INVESTIGATION OF EXON20 PIK3CA GENE MUTATION IN PLASMA OF HORMONE RECEPTOR POSITIVE BREAST CANCER PATIENTS

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ABSTRACT

Objective*:** To determine the frequency of H1047R mutation on exon 20 *PIK3CA* gene in the plasma of hormone recepter positive (HR+) breast cancer (BC) women and its relationship with clinical characteristics of these patients. *Subjects*: Plasma was separated from venous blood of 141 female HR+ BC patients, have been treating at 108 Military Central Hospital from June 2021 to June 2023. ***Method*:** Circulating free DNA (cfDNA) was isolated from plasma by magnetic beads (Thermo Fisher Scientific). The H1047R hotspot mutation on exon 20 *PIK3CA* gene was detected in 141 plasma samples by an in-house Realtime PCR assay using a wild-type allele-specific blocker. ***Results*:** 47/141 samples (33,3 %) had the H1047R mutation. The H1047R mutation was present in all disease stages, both in metastatic and non-metastatic cases. Although, the rates of gene mutation in late-stage, metastatic cancer patients were higher than those in the early-stage, non-metastatic cancer group, there was no statistically significant difference between the two groups ($p > 0.05$). Whereas it was not linked to other clinical traits of patients with HR+BC, the H1047R mutation was more common in those with liver metastases and in those received Fulvestrant ($p < 0.05$). ***Conclusion: Peripheral blood from patients with HR+BC in all stages contained the H1047R mutation on exon 20 of the PIK3CA gene. This mutation frequently developed in patients received Fulvestrant treatment and has been related to liver metastases.

Keyword: *breast cancer, PIK3CA gene, plasma, mutation, circulating free DNA*

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Date of receipt: 11/9/2023
Date of scientific judgment: 9/10/2023
Reviewed date: 16/10/2023

I. INTRODUCTION

Breast cancer (BC) is the most frequent malignant disease in both sexes and the major cause of cancer-related deaths in women, according to Globocan data in 2020 [1]. With the advancement of diagnostic technologies, most women are diagnosed in the early stages; yet, more than 20% of patients undergo recurrence and metastasis each year [2]. Endocrine inhibitor therapy is the initial choice for individuals with hormone receptor-positive breast cancer (HR+BC), which accounts for more than 70% of cases. However, clinicians continue to face obstacles such as treatment resistance, relapse, and disease progression. In recent years, identifying somatic gene mutations has provided significant information to targeted therapy to assist improve treatment quality and lengthen life for cancer patients [3].

PIK3CA is an oncogene that encodes the protein p110, a structural component of the PI3K signaling pathway, and is the second most frequently mutated gene in BC tumors. Up to 80% of this gene mutations occur in E545K (exon 9) and H1047R (exon 20) positions which are seen in 20-50% of BC patients and increase PI3K signaling pathway activation [4]. As a result, blocking the PI3K

signaling pathway is critical in BC treatment. Many clinical trials have focused on this topic in recent years, and Alpelisib has been approved by the US FDA to treat patients with metastatic BC who have HR+HER2- and PIK3CA gene mutations [5]. Identifying PIK3CA gene mutations facilitates in the selection of specific therapy regimens for individuals with advanced-stage HR+BC [3]. The relevance of PIK3CA gene variants, particularly exon 20 mutations, is currently debated in research. Some researchers discovered that PIK3CA gene mutations were frequently associated with a poor prognosis and patients with a shorter overall survival [6, 7]. Takashi Takeshitaa's research, on the other hand, demonstrates that patients with exon 20 mutations had a better prognosis than patients without mutations [8].

Somatic mutations have recently been identified not only on DNA molecules isolated from biopsy tumor tissues, but also on DNA molecules generated from tumors that circulate freely in the peripheral blood (circulating tumor DNA-ctDNA), which aids in the testing process because it is less invasive and can be substituted in cases where tissue samples are unavailable or unsuitable for molecular biological testing methods [9].

Thus, this research was conducted to determine the prevalence of the H1047R mutation in exon 20 of the PIK3CA gene in the plasma of HR+BC patients and to analyze the link between this mutation and the clinical characteristics of HR +BC patients.

II. SUBJECTS AND METHODS

2.1. Clinical samples

From June 2021 to June 2023, 141 HR+BC patients have been treated at the Cancer Institute, 108 Military Central Hospital. The patients had agreement to participate in the study and had adequate clinical and histological data.

2.2. Methods

*** cfDNA extration method from plasma:** Within 4 hours of blood collection, peripheral venous blood samples will be centrifuged at 4000g for 10 minutes to separate plasma from blood cells, and the plasma will be stored in a freezer at – 80 ^{0}C until analysis. CfDNA was extracted from plasma samples using Thermo Fisher Scientific magnetic beads. The cfDNA isolation method was carried out in accordance with the manufacturer's instructions. cfDNA samples are utilized to detect genetic changes, while the rest are kept in a freezer at – 80 ^{0}C.

*** H1047R mutation detection method:** The H1047R mutation was discovered using an in-house Realtime PCR process that combined primers intended to specifically couple with the mutant allele and a blocker designed to specifically pair with the wild-type allele. Blocker is an oligonuclotide fragment with a phosphorylated 3' end, thus in the wild-type sample reaction, this substance partners particularly with the wild-type sequence, inhibiting the polymerization reaction. In the case of a mutant DNA sample, the blocker does not couple with the mutated nucleotide; instead, the particular primer pairs with the altered nucleotide, kicking off the amplification and synthesis of the DNA chain. Figure 1 depicts the reaction principle.

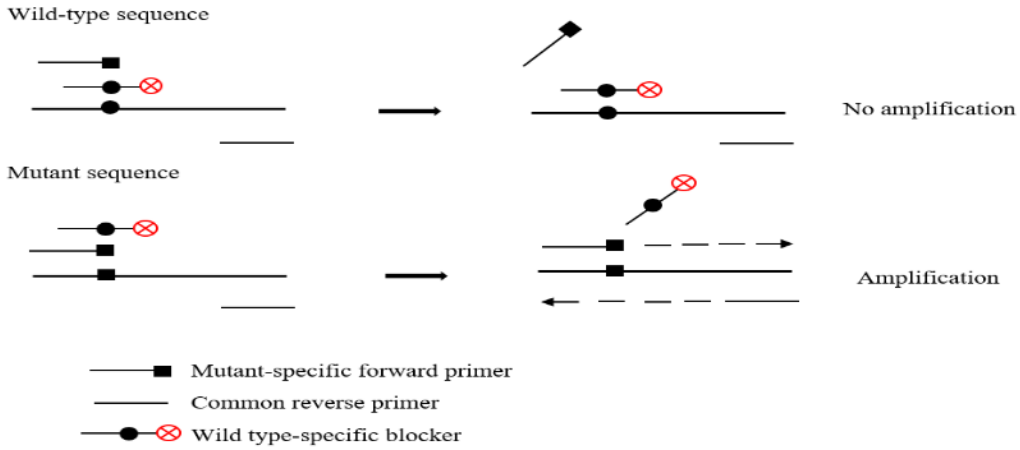

Figure 1. ***The principle of a real-time PCR process to identify the H1047R mutation.***

The final Realtime PCR assay was carried out in a reaction volume of up to 10 µl, using 2X Universal PCR Master Mix (no UNG)TM (Applied Biosystems, Foster City, CA), 40 nM of each primer, 1.2 µM blocker, 2 µl cfDNA or DNA of control samples. The following thermocycling conditions were utilized for the realtime PCR assay: 10 min. at 95°C, 50 cycles of 15 sec. at 95°C, 20 sec. at 55 °C and 20 sec at 72°C.

The comparative cycle threshold (Ct) approach was utilized to investigate fold amplification shifts. Ct values were recorded for each allele-specific experiment, and ΔCt values were computed as the difference in Ct of the same allele amplification with and without blocker. The sample was considered to have the mutation when the ΔCt values of the relative amplification were significantly lower than the ΔCt values of a wild-type control sample (figure2).

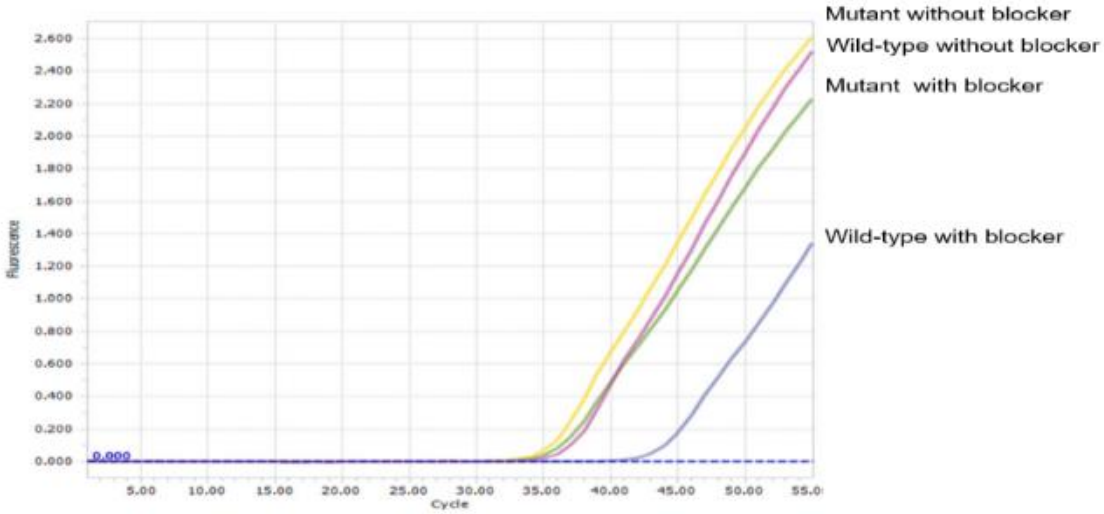

Figure 2: ***DNA amplification of negative control (wild-type DNA) and positive control (mutant DNA) samples, PCR procedure with and without blocker.***

*** Data analysis approaches**: Data was calculated using medical statistical techniques on Spss 20.00 software. Compare the difference between two proportions using the Chi-square test.

III. RESULTS

3.1. Characteristics of HR+BC patients

	Characteristics of study'population, n (%)	
Age (years)	$\overline{X} \pm SD$ (range)	51,5 ± 12,64 (26 - 81)
	< 50	72 (51,1)
	≥ 50	69 (48,9)
Family history	yes	29 (20,6)
Menopausal status	Post-menopausal	74 (52,5)
Tumor histology	Ductal	136 (96,5)
	Lobular	5 (3,5)
Stages	Early	78 (55,3)
	Late	63 (44,7)
Relapse	yes	27 (19,1)
Metastatic status	Any sites	76 (53,9)
	Lymph nodes	68 (48,2)
	Liver	5 (3,5)
	Lung	15 (10,6)
	Bone	23 (16,3)
The number of metastasis lesion	0	65 (46,1)
	≤ 2	63 (44,7)
	≥ 3	13 (9,2)
Treatment	Surgery	116 (82,3)
	Chemotherapy	125 (88,7)
	Endocrine therapy	93 (66,0)
	Radiotherapy	48 (34,0)
	Immunotherapy	50 (35,5)
H1047R mutation	yes	47 (33,3)

Comment: The participants'age ranged from 26 to 81 years old, with an average age of 51.5±12.64 years. 52.5% of the women were postmenopausal, had early stage cancer, and had ductal carcinoma. Recurrence occurred in 19.1% of patients, and metastasis occurred in 53.9% of cases, including lymph nodes, lung, liver, and bone metastases. The majority of patients (82.3%) underwent total mastectomy, axillary lymph node dissection (Patey), and chemotherapy (88.7%). Patients received endocrine therapy, radiation, immunotherapy that accounted for 66%, 34%, 35.5%, respectively . H1047R mutation was found in 47/141 plasma samples from HR+BC patients, accounting for 33.3%.

3.2. The relationship between the H1047 mutation and clinical features of HR+BC patients

Table 2. *Association between H1047R mutation and clinical features of HR+BC patients*

Patients' clinical features		H1047 R mutation status		p-value
		No n = 94 (66,7%)	**Yes n = 47 (33,3%)**	
Age (years)	< 50 ≥ 50	46 (48,9) 48 (51,1)	26 (55,3) **21 (44,7)**	0,592
Family history	No Yes	76 (80,9) 18 (19,1)	36 (76,6) 11 (23,4)	0,659
Menopausal status	Pre-menopausal Post-menopausal	42 (44,7) 52 (55,3)	25 (53,2) **22 (46,8)**	0,375
Tumor histology	Ductal Lobular	89 (94,7) 5 (5,3)	**47 (100)** 0 (0)	0,169
Stages	Early Late	56 (59,6) 38 (40,4)	22 (46,8) **25 (53,2)**	0,151
Relapse	No Yes	80 (85,1) 14 (14,9)	34 (72,3) 13 (27,7)	0,110
Metastasis	No Yes	47 (50) 47 (50)	18 (38,3) **29 (61,7)**	0,213
Lymph nodes invasion	No Yes	53 (56,4) 41 (43,6)	20 (42,6) 27 (47,4)	0,153
Liver metastasis	No Yes	93 (98,9) 1 (1,1)	43 (91,5) 4 (8,5)	**0,042***
Lung metastasis	No Yes	85 (90,4) 9 (9,6)	41 (87,2) 6 (12,8)	0,572
Bone metastasis	No Yes	81 (86,2) 13 (13,8)	37 (78,7) 10 (21,3)	0,334
The number of metastasis lesion	0 ≤2 ≥ 3	47 (50) 41 (43,6) 6 (6,4)	18 (38,3) 22 (46,8) 7 (14,9)	0,177
Received therapy				
Surgical therapy	No Yes	15 (16) 79 (84)	10 (21,3) **37 (78,7)**	0,486
Chemotherapy	No Yes	13 (13,8) 81 (86,2)	3 (6,4) **44 (93,6)**	0,263
Hormone therapy	No Yes	32 (34) 62 (66)	16 (34) **31 (66)**	1.000
Radio therapy	No Yes	67 (71,3) 27 (28,7)	26 (55,3) 21 (44,7)	0,089
Immunotherapy	No Yes	61 (64,9) 33 (35,1)	30 (63,8) 17 (36,2)	1,000

Comment: The H1047R mutation has been found in the peripheral blood of BC women at all stages of the disease, both metastatic and non-metastatic. This mutation was only discovered in ductal BC patients' blood and not in lobular group. Mutations in exon 20 of the PIK3CA gene were more common in patients under 50 years old, pre-menopausal, with metastases, and who have received surgery, chemotherapy, or endocrine therapy, but the difference of mutation rates among these groups was not statistically significant ($p > 0.05$). The H1047R mutation, on the other hand, is more prevalent in liver metastatic BC individuals ($p < 0.05$).

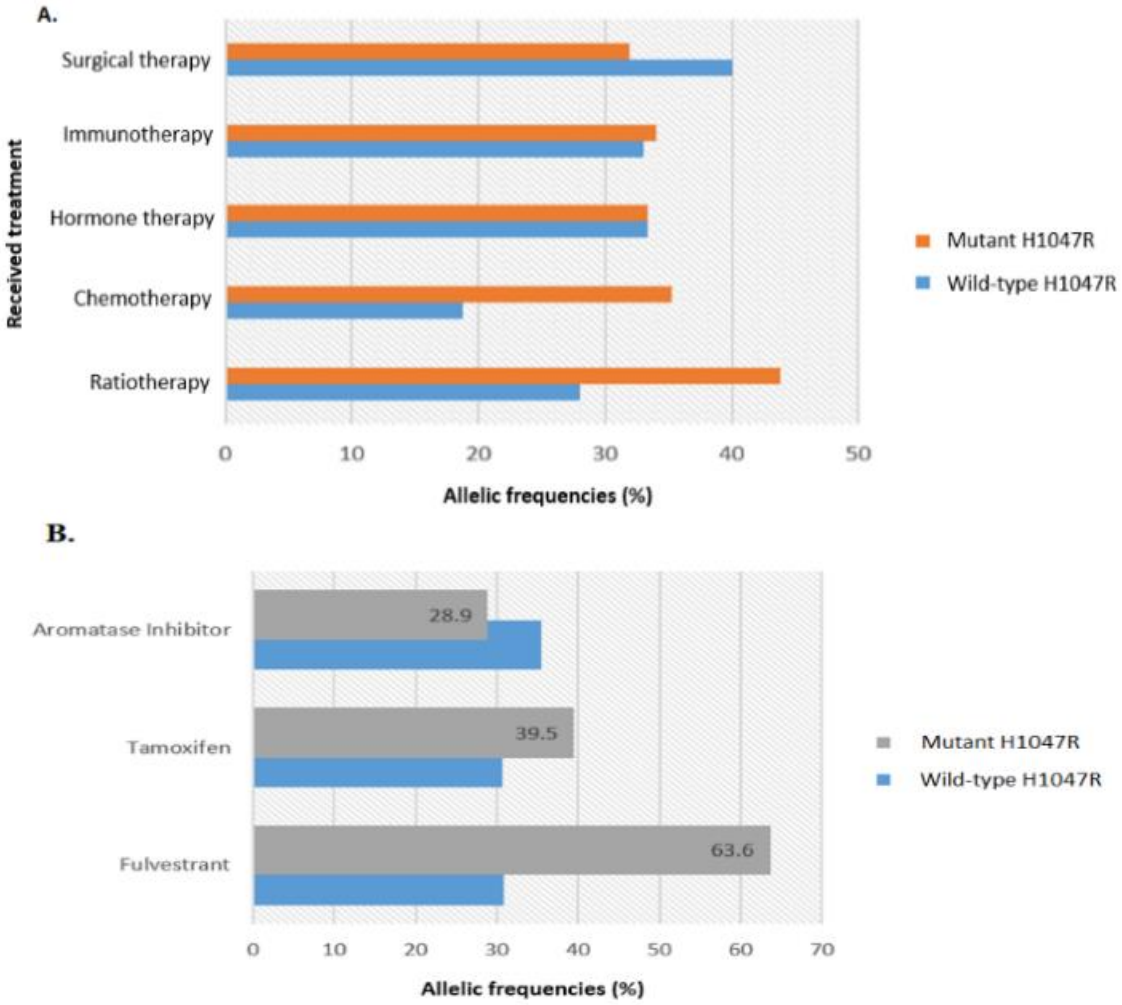

Figure 3. ***Distribution of H1047R mutation rates by treatment regimen (A) and endocrine therapy regimen (B).***

Comment: The H1047R mutation was more common in individuals who had not undergone surgery and have received chemotherapy, radiation, Tamoxifen, or Fulvestrant. Exon 20 PIK3CA gene mutation was found in a significantly higher proportion of HR+ BC patients treated with Fulvestrant than in the non-treated group ($p < 0.05$).

IV. DISCUSSION

The study's participants had an average age of 51.5 ± 12.64 years, with 48.9% being over 50 years old, 52.5% being menopausal, and 20.6% having a personal or relative history of prior malignancy. According to data in 2022, 83% of invasive BC diagnoses in the United States are above the age of 50, the average age at BC diagnosis was 62, and more than half of cases are diagnosed with early stage BC [10]. Thus, the average age of disease in the research group is younger, and the proportion of BC patients under 50 in the study is also greater than the above statistical data, nevertheless the proportion of early stage cases is similar to previously reported data [10, 11].

Published studies reported that PIK3CA gene mutations account for 20-50% of breast cancer tumors; the majority of hot spot

mutations of this gene occur in exon 20 and exon 9, with the most common frequency being H1047R, E545K, E542R, and H1047L [4, 12]. In recent years, researchers have contributed a large amount of evidence demonstrating the presence of freely circulating DNA fragments in the blood (cfDNA) originating from tumors (ctDNA), promising to become a potential tool called "Liquid Biopsy" that can replace biopsy tumor tissue because analysis of molecular abnormalities on cfDNA allows to reveal genetic information of primary and metastatic tumors, genetic changes over time of tumor development, and predict treatment response [13]. In the present research, 33.3% of HR+ BC patients had the hotspot mutation H1047R exon 20 in their blood. Our study's mutation rate is quite similar to other authors' investigations on tissue and peripheral blood samples [14]. Furthermore, several observation revealed a higher percentage of H1047R mutation than our finding, presumably due to the fact that our study population only included HR+breast cancer women [12]. The H1047R mutation was found at all stages of the disease, with the frequency the mutation increasing with disease stages. The mutation was only found in the ductal carcinoma group, not the lobular carcinoma group. On the other hand, previous researches found that PIK3CA gene mutations were present at varying frequencies in early and late stage BC, as well as in all BC subtypes [8, 11]. This disparity can be explained by the fact that our study had a smaller sample size, as HR-negative breast cancer patients were excluded from the study. The major difficulty in analyzing liquid biopsies is that the amount of ctDNA is often very low in the large number of wild-type DNA background, is greatly reduced in the blood under the influence of therapeutic interventions on the patient, and has a short half-life, requiring cfDNA isolation and analysis to be performed as soon as possible after blood collection, and at the same time it is necessary to use a sensitive testing method. So, in addition to differences in the research population, the time and mutation analysis method also greatly affect the frequency of mutations in researches [9].

Exon 20 mutation was also more common in patients with recurrence, having bone, lung, or liver metastasis; the occurrence of this mutation is more frequently encountered in people with severe disease stages and more metastatic sites. The data demonstrated that the appearance of the H1047R mutation might indicate the disease progression, especially since the difference in the rate of gene mutations between patients with liver invasion and the non affected group was statistically significant ($p < 0.05$). So, prospective research data on a larger population is required to evaluate the prognosis role of this mutation of BC patients in Vietnam.

The association between exon 20 mutations and clinical features of BC patients has been observed to differ greatly amongst studies. Mosele and colleagues discovered that PIK3CA gene mutations were more prevalent in HR+HER2- BC patients under the age of 65 who had received endocrine therapy ($p < 0.01$) [11]. Exon 20 mutation was also more common in females under the age of 55, as well as in late-stage cancer patients, postmenopausal women, and patients with lymph node metastases, although this mutation was not appeared to connect with other clinical features ($p > 0.05$) [6].

In our finding, the rate of exon 20 mutation was higher in patients who did not have surgery, chemotherapy, or radiotherapy, but the difference was not statistically significant when compared to the non-treated groups ($p > 0.05$) (Figure 3A). Furthermore,

when assessing the group of HR+ BC patients who underwent endocrine therapy, the H1047R mutation showed at a considerably greater rate in the Fulvestrant – received group compared to the non-Fulvestrant-treated group (p = 0.042) (Figure 3B).

Drug resistance to endocrine therapy has previously been observed in HR+ BC patients with PIK3CA gene mutations [15]. As a result, several targeted medicines that inhibit the PI3K signaling pathway have been studied and developed to treat endocrine-resistant individuals with PI3K mutations. Among them, when coupled with fulvestrant, Alpelisib has been demonstrated to dramatically improve disease-free survival in HR+HER2- advanced post-menopausal patients who had PIK3CA mutations [5]. It can be shown that assessing whether BC patients have PIK3CA gene mutations would assist physicians in making a prognosis and guiding the selection of appropriate treatment regimens for the patients.

V. CONCLUSION

H1047R mutation on exon 20 of the PIK3CA gene in peripheral blood of HR+ BC patients at all stages. The mutation was related with liver metastases and developed at a high rate in individuals treated with Fulvestrant.

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