FOXP3 and *HER-2/ErbB2* in Breast Cancer: Finding Regulatory Links

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Abstract

The transmembrane tyrosine kinase receptor, HER2/ErbB2, has been a subject of many studies owing to its predictive and prognostic values and its being a target of antibody-mediated treatment. Retrospective evidence strongly proposes that the over expression of HER2 is related to reduced disease free and overall survival in node positive and perhaps also node negative breast cancers. Prospective attempts showed that antibodies to HER2 can form tumor responses in advanced breast cancer women which overexpress this molecule. The existence of forkhead box protein 3 (FOXP3) in cells of breast cancer is a subject of debate. We have systematically analyzed the FOXP3 expression in 20 breast carcinoma samples at transcript levels. Recent progresses in understanding breast cancer crosstalk, homing processes, tumor cell dormancy, premetastatic niche formation and finally recognition of their micromilieu cytokines, growth factors and chemokinesmight provide the foundation of developing targeted treatment plans potentially rendering primary carcinomas and their metastases more responsive to chemotherapies. The current review has focused on the deep connections between HER-2 and FoxP3 in context of considering the determination of both of these molecules together to rationalize or more personalize the future research directions leading to better treatment for women with breast carcinoma.

Keywords: *Her-2, Breast cancer, Foxp3, Inhibit, Therapy, Trastuzamab.*

Introduction

Breast cancer is a major cause of concern in women worldwide. It is responsible for an appreciable amount of morbidity and mortality in women, with every 8th women showing symptoms which sooner or later progresses into disease. Human epidermal growth factor receptor 2-overexpressing (Her-2^{+}) breast Ca represents about 20–25% of breast cancer and has been shown to be more aggressive as compared to the Her2^{-}[1-2]. Her2 is a reliable biomarker whose overexpression is frequently correlated to poor prognosis and clinical outcomes. Trastuzumabor (Herceptin), the monoclonal antibody which targets the extracellular domain of HER2, has been long used in treatingpatients with Her2+ breast carcinoma. Nevertheless, many patients suffering fromHer2^{+} tumors possess de novo resistance without response to Trastuzamab, while others developed resistance after a period of Trastuzamab therapy. Her2^{+} signaling mechanism has been extensively studied to identify additional targets to design effective treatments to Her2^{+} breast cancer patientswith Trastuzumab resistance. One of such potential targets can be Foxp3 which is an important factor overexpressed in all cancers including breast cancer. Its relation to Her2+ signaling shows that it plays an important role in suppressing *Her2* oncogene. On the contrary, Foxp3 is also a fundamental partner of Treg cells which suppresses the immune response against tumor in tumor milieu. Studies have also shown that Foxp3 inhibition in Her2^{+}over expressing breast carcinomas could be correlated with better prognosis and relapse free with general survival[3]. Thus, Foxp3 can be envisaged as a pivotal target whose down regulation in tandem with other breast cancer targeted therapies can have astounding effects in women with breast carcinoma. FOXP3 and related mechanism have an essential role in breast cancer metastasis inhibition and such finding might introduce a new target to clinically control the metastasis of breast cancer in the

future. There is an inverserelation between expression of FOXP3 nuclei of breast tumor cellsand breast cancer metastasis. Thus, targeting Foxp3^{+}Treg cells in the microenvironment of cancer combined with anti-Her2 treatment can be a novel strategy against breast cancer particularly chemotherapy-resistant breast cancers. In this review, we have discussed the relation between Her2 and Foxp3 and their role in breast cancer with some recent therapies targeted against them.

HER2 in Breast Cancer: *Her*2 is apro oncogene is a member in the family of human epidermal growth factor.Other members of this family are HER-1,HER-3, HER-4 and HER-2 which are involved in more than ¼ of breast cancer cells[4].It is present on the long arm of the chromosome No. 17 and it encodes atransmembrane tyrosine kinase receptor.The Her 2 and the other member receptors have an extracellular ligand binding site that is rich in cysteine residues,a transmembrane domain and an intracellular domain having tyrosine kinase activity. HER2 has no direct ligand.

HER2 is normally present as a monomeric receptoron the surface of epithelial cells of mammary glands at a very low level. In tumor cells, HER2 expression increases manifolds. Studies have shown that gene amplification is the reason behind HER2 overexpression. HER2 receptor is activated by ligand binding followed by dimerisation of receptor throughhomomeric orheteromeric binding with other EFGR receptors. Extracellular domain rich in tyrosine residues is involved in this binding. HER. Neuregulin-1, a cell adhesion molecule, is a direct ligand for HER3 and HER4and binds with either HER3 or HER4. After ligand binding, HER2 forms heterodimer with HER4 or HER3. This dimerisation of receptor is followed by the autophosphorylation of the tyr residues of intracellular domain through the activation of tyrosine kinase activity of HER2, which turns up cascades of events that are involved in the activation of intracellular signaling pathway. Several signal transducers bind to the activated heterodimer through their src homology domain and initiate signal transducing pathways thereby, stimulating PI3/AKt or MAPK signaling pathways andvarious transcription factors and overproduction of VEGF. Various protooncogenes e.g c-fos, c-jun, c-mycetc are also activated.HER-2 over-expression thus activates multiple pathways involved in the process of metastasis[5].The heterodimer also degrades cell cycle inhibitor P27. Loss of P27 causes proliferation of cancer cell[6].

FoxP3 and Immunosuppressive mechanisms in tumor milieu: Forkhead (FKH) box proteins is a bigtranscription factor familyutilized in different cellular processes. The gene encoding (FOXP3) is present on the chromosome Xwhich containseleven coding exons and three non coding exons [25]. The characteristic feature of these transcription factors is the existence of a highly preserved(100) amino acids C- terminal FKH binding domains. The FKH with crystal structure domains showed that it has the DNA binding ability and described as a "wing-helix" owing to its similarity to the butterfly morphology. This highly conserved carboxy terminal FKH domain (a.a.338to421) is responsible for DNA binding ability. The N-termnal domain of FOXP3 comprising of 2 proline-rich regions implicated to mediate transcriptional repressions. The central region of Foxp3 protein comprises a zinc finger (A.A. 200-223) & a leucine zipper (LZ)-like motif (A.A. 240-261), thatpromotesFOXP3 homo-dimer or tetramer formation (Fig. **1**)[26].

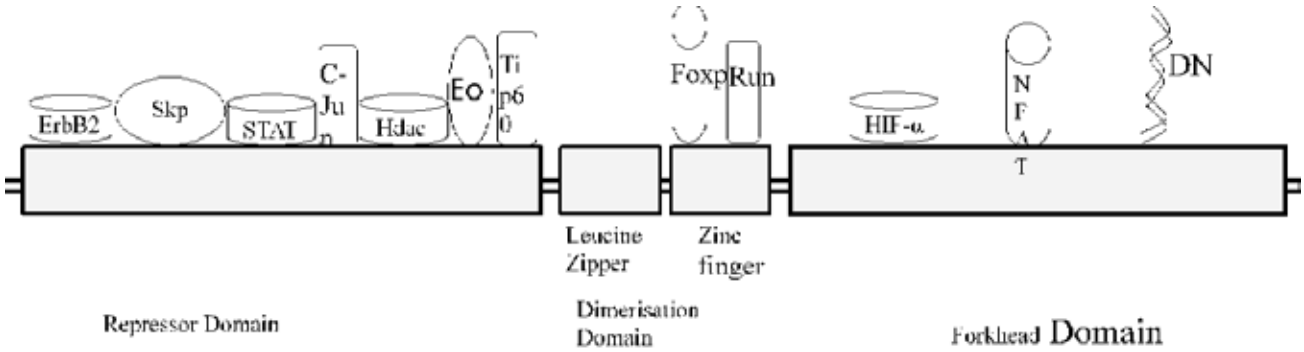

Figure 1

Figure (1): Foxp3 domains (a) N- terminal repressor domain having tumor suppressor activity inhibiting the suppression of ErbB2 (erb-b2 receptor tyrosine kinase-2), SKP2 (S-phase kinase associated protein-2), STAT3 (Signal transducer and activator of transcription 3), c-Jun (transcription factor c-Jun), HDAC7 (histone deacetylase

7), Eos (Ikaros Family Zinc Finger 4 IKFZ-4), TIP60 (Histone Acetyl TransferaseTIP60). (b) Intermediate domain or dimerization domain comprising of leucine zipper and zinc finger responsible for dimerization of Foxp3 interacts with Foxp3 and Runx1 (runt-related transcription factor 1). (c) Forkhead Domain which has DNA binding ability and interact with factors such as NFAT (Nuclear Factor Activated T-cells), the DNA or HIF-1α (Hypoxia-inducible factor1α)

The scurfy mutant mouse strain having frame shift mutation of (FKH) box transcription factor (Foxp3)sustainoing a lethal lymphoproliferative disorderscausing death within an age of 3to4 weeks. Mutations in FOXP3 resulted in various autoimmune diseases such as hepatomegaly, splenomegaly,insulitis and massive lymphatic infiltration in liver and skin. Humans (FOXP3)mutationleads to an autoimmune syndrome called IPEX (immune dysregulation, polyendocrinopathyenteropathy and X-linked disease), which is an X-linked immunodeficiency specified as insulin-dependent *Diabetes mellitus* (IDDM), thyroiditis, massive T-cell infiltrates in several organs accompanied by chronic wastings[27-28].

T *reg-mediated suppression mechanisms:* Foxp3+ CD25+CD4+ Treg (Foxp3+ Treg cells)cells mediated immune suppression is a primary mechanism in immunological self-tolerance, preventing autoimmune disease development.Treg cell development is impaired by gene mutation that encode Treg Specific transcription factor (foxp3)leading to a lethal multiple organ autoimmune diseasetermed as immuno-dysregulation, poly endocrinopathy, enteropathicand X-linked (I.P.E.X) syndrome. (Foxp-3+Treg) cell depletion by differenttechniquesresults inthe same autoimmune diseases in other normal rodent[29].Tumor tissuesof different cancers including breast are often infiltrated by huge numbers of Treg cells and this abundance of cells is usually implicated in poor clinical prognosis. The removalof (Treg) cells by implying the cell-depletion anti CD25 antibodies, by the in vivoantibody administrations histocompatible T-cell-deficient mice can efficientlyeliminate different inoculated syngeneic cancers [30]. There was an elevatedcancer infiltrated CD8+ T cells showing a strong activity to kill tumor specific, and in response to rechallengingagainst the same cancercell, caused aquicker anti-tumor response, demonstrating the existence of cancer specificimmunity[31]. A number of studies established that Treg cells removal caninduce effecient anti cancer immunity by eliminating the immunologic un-responsivenessto syngeneic cancers. Moreover, among different Treg dependent suppression mechanism, those which are essential to maintain self tolerance have the greatest effect on tumor's immunity. There are onlysmall number of molecules whose expressions are adjusted by direct or indirect Foxp3 and whose deficiencyeliminates Treg suppression functioning leading to serious autoimmunitydisorders. This candidate includes Interleukins 10, 2 and IL-2 receptors subunit, Tfg-βand C.T.L.A-4. The basicproperty of Treg cell is thehigh affinity IL-2 receptor expressions consisted of CD25, 122 and132 at highly levels,but with rare production of Interleukin-2. Thus,there is a highly dependence of Treg cellson the exogenous Interlukine-2, which is primarilyreleased by the activatedT-conv cell, for itsproliferation and survival.

IL-2 is indispensable for the function and survival of Treg cells, evident by the fact that anti-IL-2 antibodies compromise Treg survival and function, resulting in severe autoimmune disorders as produced by Treg deficiency. Foxp3combines with and attenuates NFAT and AML1 transcription factors which are necessary for IL-2 production, thereby represses IL-2 production [32-33]. Owing to high dependence of Treg cells on IL-2 and self insufficiency in its production,they absorb IL-2 from the surrounding. This limits IL-2 amount which is available for Tconv cells and as a result, leading to the activation and proliferation suppression of the Tconv cells which are essential forimmune responses[34]

C.T.L.A-4 is a high effective co-inhibition molecules that is expressed by (Treg) cell and (Tconv) cell following their activations. The C.T.L.A-4Treg-specific deletion in micecauses systemic high multiplication of (Tconv) cell and lethal autoimmunity disorders and affectsseveral bodytissue and organs[35].A heterozygous CTLA-4 mutation in humans is characterized bymany autoimmune symptom accompanied with Treg cells suppressive function's impairment[36]. CTLA-4 exhibit a high affinitiesthan (CD28)for their (CD80) and (CD86) ligands.The C.T.L.A-4 which is expressed bythe Treg cell will physically outcompete the (CD28)expressing (Tconv) cellto bindthe (CD80/CD86) on the antigen presentingcell, therefore inhibitsthecostimulation ofTconv cell[37]. The C.T.L.A-4 on Tregcells also down modulatethe (CD80/CD86)expression on the DC,hence, inhibiting the Tconv cells activations[38-39].The C.T.L.A-4 and other accessory molecules are further upregulated by the TCR stimulation of Treg cells, specially the adhesion molecules like LFA-1, whose

deficiency compromise suppressive activity. Thehigh L.F.A-1 and other accessorymoleculeexpression pribeforeor T.C.R stimulationscanlead toestablish a less threshold to T.C.R-induced activations ofthe Treg cell[40]. The attenuated T.C.R signal may savethe Treg cell from activations induced cell deaths upon their exposuresto antigen, assisting them for better survival than(Tconv) cell,since such Treg cells overrideTconvattumor sites. This combined effect of collective highly CD25 & C.T.L.A-4 expressions, relianceupon the exogenous Interleukine-2 and T.C.Rstimulations havedecisive role in the Treg mediated suppressions.

Foxp3 and cancer: A largenumber of Treg cells often infiltrate different mice & humantumors. In humans, the lung[41], liver[42], head and neck [43], breast[44], gastrointestinaltract[45] and ovary[46] tumors were shown to bear a highly numbers of tumor infiltrated Treg cell[47]. The decreased ratioof tumor infiltrating (CD8+) T cells to FOXP3+ Treg cellswere correlated with bad prognosis, mainly in breast cancer patients[48], gastric[49], and ovarian cancer[50].A previous met a-analysis published data specified that in most breast, kidneys, cervix tumors and melanoma,the highly frequent tumor-infiltrated FOXP3+ cell was associated with patient's survival negatively[51].

Interplay between HER2 and FoxP3 in breast cancer: A study by Parej*et al* on the correlation between circulating Treg cells in $Her2^+$ and $Her2^-$Bc cells showed that $Her2^+$tumors were characterized by increased levels of FoxpTreg cells in the blood of BC patients [52].The high levels of Treg cells have been frequently correlated with MAB or LABC. $Her2^+$ and $Her2^-$ tumors when subjected to chemotherapy with trastuzumab alone or in combination with other drugs caused overall reduction in the Treg cells frequencies to normal levels. Some of the Her2+ patients initially responding to trastuzumab therapy exhibited disease reoccurrence after sometime which was correlated with increase in Treg cell frequency. A correlation between changes in circulating Treg frequency and plasma HER-ECD(extracellular domain) implied that at least some of those cells canrespond to and recognize the systemically circulating (HER) proteins. Patients who respond to trastuzumab treatment by decreasing plasma HER-ECD also demonstratedlow frequency of Treg.It was noticed that HER was eliminated from blood circulation by antigen-trastuzumab complex production and uptake by phagocytic cells via combining with FcgR[53-54]. HER-trastuzumab complex formation also led to maturation, activation and reinforced antigen cross presentation by the Antigen Presenting Cell[55], and along with low Treg frequency, may possibly induce an enhanced antitumor responses.

Foxp3 isrecognized during *Scurfin* position cloning, which isthe gene responsible for X linked autoimmunesdisorders in humans and mice (Immune dysregulation, enterophathy, polyendopathy, X-linked & IPEX)[56-59]. Mice heterozygous for$Foxp3^{sf}$ spontaneously developed malignant tumors among which more than half were mammary carcinomas. To establish a link between mammary carcinomas and Foxp3 mutation mice, heterozygous for $Foxp3^{sf}$ were treated with a carcinogen, 7,12-dimethylbenz [a] anthracene (DMBA). It was observed that mutation for $Foxp3^{sf}$ but not for Otc^{spf} gene leads tomainelevation in susceptibility to the mammary carcinomas. A comparison of Foxp3 expression normal and mammary epithelium from bothwild-type and $Foxp3^{sf\ +}$ mice showed that Foxp3 mRNA was identified in normal epithelium of both WT and $Foxp3^{sf}$ mice, but not in mammary carcinomas. This decrease in Foxp3 MRNA was concurrent with significant increase in HER2 mRNA in mammary epithelium. There was also increased HER2 mRNA expression in $FOXp3^{sf/spf}$ epithelium than in wild type female mice indicating a potential gene dosage impact of in vivo Foxp3 of HER2 regulation. Repression of HER2 by Foxp3 is mediated by a direct binding between Forkhead domain of foxp3 onto the promoter region of HER2.The Foxp3 binding site deletion increased the *Her2* promoter activities and relieved Fox p3 mediated Her2 repression. In mostbreast cancers, LOH alone was enoughfor the locus inactivation, probablyowing to Xchromosomeinactivation. Silencing of *Foxp3* gene by *Foxp3* siRNA decreased *Foxp3* expression by more than (100)fold, while increasing *Her2* mRNA by 7 folds. A comparison of Foxp3+ and Foxp3− cancer samples revealed that $Foxp3^+$samples has reduced HER2 scores compared with $Foxp3^-$ specimens suggesting a decisive role for Foxp3 in repressing HER2 expression.Further results revealed that mice heterozygous to*Foxp3* mutations spontaneously developed high rates of mammary cancer. Cells where WT allele was silenced by X inactivations had inactive Foxp3 and overexpression of *Her2*.Most of these mutations concentrated onzing finger & FKH domains which inactivated tumor growth inhibition & repressor activity of Foxp3 (Fig. **2**). It could be concluded that the FKH and Zinc finger domains of intracellular transcription factor foxp3 is directly

boundwith the promoter's region of *Her2* and acts as a restraint on the transcription of her 2. Any deletion or mutation in foxp3 relieves *Her2* of this restraince resulting in its overexpression ultimately causing tumorigenesis [60].

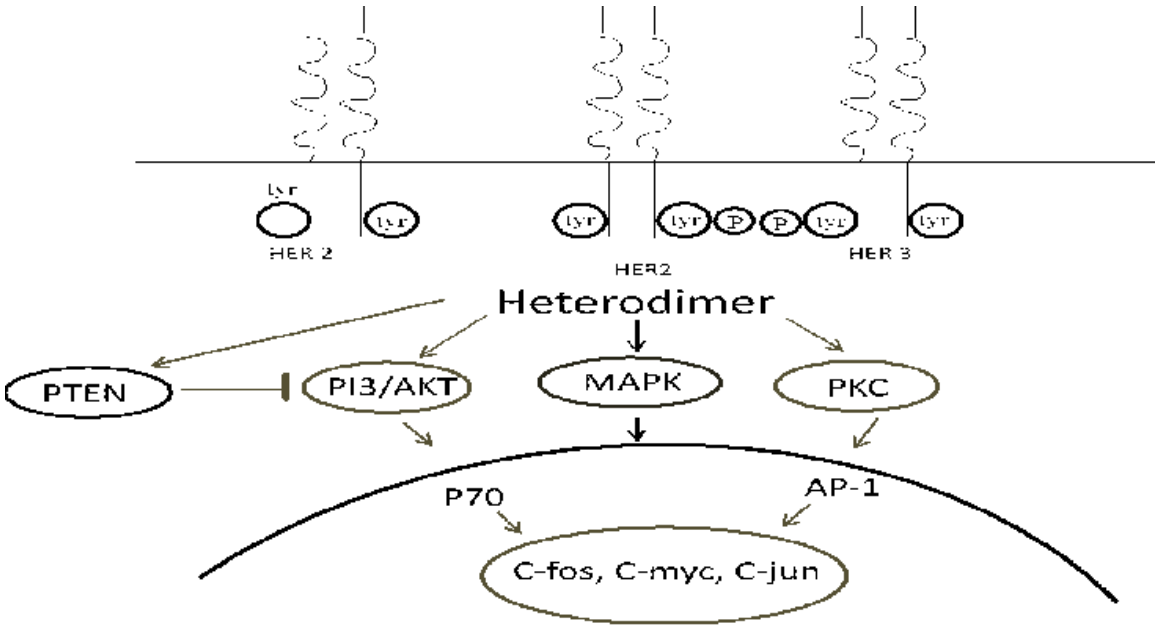

Figure 2

Figure (2): Schematic representation of HER2 signalling mechanism. Receptor tyrosine kinases cause dimerization of HER2 by phophorylating at tyrosine residues which further activate various downstreaming pathways resulting in cancer..This heterodimerisation activate PI3/AKT,MAPK,PTEN and PKC pathways which further activate downstream signalling molecules P70 and AP-1 that in turn activates proto-oncogenes c-fos,c-myc and c-jun involved in cell proliferation. However PTEN has antagonistic effects on thePI3/AKT pathway.

FOXP3 prevents metastasis and inhibits angiogenesis in breast carcinoma cells: The nuclear expression levels of the protein (Foxp3) were evaluated in the cell line of breast carcinoma. Among these cell lines, S.K.B.R-3 & M.D.A-M.B-231, expressed relatively lower Foxp3 level,while T47D and M.D.A-M.B-361, expressed relatively higher Foxp3 levels. Up regulation of *Foxp3* in S.K.B.R-3 and M.D.A-M.B-231 cells decreased the breast Ca cellinvasion capacity, whereas, Foxp3-siRNA mediated silencing in T47D and M.D.A-M.B-361 cell had about two fold elevation in the rate of tumor invasion in comparison with the control group cells. The results suggested that Foxp3 overexpression has an inhibitionimpact on breast Ca cellsinvasions & adhesions, while Foxp3 silencings possesses an opposite impact. This could be due to the gene expression change viz. C.D.44 R.O.C.K2, E.C.M.1, D.L.G.A.P.5 and Serpine1 associated with cellular movement. Downregulation of these genes, particularly CD44, was most significant in reducing BC invasion and metastasis. C.D.44, the hyaluronic acid cell surface receptor is an adhesion molecule which has a long standing correlation with breast Ca cell invasions & metastasis [61-62]. Foxp3 mediated its inhibitory effect on breast Ca metastasis vialinkage with the promoters of gene encoding C.D.44 thereby inhibiting expression of breast Ca cells[63]. An inverselyrelation between CD44 expression and foxp3 reveals that FOXP3 inhibits metastasis via downregulation of CD44 expression[64]. Chemokine CXCL12 and its receptor CXCR4 (CXCL12/CXCR4) play amain role in regulating malignant cell growth, invasion, metastasis and secretion. Foxp3 regulates CXCL12/CXCR4 expression demonstrating an inverse correlation between Foxp3 and CXCL12/CXCR4. Foxp3 showed low mRNA and protein levels in MCF-7 and MDA-MB-231 BC cell lines compared to normal breast epithelial cells. However, stable Foxp3 over expression in MDA-MB-231 cells led toreduced CXCR4, ErbB2/HER2, SKP20 and c-MYC expression.

Thus, Foxp3 regulates breast Ca metastasis by down-regulating the expression of some metastasis-related molecule including C.D.44 and C.X.C.R.4[65].

In addition to its inhibitory role in metastasis, fox p3 has also been implicated to inhibit VEGF- mediated angiogenesis. It was observed that ectopic expressions of Foxp3 in M.C.F-7, T47D and M.D.A-M.B- 231 cell lines downregulates VEGF expression and that silencing endogenous FOXP3 by shRNA upregulated VEGF expression at both the mRNA and protein levels. High Foxp3 expression was a protective factor for breast cancer survival, while high VEGF expression enhances breast cancer survival (Fig. 3). Foxp3-positive samples also showed a lower blood vessel density when compared with nuclear Foxp3$^-$ negative samples. These data hypothesize that Foxp3 is negatively related to angiogenesis in breast Ca. Foxp3 inhibits breast Ca angiogenesis invitro and in vivo. This foxp3 mediated downregulation of VEGF took place by a direct interaction of forkhead-binding motifs of Foxp3 with the VEGF promoter 1.2 kb upstream of transcription start site, thus inhibiting VEGF promoter transcription and activity[66].

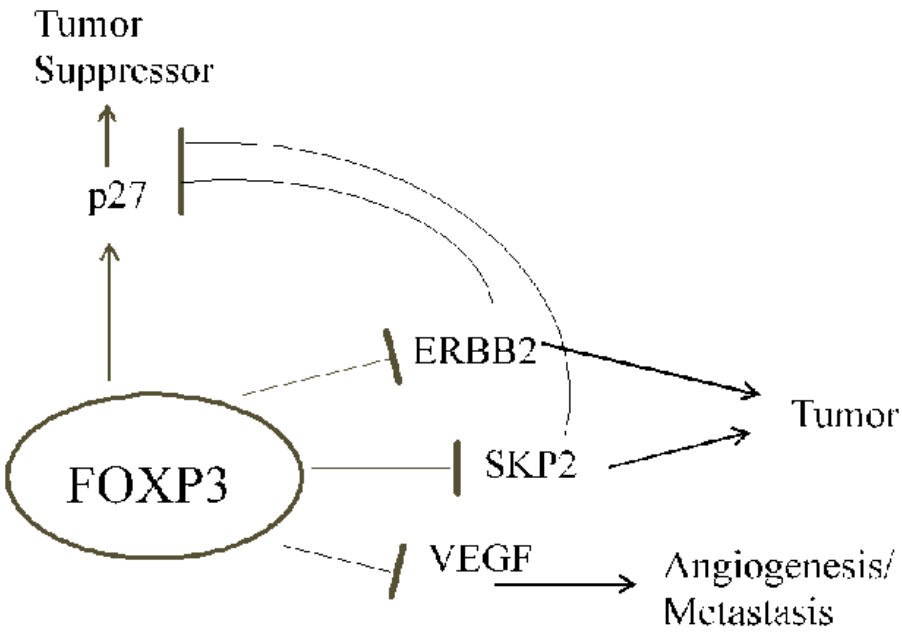

Figure 3

Figure (3): Schematic representation of role of Foxp3 as a inhibitor of oncogenes ERBB2 and SKP2 which cause breast cancer. Foxp3 also inhibits VEGF which is an important factor responsible for angiogenesis and metastasis to distant organs. Contrary to these Foxp3 maintains the expression of p27 a cell cycle inhibitor gene, thus exerting tumor suppressive function. Both ERBB2 and SKP2 inhibit expression of p27.

Therapies targeting FOXP3: In immunotherapy, the major hurdle is the T cell expressing Foxp3. Foxp3 is an intracellular transcriptional factor. It has been observed that the regulatory T cells elevate the breast cancer by suppressing the antitumor immunity. According to the research work carried out by Takeuchi, the tumors which are not recognized earlier by regulatory T cells triggers a strong antitumor response resulting in the blockage of several immune checkpoints. Thus, in order to elevate the efficacy of vaccines, Foxp3 expressing T regulatory cells are needed to be suppressed[67].

Antigen, antibody, peptide or protein: In their study, Devi and Nath prepared novel vaccines containing synthetic mRNA encoding Foxp3 antigen and delivered them into dendritic cells. Upon transfection or pulsation the peptides of tumor antigens, total protein or total mRNA isolated from tumor cell, DNA encoding specific tumor antigens or synthetic tumor-antigen-encoding mRNA entered into dendritic cells. These tumor antigen-associated dendritic cells resulted in

subsequent generation of mature dendritic cells, which could efficiently elicit tumor antigen specific cytotoxic T lymphocytes fundamental in destruction of cancer cells[68].

A synthetic peptide P60 and antitumor DC vaccines were used to treat mice bearing LM3 and 4T1 tumors. P60 monotherapy was shown to inhibit tumor growth in immunocompetent in addition tothe immuno-compromised animals, primarily by inhibiting the secretion of immune-supressive cytokine IL-10 secretion in FOxp3 expressing breast carcinoma cells. Furthermore, combined treatment with antitumor dendritic cell vaccines and P60 increased the therapeutic efficacy of these vaccine in experimental models[69].

Conclusion

The important role of FOXP3 as a tumor suppressor is evident by the fact that it not only represses HER2 and SKP2 breast cancer oncogenes, but also inhibits VEGF a pivotal player causing in angiogenesis and metastasis. However, it has totally contrary role in tumor milieu where its presence on the tumor infiltrating Treg cells results in suppression of anti-tumor response. As such patients initially respond to chemotherapeutic drugs develop resistance over a period of time. Patients who have Her-2^+ over expressing breast cancer with primarily response to gradual trastuzumab acquired resistance. Foxp3 mediated inhibition of Her2 signaling mechanism has been extensively studied to identify additional targets to design effective treatmentsto those patients with drug-resistant Her2^+breast Ca patients. Studies have also shown that Foxp3 inhibition in Her2^+ overexpressing BC could be correlated with better prognosis and overall and relapse free survival. Thus, Foxp3 can be envisaged as a pivotal target whose down regulation in tandem with other breast cancer targeted therapies can have astounding effects in BC patients. A synergistic inhibition of Foxp3 in tumor milieu and anti-HER2 therapy on similar lines with anti-HER-2 and anti-VEGF therapy in HER2-positive breast carcinoma can be developed. Foxp3 can be envisaged as an important future candidate in designing an effective therapy against HER2- overexpression particularly drug resistant HER2- overexpressing breast cancer.

Conflict of Interest: None

Source of Findings: None

Ethical Clearance: None

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