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INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/11234
DOI URL: <http://dx.doi.org/10.21474/IJAR01/11234>



RESEARCH ARTICLE

PROTEOMIC ANALYSIS OF ERBB2 - A POTENTIAL BREAST CANCER MARKER: AN INTEGRATED BIOINFORMATICS STRATEGY

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Manuscript Info

Manuscript History

Received: 20 April 2020
Final Accepted: 25 May 2020
Published: June 2020

Key words:-

Breast Cancer, ERBB2, Tyrosine Kinase Receptor, Protein-Protein Interaction, Post Translational Modification.

Abstract

Background: Breast cancer is one of the most common malignant cancers in women around the world. Until now, despite great improvements in treatment the high incidence rate and mortality worldwide are exponential. Thus identification of novel molecular cancer drivers and evaluation of existing breast cancer biomarkers is a critical step.

Objective: This study aims to explore one of the driven genes-ERBB2 receptor- a good predictive cancer biomarker, its specific TK receptor domain mutations, biological pathways, and oncoproteins challenging the apoptotic process, involved in the progression of breast cancer using *In silico* approach.

Methods: We used the multiple advanced bioinformatics tools performing structure-based Domain architecture analysis with predicted functional protein association of ERBB2 gene and the role of post-translational modification-- in instigating TK receptor domain mutations providing an understanding of the underlying mechanism of tumor invasion and metastasis.

Results: Considering all approaches as complementary the TK inhibitors and the role of the ERBB2 gene can improve drug targeting prediction strategy highlighting the importance of driver genes. Our results suggest that functional validation is a positive prediction and we provided answers to some of the imperative questions although important concerns in the field remain unresolved like the implications for therapeutics etc. for future biological and clinical accomplishments.

Conclusion: The evaluation of the existing cancer candidate gene and the interacting proteins and pathways particularly ERBB2- downstream signaling pathway has the potential to generate novel hypotheses in oncology. Thus provide a baseline to identify target protein-based pathways involved through wet experiments.

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Introduction:-

The high prevalence of breast cancer mortality has recently been reported among Saudi Arabian women. The incidence of morbidity is increased 10 –fold in the past few years with the reported prevalence of 21.8%, having >9.48% in the Qassim region [1-2]. It is the second most prevalent and leading cause of death in women and the fifth reported cancer deaths worldwide due to late diagnoses and poor treatments [3-5].

The advancements in genomic technologies strongly boost up the research studies. In silico approach is used in the present study, to uncover the molecular basis of breast cancer-associated – the "ERBB2" gene. This is a membrane tyrosine kinase gene that is overexpressed and gene amplified in about 20% characterized by prompt tumor growth, increased disease progression with decreased survival rate. Recently been a useful treatment target for breast cancer, but predictive of poor clinical outcome [6].

ERBB-2 is a receptor tyrosine-protein kinase also recognized as TKR1, HER-2, CD340, NEU, HER-2/neu, MLN 19, and NGL. Gene ID: 2064 - the gene code depicts that this gene belongs to the epidermal growth factor (EGF) family. Plays a significant role in cell growth, production, existence, and motility, any mutation of ERBB2 will cause cancers and the over-expression will cause activation of downstream signaling. The underlying mechanism involved in ERBB2 oncogenic activity is a complex signaling network, as both homo/heterodimers upon ligand binding tightly regulate migration, metastasis, and invasion of the malignant cell [7-8]. Humanized monoclonal antibodies and receptor tyrosine kinase inhibitors are the recently proposed anti- ERBB2 therapies for breast cancer treatment - whose overexpression alters cell proliferation and survival [9-10].

The effectiveness of protein-protein interactions in determining gene-disease associations, usually proposed by computational approaches, reveals two sources of information either to predict associated genes with diseases and linkage intervals/physical interaction networks. Different techniques uncover the associations between gene and disease taking an integrative approach such as; gene expression, biological pathways, protein sequences, and numerous phenotypic traits of diseases. The assorted mechanism of post-translational modifications that modulate protein's cellular function is an additional target of the current study, critically involved in the regulation of cell signaling at a molecular level [11-14].

The present study is designed to explore the association of the ERBB2 gene with breast cancer by using advanced in silico analysis. The preceding bioinformatics tools such as SMART, KEGG pathway, STRING, Cytoscape, LENS, NEXT/UNIPROT, and PTM code 2 databases were used to find the protein-protein interactions, their metabolic pathways, and related post-translational modifications of the ERBB2 gene.

Methods:-

The present study depicts an in silico approach to evaluate an in-depth analysis of ERBB2-a potential marker for breast cancer. The amino acid sequence of ERBB2 was retrieved from the genome database at NCBI. The complete protein data and reference proteome sets were determined using the Uniprot/ Swissprot/Pfam/SMART database. The biological associations of differentially expressed protein and their functional interactions (knowledge base) were identified by STRING 8.3 (<http://string-db.org/>) tool. The identified interactions were then subjected to Cytoscape software (www.cytoscape.org/) for visualization. Out of so many plugin analysis tools available in Cytoscape, CytoHubba is used for further analysis of the degree of top 10-protein node and genes, hub genes, and also the Molecular Complex Detection-MCODE, another plugin was used to identify the modules of the PPI network using the standard criteria.

To identify associated pathways -the KEGG- database is used (<http://www.genome.jp/kegg/>) along with SMART- the Simple Modular Architecture Research Tool (<https://smart.embl.de/>) that predicts molecular pathways with their associated biological functions.

SMART (Genome) and GPS-5.0 are used to predict potential post-translational modification of the ERBB2 using the FASTA sequence. For the prediction of the S-nitrosylation site (<http://csb.cse.yzu.edu.tw/SNO>) GPS-SNO 1.0 on the medium threshold while NetPhosK1.0 Program (<http://www.cbs.dtu.dk/services/NetPhos>) is used to predict the phosphorylation sites and the YinOYang 1.2 (<http://www.cbs.dtu.dk/services/YinOYang/>) is used for computational prediction of O-glycosylation sites in ERBB2 proteins [4, 14-16].

Results:-

The ERBB-2 gene sequence was obtained directly from the NCBI- (National Center for Biotechnology Information) database. ERBB2 receptor tyrosine-protein kinase protein sequence (FASTA) consists of 1255 amino acids and 3768 nucleosides (**Fig.1a RefSeq**)>hsa:2064 K05083 [EC:2.7.10.1] while the chromosomal location of the ERBB2 gene has been actuated by the gene card on chromosome 17 at position 17q12 (**Fig.1b**). Sequence Alignments (Homo sapiens-P04626) of ERBB2 was obtained by the Swiss Model Repository (**Fig.2**) along with the ERBB2 predicted Protein Structure.

ERBB2- SMART- Domain Architecture Analysis (**Fig.3**) shows seven different domains. Pfam-receptor L domain-2, FU-furin-like cysteine-rich domain-3, TM-transmembrane receptor domain-1, and a Tyrkc-tyrosine kinase- analyzed by PTM code 2- (an online resource) of the interacting proteins having functional associations with both known and predicted PTMs. There are 83 post-translational modifications annotated (33 propagated) in the given gene (**Fig.3**). Five observed PTMs sites with their counts correspond to 54-phosphorylation, 16-ubiquitination, 7-N-linked glycosylation, 5-nitrosylation, 1-acetylation. ERBB2-Protein Interaction Network-via-PTM code 2 algorithm analysis (**Fig.4**) display 53 interacting proteins, 43 proteins with PTM association, and 32539 total PTM associations for this ERBB2.

The predicted ERBB2 post-translational modifications (**Fig.5**) were used for the prediction of the S-nitrosylation site, demonstrating three nitrosylation sites in the given gene while the prediction of phosphorylation sites depicts the highest count. The potential phosphate alteration represented in the graph as vertical lines are the phosphorylated Ser residues (Red); the phosphorylated Thr residues (Green); the phosphorylated Tyr residues (Blue). The pink flat line is the threshold for alteration potential. **Fig.5b**-Yin0Yang 1.2: Predicted O-(beta)-GlcNAc sites in the ERBB2 receptor protein sequence show both O-GlcNAc and NetPhos predictions. O-GlcNAc potentials represented by green vertical impulses while blue wavy horizontal line depicted the threshold for predicted O-GlcNAcylated sites conversely, the YinYang sites are indicated by small red stars on the green impulses.

The system-wide quantitative proteomic study of breast cancer tumors (**Fig.6a**) is suggestive of the association of ERBB2 and single-nucleotide variations (SNVs) in breast cancer (BioMuta) [17]. Exploring further the cancer type vs. nsSNV frequency plot, suggest the highest frequency of variation of ERBB2 gene mutation in breast cancer (**Fig.6b-c**). The integrated BioMuta dataset indicates the frequency of nsSNVs for ERBB2 on the y-axis and the type of cancers on the x-axis.

The overlapping network of all canonical pathways implicated in ERBB2 signaling representing interconnection between altered canonical pathways obtained by Cytoscape (WP673-ERBB Signaling Pathway- Homosapiens) highlighting the involvement of signaling pathway in cancer by overexpression of ERBB2 (**Fig.7**).

Protein Interactome reveals converging molecular pathways of Breast Cancer Gene- ERBB2. The PPI network of ERBB2 created by the Cytoscape for visualization (**Fig.8a**). 32 nodes and 66 edges by Cytohubbe, also correspond to the interaction of top 10 functional protein Hub nodes of ERBB2. The degree of connectivity between top 10 genes in the PPI network appears in red-orange includes KRAS, HRAS, GRB2, ERBB2, SHC1, PIK3R2, PTPN11, GRB7, EGF and PIK3CG. While the Protein-Protein Interaction Network via STRING predicted the ten functional protein partners that interact with the ERBB2 comprising EGF, GRB2, PTPN11, HSP90AA1, SHC1, NRG1, GRB7, HRAS, KRAS, and BTC (**Fig.8b**).

The specified functions of the predicted protein partners include; EGF (Pro-epidermal growth factor) known to be involved in stimulating different epithelial and epidermal tissue growth. GRB2 (Growth factor receptor B2) that functions as an adapter protein between cell surface receptors and signaling pathways. PTPN11 (Tyrosine-protein phosphatase non-receptor type 11) a downstream regulator of signal transduction tyrosine kinases receptors dephosphorylation. HSP90AA1 (Heat shock protein 90-alpha A1) a molecular chaperone involved in the regulation of signal transduction and control different cell cycle stages of specific target proteins. SHC1 (transforming protein 1) a signaling adapter that links signaling pathways and growth factor receptors activation. NRG1 (Pro neuregulin-1) a membrane-bound direct tyrosine kinase receptors ligand for ERBB3-4 and co-receptor ERBB1-2 causing receptor activation via tyrosine phosphorylation. GRB7 (Growth factor receptor-bound protein 7) interacts with several receptor kinases to regulate downstream signaling via the cytoplasmic domain. HRAS- (GTPase-HRas) involved in signal transduction by Ras protein activation. KRAS (GTPaseKRas) a Ras protein having GTPase activity primarily

regulate cell proliferation and also involved in stimulating oncogenic events. BTC (Probetacellulin) is an established growth factor and a potent mitogen for various epithelial/ vascular cells.

Cytoscape–Network module of PP1 merged ERBB2 pathways (**Fig.9**) illustrate the Strings- Breast cancer PPI network having all the available functional proteins involved in the disease (a) along with the substantial module (b) displays a total of 7nodes and 15 edges in the ERBB2 construct Protein-Protein Interaction network by Cytoscape-Mcode analysis., also show subnetwork and close functional protein partners. While the Interactome of the merge ERBB2 pathways: WP673-ERBB2 Signaling & WP-4262-Breast Cancer Pathway (c) using Cytoscape-Merge tool analysis, to find the Interactome between breast cancer and the ERBB2.

Figure.1

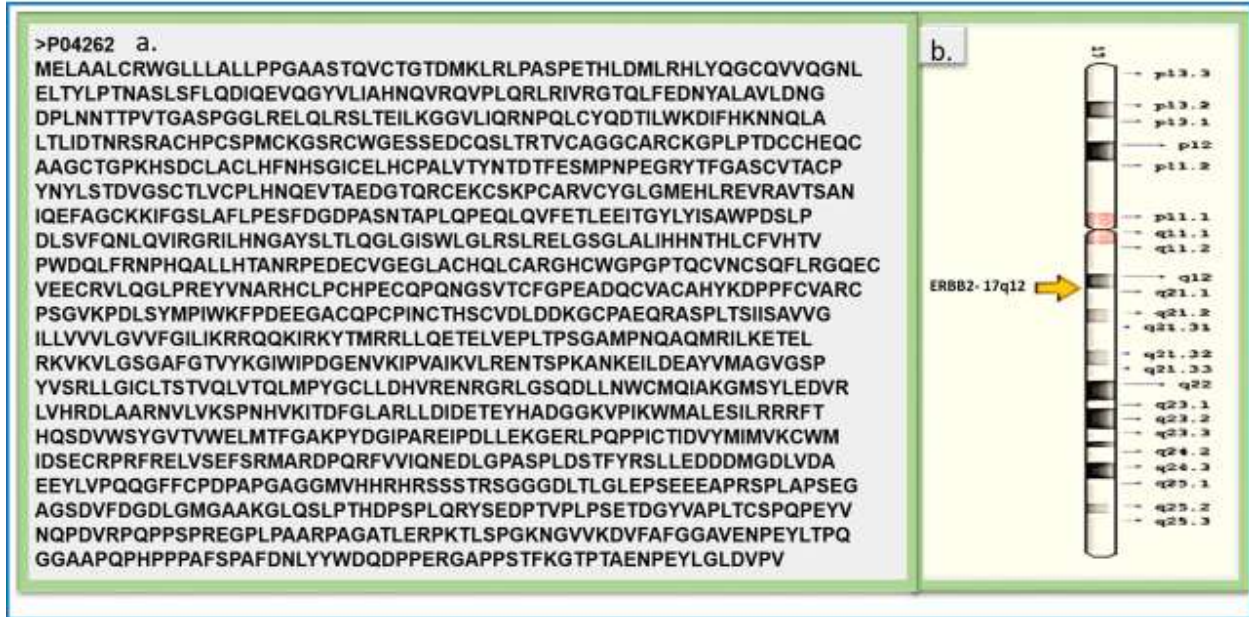


Figure 1:- ERBB2 receptor tyrosine-protein kinase Protein Sequence (FASTA) - 1255aa/3768nt (RefSeq) >hsa:2064 K05083 [EC:2.7.10.1] via UNIPROT. b. Chromosomal Location of ERBB2 Gene -17q12.

Figure.2

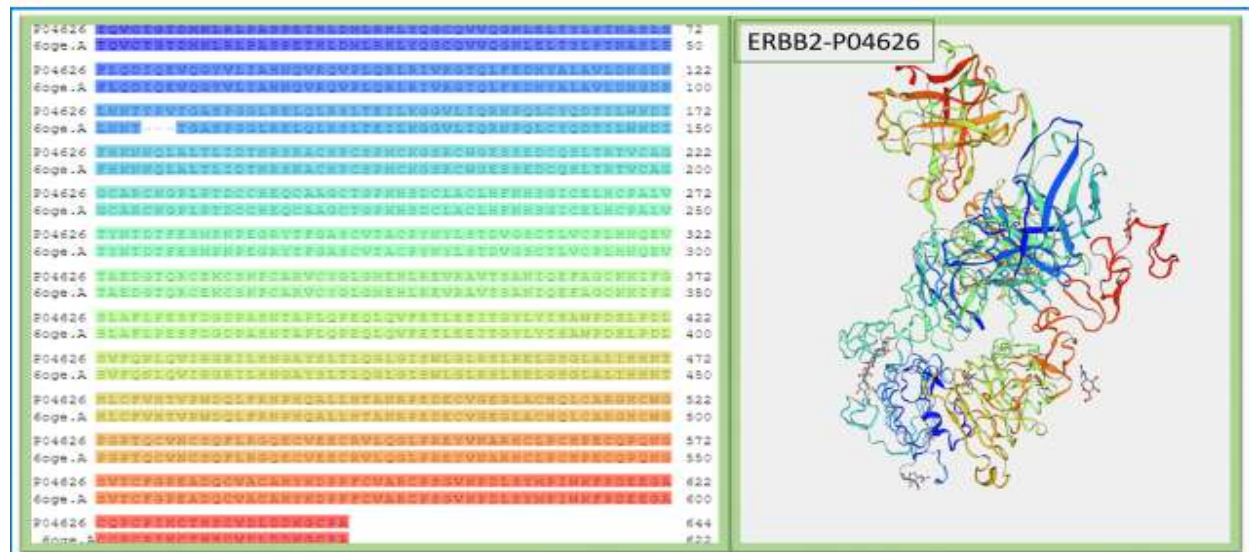


Figure 2:- ERBB2 Sequence Alignments (Homo sapiens-P04626) Via SWISS-MODEL Repository. Illustrate

Receptor tyrosine-protein kinase ERBB-2 sequence alignment each conserved domain is given in boxes with different colors, numbers refer to the amino acids separating the various domains. The colors complementary to the sequence alignment showed in ERBB2 secondary structure prediction using PDB id: 6oge. (Identical sequences: Homo sapiens: X5DNK3 -Cryo-EM Structure of HER2)

Figure.3

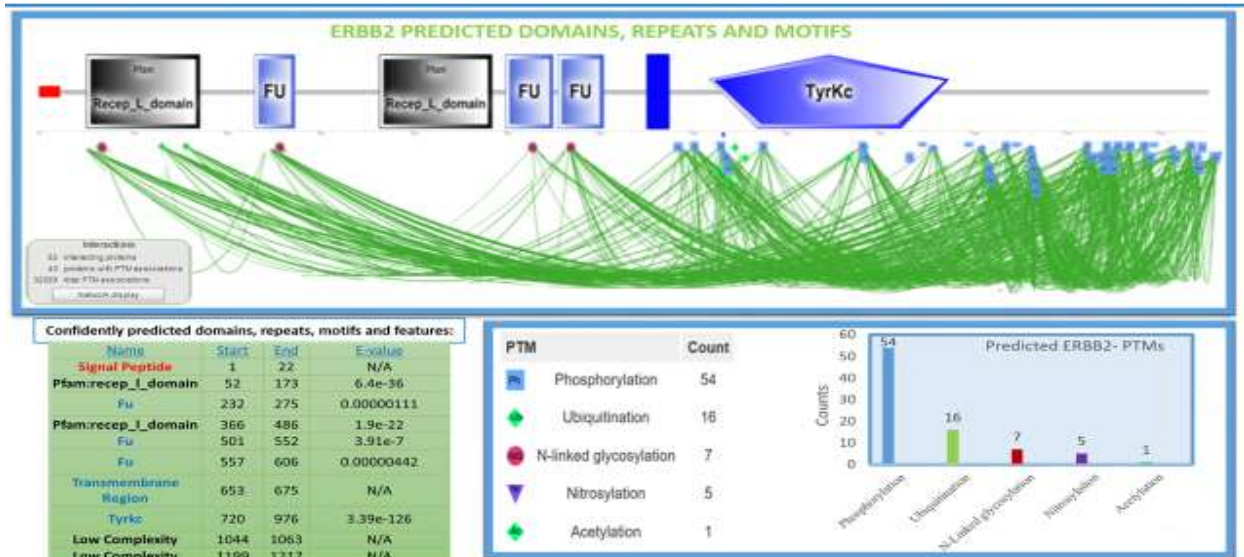


Figure 3:- SMART analysis of ERBB2.Predicted Protein Domains (Homo sapiens-P04626) and their associated Post Translational Modifications Using PTM code 2. Diagram illustrates the ERBB2 predicted protein domains, repeats and motifs, count, and sites of post-translational modifications. The green lines indicate the interaction network with different post-translational modifications.

Figure.4

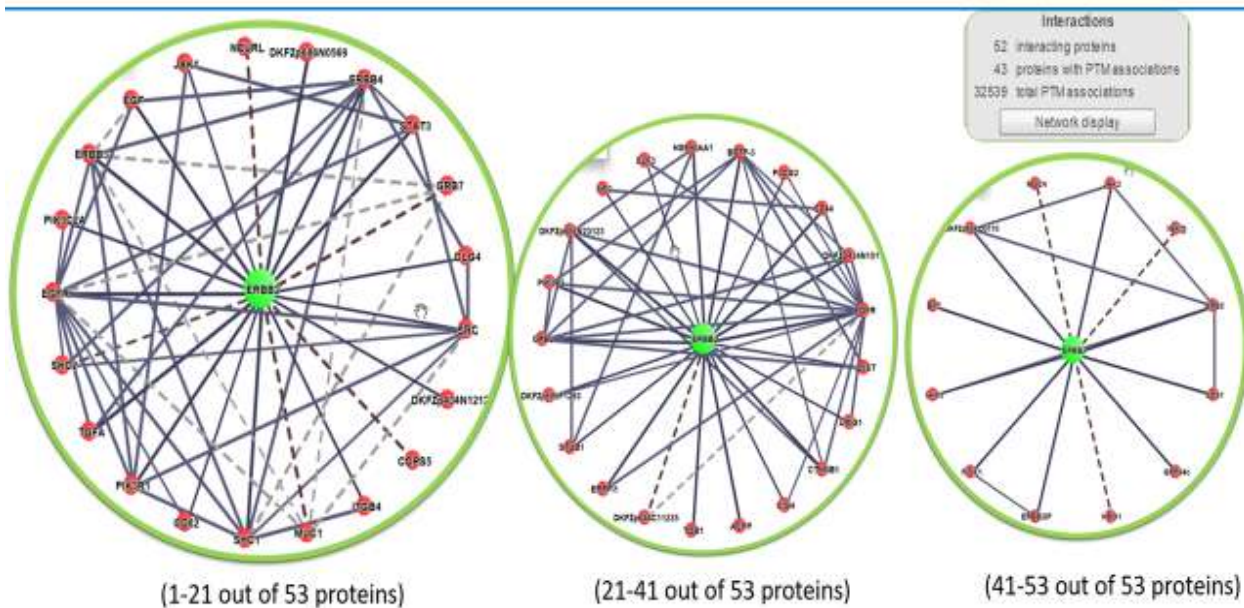


Figure 4:- ERBB2 protein interaction Network. PTM Code 2 algorithm analysis demonstrates 3 clusters based on the number of interactions in the large network of protein-protein interactions. The network displays the interacting proteins represented by red color while the green circle represents the Hub protein - the ERBB2.

Figure.5

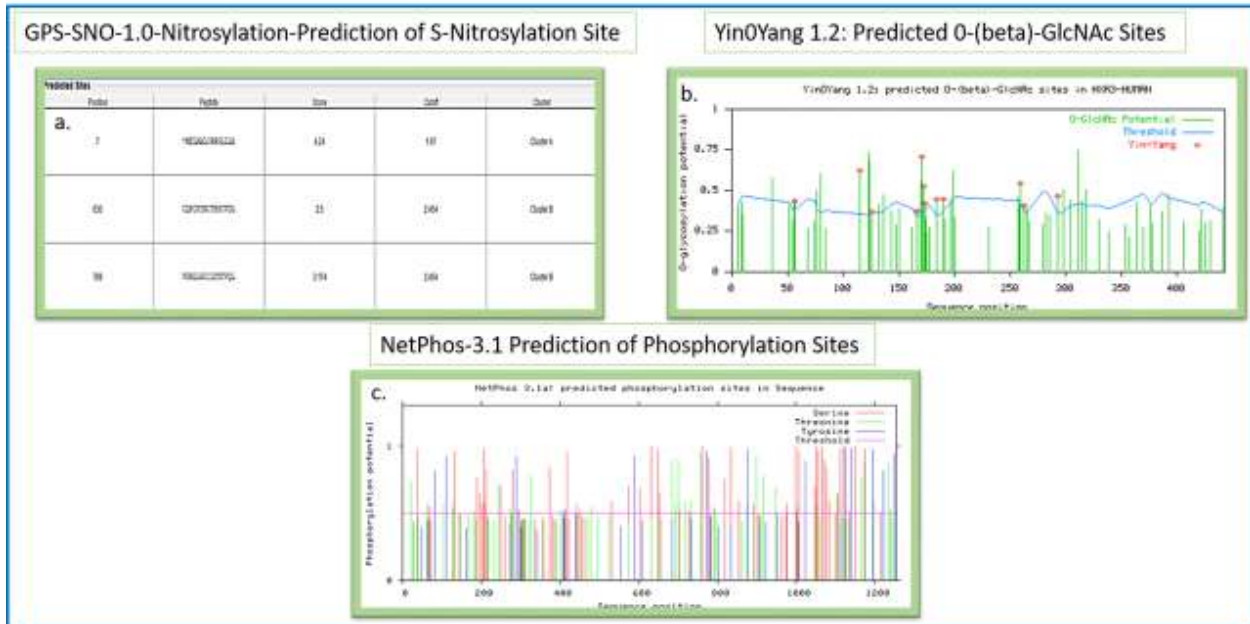
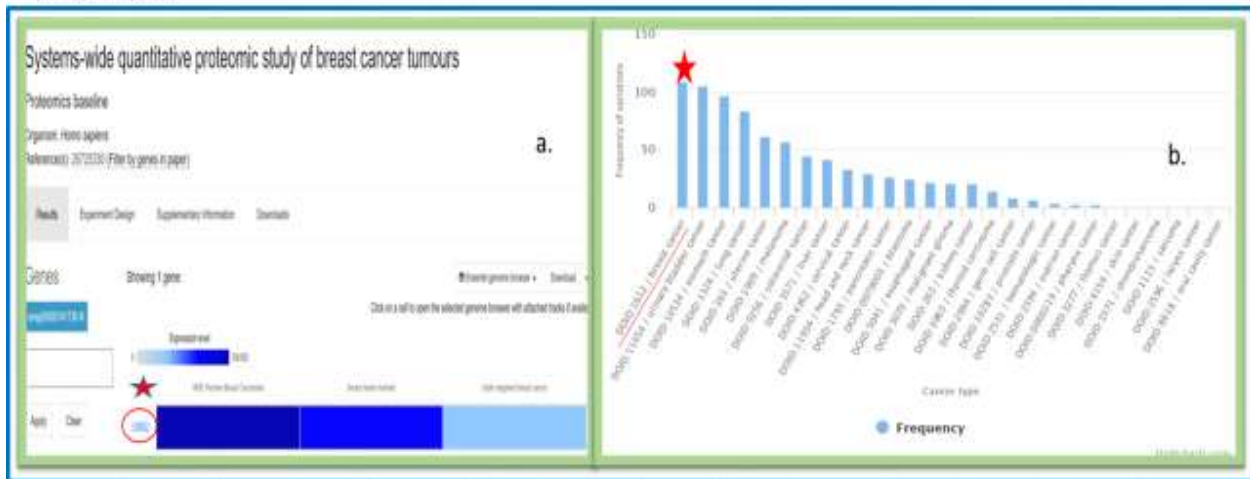


Figure 5:- ERBB2 Predicted Post Translational Modifications. a) Nitrosylation b) Phosphorylation & c) Glycosylation Via GPS-5.0.

Figure.6



C.	Chr	Chr Position	Ref Base	Alt Base	Protein Position	Ref Residue	Alt Residue	Cancer Type	UBERON ID	Frequency	Source
	17	37,863,275	C	T	36	P	S	DOID:1612 / breast cancer	UBERON:0000310	3	COSMIC

Figure 6:- ERBB2 and single-nucleotide variations (SNVs) in breast cancer via BioMuta. b. Cancer type vs. nsSNV frequency plot. c. DOID:1612/Brest Cancer.

Figure.7

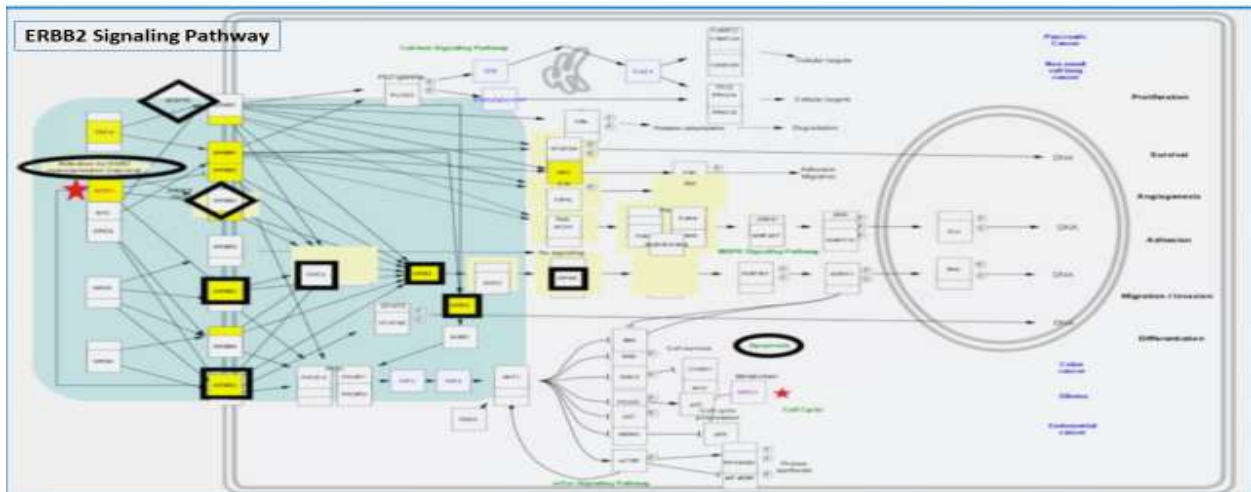


Figure.7:- An overlapping network of all canonical pathways implicated in ERBB2 signaling. Overlapping network representing interconnection between altered canonical pathways obtained by Cytoscape (WP673-ERBB Signaling Pathway- Homosapiens). Activation of ERBB2 overexpression finally leads to apoptosis represented by Oval Shape while Square and diagonal shapes indicate the disease-associated proteins subjected to result in a list of altered canonical pathways. Black lines represent interconnections in pathways therefore an increase in the number of Black lines arising from a node signifies an increase in pathway interconnections. Green color indicates different pathways and blue colors indicate their involvement in different types of cancers. All proteins should be mentioned with yellow represents the first neighbors of ERBB2 and white represents other interacting functional proteins involved in these pathways.

Figure.8

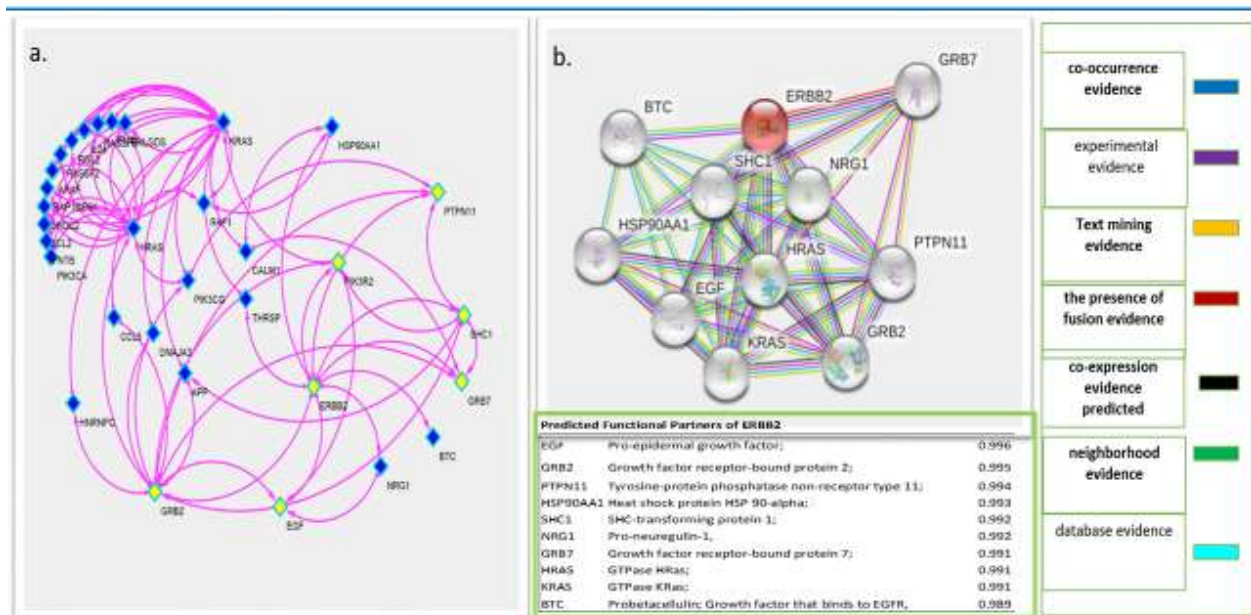


Figure 8:- Protein Interactome Reveals Converging Molecular Pathways of Breast Cancer Genes ERBB2. Pink lines represent edges; green-yellow nodes indicate first neighbors of the selected node while blue nodes represent other interacting partners. b.ERBB2Protein-Protein Interaction Network-via-STRINGS, the nodes represent the functional protein partners while the color lines indicate edges suggesting the type of interaction.

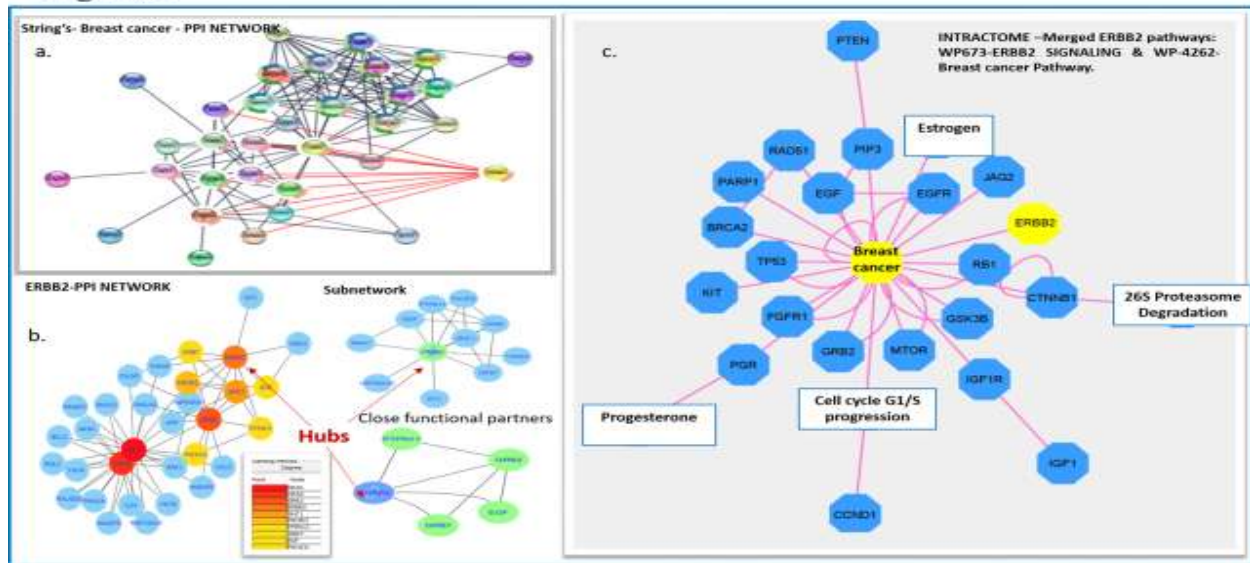
Figure.9

Figure 9:- Cytoscape–Network module of PPI Merged ERBB2 pathways. a. Illustrate the Strings- Breast cancer PPI network. b. ERBB2 –PPI network, subnetwork, and close functional partners. c. Interactome of the merge ERBB2 pathways: WP673-ERBB2 Signaling& WP-4262-Breast Cancer Pathway. The node represents proteins and the connecting lines (edges) the functional links. The color of each protein red, orange, yellow, blue represents the first neighbor's interactions while the edges connecting the nodes indicate the first neighbors interacting molecules.

Discussion:-

The current study evaluated the integration of advanced computational technology with the cancer biomarkers particularly the ERBB2 gene and its involvement with breast cancer. As a high prevalence of breast cancer mortality among Saudi Arabian women confirmed by the descriptive analysis[1], this approach may help to establish novel breast cancer markers and identify potential therapeutic targets, essentially needed for the early diagnosis and treatment of breast cancer.

The prediction of individual gene function made easier by using computational analysis that aids in finding cellular interaction networks, and their associated cellular physiological mechanisms. The activation of ERBB2 associated downstream signaling pathways [7,9], will help in understanding the molecular mechanisms leading to breast cancer. Genomic location, function, catalytic activity, Protein structure and domains, interactive functional partner's, and post-translational modifications of the ERBB2 gene have been accumulated by Bioinformatics data analysis.

ERBB2 is recognized as an oncoprotein associated with the family of EGFR-epidermal growth factor receptor, amplification, or protein overexpression has been a recognized mechanism involved in breast cancer. Approximately 20% of breast cancers invasion and metastasis reported correlated with poor patient survival [18-19]. The physiological implications of ERBB2 include the regulation of molecular and genetic events during embryonic development and in adult tissue maintenance. The underlying mechanism of cell adhesion and migration in ERBB2-positive tumor invasion and metastasis are still obscure despite enormous investigations. Deregulated ERBB2 pathways help in the development of ERBB2-targeted therapies in breast cancer pathogenesis [20].

The protein-based approach may directly reflect cellular functions and functional networks involved in breast cancer rather than only genes and transcripts. Moderate affinity and promiscuity of recognition still a proven challenge for targeting Protein-Protein Interactions (PPIs), despite the enormous research in the discovery of anticancer drugs using ERBB2/p130C. However, resistance to ERBB2-targeted treatments was reported due to the contribution of various factors[7,9-10].

One important factor is phosphorylation, which is vital for protein-protein interactions and initiation of the signaling pathways as evident from the cytoplasmic domain of ERBB2 having numerous phosphorylation sites particularly

tyrosine phosphorylation. The homo/heterodimerization binding with the EGFR family leads to the activation of different ERBB2 pathways including downstream signaling pathways known to be involved in overexpression/amplification of the gene. The SMART diagram represents a summary of the ERBB2 protein domains and different post-translational modifications(**Fig.3**), particularly the predicted hyperphosphorylation (**Fig.5**). Suggesting that this ERBB2 gene contains a protein receptor tyrosine kinase that regulates outgrowth and stabilization of peripheral microtubules (MTs) by binding with its potential ligand - GP30,thus the associated signaling pathway elicited phosphorylation, causing inhibition of GSK3B at the cell membrane to inhibit the phosphorylation of antigen-presenting cells (ACP)[7,21].

SMART- Domain Architecture Analysis of ERBB2 displays seven different domains particularly the Tyrkc-tyrosine kinase-1 domain, any mutation in this region is associated with different types of cancer. The system-wide proteomic study of breast cancer tumors suggests the highest frequency of variation of ERBB2 gene mutation and single-nucleotide variations (SNVs) in breast cancer. The oncogenic activity depends on several types of somatic mutations in the ERBB2 kinase domain mainly encourage the phosphorylation of cellular signaling proteins in different types of cancers particularly in breast cancer, as these mutations are more proficient to prevent the effect of apoptosis or have altered catalytic activity[10,22]. These oncogenic insertions activate mutations that may cause auto-inhibition by conformational alteration that subsequently alters the Adenosine triphosphate (ATP)-binding cleft to enhanced kinase activity by increasing ATP binding attraction and turnover number thus contributes in the subsequent phosphorylation events.

The molecular subtypes of breast cancer indicate that specific hormone receptors such as estrogen and progesterone subtypes and ERBB2 gene manifestations subsequently lead to breast cancer[23]. According to the reported research, greater than 10% frequency of all breast cancers have somatic alterations only in three of the genes TP53, PIK3CA, and GATA3 however, ERBB2 activates the PI3K/AKT and the RAS/RAF/MAPK pathways and induce cell development and survival[8,24-26].

ERBB2 activating mutations particularly in the tyrosine kinase region reported in the preclinical data suggest the response of HER2 tyrosine kinase inhibitors in several tumor types. However, resistance to ERBB2-targeted treatment is, unfortunately, becoming a major limitation[27-28]. Further clinical investigation is needed for an enhanced understanding of the mechanisms of ERBB2-mutational driven therapy as new therapeutic targets to improve cancer patient survival and care.

Conclusion:-

In silico approach in the current study expressed distinctive opportunities to enhance understanding of the biological function of ERBB2- a potential marker for breast cancer. The current approach of molecular networking and protein interaction of ERBB2 in breast cancer has revolutionized the importance not only of the proteins themselves but of their inter-relationships. Breast cancer is mostly caused by the ERBB2 gene mutations with the prevalence of 20% breast cancer in females, these gene-specific tyrosine kinase domain mutations activate the oncogenic activity of the gene resulting in phosphorylation of cell signaling protein thus affecting resistance to programmed cell death (apoptosis).

In conclusion, the evaluation of the existing cancer candidate gene and the interacting proteins and pathways particularly ERBB2- downstream signaling pathway has the potential to generate novel hypotheses in oncology. Thus provide a baseline to identify target protein-based pathways involved through wet experiments and can in future be investigated in more detail which can reveal new targets for treatment.

Abbreviations:

TK: Tyrosine kinase receptor

PPI: Protein-Protein Interaction

PTM: Post Translational modification

SNVs: single-nucleotide variations

ATP: Adenosine triphosphate

MTs: Microtubules

EGFR: Epidermal growth factor receptor

ACP: Antigen-presenting cells

SMART: The Simple Modular Architecture Research Tool

EGF: Pro-epidermal growth factor
GRB2: Growth factor receptor B2
PTPN1: Tyrosine-protein phosphatase non-receptor type 11
HSP90AA1: Heat shock protein 90-alpha A1
SHC1: Transforming protein 1
NRG1: Pro neuregulin-1
GRB7: Growth factor receptor-bound protein 7
BTC: Probetacellulin
TP53: Tumor protein p53
PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
GATA3: GATA binding protein 3
PI3K/AKT: Phosphatidylinositol 3-kinase/Protein Kinase B
RAS/RAF/MAPK Cascade: RAS/RAF/mitogen-activated protein kinase Cascade

Ethics Approval and Consent to Participate:

No ethics approval was required for the study.

Conflict of Interest:

The author(s) confirm that this article content has no conflict of interest.

Acknowledgments:-

Declared none.

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