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## Clinicopathological significance of *FANCA* mRNA expression in Thai patients with breast cancer

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### Abstract

The Fanconi anemia complementation group A (*FANCA*) gene is a potential tumor suppressor gene due to the repair of DNA damage mechanisms, and it remains a candidate as a cancer predisposition gene of breast cancer. In this study, the altered *FANCA* mRNA expression and the association with their clinicopathological data were investigated in patients with breast cancer. A total of 79 breast tumors from patients who did not receive chemotherapy or radiotherapy and their corresponding normal breast tissues were determined for the *FANCA* mRNA expression level using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The association between *FANCA* mRNA expression and patient clinicopathological features were analyzed using Chi-square tests and survival analysis was examined by the Kaplan-Meier method and Cox regression analysis. The *FANCA* mRNA expression level was overexpressed and underexpressed in breast cancers, with the frequency of 20 out of 72 (27.8%) and 7 out of 59 (11.9%), respectively. The *FANCA* over-expression was found associated with triple-negative breast cancer patients ( $P=0.048$ ), Odds ratio; 95%CI (3.15; 0.98-10.09). Additionally, *FANCA* under-expression has been observed with low tumor grade I ( $P=0.035$ ). This study concluded that the over-expression of *FANCA* mRNA expression used for screening hormone receptor status potentially was associated with triple negative-breast cancer while under-expression used for tumor grading biomarkers potentially in breast cancer patients was associated with low tumor grade.

**Keywords:** breast cancer; clinicopathological significance; *FANCA* mRNA expression; tumor suppressor gene.

### 1. Introduction

Breast cancer is the most common cancer in women worldwide and is one of the most common causes of cancer death in both the developed and developing world. In Thailand, the age-standardized incidence rate of breast cancer in women was about 34.2 per 100,000 populations (Rojanamatin et al., 2021). Major risk factors involve breast carcinogenesis including genetic variations such as mutations, deletions, amplifications, genetic polymorphisms as well as

epigenetic factors. These mechanisms affect protein function in breast cancer (Feng et al., 2018; Lin, & Hsieh, 2001; Paluszczak, & Baer-Dubowska, 2006; Saelee et al., 2014).

The Fanconi anemia complementation group A (*FANCA*) gene is a tumor suppressor gene located at 16q24.3, and primarily recognized as a gene involved in Fanconi anemia (FA), which is a rare hereditary disorder associated with multiple congenital abnormalities, bone marrow failure, and predisposition to malignancy (Solomon et al.,

2015; Thompson et al., 2005). There are 14 FA or FA-like genes, included FANC-A, -B, -C, -D1 (or BRCA2), -D2, -E, -F, -G, -I, -J, -L, -M, -N and -P (or BTBD12) and one FA-like complementation group (FANCO) (Crossan, & Patel, 2012; Yin et al., 2015). The FA genes are closely associated to each other in molecular pathways involving DNA repair, and DNA interstrand crosslinking in homologous recombination repair (Han et al., 2015).

Several studies have found that genetic variations of FA genes have been reported associated with several cancer risks including a contribution to the development of breast cancer (Dörk et al., 2019; Fang, Wu, Zhang, Liu, & Zhang, 2020). Moreover, the familial history of prostate cancer is reported to be associated with the loss of FANCA function (Hongo, Kosaka, Aimono, Nishihara, & Oya, 2020). Mutation screens of FANCA in adult sporadic cases of acute myeloid leukemia detected a low frequency of both missense mutations and heterozygous exonic deletions (Condie et al., 2002; Tischkowitz et al., 2004), as well as the altered expression of FANCA gene is frequently found to be affected by the chemotherapeutic performance of patients (Liu et al., 2020). However, the alteration of the effect of FANCA gene expression on breast cancer susceptibility remains unclear.

In a previous study on gene expression by microarray method, the results showed that the FANCA gene was overexpressed (data not shown); hence, the present study was conducted to confirm the FANCA gene expression in breast cancer patients and correlated the altered FANCA mRNA expression level and clinicopathological significance of breast cancer, including survival analysis, among a population of Thai women patients.

## 2. Objectives

To investigate the alteration of FANCA mRNA expression using RT-qPCR and analyze the association of its altered expression with their clinicopathological data in Thai women patients with breast cancer.

## 3. Materials and methods

### 3.1 Tissue samples

Primary breast carcinoma tumors and corresponding normal breast tissues were collected from 79 patients who underwent resection at the

National Cancer Institute, Bangkok, Thailand, between 2007-2011. The normal breast tissues were used as the basal level of FANCA mRNA expression to compare the level of FANCA mRNA expression with breast carcinoma tumor. All patients, who did not receive chemotherapy or radiotherapy treatment, were recruited into this study. This study was approved by the Institutional Review Board (IRB) of the National Cancer Institute, Bangkok, Thailand (Protocol Number: EC COA 009/2012). Tissue samples were preserved in RNAlater™ Stabilization Solution (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and kept at -80 °C until used. Patients' clinicopathological characteristics was assessed including age at diagnosis, tumor size, histological grade, axillary lymph-node status, number of lymph nodes, staging, triple-negative tumor (ER-, PR- and HER2-), immunohistochemistry staining of ER, PR and HER2, chemotherapy treatment (anthracycline and anthracycline+taxane), distant metastasis (lung, bone and liver), and the length of postoperative survival were collected from patient files.

### 3.2 RNA isolation and cDNA synthesis

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and mRNA was purified by Oligotex mRNA purification kit (QIAGEN, Gmbh, Germany) according to the instruction manual. cDNA synthesis for reverse transcription step was carried out by the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) for RT-qPCR analysis.

### 3.3 FANCA expression analysis by RT-qPCR

Alterations of FANCA mRNA expression levels were analyzed by LightCycler® 96 Instrument (Roche Applied Science, Penzberg, Germany). In a total volume of 10 µl, the PCR reaction contained 20 ng of cDNA, 1x LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany), 4 mM MgCl<sub>2</sub>, 0.5 µM forward and reverse primers. The primers for the human FANCA gene and β-globin housekeeping gene were designed by Primer3-Plus (Untergasser et al., 2007). The primer sequence for FANCA gene were used as a target gene (forward: 5'-TATAGGCTCTGCTTTGCAGGAT-3', and reverse: 5'-TTTCTCTGCTCCACAGTCAGC-3')

and  $\beta$ -globin housekeeping gene was used as an internal reference gene (forward: 5'-ACACAACACTGTGTTCACTAGC-3', and reverse: 5'-CAACTTCAT CCACGTTACC-3') to obtain relative expression values. The thermal amplification conditions for qPCR were as follows: 95 °C for 5 min (to activate the FastStart *Taq* polymerase), followed by 40-cycles amplification (95 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s). After the qPCR step, each amplification reaction was analyzed using the melting curve using LightCycler software (Roche Applied Science, Penzberg, Germany). Relative gene expression was determined using the  $2^{-\Delta\Delta Ct}$  method as previously described by Livak and Schmittgen (2001). The relative *FANCA* mRNA expression between primary breast carcinoma tumor and corresponding normal breast tissues in the same patient with more than 3.0-fold was assigned as a group of over-expression while its counterpart with less than 0.3-fold was assigned as group of under-expression.

#### 3.4 Statistical analysis

A Chi-square test was used to analyze the association between *FANCA* mRNA expression level and clinicopathological parameters such as age at diagnosis, tumor size, histological grade,

axillary lymph-node status, number of metastatic lymph nodes, staging, triple-negative tumor (ER<sup>-</sup>, PR<sup>-</sup> and HER2<sup>-</sup>), immunohistochemistry staining of ER, PR and HER2, chemotherapy treatment (anthracycline and anthracycline+taxane), distant metastasis. The Kaplan-Meier method and the Log-rank test was used for overall survival analysis and the Cox regression method was utilized to assess the prognostic effect for the altered *FANCA* mRNA expression on breast cancer patient survival. The P value of less than 0.05 was considered a significant correlation.

#### 4. Results and discussion

##### 4.1 Association between alterations of *FANCA* mRNA expression and clinicopathological significance

The *FANCA* mRNA expression level of the 79 breast tumors and paired normal breast tissues were determined by RT-qPCR. 20 of 72 (27.8%) tumors showed *FANCA* mRNA over-expression and correlated with triple-negative breast cancer patients (P=0.048, Table 1), Odds ratio; 95%CI (3.15; 0.98-10.09). While 7 of 59 (11.9%) tumor cases showed *FANCA* mRNA under-expression and were observed in low tumor grade I (P=0.035, Table 2).

**Table 1** Association between *FANCA* mRNA over-expression and clinicopathological characteristics in 72 breast cancer patients by quantitative real-time reverse transcription-PCR.

Parameter	No.	<i>FANCA</i> mRNA expression		Odds ratio, (95%CI)	P
		No altered n (%)	Over-expression n (%)		
<b>Age</b>					
≤50	36	24 (66.7)	12 (33.3)	0.57, (0.20-1.63)	0.293
>50	36	28 (77.8)	8 (22.2)		
<b>Tumor size(cm)</b>					
≤2	9	6 (66.7)	3 (33.3)	0.74, (0.17-3.29)	0.701
>2	63	46 (73.0)	17 (27.0)		
<b>Histologic grade</b>					
I	5	5 (100.0)	0	-	0.356
II	30	21 (70.0)	9 (30.0)		
III	37	26 (70.3)	11 (29.7)		
<b>Tumor stage</b>					
I, IIA, IIB	45	33(73.3)	12 (26.7)	1.16, (0.40-3.34)	0.786
IIIA, IIIB	27	19 (70.4)	8 (29.6)		
<b>Lymph-node status</b>					

<b>FANCA mRNA expression</b>					
<b>Parameter</b>	<b>No.</b>	<b>No altered n (%)</b>	<b>Over-expression n (%)</b>	<b>Odds ratio, (95%CI)</b>	<b>P</b>
Negative	30	25 (83.3)	5 (16.7)	2.78, (0.88-8.76)	0.075
Positive	42	27 (64.3)	15 (35.7)		
<b>Lymph Nodes (no.)</b>					
0-2 positive	46	34 (73.9)	12 (24.5)	1.26, (0.44-3.64)	0.670
>2 positive	26	18 (69.2)	8 (26.7)		
<b>Immunohistochemical</b>					
<b>ER status</b>					
Negative	26	15 (57.7)	11 (42.3)	0.36, (0.12-1.05)	0.058
Positive (1+,2+,3+)	43	34 (79.1)	9 (20.9)		
<b>PgR status</b>					
Negative	34	22 (64.7)	12 (35.3)	0.46, (0.16-1.32)	0.255
Positive (1+,2+,3+)	35	27 (77.1)	8 (22.9)		
<b>HER2 status</b>					
Negative	49	32 (65.3)	17 (34.7)	0.36, (0.09-1.40)	0.150
Positive (1+,2+,3+)	19	16 (84.2)	3 (15.8)		
<b>Triple negative tumor</b>					
ER, PR, HER2 positive	50	39 (78.0)	11 (22.0)	3.15, (0.98-10.09)	0.048*
ER, PR, HER2 negative	17	9 (52.9)	8 (47.1)		
<b>Chemotherapy treatment</b>					
Antracycline	34	22 (64.7)	12 (35.3)	0.66, (0.19-2.26)	0.502
Antracycline+taxane	19	14 (73.7)	5 (26.3)		
<b>Distant metastasis</b>					
No	43	30 (69.8)	13 (30.2)	1.03, (0.27-3.94)	1.000
Yes	13	9 (69.2)	4 (30.8)		

CI = confidence interval; no altered = a case without under-expression

**Table 2** Association between *FANCA* mRNA under-expression and clinicopathological characteristics in 59 breast cancer patients by quantitative real-time reverse transcription-PCR.

<b>FANCA mRNA expression</b>					
<b>Parameter</b>	<b>No.</b>	<b>No altered n (%)</b>	<b>Under-expression n (%)</b>	<b>Odds ratio, (95%CI)</b>	<b>P</b>
<b>Age</b>					
≤50	29	24 (82.8)	5 (17.2)	0.34, (0.06-1.93)	0.254
>50	30	28 (93.3)	2 (6.7)		
<b>Tumor size(cm)</b>					
≤2	7	6 (85.7)	1 (14.3)	1.67, (0.35-7.99)	1.000
>2	52	46 (88.5)	6 (11.5)		
<b>Histologic grade</b>					
I	7	5 (71.4)	2 (28.6)	-	0.035*
II	26	21 (80.8)	5 (19.2)		
III	26	26 (100.0)	0		

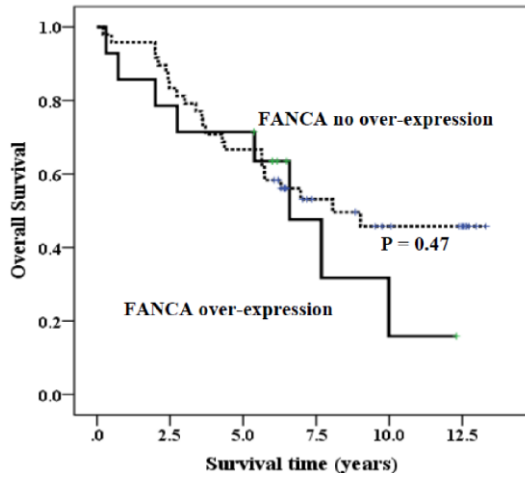
<i>FANCA</i> mRNA expression					
Parameter	No.	No altered	Under-expression	Odds ratio, (95%CI)	P
<b>Tumor stage</b>					
I, IIA, IIB	36	33 (91.7)	3 (8.3)	2.32, (0.47-11.47)	0.415
IIIA, IIIB	23	19 (82.6)	4 (17.4)		
<b>Lymph-node status</b>					
Negative	27	25 (92.6)	2 (7.4)	2.32, (0.41-13.03)	0.437
Positive	32	27 (84.4)	5 (15.6)		
<b>Lymph Nodes (no.)</b>					
0-2 positive	37	34 (91.9)	3 (8.1)	2.52, (0.51-12.50)	0.407
>2 positive	22	18 (81.8)	4 (18.2)		
<b>Immunohistochemical</b>					
<b>ER status</b>					
Negative	15	15 (100.0)	0	-	0.171
Positive (1+,2+,3+)	41	34 (82.9)	7 (17.1)		
<b>PgR status</b>					
Negative	23	22 (95.7)	1 (4.3)	4.89, (0.55-43.71)	0.220
Positive (1+,2+,3+)	33	27 (81.8)	6 (18.2)		
<b>HER2 status</b>					
Negative	37	32 (86.5)	5 (13.5)	1.03, (0.18-5.78)	1.000
Positive (1+,2+,3+)	18	16 (88.9)	2 (11.1)		
<b>Triple negative tumor</b>					
ER, PR, HER2 positive	46	39 (84.8)	7 (15.2)	-	0.585
ER, PR, HER2 negat	9	9 (100.00)	0		
<b>Chemotherapy treatment</b>					
Antracycline	25	22 (88.0)	3 (12.0)	1.05, (0.16-7.08)	1.000
Antracycline+taxane	16	14 (87.5)	2 (12.5)		
<b>Distant metastasis</b>					
No	34	30 (88.2)	4 (11.8)	2.50, (0.47-13.31)	0.355
Yes	12	9 (75.0)	3 (25.0)		

CI = confidence interval; no altered = a case without over-expression

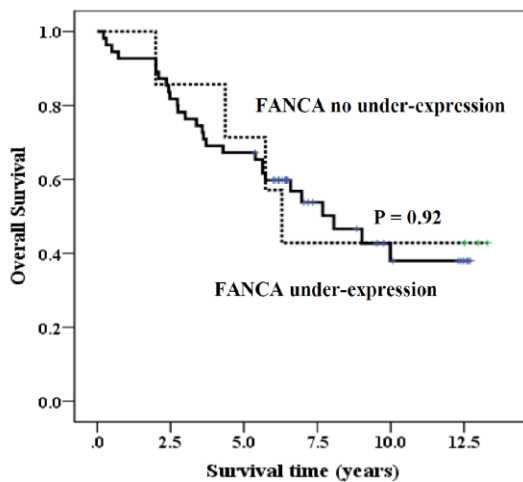
#### 4.2 Association between alterations of *FANCA* mRNA expression and survival of breast cancer patients

The survival curve was analyzed by the Kaplan-Meier method and the different survival time between mRNA over-expression or under-expression of patients and without them was compared by the Log-rank test. The data showed

that the correlation of *FANCA* mRNA over-expression and under-expression and overall survival time were not statistically significant (P= 0.47 and P= 0.92), respectively, see Figures 1 and 2. Additionally, Multivariate Cox regression analysis showed significant correlation between overall survival and tumor stage (HR = 4.251, 95%CI = 1.74-10.36, P=0.001, see Table 3).



**Figure 1** Overall survival was analyzed by Kaplan-Meier method and the Log-rank test was used to compare between FANCA over-expression and without (P=0.47).



**Figure 2** Overall survival was analyzed by Kaplan-Meier method and the Log-rank test was used to compare between FANCA under-expression and without (P=0.92).

**Table 3** Multivariate Cox regression analysis of prognostic biomarkers for survival of breast cancer patients.

Clinical variables	HR	95% CI	P
Age; >50 vs ≤50	0.845	0.40-1.78	0.658
Tumor size(cm); >2 vs ≤2	0.467	0.07-2.98	0.421
Histologic grade; III vs I+II	1.457	0.68-3.12	0.331
Tumor stage; IIIA, IIIB vs I, IIA, IIB	4.251	1.74-10.36	0.001*
Lymph Nodes (no.); >2 vs ≤2	1.943	0.73-5.18	0.184
Triple negative tumor; ER, PR, HER2 negative vs ER, PR, HER2 positive	1.901	0.68-5.34	0.223
FANCA mRNA alterations; Alterations vs without	1.269	0.58-2.76	0.548

HR = hazard ratio; CI = confidence interval

In conclusion, the Fanconi anemia (FA) pathway plays a central role in DNA repair, and DNA interstrand crosslinking in homologous recombination repair (Han et al., 2015). Genetic

variations of FA genes were designated by mutations, deletions or loss of function that inducing genomic instability responsible for cancer cell transformation, cancer progression and

confer sensitivity to DNA-damaging treatments. Whereas, FA gene amplification or gain of function may offer an advantage to cancer cells by diminishing replication stress and alleviating DNA damage induced by chemotherapeutics (Niraj, Färkkilä, & D'Andrea, 2019). Various mutant forms or deletions of *FANCA* affect DNA damage and are strongly associated with a predisposition to breast cancer (Chen, Zhang, & Wu, 2014; Solyom et al., 2011).

Currently, we evaluated 79 invasive ductal breast carcinomas for the aberrant mRNA expression level of *FANCA* genes. We observed the alteration of *FANCA* mRNA expression in breast cancer, then evaluated the association between them and clinicopathological significance. The *FANCA* mRNA over-expression was identified in tumor cases with a frequency of 27.8% in tumor samples and it was significantly associated with triple-negative breast cancer patients ( $P=0.048$ ). Several previous studies had reported *FANCA* mRNA expression were up-regulated in tumor tissues, for example the up-regulation of *FANCA* mRNA expression in melanoma was found and it possibly contributes to melanoma progression and the dataset from GEO (Gene Expression Omnibus) found that nine FA genes, including *FANCA*, were transcriptionally up-regulated in melanoma tissues (Kao et al., 2011; Yin et al., 2015). However, this study found that no significant association was observed between *FANCA* mRNA over-expression and chemotherapy treatment and survival of cancer patients. The data differ from the previous report that found the other types of FANCA affect survival and chemotherapy status of breast cancer patients such as gene amplification and over-expression in FA genes may offer an advantage to cancer cells with resistance to chemotherapy (D'Andrea, 2010; Niraj et al., 2019) and high *FANCA* expression determines a worse prognosis in chronic lymphocytic leukemia and lung carcinoids (Bravo-Navas, Yáñez, Romón & Pipaón, 2019; Swarts et al., 2013).

Moreover, we also found an under-expression of *FANCA* mRNA, which showed a frequency of 11.9% in tumor cases, significantly associated with low tumor grade I ( $P=0.035$ ). These results suggest that the absence of *FANCA* mRNA expression induced the progression of breast cancer. Consistent to previous experiments, the downregulation of *FANCA* mRNA expression

has been reported in acute myeloid leukemia samples and the downregulation of *FANCA* mRNA expression was rare in head and neck squamous cell carcinoma (Tischkowitz et al., 2004; Wreesmann, Estilo, Eisele, Singh & Wang, 2007). However, our data could not find the correlation of *FANCA* mRNA under-expression and survival status of patients ( $P=0.92$ ) and no difference in survival period between *FANCA* mRNA under-expression and without.

The association between alteration of *FANCA* mRNA expression and overall survival analysis is not significant. However, genetic variants in the *FANCA* gene are related to overall melanoma survival (Yin et al., 2015). So, the level of *FANCA* mRNA expression affected the amount of *FANCA* protein and the cause of relationship between gene expression and some clinicopathological breast cancer, while the variant of *FANCA* gene affected the function and cause of the relationship between gene variation and overall survival. As the results, multivariate Cox regression analysis showed the tumor stage is correlated to overall survival significantly. Therefore, the tumor stage of breast cancer patients plays a key role in the prognosis biomarkers for survival of breast cancer patients (Jacobson et al., 1995; Dong et al., 2014).

## 5. Conclusion

This study found that alterations of *FANCA* mRNA expression were a complex function of over-expression and under-expression. This study concluded the altered *FANCA* mRNA expression correlated with breast cancer susceptibility and might be contributed to a biomarker in breast cancer patients.

## 6. Acknowledgements

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