Saudi Journal of Biological Sciences 29 (2022) 2828–2835

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Mutational pattern of PIK3CA exon 20 in circulating DNA in breast cancer

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ARTICLE INFO

Article history:
Received 10 August 2021
Revised 28 December 2021
Accepted 2 January 2022
Available online 6 January 2022

Keywords:
Mutational signature
Breast cancer
PIK3CA
Nucleotide substitution
Cancer etiology

ABSTRACT

Breast cancer (BC) is one of the most common cancers with diverse mutations, etiology and causes. Mutational signature of the driver genes could allow for better understanding disease etiology and progression. This study aims to assess PIK3CA Exon 20 somatic mutational signature in relation to potential underlying etiology. Circulating DNA of 71 Egyptian BC patients was isolated, amplified for PIK3CA Exon 20, and sequenced. Mutational signature was determined according to COSMIC v2 signature. Public BC dataset was analysed to assess PIK3CA mutations effect on the transcriptomic profile. Somatic mutations of PIK3CA exon 20 were found in 66.2% of the study cohort. Nucleotide substitution patterns were similar to general nucleotide substitution patterns in BC. Signature 3 and 9 were the most common signatures in the studied BC patients. Signature of Aristolochic acid exposure was found in some cases. The most common nucleotide substitution was T > A transversion, but substitutions T > G and T > C were correlated to each other and to the total mutation number. PIK3CA mutations were found to disrupt several pathways including RAC1, PDGF, Wnt, and integrin signalling. PIK3CA exon 20 mutational signatures in Egyptian BC patients could suggest a disease etiology involving homologous recombination deficiency (HRD) and polymerase eta (Pol η). Nucleotide substitution patterns could indicate the role of exposure to oxidative stress and some carcinogens such as 4-aminobiphenyl and Aristolochic acid.

1. Introduction

Cancer diagnosis and treatment is traditionally based on tumor morphology, clinical signs, and the primary site of cancer. More recently, systematic investigations of cancer genomes had shown various somatic mutation profiles in each type of cancer, allowing better understanding for disease etiology, prognosis, and therapeutic approaches (Van Hoeck et al., 2019). Genomes of cancer patients usually show 10^3–10^5 somatic mutations. Computational analysis of mutation patterns in somatic genomes has provided unbiased perception of mutagenic processes and pharmacogenomics consequences in cancer (Hu et al., 2020). In different cancer types, some mutations significantly associate with tumorigenesis and are known as driver mutations. Driver mutations induce tumorigenesis through accumulation of more driver and non-driver mutations (Stratton et al., 2009).

Genetic testing for driver genes could help better understanding of tumorigenesis and aid personalized treatment approaches, given the growing number of drugs targeting mutant genes' proteins. It has recently been revealed that mutational signatures could show clinical significance in predicting treatment response in cancer (Van Hoeck et al., 2019). Nucleotide substitutions are usually expressed by the pyrimidine change, giving six possible substitutions for every nucleotide. For each of the six types of substitutions, somatic mutation patterns could be expressed as sixteen different patterns, giving a total number of 96 mutation patterns. This is obtained by determining each nucleotide substitution using its immediate sequence context at 5′ and 3′ ends (Alexandrov and Stratton, 2014).

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2022.01.002
1319-562X/

The most recurrent primary tumour in women is breast cancer (BC) with about 1.7 million cases diagnosed annually. It is the fifth major cause of death in women. Breast cancer is a heterogeneous disease on histopathological and genetics levels. Determination of driver mutations implicated in tumour development could help in designing efficient therapeutic protocols and prediction of disease prognosis. Hence, mutations in BCs are being studied extensively using DNA sequencers (Watanabe et al., 2018). One of the most important driver genes in BC is Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), which contains common mutational hotspots at exon 9 and exon 20 (Zhang et al., 2017). Genetic hyperactivation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signalling has been recognized as the most frequent driver mechanism in many types of cancers (Thorpe et al., 2015). PIK3CA and Phosphatase and TENsin homolog (PTEN) were identified as most frequently genes harbouring somatic point mutations in>12 solid tumours of different types (Lawrence et al., 2014). Examples include breast (>30%), endometrial (>30%), bladder (>20%), colorectal carcinoma (>17%), and head and neck squamous cell carcinoma (>15%). Most mutations cluster in exon 9 and exon 20 hotspots (Zhang et al., 2017).

This study aims to assess PIK3CA Exon 20 somatic mutational signature in relation to potential underlying etiology and disease progression. Mutational signature is determined through analysis of the nucleotide substitution pattern in the DNA sequence. Different patterns suggest different potential underlying causes of the mutations.

2. Material and methods

2.1. Ethical statement

The study plan and clinical data sheet forms were approved by the Institutional Review Board (IRB) of Al-Azhar University Hospital in Damietta. The study protocol was approved by the Scientific Research Ethical Committee of Faculty of Pharmacy (Girls), Al-Azhar University, abiding by the Declaration of Helsinki (Approval no.51/9). Written informed consent was obtained from each participant enrolled in this study.

2.2. Participants

This study included 71 female patients with de novo malignant breast tumor admitted at the Department of Surgery in Al-Azhar University Hospital (Damietta). Inclusion criteria were: new diagnosis with primary BC with no prior treatment and female gender. Patients with benign tumors, recurrent BC, and males with BC were excluded. For each participant, age of diagnosis, tumor grade, tumor histology, tumor metastasis, tumor size, lymph node metastasis, type of treatment, disease free survival (DFS) and overall survival (OS) were recorded. The mean age of enrolled patients was 48.27 ± 10.18. The body mass index (BMI) ranged from 25-29 kg/m^2, and none of the enrolled patients is a smoker. The patients were selected based on clustered randomization.

2.3. Samples

Five ml blood sample was collected from each patient for DNA extraction along with a sample of the tumor tissue obtained by core biopsy. Immunohistostaining for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) were performed on 10% formalin-fixed paraffin embedded blocks for each patient using monoclonal antibodies (Dako), following manufacturer instructions.

Treatment protocols: After radical or conservative surgery was performed for patients with early BC, adjuvant chemotherapy was given to each patient following standard protocols. Patients were followed up to assess treatment response in the form of DFS and OS.

3. DNA extraction and PCR amplification of PIK3CA exon 20

Cell free DNA (cfDNA) was extracted from peripheral blood samples of breast cancer patients using a phenol / chloroform method that involves the addition of glycine as described in Ibrahim et al., (Ibrahim et al., 2016). The PCR reaction was performed using Thermo Scientific™ DreamTaq™ DNA Polymerase in a 25-µL reaction volume mixture containing 200 ng of cfDNA. Cycling conditions were as following: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s, and a final elongation at 72 °C for 7 min. Primers were designed using **Primer Blast** as following: Forward primer (5′->3′): AGTCAGTCAACCATAATCACCTTG, and Reverse primer (5′->3′): CTATGCAATCGG TCTTTGCCTG. The length of the target sequence is 806 nucleotides.

3.1. Sequence analysis

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced by Sanger sequencing using the same primer under standard technique (ABI PRISM 3730 automated sequencer, Invitrogen). Analysis of the sequence by BLAST (reference GRCh38.p13) and Mutation Finder (Hijikata et al., 2010) was performed. Afterwards, nucleotide substitution collectively was analysed using Signal® (Degasperi et al., 2020) to determine the predominant mutational signatures (including COSMIC v2 and Environmental Mutagen signatures), in the study group. Also, samples were analysed individually to eliminate the possibility of inter-sample bleeding errors (Maura et al., 2019).

3.2. Analysis of public datasets

Affymetrix microarray dataset GDS4053 (Cizkova et al., 2010) was analysed to evaluate the effect of PIK3CA mutations on the transcriptomic profile in BC. The dataset was analysed for differential expression between BC patients with and without PIK3CA mutations using Student's *t* test. The P value was corrected using the Benjami - Hochberg (BH) method (Benjami and Hochberg, 1995). Top 500 genes with significant difference of expression were analysed by PANTHER (Thomas et al., 2003) (RRID SCR: 004869) for pathways and molecular functions. P value < 0.05 and FDR < 0.05 were considered significant.

4. Results

This study was comprised of 71 confirmed *de novo* malignant BC cases. Tumour size grade classification showed that most of them were T1, T2 (24, 35 respectively). PIK3CA exon 20 mutations were recorded in 47 (66.2%) patients. Most patients with mutations (n = 41) showed multiple mutations.

A total of 408 Substitutions were recorded. Patterns of the 408 recorded substitutions were represented as described before (IACR, 2021) to determine the mutation signature (Fig. 1, a). Signal® mutational signature analysis revealed predominance of signature 3 (30.1%), signature 9 (26.5%), and to a lesser extent signature 1 (11.3%).

To eliminate the possibilities of sample- bleeding in mutation signature analysis, mutational signature analysis was run for individual samples and similar results were obtained in most cases. In

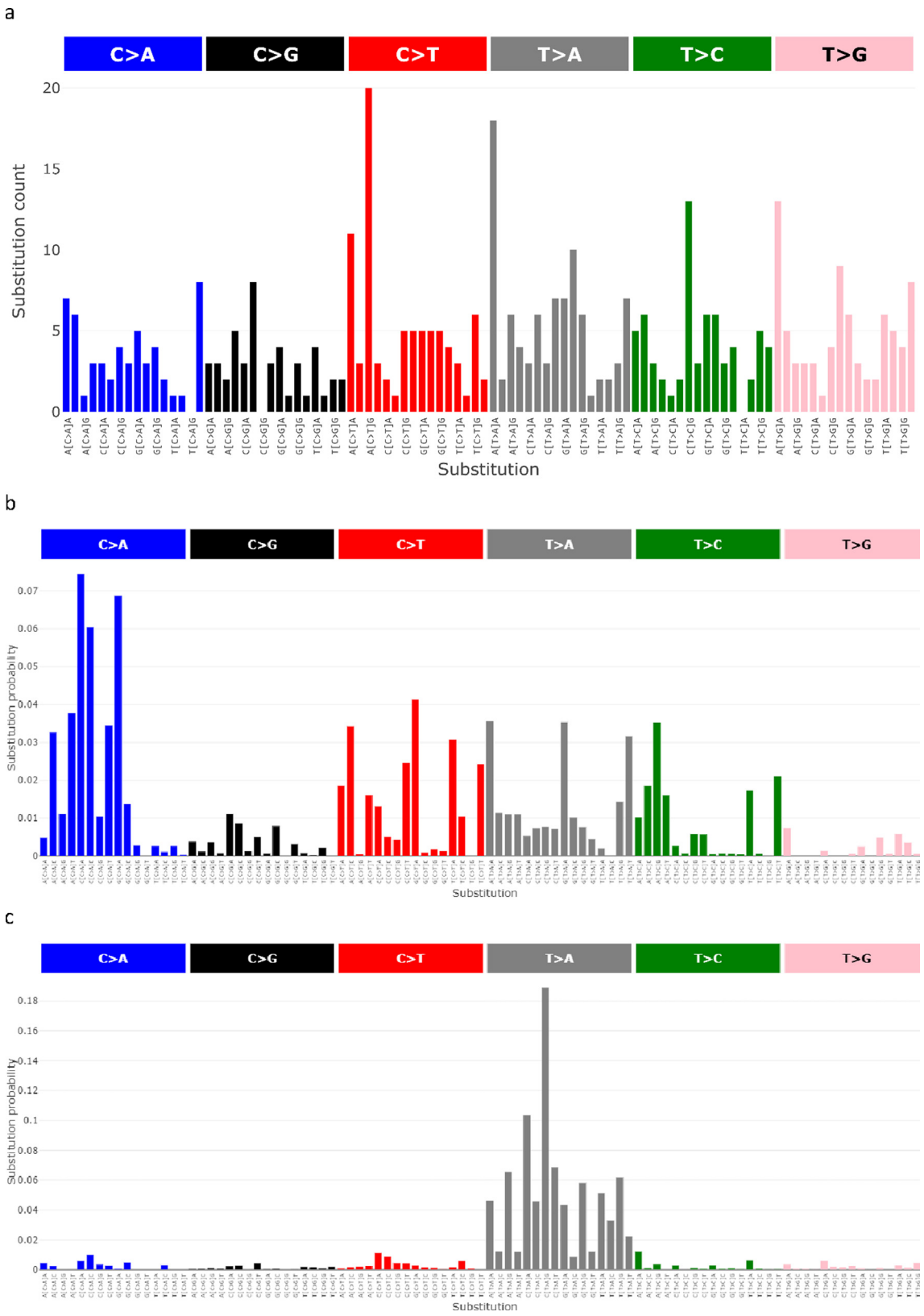

Fig. 1. (a): Mutational signature of PIK3CA exon 20 in the studied Egyptian BC patients. **(b):** Signature of 4-Aminobiphenyl. **(c):** Signatures of Aristolochic acid.

addition, 4 cases showed signature 22 (all of them were ER +). Patients with Signature 3 were predominantly HER + while patients with signature 9 were predominantly ER- PR- (OR 1.3×, 95% CI 1.1–1.6).

None of the cases showed signatures 2 or 13.

Signature of Environmental Mutagens showed signature of several environmental carcinogens. Signature of 4-Aminobiphenyl was observed (22.7%), and to a lesser extent, signatures of Aristolochic acid (5.7%) (Fig. 1 b and c, respectively) followed by aflatoxin and dibenzo (a,l) pyrene.

Regarding nucleotide substitution patterns, T > A was the most common followed by C > T. It was noted that T > C, T > G were the highest significantly correlated substitutions to the total number of mutations (r = 0.766, and 0.764 respectively, P value is < 0.00001.). On the other hand, cytosine substitutions were of weak to moderate positive correlation to total substitutions in each patient.

Regarding the co-occurrence of specific nucleotide substitutions, there was a strong positive correlation between T > G and T > C (P value < 0.00001. r = 0.75). Also there was a week to moderate positive correlation in: T > C and C > G (P value = 0.016, r = 0.35), T > G and C > G (P value = 0.0197, r = 0.34), T > A and T > G (P value = 0.01, r = 0.36), and T > C and T > A (Fig. 2) (P value < 0.00049, r = 0.49).

Analysis of nucleotide substitutions profile of several loci on chromosomes 10, 14 in the same BC patients showed significant correlation between PIK3CA substitution profile in BC and general substitution profile in BC (Fig. 3), (P value = 0.028028, r = 0.8658.).

Patients with poor treatment response showed no significant difference in COSMIC v2 mutational signature compared to good responders. Upon considering nucleotide substitution, patients with predominant C > A substitution were in the short DFS group (DFS < 2 years). However, the small number of patients with this pattern didn't allow for proper statistics.

To evaluate the molecular mechanisms by which PIK3CA mutations drive BC, analysis of GDS4053 expression array revealed dysregulation of many genes of several pathways in BC patients with PIK3CA mutations compared to BC patients without PIK3CA mutation (Fig. 4).

5. Discussion

In Egypt, BC is the most common women malignancy accounting for 32.4% of all cancer cases (Alexandrov and Stratton, 2014). Generally, mutations in cancer could follow a specific pattern according to both the cause and the site of the tumour (IACR, 2021). However, to date, no study focused on the mutation pattern of driver genes in Egyptian BC patients.

PIK3CA activating mutations were reported to be an early event in BC development and likely play a role in tumour initiation more than in invasive progression (Miron et al., 2010). Although PIK3CA mutations are known as driver mutations of BC, their prognostic value of PIK3CA mutation in BC is still unclear. (Kalinsky et al., 2009) suggested that mutations in PIK3CA gene were associated with better recurrence-free survival (Kalinsky et al., 2009). (Barbareschi et al., 2007) showed that early recurrence and death

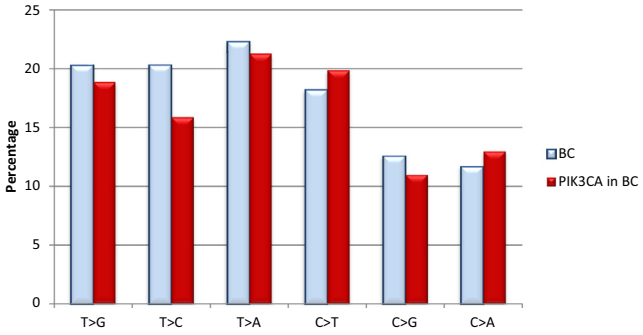

Fig. 3. Comparison of substitutions profile (as percentage from total mutations) of PIK3CA and other genes on several loci of chromosomes 3, 10, 14 in the studied Egyptian BC patients.

were associated with PIK3CA exon 9 mutations, and favourable outcomes were associated with exon 20 mutations (Barbareschi et al., 2007). It is noteworthy that the clinical consequences of exon 20 PIK3CA-mutated tumors might vary according to the status of molecular markers in BC, namely ERα, PR, and erythroblastic oncogene B receptor Tyrosine Kinase 2 (ERBB2) (Loi et al., 2010).

The current study focused on the mutational pattern of PIK3CA exon 20. It was found that 66.2% of studied BC patients had somatic mutations of PIK3CA in exon 20, most of them showed multiple mutations. This high prevalence of PIK3CA mutations came in agreement with previous studies reporting that PIK3CA mutations were common in BC patients, predominantly at exon 20 (Mangone et al., 2012). On the other hand, some studies reported lower incidence of the PIK3CA mutations. For example, (Mosele et al., 2020) showed that only 22% of the overall population presented a PIK3CA mutation (Mosele et al., 2020).

Mutational signature analysis (Fig. 1) for patients with PIK3CA exon 20 mutations revealed predominance of COSMIC v2 Signature 3 (30.1%). COSMIC v2 Signature 3 is reported to correlate with homologous recombination deficiency (HRD). Homologous recombination is a conserved pathway that synchronizes accurately the repair of double-stranded DNA breaks and primarily functions during the late S and G2 phases of the cell cycle (Ceccaldi et al., 2016).

In the current study, most patients with this signature were HER2 + . Similarly, COSMIC v2 Signature 3 was reported to be correlated with high mutation burden through several mechanisms including cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), programmed death-ligand 1 (PD-L1), immune suppressor indolea-

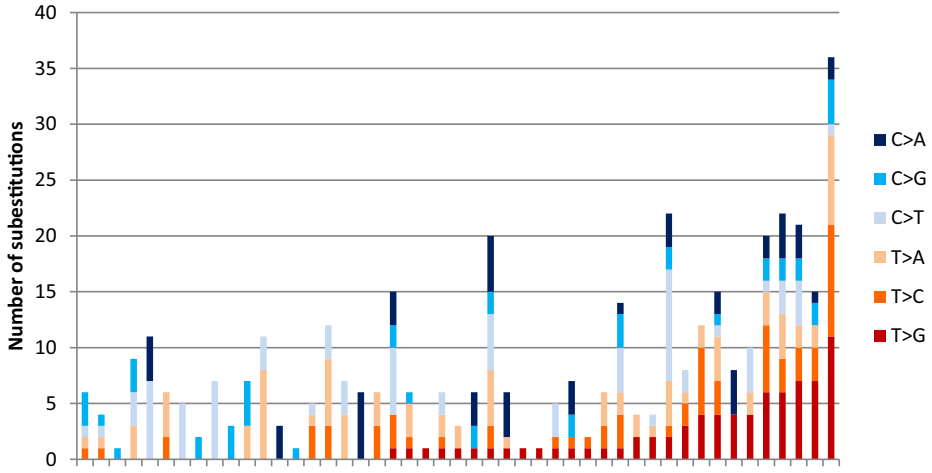

Fig. 2. Substitutions in the studied Egyptian BC patients. The most prevalent nucleotide substitutions were C > T and T > A.

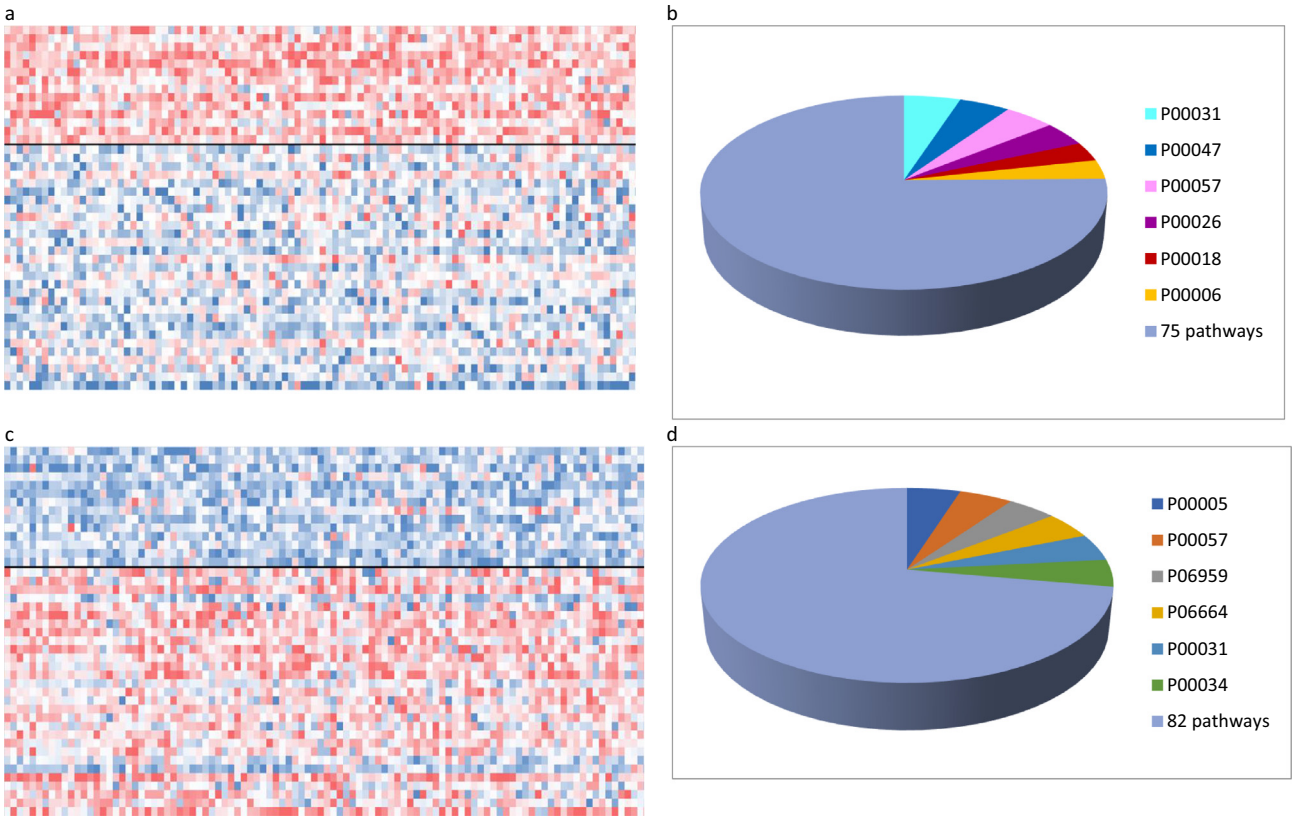

Fig. 4. (**a**) Heat map of DEGs showing the top 100 overexpressed genes in dataset GDS4053. **(b):** The most upregulated pathways (Inflammation mediated by chemokine and cytokine signalling pathway (P00031), PDGF signalling pathway (P00047), Wnt signaling pathway (P00057) Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha mediated pathway (P00026), EGF receptor signalling pathway (P00018), Apoptosis signalling pathway (P00006)). **(c):** Heat map of DEGs showing the top 100 under-expressed genes in dataset GDS4053. **(d):** The most downregulated pathways (Lower right) (Angiogenesis (P00005), Wnt signalling pathway (P00057), CCKR signalling map (P06959), Gonadotropin-releasing hormone receptor pathway (P06664), Inflammation mediated by chemokine and cytokine signaling pathway (P00031), Integrin signalling pathway (P00034)). BC patients without PIK3CA mutations were shown in the upper panel of each heat map. P value of expression levels between normal and tumor cells were corrected using Benjamini-Hochberg method.

mine 2,3-dioxygenase-1 (IDO-1) and strong Interferon-gamma (IFN-γ) signature in both basal and HER2-enriched BC subtypes (Min et al., 2020).

In the current study, the second common signature was COSMIC v2 signature 9. Formerly, signature 9 was detected in many cancers other than BC, but recently it was reported in BC, Its levels were shown to be comparable to, or even exceed, the levels of previously known BC signatures (Huang et al., 2018). This signature correlates with failure in polymerase eta (Pol η), an error-prone polymerase that participates in activation induced cytidine deamination process (Nik et al., 2016). Several genes stimulate Pol η-dependent DNA synthesis including breast cancer 2 (BRCA2), where Pol η-mediated DNA repair initiates recombination-associated DNA synthesis, suggesting its crucial role in BC (Buisson et al., 2014).

Upon studying the correlation of signature 9 and clinicopathological parameters in the current study, it was found that the patients were predominantly ER-/ PR-. This could suggest a correlation with poor prognosis and low DNA repair capacity (DRC) values. ER signalling works through increasing proliferation and initiating mutations when DNA repair is dysregulated. Such dysregulation is the main cause of breast carcinogenesis with aggressive phenotype (Matta et al., 2016). Also, ER was revealed to have a bidirectional relationship with tumor protein 53 (TP53), which affects many repair pathways. ER was reported to shift the PI3K pathway associated repair signalling (Serra et al., 2011). Dysregulation of growth factor receptors, including ER, can stimulate several signalling pathways, this could lead to hormonal therapy resistance (Miller et al., 2010). Collectively, these studies could explain the correlation of ER-/ PR- with signature 9. The Polη failure characteristic of signature 9 could be acting synergistically with impaired ER signalling to hinder DNA repair leading to increased mutational burden.

In the current study, COSMIC v2 Signature 1 was observed, suggesting an endogenous mutational process which is initiated by deamination of 5-methyl-cytosine (Pfeifer, 2006).

Interestingly, 4 of the studied patients showed COSMIC v2 Signature 22 which is basically related to aristolochic acids exposure (AAs). Aristolochic acids could be present as a contaminant in some herbs used in traditional medicine. The carcinogenicity of AAs to human was confirmed by the detection of highly persistent DNA-AAs adducts in both animal models exposed to AAs and in AAs-intoxicated patients (Han et al., 2019). Without efficient DNA repair, A > T transversions and C > T transition were reported on the non-transcribed gene strand of TP53 tumor suppressor gene in high frequency, contributing to AAs carcinogenicity (Hoang et al., 2013). Sources and duration of exposure to AAs in Egyptian BC patients needs further investigations.

All patients with signature 22 were ER+, raising questions about the potential response to hormonal therapy given the role of estrogen in renal diseases along with the nephrotoxicity of AAs in patients with signature 22 (Gluhovschi et al., 2012). The small number of such cases in the current study leaves the questions open for further studies.

Signatures 2 and 13 are connected to Activation Induced Deaminase/ apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (AID/APOBEC) family of cytidine deaminases (Nik-Zainal

et al., 2012). None of the cases in the current study showed signatures 2 or 13, suggesting that APOBECs might not be of a major role in BC mutation in Egypt. Yet this point needs further studies on the mutational pattern in Egyptian BC patients from several governorates.

In addition to AAs signature, two other environmental mutagens signatures were observed in this study. The first signature was of 4-aminobiphenyl, which is an aromatic amine that undergoes metabolic activation to form a reactive mutagenic species such as lacto-peroxidase that binds to DNA forming adducts. These DNA adducts could lead to BC (Sheikh et al., 2017).

The second signature was dibenzo (a, l) pyrene signature. Dibenzo (a, l) pyrene is a product of tobacco smoke. The relation between BC and tobacco smoking has been controversial. Human cancer mutation signatures related to tobacco smoking are potentially induced by polycyclic hydrocarbons and subsequent DNA adducts production (Alexandrov et al., 2013). As none of the study cohort was a smoker, there might be a potential role of second-hand smoking in the BC development.

Regarding nucleotide substitution patterns (Fig. 2), T > A transversion was the most common followed by C > T transition. T > A transversion was reported to be common in cancers and likely due to direct and indirect outcomes of activation-induced cytidine deaminase mutagenesis and trans lesion DNA synthesis by error-prone polymerases (Kasar et al., 2015). On the other hand, C > T transition was formed by replicative polymerases favouring the insertion of adenine facing the non-informative templates (Strauss, 2002).

In the current study, high mutation rate was found to correlate with T > C and T > G substitutions. These substitutions were reported to be clock-like, as the number of mutations belonging to this signature correlates with age of diagnosis in many cancer types (Alexandrov et al., 2016). Signature 9 is distinguished by T > G transversions at ApTpN and TpTpN trinucleotides. These transversions were reported to be commonly generated by oxidative DNA damage (Rünger, 2008). Co-occurrence of specific nucleotide substitution was found in the current study between T > G and T > C. This could be indicative of oxidative DNA damage accumulation with age.

Furthermore, significant moderate to weak correlations of T > C with C > G and T > G with C > G were observed. These patterns are also described in single base substitutions (SBS37) signature and related to error-prone DNA polymerases Pol η (Shen et al., 2019). However, no enough data is available about the prevalence of SBS37 signature in BC to date.

While the distribution of COSMIC v2 Signatures was the same in long and short DFS groups (as a marker of treatment response), it was noted that patients with predominant C > A substitution were in the short DFS group (DFS < 2 years). C > A mutations could be due to exposure to 4- aminodiphenyl, and dibenzo (a,I) pyrene which are tobacco smoke carcinogens (Alexandrov et al., 2016). Signature of 4-aminodiphenyl, and dibenzo (a,I) pyrene exposure was found in environmental toxin signature profiling of the current study cohort.

In the current study, the nucleotide substitution pattern of PIK3CA in BC is similar to the general nucleotide substitution pattern in BC (Fig. 3). This is consistent with the previous study suggesting that the nucleotide substitution pattern is related to etiology rather than a particular gene. Thus, mutations in BC could be following a specific nucleotide substitution pattern regardless of the passenger genes (non-driver) involved (IACR, 2021).

To evaluate the impact of PIK3CA mutations in BC, an expression array public dataset was studied to determine differentially expressed genes (DEGs) and differentially upregulated and downregulated pathways.

Upregulated pathways in patients with PIK3CA mutations include platelet-derived growth factor (PDGF) signalling pathway. In most tumors, angiogenesis pathways are activated involving vascular endothelial growth factor (VEGF), PDGF, fibroblast growth factors (FGFs), interleukin (IL) 8, and angiopoietin signals. The induction of angiogenesis and vessel integrity are regulated by the PI3K-Akt pathway through several mechanisms (Karar and Maity, 2011).

Wingless-type mouse mammary tumor virus, (MMTV), integration site family member (Wnt) signalling was found to be dysregulated in cases with PIK3CA mutations. Wnt signalling pathway is vital for regulation of proliferation, growth, differentiation, and survival from embryo stage. Crosstalk of complex pathways of Wnt signalling has been detected leading to various effects in different types of cancer (Ford et al., 2009). Therefore, activation of some Wnt pathway genes has an important consequence of PIK3CA mutations in BC in conserving crosstalk between the PI3K/Akt and Wnt pathways (Baryawno et al., 2010). On the other hand, other Wnt pathway genes are downregulated in patients with PIK3CA mutations, most of these genes were previously reported to be Wnt pathway inhibitors (Li et al., 2016).

Gonadotropin-releasing hormone receptor pathway and integrin signalling pathway were found to be downregulated in cases with PIK3CA mutations. This is unexpected as these pathways are generally upregulated in cancer (Hanker et al., 2017). Upon analyzing the list of downregulated genes in these pathways, it was observed that they are regulatory genes in several pathways including PIK3R1 and ras-related C3 botulinum toxin substrate 1 (RAC1). Underexpression of phosphoinositide-3-kinase, regulatory subunit 1 (PIK3R1) might be related to PI3K pathway activation and stimulation tumor development and progression. It was reported that PIK3R1 underexpression was detected in 61.8% of BC tumors and poor metastatic free survival (Magdalena et al., 2013).

6. Conclusions

Somatic mutations of PIK3CA exon 20 could play a crucial role in BC prevalence in Egypt. The similarity between the nucleotide substitution patterns of PIK3CA exon 20 and general nucleotide substitution pattern in BC suggesting that the nucleotide substitution pattern is related to etiology rather than to particular gene. Most common mutational signature in the studied BC Egyptian patients were COSMIC v2 Signature 3 and 9, related to HRD and Pol η, respectively. Signature 9 cases were ER-/ PR- suggesting its correlation with poor prognosis. Aristolochic acids exposure could be implicated in BC development and potential response to hormonal therapy. Error-prone polymerases could play crucial role in BC as the most common substitution was T > A transversion. Also, the co-occurrence of T > G and T > C and their relation to the total mutations number could indicate oxidative DNA damage accumulation with age. PIK3CA mutations could drive BC via disrupting several pathways including RAC1, PDGF, Wnt and integrin signalling.

Authors' Contributions

All authors participated in the study conceptualization, design, funding, analysis of the data and interpretation of results. IHI conducted experiments, conducted the bioinformatics study, HGA and NNLA conducted experiments and drafted the manuscript. HSA supplied blood samples of breast cancer patients and clinical examination results (including tumor size, grade, immunohistochemical tests and patients' survival data). All authors reviewed and approved the manuscript.

Ethical Approval

This study protocol was reviewed by the Institutional Review Board (IRB) of Al-Azhar University Hospital (Damietta) and approved by the Scientific Research Ethical Committee of Faculty of Pharmacy (Girls), Al-Azhar University, Code no. 51/2015.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data availability

Data are available on researcher request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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