Research Square

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Investigation of Hotspot Mutations of PIK3CA in Iranian Women with Sporadic Breast Cancer

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Abstract

Studies show that PI3K/AKT signaling pathway is frequently altered in many malignancies including breast cancers. A major part of mutations that altered this pathway are located in *PIK3CA* gene. We investigated the frequency of hotspot *PIK3CA* mutations in 90 Iranian women patients with breast cancer and examined possible association between mutations, P110α tissue expression and microvessel density (MVD). Mutation was detected in 15 samples (16.7%). Fifty-eight (64.4%) samples showed overexpression in P110α. For CD34, 13 patients had a score of + 3 (high MVD), 36 patients had score 2+ (intermediate MVD) and 41 patients had score 1+ (low MVD). We have identified no significant relationship between PIK3CA, p110α protein expression and CD34.

Introduction

Breast cancer (BC) is the most common malignancy in women and an important cause of cancer related mortalities among women worldwide [1–5]. One of the most important steps in tumor progression is angiogenesis .When a tumor reaches the size of 2 mm, it needs more oxygen and nourishment so it starts to induce new blood vessels from the endothelium of the existing vasculature, malignant cells penetrate into the blood circulation and may therefore lead to metastasis, which is

necessary but not sufficient to the development of metastasis [5–7]. Angiogenesis is regarded as a putative target for cancer treatment [8, 9]. Micro-vessel density assessment is a common technique to quantify angiogenesis in breast cancer. In this technique immunohistochemical staining of new blood vessels is performed in fixed biopsies of specimens [10–12].

Data show that increased MVD is associated with a higher incidence of metastasis and a poor prognosis in different types of malignancies, including breast cancer [5]. Although significant improvements in breast cancer treatment have been achieved within the last decades, the need for novel therapeutic approaches aiming

at specific tumorigenic cells and their key oncogenic pathways

has become obvious [13].

One of the most important pathways which has a key role in angiogenesis is PI3K/AKT/mTOR [6]. Cellular pathway begins with attachment of growth factors to tyrosine kinase receptors (RTK) such as HER2, MET, EGFR and IGFR. The pathway continues by phosphorylation of phosphatidylinositol 4, 5 bisphosphate (PIP2) by PI3K and form phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) as a second messenger that controls many cellular processes like proliferation, survival, cell growth and angiogenesis [14–19].

PI3Ks are heterodimer proteins that are classified into three classes (I–III) based on their structures and specificity to substrate. Class I PI3K has two subgroups: IA and IB. Class IA PI3K is referred to as “PI3K” and is involved the most in cancer pathogenesis. PI3K consists of two subunits: a catalytic domain

named p110 and p85; a regulatory domain. Catalytic subunit has three isoforms: α, β and ¥. P110α is encoded by *PIK3CA* gene which is located in 3q26 and is frequently mutated or amplified in many tumors such as breast cancer [14, 20, 21]. *PIK3CA* gene mutations enhance enzyme activity of catalytic subunit of PI3K. Thus, *PIK3CA* is considered as an oncogene, and may be used as a potential therapeutic target in cancer. The frequency of *PIK3CA* mutation in breast cancer is about 8 to 40%. Over 80% of mutations are located in 3 'hot spots', and they are reported in many cancers like colorectal, gastric, brain, breast and lung cancers. Two of these mutations are in exon 9; located in the helical domain (E542K, E545K), and one of them is located in exon 20 (H1047R) and in kinase domain of p110α [13, 14, 22–30].

Materials and Methods

- **Tissue sampling**

Tissue samples were selected from patients referred to Mehrad hospital- Tehran, Iran, regardless to age, stage, grade or histopathological subtype. Tissue paraffin blocks were already prepared; 90 samples were selected for the study. DNA extraction was done using The QIAamp DNA FFPE Tissue Kit Cat No. /ID: 56404

- **PCR**

primers were designed for hotspot mutation area. Because of a pseudogene in chromosome 22 that has >98% homology with exons 9 in *PIK3CA* , primers were designed with a mismatch at 3' end of the exon 9 reverse and forward primers between the gene and the pseudogene to specifically amplify the gene.

Primer details (table 1) and condition of PCR (table 2) are described as below:

Table 1

Table 2

PCR products were sequenced with forward primers. The Sanger sequencing results were analyzed by codon code aligner software (v.6.0.2).

- **Immunohistochemistry**
  Immunohistochemistry for p110α was performed with the Abcam kit (Abcam ab71090) according to the protocol. Four μm thick sections obtained from paraffin embedded tissue blocks. The slides were deparaffinized and rehydrated in graded alcohols and then boiled in 10 mM Tris, 1 mM ethylenediamine tetra acetic acid (EDTA) buffer (pH 9.0) in a microwave oven for 10 min at high level and 5 min at low level for antigen retrieval .Sections were incubated with 1 % bovine serum albumin (BSA) and Tris-buffered saline (TBS) and 10% normal serum for 1 hour, to reduce the background. The slides were then incubated overnight with anti-p110α antibody 1:200 dilution at 4°C. In the second day, after blocking the endogenous peroxides with 5 % hydrogen peroxide in methanol, sections were

washed in water and incubated with secondary antibody (DAKO: k5007) for 1h. Coloring reaction was performed using 3, 3’-diaminobenzidine (DAB) (Sigma-Aldrich: 91-95-2), and the tissue slides were stained with hematoxylin. Negative control was applied by elimination of primary antibody. Scoring performed based on the number or the intensity of the stained cells: negative (0), weak (1+), moderate (2+), and high staining (3+).CD34 immunohistostaining was also done using Dako, Clone: QBEnd 10 kit according to manufacturer's protocol to evaluate tumor angiogenesis. For scoring of CD34, with a light microscope under low magnification, 4 hotspots were determined, and new micro-vessels were counted under high magnification and the average MVD of that sample was determined. The results were reported as: score 1+: Low MVD (0- 19.9), score 2+: Moderated MVD (20-29.9) and score 3+: high MVD (more than 40). Normal controls (breast with benign changes) and negative controls (staining without primary antibody) were also included in the experiment.

- Statistical analysis
  Statistical analysis was done using SPSS 19.0 software. Relationship between hotspot mutations and PI3K protein expression, PIK3CA gene mutations and MVD and PI3K protein expression with MVD were studied using either a chi-square test or Fisher’s exact test. A P-value of <0.05 was considered statistically significant.

Results

- **Sanger Sequencing**

PIK3CA mutations were identified in 15 (16.7%) of 90 cases. Two of mutations were E542K c:1624 G>A and located in exon 9, 13 of mutations were H1047R c:3140 A>G and located in exon 20.

Figure1

- **Immunohistochemistry results**

We estimated expression of p110α and MVD in 90 breast carcinoma tissues. For expression of p110α the immunohistochemical scores of 0 and 1+ staining were considered as negative, and 2+ and 3+ staining were considered positive. Based on the results, 58 out of 90(64.4%) of breast carcinoma samples have positive staining for p110α, and 32 samples (35.6%) were negative.

In MVD assessment 13 (14%) patients had score3+ (high MVD), 36 (40%) patients had score 2+ (intermediate MVD) and 41(45%) patients had score 1+ (low MVD). Negative (0), weak (1+), moderate (2+) and high staining (+3) of p110α protein in breast carcinoma tissue are shown in figure 2.

Figure 2
To determine association between mutations of the PIK3CA gene and PI3K expression, we performed a chi-square test on all 90 patient samples. Our analysis showed no statistically significant correlation between PIK3CA gene mutations and PI3K expression (p =0.84), suggesting that the level of PI3K expression is not associated with PIK3CA mutations.

Although *PIK3CA* expression was considered to increase likelihood of increased MVD as a factor for angiogenesis and metastasis, MVD was not significantly associated with the PIK3CA mutations and expression. ($p = 0.797$, $p = 0.354$, respectively).

Discussion

The PI3K/Akt pathway is one of the most important intracellular signaling pathways. It has an important role in regulation of apoptosis, proliferation, and tumorigenesis. Abnormal activation of this pathway can lead to different types of malignancies. Studies mark PI3K as the most implicated protein in cancer. 97% of mutations of this pathway are located in PIK3CA gene. PIK3CA gene encodes P110a, the catalytic subunit of PI3K. Over 80% of mutations in the PIK3CA gene are localized in exon 9 (helical domain) and exon 20 (kinase domain) in three hotspots. E542K and E545K in exon9 and H1047R in exon 20.The mutation rate of PIK3CA has a wide range in various cancers (see Catalogue of Somatic Mutations in Cancer, http://www.sanger.ac.uk/genetics/CGP/cosmic).This high mutation rate has made it an attractive topic to the researchers.

In our study, about 16.4% of patients with breast cancer carried PIK3CA mutations. These results are slightly lower than findings in other populations (Table 3); As Reinhardt et al. who found a 26.7% prevalence of PIK3CA in German population. They also concluded that it was significantly more frequent in hormone receptor positive-HER2 negative, as well as G1 and G2 tumors, but it was not associated with recurrence free survival [36]. Mosele et al. on the other hand, found PIK3CA mutated-HR+/HER2- tumors to be resistant to chemotherapy and have a poor outcome, while it was associated with better overall survival when occurred in triple negative tumors [37]. PIK3CA mutation was found in 35.7% of breast cancer patients in Martínez-Sáez et al.'s study, who used therascreen PIK3CA mutation assay in Spanish population. They also concluded that PIK3CA mutation rates are lower in triple negative tumors, but the main PIK3CA mutations (H1047R, E545K, E542K, N345K, and H1047L) were equally distributed among all subtypes [38].

Table 3

There have been two other studies about PIK3CA in Iran. They have reported a frequency of 14%[39] and 40%[40] for PIK3CA mutations. The difference between our study and the latter could be a result of these factors: They have investigated all the mutations in exons 9 and 20, while we examined just hotspot mutations (E542K, E545K and H1047R); In addition, pseudogene of exon 9 could affect the results.

Table 4

We also observed that mutations in exon 20 (86.6%) are more frequent than mutations in exon 9 (13.4%), in concordance with other studies that have shown a higher frequency in mutations of exon 20 than exon 9 [29].

Our study shows the overexpression of PI3K in 64.4% of patients. These results are in keeping with previous findings in breast cancer and other types of cancers [41-44].

The correlation between PI3K expression and PIK3CA mutations have been assessed in three other studies in breast and colorectal cancer, which none of them were statistically significant as our data shows [41, 43, 45]. This inconsistency may be caused by the high occurrence of PI3K overexpression against a much lower PIK3CA mutation rate.

We also didn't find any correlation between MVD (CD34) as an indication of angiogenesis and over expression of PI3K or mutation of PIK3CA. This could be due to the role of other proteins of this pathway like HER2, PTEN as a tumor suppressor gene, and other pathways in angiogenesis.

The association of PIK3CA and prognosis of breast cancer was also studied by authors.

In conclusion, these results suggest that overexpression of PI3K and PIK3CA gene mutations have an important role in the initiation and progression of tumors. Thus, both PI3K expression level and PIK3CA mutations can be putative biomarkers for diagnosis, and the PI3K/AKT pathway may be a good candidate for targeted therapies.

Declarations

Acknowledgments

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Declaration of interest

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References

1. Youlden DR et al (2014) Incidence and mortality of female breast cancer in the Asia-Pacific region. Cancer biology Med 11(2):101
2. Parkin DM et al (2005) Global cancer statistics, 2002. Cancer J Clin 55(2):74–108
3. Mathers C (2008) The global burden of disease: 2004 update. World Health Organization
4. DeSantis CE et al (2016) Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. Cancer J Clin 66(1):31–42
5. Stewart B, Wild CP (2017) World cancer report 2014. Health
6. Jiang B-H, Liu L-Z (2008) *PI3K/PTEN signaling in tumorigenesis and angiogenesis.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. 1784(1):150–158

7. Vaupel P, Mayer A, Höckel M (2004) *Tumor Hypoxia and Malignant Progression*, in *Methods in Enzymology*. Academic, pp 335–354
8. Weis SM, Cheresh DA (2011) Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med 17(11):1359–1370
9. Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473(7347):298–307
10. Uzzan B et al (2004) *Microvessel Density as a Prognostic Factor in Women with Breast Cancer.* A Systematic Review of the Literature and Meta-Analysis. 64(9):2941–2955
11. Martin L et al (1997) Examining the technique of angiogenesis assessment in invasive breast cancer. Br J Cancer 76:1046
12. Toi M et al (1995) Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. Breast Cancer Res Treat 36(2):193–204
13. Meier-Stiegen F et al (2014) Analysing the mutational status of Pik3ca in circulating tumor cells from metastatic breast cancer patients. Oncol Res Treat 37:5
14. Mukohara T (2015) PI3K mutations in breast cancer: prognostic and therapeutic implications. Breast Cancer: Targets Therapy 7:111
15. Vara JÁF et al (2004) PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 30(2):193–204
16. Carnero A et al (2008) The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. Curr Cancer Drug Targets 8(3):187–198
17. De Luca A et al (2012) The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. Expert Opin Ther Targets 16(sup2):S17–S27
18. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-kinase–AKT pathway in human cancer. Nat Rev Cancer 2(7):489–501
19. Karar J, Maity A (2011) PI3K/AKT/mTOR pathway in angiogenesis. Front Mol Neurosci, 4
20. Karakas B, Bachman K, Park B (2006) Mutation of the PIK3CA oncogene in human cancers. Br J Cancer 94(4):455–459
21. Zhao L, Vogt PK (2008) Class I PI3K in oncogenic cellular transformation. Oncogene 27(41):5486–5496
22. Kalinsky K et al (2009) PIK3CA Mutation Associates with Improved Outcome in Breast Cancer. Clin Cancer Res 15(16):5049–5059
23. Samuels Y et al (2004) High frequency of mutations of the PIK3CA gene in human cancers. Science 304(5670):554–554
24. Campbell IG et al (2004) Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 64(21):7678–7681

25. Bachman KE et al (2004) The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 3(8):772–775
26. Arsenic R et al (2014) Analysis of PIK3CA mutations in breast cancer subtypes. Appl Immunohistochem Mol morphology 22(1):50–56
27. Tserga A et al (2016) Mutation of genes of the PI3K/AKT pathway in breast cancer supports their potential importance as biomarker for breast cancer aggressiveness. Virchows Arch 469(1):35–43
28. Samuels Y, Waldman T (2010) *Oncogenic mutations of PIK3CA in human cancers*, in *Phosphoinositide 3-kinase in Health and Disease*. Springer, pp 21–41
29. Samuels Y, Velculescu VE (2004) Oncogenic mutations of PIK3CA in human cancers. Cell Cycle 3(10):1221–1224
30. Aleskandarany MA et al (2010) PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. Breast Cancer Res Treat 122(1):45–53
31. Stemke-Hale K et al (2008) An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 68(15):6084–6091
32. Loibl S et al (2014) PIK3CA mutations are associated with lower rates of pathologic complete response to anti–human epidermal growth factor receptor 2 (HER2) therapy in primary HER2-overexpressing breast cancer. J Clin Oncol 32(29):3212–3220
33. Majewski IJ et al (2015) PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2–targeted therapies in breast cancer. J Clin Oncol 33(12):1334–1339
34. Papaxoinis G et al (2015) Significance of PIK3CA mutations in patients with early breast cancer treated with adjuvant chemotherapy: a hellenic cooperative oncology group (HeCOG) study. PLoS ONE 10(10):e0140293
35. Branch K (2015) *Prognostic value of PIK3CA mutation status, PTEN and androgen receptor expression for metastasis-free survival in HER2-positive breast cancer patients treated with trastuzumab in adjuvant setting.* proteins (EGFR3, EGFR4, EGFR1). 6(8):9–11
36. Reinhardt K, Stückrath K, Hartung C, Kaufhold S, Uleer C, Hanf V, Lantzsch T, Peschel S, John J, Pöhler M, Bauer M, Bürrig FK, Weigert E, Buchmann J, Kantelhardt EJ, Thomssen C, Vetter M (2022) PIK3CA-mutations in breast cancer. Breast Cancer Res Treat 196(3):483–493 Epub 2022 Oct 24. PMID: 36279023; PMCID: PMC9633529
37. Mosele F, Stefanovska B, Lusque A, Tran Dien A, Garberis I, Droin N, Le Tourneau C, Sablin MP, Lacroix L, Enrico D, Miran I, Jovelet C, Bièche I, Soria JC, Bertucci F, Bonnefoi H, Campone M, Dalenc F, Bachelot T, Jacquet A, Jimenez M, André F (2020) Outcome and molecular landscape of patients with PIK3CA-mutated metastatic breast cancer. Ann Oncol 31(3):377–386 Epub 2020 Jan 24. PMID: 32067679
38. Martínez-Sáez O, Chic N, Pascual T, Adamo B, Vidal M, González-Farré B, Sanfeliu E, Schettini F, Conte B, Brasó-Maristany F, Rodríguez A, Martínez D, Galván P, Rodríguez AB, Martinez A, Muñoz M,

Prat A (2020) Frequency and spectrum of PIK3CA somatic mutations in breast cancer. Breast Cancer Res 22(1):45. 10.1186/s13058-020-01284-9. PMID: 32404150; PMCID: PMC7222307

39. Gharbi S, Faghihi M, Tavassoli M (2011) A novel PIK3CA hotspot mutation in Isfahanian breast cancer patients. Cancer Invest 29(4):313–317
40. Azizi Tabesh G et al (2017) The High Frequency of PIK3CA Mutations in Iranian Breast Cancer Patients. Cancer Invest 35(1):36–42
41. Palimaru I et al (2013) Expression of PIK3CA, PTEN mRNA and PIK3CA mutations in primary breast cancer: association with lymph node metastases. SpringerPlus 2(1):464
42. Jehan Z et al (2009) Frequent PIK3CA gene amplification and its clinical significance in colorectal cancer. J Pathol 219(3):337–346
43. Abubaker J et al (2009) PIK3CA alterations in Middle Eastern ovarian cancers. Mol Cancer 8(1):51
44. Akagi I et al (2009) Overexpression of PIK3CA is associated with lymph node metastasis in esophageal squamous cell carcinoma. Int J Oncol 34(3):767–775
45. Kim J-Y et al (2017) Clinical implications of genomic profiles in metastatic breast cancer with a focus on TP53 and PIK3CA, the most frequently mutated genes. Oncotarget 8(17):27997

Tables

Table 1
primer information

Exon	Sequence(5'->3')	Product Size	Annealing temperature
20	Forward: TCATTTGCTCCAAACTGACCA Reverse: GGTCTTTGCCTGCTGAGAGT	388	60
9	Forward : GGCTAACTTCAGCAGTTACTATTCTGTGAC Reverse : GAAAAAGCATTTAATGTGCCAACTACC	616	58

Table 2
PCR condition

94°C For 4min	**Initial Denaturation**	
94°C For 40s	Denaturation	35 Cycles
60°C For 30s	Annealing	
72°C For 40s	Extension	
72°C For 5 min	Extension	
4°C	Hold	

Table 3
frequency of PIK3CA mutations in different studies of breast cancer

Author	**Year**	**Mutation frequency**
Stemke et al [31]	**2008**	**26%**
Arsenic et al[26]	**2014**	**16%**
Sibylle Loibl et al[32]	2014	21.4%
Ian J. Majewski et al[33]	2015	23%
George Papaxoinis et al[34]	2015	23%
Adamczyk et al[35]	2015	15%

Table 4
frequency of PIK3CA mutations in different studies of breast cancer in Iran

Author	**Year**	**Mutation frequency**
Gharbi, S., M et al[36]	**2011**	**14%**
Azizi Tabesh, et al [37]	**2017**	**40%**
Our study	2017	16%

Figures

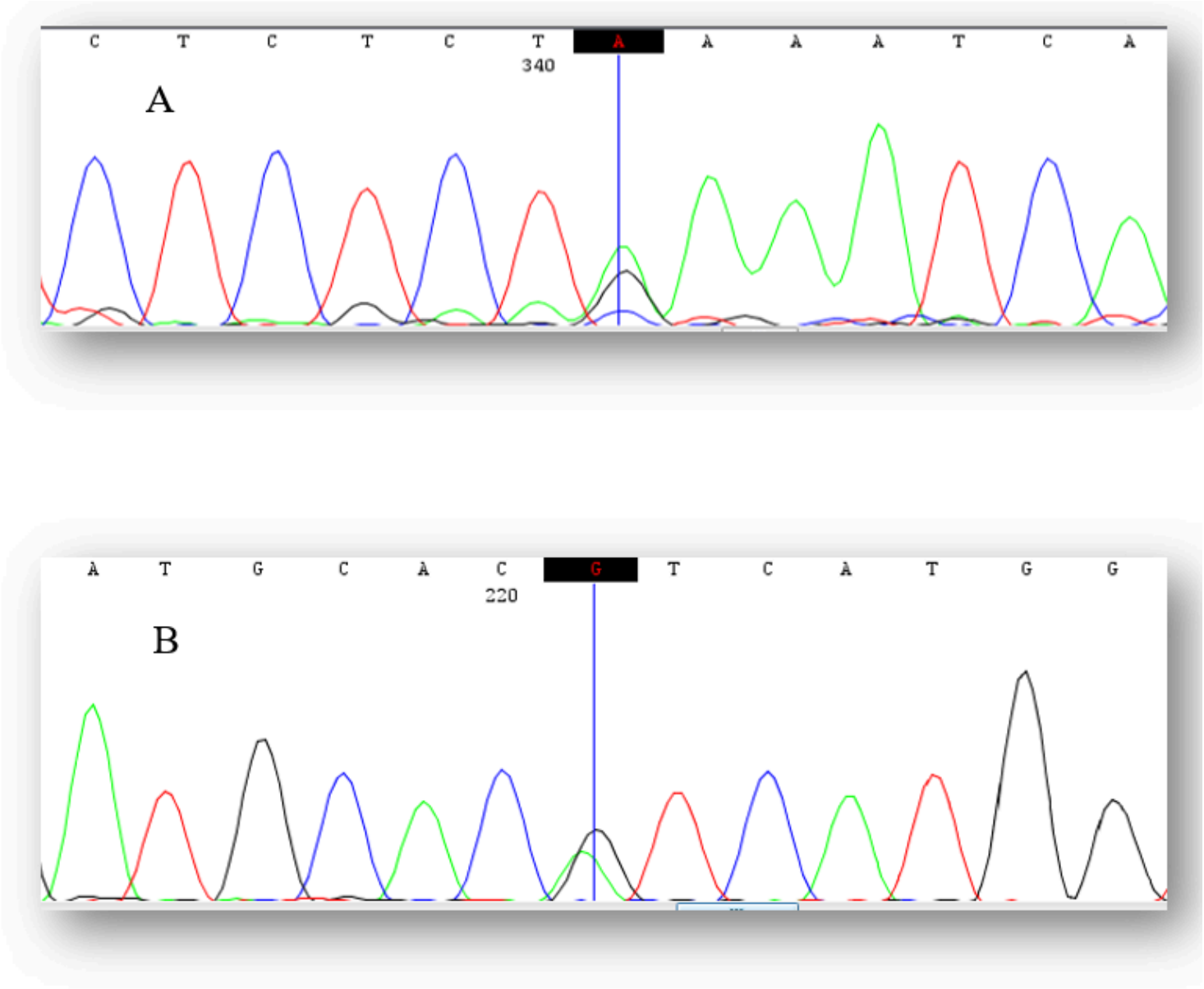


Figure 1

Illustrative electropherograms of PIK3CA mutations. A, an E542k (c: 1624 G>A) missense mutation in exon 9. B, an H1047R (c: 3140 A>G) missense mutation in exon 20.blue lines shows the position of the mutations.

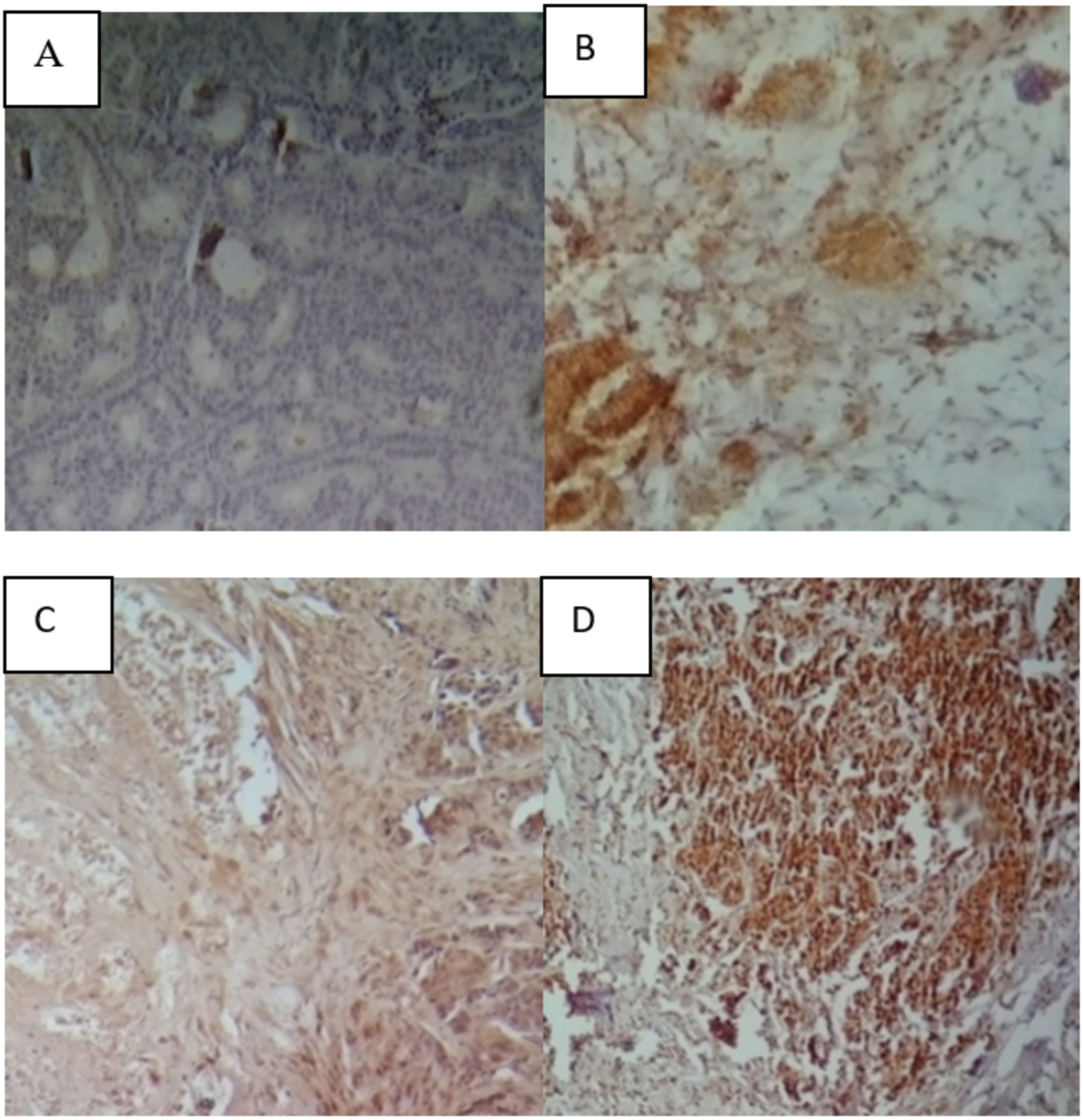


Figure 2

A) negative (0), B) weak (1+), C) moderate (2+) and D) high staining (+3) of p110α protein in breast carcinoma tissue