011; Zhao et al., 2013; Chandran et al., 2014; Farvid et al., 2014). Our study showed a significant reduction in the *AURKA* expression level in BC subjects with frequent red meat consumption by 0.54-fold ($N = 8$; Bonferroni-corrected $P < 0.025$), non-smokers by 0.52-fold ($N = 12$; Bonferroni-corrected $P < 0.025$), and without family cancer history by 0.66-fold ($N = 9$; Bonferroni-corrected: $P < 0.017$) (Table 3). However, the *CENPW* and *TIMP1* expressions in BC were not significantly different in any of the etiological factors investigated, such as family cancer history, smoking habits, fast food intake, and red meat intake (Table 3). The by-products of red meat and smoking such as heterocyclic amines and polyclinic aromatic hydrocarbons are carcinogenic agents (De Stefani et al., 1997; Steck et al., 2007). Previous studies reported distinct gene expression signatures between high and low intakes of red meat in lung cancer individuals but did not find significantly altered expression levels of the *AURKA*, *CENPW*, and *TIMP1* genes (Lam et al., 2014). Therefore, our results led us to hypothesize that the smoking status and red meat consumption alter the *AURKA* gene expression in human peripheral blood and influence the cell cycle process that leads to an increased risk of BC.

Despite the limitation of a small sample size, this study reports a significant reduction in the *AURKA* expression levels in the peripheral blood of BC patients, particularly in subjects who are non-smokers, consume red meat, and have no family cancer history. This study showed an early insight for the possibility of an early detection and diagnosis of BC.

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