Interpreting the Role of *INS* and *GLUT4* in Type 2 Diabetes Mellitus and Breast Cancer Patients

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of MS Healthcare Biotechnology

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List of Acronyms

BC	Breast Cancer
T2DM	Type 2 Diabetes Mellitus
GLUT4	Glucose transporter 4
SLC2A4	Solute carrier family 2, facilitated glucose transporter member 4
INS	Insulin
HIF1α	Hypoxia-inducible factor 1-alpha
VEGF	Vascular endothelial growth factor
Akt	Protein kinase B, serine/threonine-specific protein kinase
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
HER2	Human epidermal growth factor receptor 2
FOXO	fork-head box transcription factors
TNFα	Tumor Necrosis Factor
ROS	Reactive oxygen species
IRS	Insulin resistance syndrome
IGF	Insulin-like Growth Factor
IGFBPs	Insulin-like Growth Factor Binding Proteins
PPARɤ	Peroxisome proliferator-activated receptor

shRNA	short hairpin RNA
INSR	Insulin Receptor
UBX	Ultrabithorax
Daxx	Death-Associated Protein 6
OGTT	Oral glucose tolerance test
FPG	Normal fasting glucose
HbA1C	Oral glucose tolerance test
ORG	Oral glucose tolerance test
WHO	World Health Organization
ATLAS	Atmospheric Laboratory for Applications and Science
IDF	International Diabetes Federation
ECOG	Eastern Cooperative Oncology Group
KEGG	Kyoto Encyclopedia of Genes and Genomes
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TRIzol	Total RNA Isolation
DEPC	Diethyl pyro-carbonate

Abstract

Type 2 Diabetes Mellitus and breast cancer are two most quickly prevailing morbidities that often exist parallel to each other. Along with environmental factors genetic factor is the huge one in such conditions. INS and GLUT4 are the genes that are dysregulated in both diseases. INS codes for insulin secretion through pancreatic beta cells that helps the body to utilize the glucose while GLUT4 is insulin dependent glucose transporter. According to literature INS is overexpressed in cancer and early stage of T2DM. While GLUT4 is down-regulated in T2DM and up-regulated in Breast Cancer.

The aim of this study was to analyze the expression of two genes in such disorders and linking the disease occurrence. The purpose is providing common biomarkers for prognosis, diagnosis, and therapeutics. String and Enrichr web analysis were done to confirm the role of the two genes in T2DM and breast cancer pathways. Reverse Transcriptase PCR was done for amplification of gene-primers. Real Time PCR was done for expression comparison in four study groups i.e. breast cancer, T2DM, breast cancer + diabetes Mellitus group and the control group. 5 samples of BC, 4 samples of DM, 2 samples of BC+DM and 7 control samples were processed. Our results evaluated that the GLUT4 and INS expression was same as mentioned in literature and the expression difference in disease vs control was statistically significant.

1. Introduction

1.1. Type 2 diabetes Mellitus

Type 2 diabetes mellitus is concerned with persistent hyperglycemia due to insulin resistance. It poses as a cause of many other complications and if it's not controlled it leads to death. T2DM is the most common form of diabetes, more than 90 percent (Glovaci, Fan, & Wong, 2019). Occurrence is among the adults (above 20s), mostly (Kharroubi & Darwish, 2015). It has become the most pressing issue of the world in the last few decades and 5.2 million deaths attributed to diabetes, globally.

There are two things regarding beta cells in T2DM. First, beta cell producing enough insulin but there is further insulin resistance and the second one is beta cells become inefficient or damaged after a long period of burden while fulfilling the cellular need of insulin, in the presence of insulin resistance. The factors fueling T2DM are physiological, environmental, and genetic. This kind of diabetes mellitus is mostly the result of obesity and physical inactivity. (WHO)

1.2. Epidemiology of Type 2 diabetes mellitus

The alarming prevalence of diabetes in the world has seek so much attention and from all other diabetes types of the Type 2 Diabetes mellitus is on the top. According to epidemiologist individuals who migrate from low-prevalence areas to developed countries have an increased risk of Type 2 Diabetes Mellitus. (Forouhi & Wareham, 2019). The reason behind is availability of high income, food and diagnostics. On the other hand, in the underdeveloped countries there is also lack of diagnosed cases in the world data of diabetes.

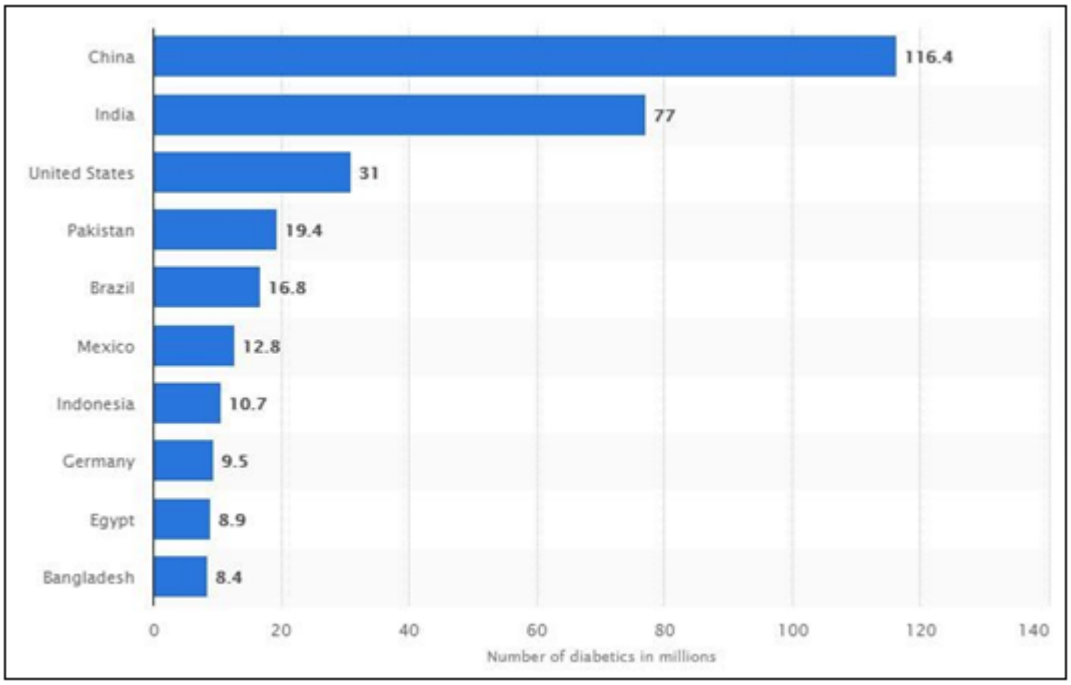


Figure 1.1. This fig show the incidence level in millions among the countries. Age estimate of 20 to 79 year (Federation, 2019)showing that Pakistan is in top ranking among other countries.

1.3. Clinical Diagnosis of T2DM

The symptoms of type 2 diabetes mellitus have resemblance with the type 1 diabetes mellitus but are less marked, frequently. Many diagnostic criteria are used for T2DM. Oral glucose tolerance test (OGTT) used to check how your body handles food saccharides. It tells a person have already diabetes or on risk. For this a person have to drink 8 ounces of a syrupy glucose solution containing 2.6 ounces of sugar after 8 hours of fasting and 2 hours before checkup (Staff, 2020a).

IFG impaired fasting glucose FPG normal fasting glucose this checks your blood glucose level after 8 hours of fasting. IFG refers to a metabolic stage intermediate between normal glucose homeostasis and diabetes, now referred to as pre-diabetes (Michael Dansinger, 2020).

Glycated hemoglobin test HbA1C is the most efficient way which measures the level of glucose attached to hemoglobin and used to check both diabetes and pre-diabetes. This test not only measure the blood glucose concentration but also monitor for over 8-12 weeks (Schauer et al., 2003).

Table 1.1: Three ways of testing diabetes.

	OGTT (mmol/L)	FPG (mmol/L)	Hb1AC (Percent)
Normal	< 7.8	≤ 6	4 - 5.9
Pre-diabetes	7.8 – 11	6.1 - 6.9	5.7 - 6.4
Diabetes	≥ 11.1	≥ 7	≥ 6.5

1.4. Breast cancer and its possible types

Uncontrolled growth and progression of breast cells produce the malignant form of tumor that we call breast cancer. It has increased dramatically, heightening the concern of physicians and women. When it remains in the same position of breast and doesn't spread it is called noninvasive breast cancer. When it spreads only to nearby tissues it's called locally advanced breast cancer. While the metastatic breast cancer is the condition in which cancer invade distant body parts from the breast. While talking about kinds of breast cancer 'angiosarcoma' is the condition in which blocking of lymphatic vessels in breast skin results in swelling and redness of breast and it's not much common. Ductal carcinoma in situ(DCIS); Cancer of milk ducts in

breast. It's the earliest condition of breast cancer that is mostly not invasive. It is revealed through mammogram test. Inflammatory breast cancer; Cancer of blood and lymph vessels linings. On the breast skin it appears like bruise and lesions. It is named after its inflamed appearance. It is aggressively growing cancer and rarely occurred. It comprises of 1 to 5 % of all breast cancers. Invasive lobular carcinoma; Lobular carcinoma in situ(LCIS.) It's the rare condition in which abnormal proliferation of cells occur in breast lobules that are milk glands. It's not the cancer but can be invasive at greater risk. It can't be found through mammogram. Male breast cancer; Few males can also develop breast cancer its mostly in old men. There are several causes for developing breast cancer in male which include the increase of female hormone like estrogen due to medications or syndromes in men. Ductal carcinoma; is most common one but least common is lobular one because male has blind ended ducts in their breast not the ducts ending in lobules. Paget's disease of the breast; It's different from the Paget's disease of the bones. In such condition the cancer occur in nipple and areola, around the nipple.

It confined just to nipple in very few cases. Nipple may be inverted or flattened, scaly and crusty appearance of areola are the common symptoms.

1.5. Breast Cancer epidemiology

It is the leading cancer type among women all over the world. The incidence rates are different in developed and under developed countries. Although the incidence rate is high in developed countries but its decreasing there comparable to less developed countries where the mortality rate is also high because of unavailability of resources for early diagnosis and treatments. . (Cancer atlas, 2018.)

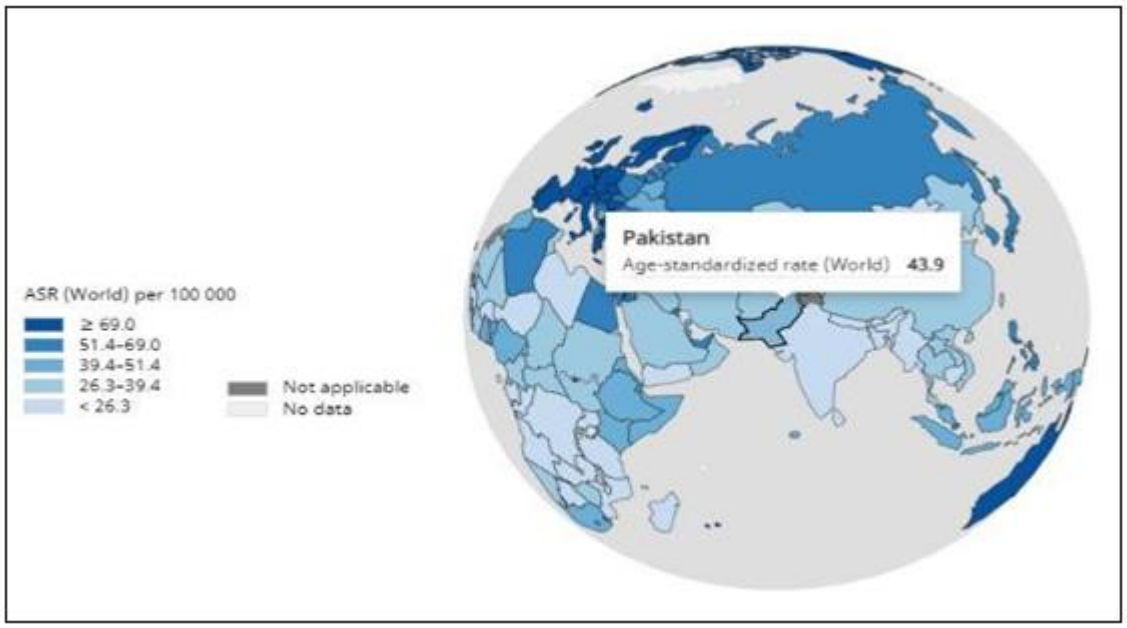


Figure 1.2. Estimated worldwide incidence of breast cancer in females of all ages (Thomas, 2018). Pakistan suffering from 43.9 % breast cancer incidences.

1.6. Clinical diagnosis of breast cancer and its staging

For the medical diagnosis of breast cancer first breasts and lymph nodes in armpits are examined by the doctors to check the presence of nodule or any other abnormality. Then screening mammogram or X-ray of breast is done to screen any aberration in breasts. Further evaluation is done by diagnostic mammogram. Ultrasound is done in which sound waves are used to produce images of deep body structures. It may also be used to determine whether a breast lump is a solid mass or a fluid-filled cyst. Biopsy is the definitive way of testing in which tissue core is extracted with the help of specialized needle device guided by any imaging technology. The extracted piece is then analyzed in laboratory and checked whether the cells are cancerous or not and at what level of aggression. MRI magnetic resonance imaging use a magnet and radio waves to create pictures of breast interior. Before imaging a dye is injected so that but if a patient has previously done biopsy, he/she must have a metal marker left used for imaging so no need of a dye here.

For staging of breast cancer blood tests, such as a complete blood count, Mammogram of the other breast to look for signs of cancer, breast MRI, Bone scan, Computerized tomography (CT) scan, Positron emission tomography (PET) scan is done not all these tests are required for every patient it is recommended by the doctor that which test should be done (Staff, 2019).

1.7. Cellular metabolism in cancer (Normal cells become cancerous cells)

Warburg Effect: The scientist named Otto Warburg observed this effect in 1926. When certain cells prone to cancerous condition they alter their cellular metabolism. In which aerobic glycolysis instead of oxidative phosphorylation is at primary importance, it's called the Warburg effect (Warburg, Wind, &Negele in, 1927). In this phenomenon cells produce more lactate even there is enough oxygen. Tumorigenesis require more ATP and metabolites for biosynthetic pathways, so this requirement is fulfilled by glycolysis (Garrido et al., 2015).

This alternative path of cellular metabolism associated with the more oncogenes' activation and tumor-suppressants loss. Moreover, the mitochondrial deficiency is the primary feature of tumorigenesis which cannot fulfill the ATP requirement. But it is clear that this deficiency of mitochondria is not the cause of Warburg effect (Sutherland &Woodhead, 2012; Ward & Thompson, 2012) (Ferreira, 2010).

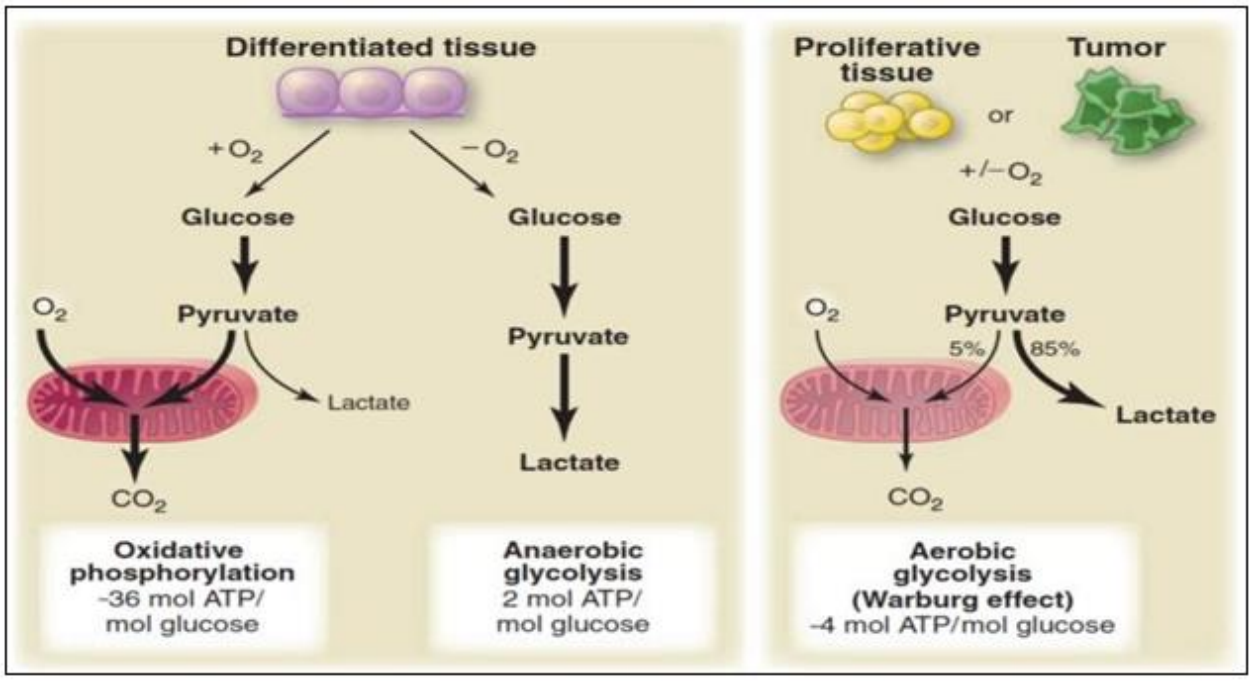


Figure 1.3. Cellular metabolism in normal cells vz in tumor cells. The figure is explaining the presence of oxygen as well as hypoxic condition during high activity in normal cells shifting the cell from oxidative phosphorylation to anaerobic glycolysis but in tumor cells although oxygen is available but cells have to maintain high metablolsim so the process used is aerobic glycolysis that is named as Warburg effect. (Vander Heiden, Cantley, & Thompson, 2009)

1.8. Recurrence of breast cancer

After the initial treatment, the patient again develop cancer after months or years. It is because some cancerous cells still survive after treatment. Gradually, they increase in number and cancer diagnosed after sometime. The recurrence may be local or distant from the original cancer place (Staff, 2020b).

According to a study, after the surgical therapy of breast cancer there was 13.3% chances of recurrence in the second year and it reduces slowly till 5th year and finally its reduction was tremendous till 12th year but after that there was no decrease in recurrence to make it zero (Saphner, Tormey, & Gray, 1996).

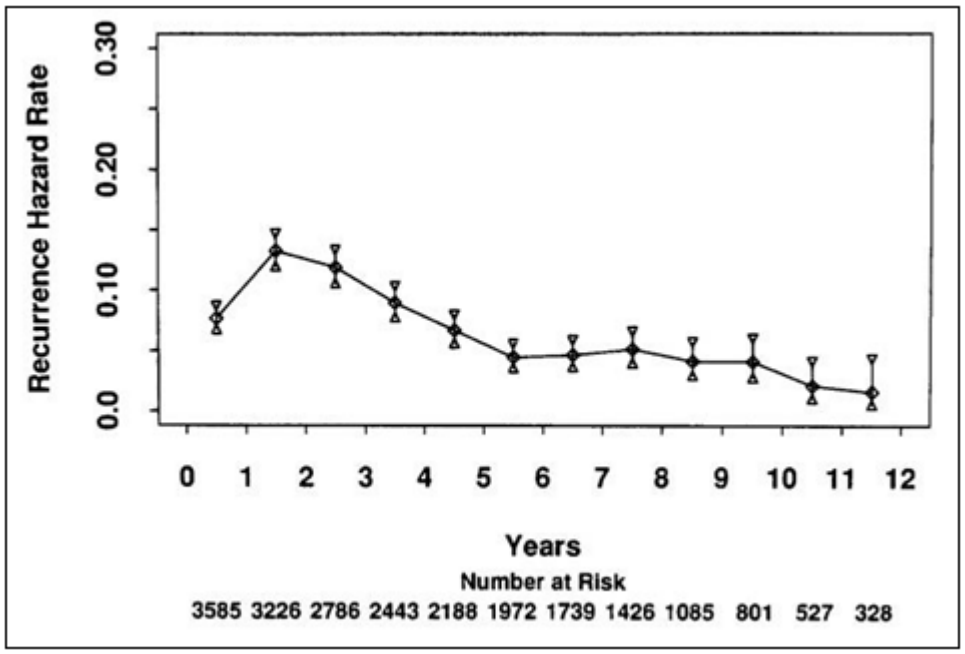


Figure 1.4. Annual hazard of recurrence for 3,585 patients entered on seven ECOG studies; Where ECOG stands for **Eastern Cooperative Oncology Group.** ECOG performance status determines ability of patient to tolerate therapies in serious illness, specifically for chemotherapy.

1.9. Aetiological factors in association with T2DM and Breast cancer

Cancer and diabetes both are metabolic disorders. Risk factors are much more similar in these two diseases like obesity, unhealthy diet (Alcohol, drugs and tobacco consumption), age, sluggish life-style, physiological stress (Abrupt emotional and hormonal changes),environmental factors (Radiations, pollutants), genetic and epigenetic factors and infections. Approximately 15% of cancers are caused by infections. In transitioning countries its rate is high. (Cancer atlas, 2019). As both has many common genetic factors. The expression of GLUT4 and INS gene is also being effected synergistically.

1.10. Genetic factor in association with T2DM and Breast cancer

The fuel leading to such two morbidities include a combination of both metabolic as well as genetic factors. Disturbance in genetic regulation occur in which many gene expressions are down-regulated and many up-regulated. More than 400 genetic variants contributing to diabetes

risk have been identified (Forouhi& Wareham, 2019). About 50% of all the tumors in human contain genetic alteration that inactivate the tumor suppressor genes or activate the oncogenes (Fadaka et al., 2017). Common biomarkers create the bridge between these co-morbidities and at molecular level signaling pathways are also linked. In this study GLUT4 and INS expression will be discussed thoroughly.

1.10.1. Insulin (INS)

It is Homo sapiens gene that encodes for insulin production. It is located on chromosome band 11p15.5. Increase in blood glucose level trigger the releasing of Insulin that is the peptide hormone from beta cells in pancreas. Dysregulation of INS gene can result in diabetes, uncontrolled growth of cells and inherited disorder of tumor predisposition syndrome. It further control many genes specially that are participating in glucose metabolism, growth and metastasis. The matrix metalloproteinase-2 (MMP-2) that is famous for its metastatic activity is activated by insulin. The hormone named Insulin-like growth factor (IGF) has the molecular structure like insulin and is famous for its anabolic effects in adults.

Insulin participate in many signaling pathways that involve Type 1 and 2 diabetes mellitus, prolactin signaling pathway, insulin resistance, regulation of lipolysis in adipocytes, Akt,FOXO, AMPK, HIF1α signaling pathways, autophagy and oocyte meiosis pathway, ovarian steroidogenesis, MODY, Cancer pathway and mainly Insulin signaling pathway. (Chen et al., 2013; "Enrichr," 2019; "KEGG pathways," 2019; Kuleshov et al., 2016).

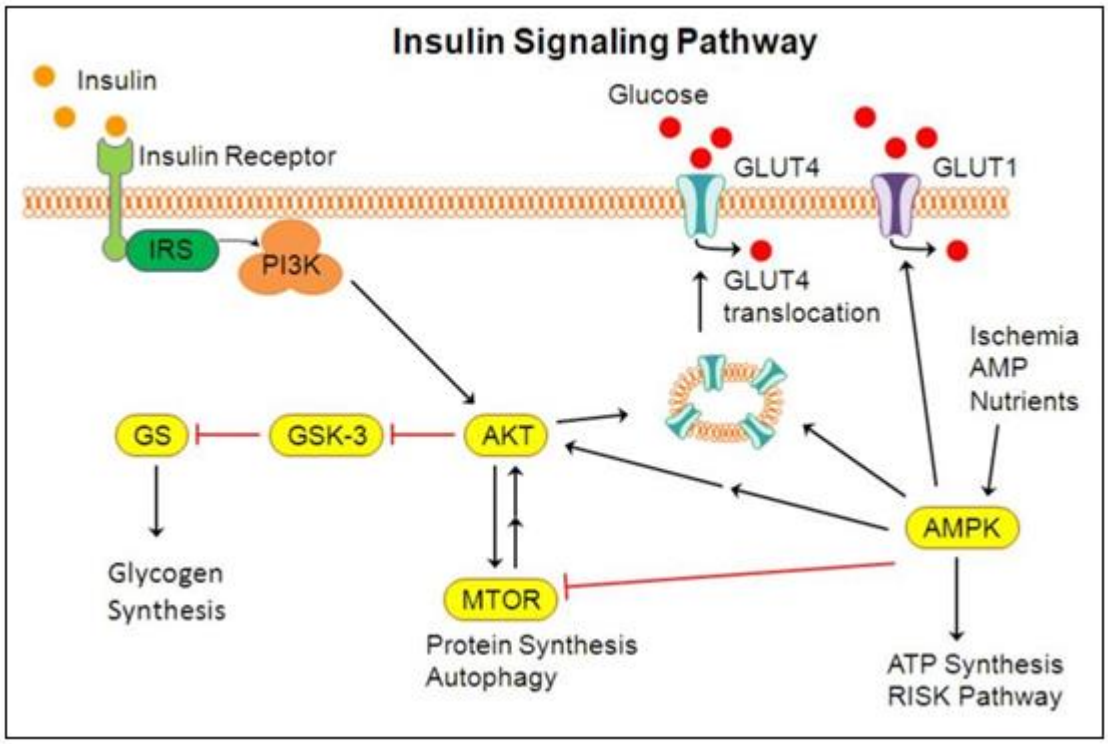


Figure 1.5. As shown in figure that INS participate in Glycogen synthesis from glucose in protein synthesis,autophagy and ATP synthesis by translocating the GLUT4 to cellular surface that plays its role in glucose uptake (Elmadhun, Lassaletta, Chu, Soh, &Sellke, 2012).

1.10.2. GLUT4 and other Glucose transporters

As you know sugars are the main source of metabolic energy in mammalian cells. The impermeability of plasma membrane needs carrier proteins in them to transport the polar molecules. For sugar transportation there are two definite transporter families one is SGLT (solute carrier family 5) that is sodium dependent sugar transporter and the other is GLUT that is Na^+ independent sugar transporters. There are total 14 GLUT proteins which are classified into 3 classes. Class 1 includes GLUT1-4 and GLUT14. Class 2 includes GLUT5, GLUT7, GLUT9, and GLUT11. Third and last class consist of GLUT6, GLUT8, GLUT10, GLUT12 and HMIT. (Calvo, Figueroa, Pulido, Campelo, &Aparicio, 2010).

GLUT4

An insulin dependent glucose transporting protein that's also called solute carrier family 2(facilitated glucose transporter member 4) - in human that is encoded by SLC2A4 gene that was discovered by David James in 1988 (Kern et al., 1990) (James et al, 1988). GLUT4 is the part of class 1. It is mainly expressed in striated muscle and adipose tissues. As these tissues constitute large portion of body mass so GLUT4 is 90% of all the other GLUTs. The specific arrangement of 509 amino acids make it to transport glucose across the cellular membrane. Its N-terminus contain phenylalanine and COOH-terminus contain two leucine residues that help in its endocytosis and exocytosis. It contains UBX domain that interact with Daxx and thus it plays part in apoptotic signaling (Buchberger, Howard, Proctor, & Bycroft, 2001). GLUT4 is involved in many signaling pathways like Type 2 diabetes mellitus, Adipocytokine signaling pathway, Insulin resistance, Insulin signaling pathway, Breast cancer, FOXO signaling pathway and AMPK pathway ("KEGG pathways," 2019).

1.10.3. Insulin and GLUT4 Interaction

When the blood glucose level increases, pancreatic cells release insulin which then through the release of proteins cause the movement of intracellular vesicles in which GLUT4 is sequestered towards the plasma membrane and the vesicles fuse with the membrane so that GLUT4 become available for glucose transport to cell and metabolize ("<Principles-of-Biochemistry-by-ALbertLeningher.pdf>,") (L et al., 2005).

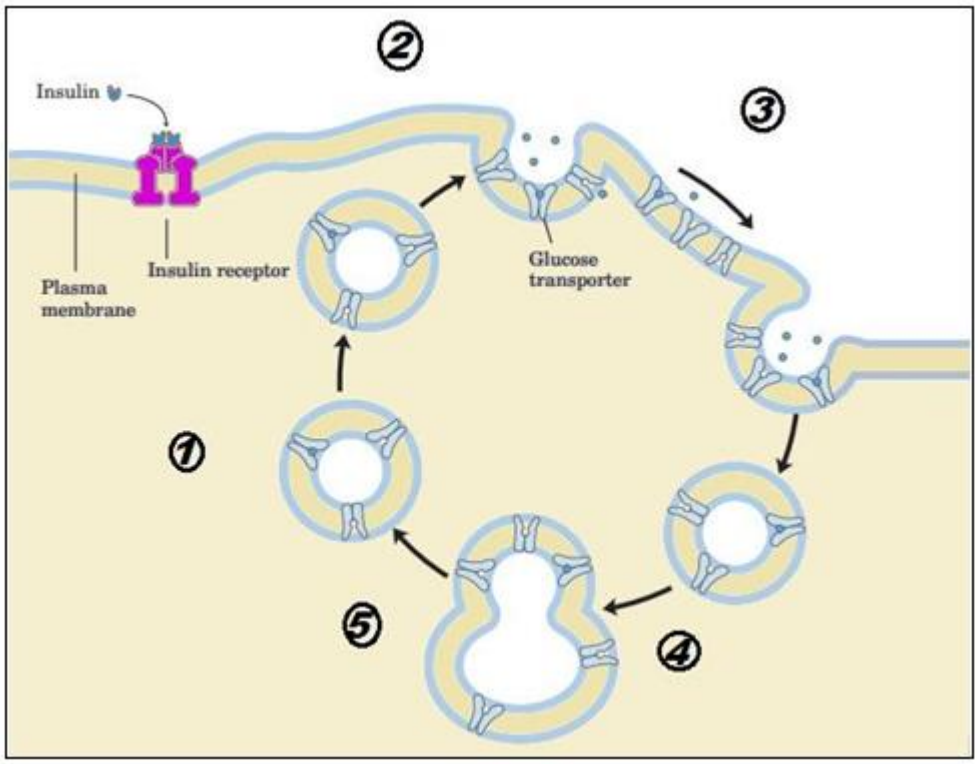


Figure 1.6. 1) Glucose transporters inside the cell; residing in membrane vesicles. 2) After insulin interaction with its receptor, the vesicles fuse with plasma membrane for GLUTs availability. 3) After dropping the insulin level, the GLUTs removed from surface through forming endocytic vesicles. 4) Small vesicles then fuse with large vesicle i.e. endosome. 5) Small vesicles budded off from endosome containing GLUTs ready to repeat the process.

Aim and Objectives

Its goal is to beat the public health threat by broadening the range of knowledge of these genes expression in both metabolic disorders. And increase its epidemiological data in Pakistan. This will take more towards the genetic based diagnosis and treatment.

- To evaluate the pivotal role of GLUT4, INS as potential biomarkers in breast cancer anddiabetic patients.

- To conduct gene expression profiling of candidate genes that may participate in linking both the diseases.

2. Literature review

Cancer cells have high expression of GLUT 4 for intense metabolism. But we also learned that Insulin resistance bring impaired GLUT4 trafficking because GLUT4 is insulin dependent transporting protein. Then how type 2 diabetic patients are on more risk of developing cancer?

2.1. Genetic stress of GLUT4 and INS in diabetes and Breast Cancer

2.1.1. Insulin resistance

The case of type 2 diabetes mellitus in which cells cannot response normally to insulin hormone, the condition called insulin resistance. Insulin resistance could be of many forms it can be due to excess membrane cholesterol, receptor modifications in expression, binding, phosphorylation state or tyrosine kinase activity (Pessin & Saltiel, 2000). It may be the blocked phosphorylation site that inhibit the insulin action. It can be induced due to several reasons; hormonal imbalance, medication, sedentary lifestyle, genetics, inflammation and certain diseases. So, many epidemiological studies have consistently revealed that insulin resistant patients are on greater risk for several kinds of cancer.

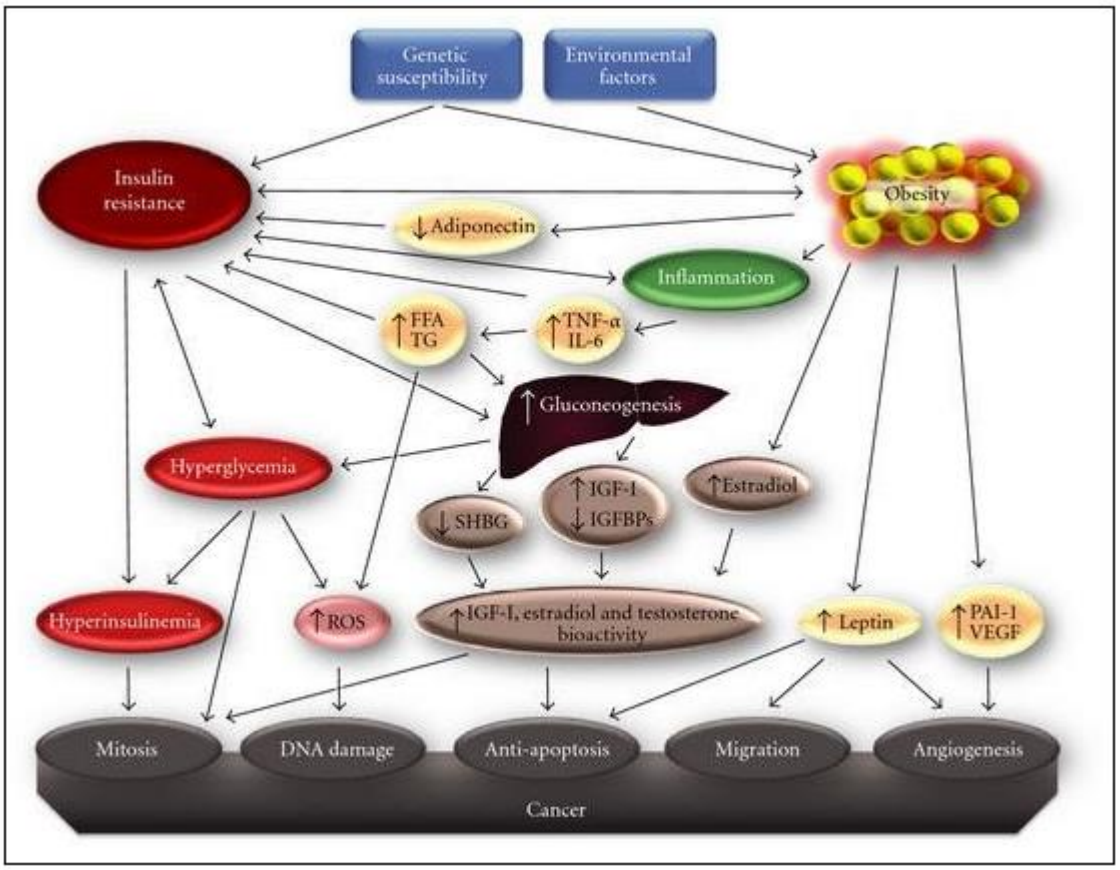


Figure 2.1. A multidimensional model suggesting insulin resistance and inflammation as driving forces for cancer (Arcidiacono et al., 2012).

2.1.2. Insulin receptor

The integral membrane glycoprotein to which insulin interact and starts its function. It binds to INSR extracellular alpha subunit that activate tyrosine kinase at beta subunit that phosphorylate various intracellular effector molecules. In tumors it involves INSR A isoform that is also able to bind with IGF 2 with high affinity and expressed in mitogenic response signaling. The deregulated expression of INSR then spur the neoplastic events to occur in epithelial tissues. In type two diabetic patients mutations in INSR occurs due to constitutive hyperinsulinemia, high expression of INSR then leads to abnormality in cellular signaling cascades and thus enhancing the growth factor dependent proliferation. Thus this insulin resistance will take part in neoplastic transformation. Many epidemiological and clinical studies have showed that the anti-diabetic

drugs proposed as auxiliary anticancer therapy. In a study (in vivo and in vitro) of MCF-7 human breast cancer cells and 3T3-L1 adipocytes with high amount of PPARγ production, an antidiabetic drug that increase insulin sensitivity named Thiazolidinediones (TZDs) act as antiproliferator and act as antagonist for PPARγ (Peroxisome proliferator-activated receptor). On molecular basis, PPARγ interacts with SP1, AP2-α and C/EBPβ and perturb the gene expression of INSR by averting the binding of AP2-α to SP1 and SP1 binding to INSR. In short, INS itself down-regulate its receptor (Arcidiacono et al., 2012). So when there is insulin resistance the receptor may overexpress and activate further the intracellular molecules in which there might be GLUT4 that's transportation increases to cell surface and more glucose uptake occur in tumor cells that potentiate cells for proliferation and metastasis.

2.1.3 Optimal activity of INS:

It has been observed that not only its super-physiological concentration but also in its low concentration INS has been acutely effective on cellular growth. The reason for this might be in the interaction of INS hormone with other hormone's receptors such as insulin-like growth factor. Presence of INS in blood for longer time period evoke the binding of INS to IGFR and thus stimulate the growth. It is tested through the INS ability to stimulate the 3H-thymidine incorporation to 3T3 cell's DNA and induce cell proliferation through DNA synthesis of the arrested cell. It has also done in fibroblasts, ZR-75-1 and MCF-7 human mammary tumor cells

(Barnes & Sato, 1979).

In insulin resistant patients more insulin produces to cope the normal glucose level in blood but continuous resistance leads to hyperinsulinemia this trigger the insulin like growth factors thus resulting in tumor progression. INS and IGF-1 both cause to increase the production of sex

steroids in ovaries that will stimulate the cellular proliferation and inhibit apoptosis of epithelium and endometrium wall of breast. The anti-apoptotic and mitogenic effect of INS operating with the increase of IGF-1 develops high risk of tumors formation (Arcidiacono et al., 2012).

2.2. Cellular growth and proliferation in association with GLUT4 and INS

2.2.1. GLUT4 and proliferation

Apoptosis is the natural phenomenon of cell death that is important for maintaining homeostasis. Cancer cells escape this phenomenon and manage to proliferate. As it is well known that GLUT4 is facilitated glucose transporter used for more intake of glucose. In case of cancer, GLUT4 expression increases because the cellular metabolism have to boost up for proliferation and avoiding apoptosis. But in natural killer cells whose cytotoxic activity brings apoptosis, GLUT 4 expression lowers down to promote cancer cell survival.

Tumor suppressor p53 down-regulates GLUT1 and GLUT4 promoter activity ~50% from the normal activity level (Schwartzenberg-Bar-Yoseph, Armoni, &Karnieli, 2004). p53 is tumor suppressor protein as it participate in controlling cell cycle and apoptosis (Calvo et al., 2010).

2.2.2 INS and proliferation

When there is more insulin in blood it triggers more growth factors that will result in more cell growth and proliferation. INS act synergistically with other hormones and growth factors. It stimulate the cell cycle progression of G_0/G1 arrested cell by triggering DNA synthesis. It is proven through a study that FGF (fibroblast growth factor), vasopressin and dexamethasone etc active even in their low concentrations in the presence of insulin while on the other they were not active even at respectively high concentrations, when the insulin was absent (Straus, 1981).

2.3. IGF signaling system (Insulin-like-growth factor system)

IGFs are involved in bridging insulin resistance to cancer (Djiogue et al., 2013). Hormonemediated cancer is majorly concerned with IGF system because of IGF-1's mitotic properties. With sustained hyperinsulinemia, consistent high expression of GH receptors, INSR and IGF-1R expressed in malignant cells that triggers the dysregulated signaling cascades MAPK and P13K/Akt. FOXO family transcription factors are directly phosphorylated by Akt thus affecting cell metabolism, differentiation, apoptosis, cell cycle arrest and DNA repair in cancer. Akt also inactivates the cell growth inhibitor; tuberin. Akt leads to the activation of raptor-mTOR complex which plays a central role in cellular metabolism and growth. PTEN, a lipid phosphatase that is a tumor suppressor also disrupted in such case. (Belfiore&Frasca, 2008)

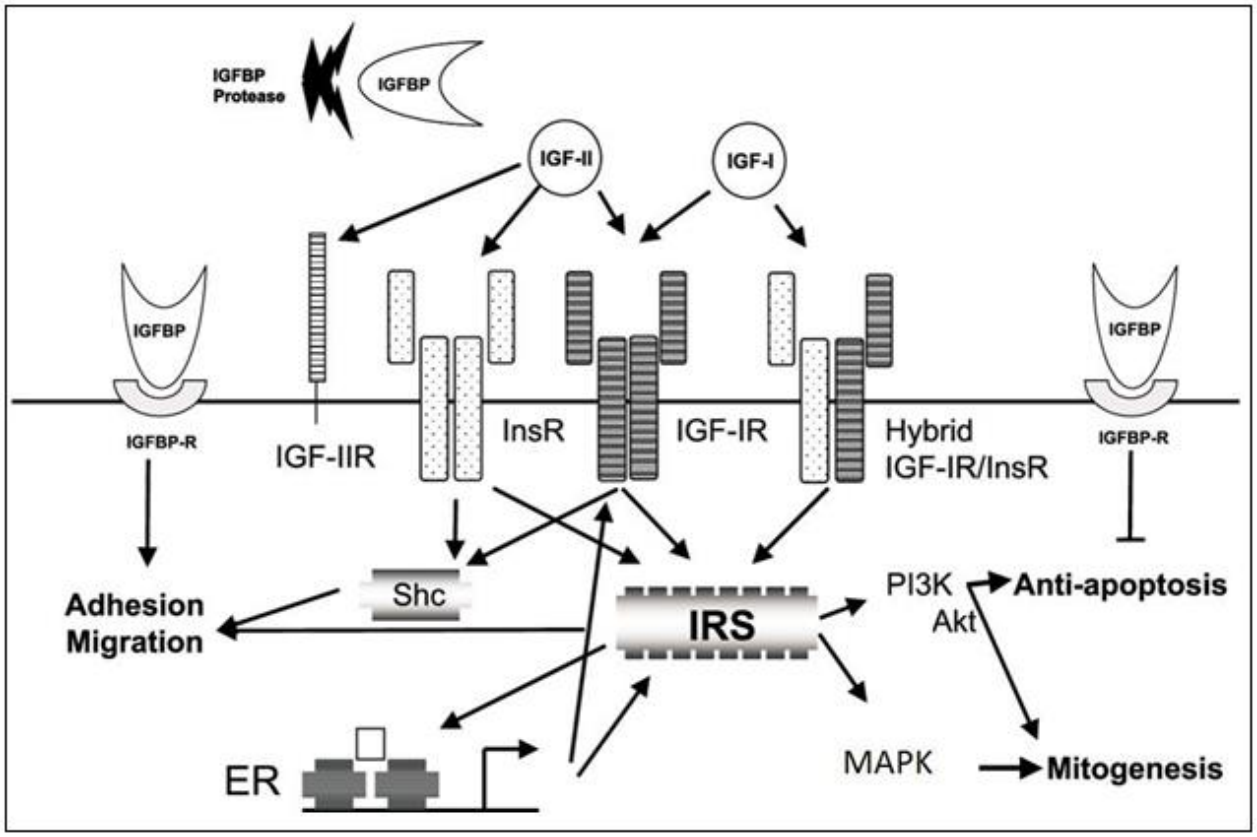


Figure 2.2. The purpose of this figure is to reveal the role of INS and Insulin-like-growth-factors in cellular migration, antiapoptosis process, growth, proliferation and mitogenesis (Sachdev& Yee, 2001).

2.4. Interaction of GLUT4 and INS with female hormones

As it is well known that lactogenic hormones up-regulate the beta cells production and insulin secretion (Petryk, Fleenor, Driscoll, &Freemark, 2000). In case of breast cancer there is high level of prolactin in blood as you see the location of breast tumors are milk producing lobules and transferring ducts. So, in diabetic patients when cells are already resistant to insulin and beta cells are producing more and more insulin that will result in hyperinsulinemia. Prolactin further increasing INS level. Thus boosting the breast cells proliferation.

Actually what happens in breast cancer, estrogen level rise to its maximum. INS and IGFs as blood serum variables cause ovaries to secrete more sex steroids like estrogen that effect the epithelial and endometrial wall cells of breast to proliferate.

Estradiol is estrogen steroid hormone that participate in breast development. Its level increases in breast cancer tissues. Like prolactin it controls insulin production. Boost up the metabolism and mitogenic pathways including insulin signaling pathways. High level of estradiol in breast cancer case will upsurge the GLUT4 translocation to membrane surface via PI3K/Akt signaling pathway (Morán, Garrido, Alonso, Cabello, & González, 2013).

2.5. Metastasis in association with GLUT4 and INS

The phenomenon in which cancer cells move away from its origin. They can metastasize through blood vessels or lymphatic vessels or through the body lining to cavities. To metastasize through blood or lymphatic vessels these vessels need to be available around the tumor cells so angiogenesis or lymphangiogenesis takes place in which VEGF play its main role (institute, 2020). Cancer cells create hypoxic condition for survival in which HIF1α is activated. This activated HIF1α up-regulate GLUT4 expression. As you know cancer cells depends on

glycolytic pathway for ATP production that is essential for cancer cells growth and motility. So by inhibiting the HIF1α, GLUT4 expression will be down-regulated so that no high production of ATP occurs and thus leads to rearrangement of actin cytoskeleton and suppression of microtubules dynamics that will result in mitotic arrest. So finally by suppressing the GLUT4 expression body get rid of cancer cells growth and metastasis (Torres et al., 2012). One more thing to know is when the cells of an organ metastasize to another organ they still named after first organ e.g., when breast cancer cells metastasize to lungs, they still called breast cancer cells there.

In pre-clinical breast cancer models using 'paclitaxel' that is a chemotherapeutic agent used to cure the breast cancer, in combination with IGF hindering revealed a significant suppression in tumor cell proliferation and its metastasis to lungs contrary to paclitaxel monotherapy (Ireland et al., 2018).

2.6. Fact of insulin intake in cancer therapy

Insulin use as adjuvant drug along with the chemotherapeutic agent 5-fluorouracil (5FU) and cyclophosphamide (CPA) on MCF-7 breast cancer cells reported in a study. It showed that low dose of MCF-7 and CPA didn't work but with the presence of insulin it worked. Likewise alone insulin couldn't inhibit the cellular growth. INS was reducing the chemotherapeutic resistance. Higher expression of pro-apoptotic Bax protein, higher protein expression of Caspase 8, autophagy-related protein 7, Atg-7, was detected in cells treated with insulin and drugs combinations. As adjuvant agent INS also hindered the breast cell proliferation and motility. INS –CPA treated cell show less intracellular ROS formation. Insulin could enhance permeability of cancer cells membrane, which leads to an increased uptake of cytotoxic agents

even in low doses this is called as Insulin-mediated-endocytosis. Induced INS then enhances the expression of IR, IGF that activate the P13K and MAPK signaling pathways that promote cellular metabolism by affecting GLUT transporters expression. So that fast metabolism result in higher susceptibility of cancer cells to cytotoxicity and apoptosis through consecutive administration of chemotherapeutic agents (Agrawal et al., 2017).

2.6.1. Metformin

Metformin is a very popular medicine used for treating T2DM. Its marketed name is Glucophage. Diabetic patients who intakes insulin glargine are at higher risk of cancer development than rest of insulin types (Michael Pollak, 2008). Although other insulin varieties also increase cancer risk but not more than glargine. But in a study it is revealed that use of metformin i.e. antioxidant along with low dose of insulin uptake cancel the risk of cancer while in case of high dose insulin consumption it halves the cancer risk (Currie, Poole, & Gale, 2009; Kalvaitis, 2009).

Metformin inhibit the gluconeogenesis in hepatocytes by stimulating AMPK pathway, this brings the reduction in the blood glucose level along with the secondary decline of insulin and IGFs level in blood (De Censi et al., 2010). In neoplastic cells it activates AMPK that leads to inhibition of mTOR signaling, protein synthesis and thus cellular proliferation (M. Pollak, 2008).

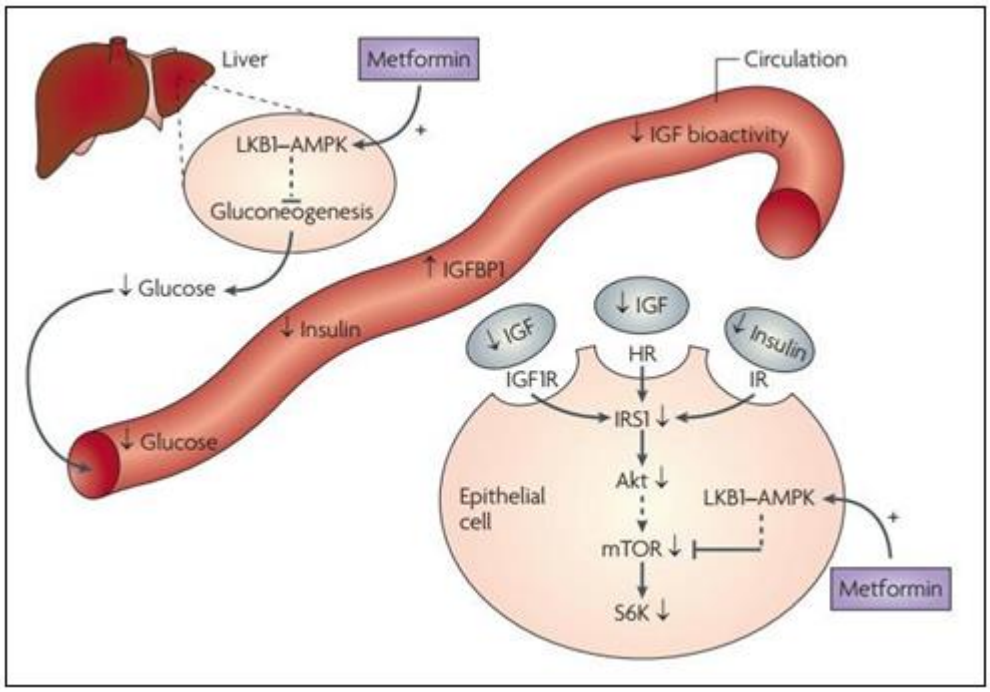


Figure 2.3. As concerning to metformin action. It doesn't act AMPK directly but in the presence of LKB1 (Liver Kinase B1 also called Serine/Threonine Kinase 11. After activating AMPK in hepatic cells metformin suppress gluconeogenesis thus reducing blood glucose level and finally insulin level in blood. This AMPK activation will bring mTOR inhibition and so that of protein synthesis and proliferation (M. Pollak, 2008).

2.7. Obesity, Insulin resistance and breast cancer

Excess of body weight associated with hyperinsulinemia is the considerable risk factor for breast cancer. So while discussing obesity adipocytes are on the top of list which play here the major role. They produce adipokines that's circulating concentration is directly proportional to BMI except adiponectin (Vona-Davis & Rose, 2007). Adipocyte secreted hormones and proteins that are involved in anti-apoptotic programs and proto-oncogenes stabilization stimulate mammary tumorigenesis, directly (Iyengar et al., 2003). Leptin (adipocytokine) with its pro-inflammatory property oppose insulin action (Paz-Filho, Mastronardi, Franco, et al., 2012; Paz-Filho, Mastronardi, Wong, &Licinio, 2012) and its high level in obese/insulin resistant patients induces high oxidative stress in endothelial cells (Fantuzzi, 2005) thus participating proliferation in breast endometrium. It influence STAT3, AP-1, ERK2 and MAPK so that involved in survival

and proliferation of breast cancer cells. Incidence of breast cancer through low level of adiponectins with its anti-inflammatory and insulin-sensitizing characteristic play role in PPARɤ pathway activation that regulate cell proliferation and differentiation so hyper expression of

BRCA1 (Breast cancer type 1) is also reported in MCF-7 breast cancer cells (Pignatelli, Cocca, Santos, & Perez-Castillo, 2003).

In another study it is revealed that it's not the overweight that increase the breast cancer risks but in fact the metabolic abnormalities that occur due to overweight results in breast cancer. Through epidemiological research it is said that overweight women along with insulin resistance had 84% more chances of breast cancer risks than obese women without insulin resistance. It was also reported that normal weight postmenopausal women were on greater risk of developing breast cancer than obese premenopausal women. It means that hormone levels matters more than excess body weight (Doheny, 2018).

Moreover Triglycerides (TG), prolactin, non-esterified fatty acids (NEFA) and VEGF are the associated markers of obesity and breast cancer (Luque et al., 2017). Actually, these markers of BMI influence the insulin resistance in person and is associated with breast cancer.

2.8. Oxidative stress

It is the state of imbalance between the availability of free radicals and body ability to detoxify them by neutralization. It is the common most condition in both the disorders i.e. diabetes and cancer. The free radicles then brings up the DNA, proteins and lipids damage. The increase production of ROS modulate the insulin signaling that may result in insulin resistance (Erejuwa, 2012). Triggering of P13/Akt signaling cascades with overproduction of ROS causes DNA

mutations for cancer development and progression. In immunological inflammation NFkB coordinate it with cell survival. NFkB also drives Cav-1 loss that reverse the Warburg effect. Oxidative stress in cells bring oxidative mitogenic pathways stimulation and also produce mitogens that leads to ROS implication in cell signaling (Klein & Ackerman, 2003). So antioxidants like metformin, few vitamins and quercetin are also beneficial for cancer treatment as used in diabetic therapy (Arcidiacono et al., 2012; Mirabelli et al., 2020).

2.9. Modern approaches for Diagnosis and therapy

2.9.1. Genomic markers

To avoid chronic side effects of systemic and traditional therapies and cost of such probable unnecessary treatments genetic testing has evolved for breast cancer screening and therapy (Serena Bertozzi et al 2018). Genetic testing is more efficient as it allow the prognosis, personalized diagnosis, treatments and their response evaluations. Blood testing for many biomarkers that are present in blood is done in such case. As this study is focusing on two common biomarkers 'GLUT4 and INS' for breast cancer and diabetes. So that expression profiling for these genes will reveal the susceptibility to diabetes and breast cancer. Thus, moving towards DNA analysis for checking the changes in such genes and diagnosing the disease in subject and finally genetic treatments evolution in these conditions.

After knowing all the above-mentioned importance of GLUT4 and INS in bridging the gap between diabetes and cancer. It is practical to target these two genes for abrogating both disorders.

Targeting the genes can be at different stages and for different purposes. The purpose may be to silent the gene, to down-regulate or to knock-down the gene and it is done at different levels of

gene regulation pathways. It can be inhibited at mRNA level or protein level. By restraining the binding of GLUT4/INS with DNA, by directly degrading these serum variables, by hampering the transcriptional activity.

After the population, clinical and laboratory research pharmacological strategies tended to use the receptor specific drugs.

2.9.2. Anti-ligand and activators

INS, IGF1 and IGF2 are the serum variables. INS is produced in pancreas and interact with neoplastic tissues through endocrine signaling. While IGFs produced in liver and influence the neoplastic tissue through autocrine, paracrine and endocrine mechanism. While targeting these ligands their bioactivity can be reduced. These involves pharmacological measures to reduce the ligand concentration or use of ligand-specific antibodies. Activators of AMPK are also under consideration because they lower the amount of circulating INS and act as anti-proliferative agents by decreasing signaling downstream of insulin and IGF1Rs.

2.9.3. Anti-receptor

Several receptor-specific antibodies are being evaluated simultaneously in many ongoing clinical trials. INSR, IGF1R and IGF2R. INSR and IGF1R are much similar in their downstream signaling pathways but still different from each other as INSR involved in carbohydrates metabolism while IGF1R is involved in proliferation control. There is also the specificity difference of tyrosine kinase activities of the two receptors but many receptor-specific antibodies have been developed that effectively block the INSR-IGF1R hybrids. Receptor inhibitor like HSP90 (heat shock protein- Chaperon protein) target these receptors for anti-neoplastic activity purpose.

2.9.4. Tyrosine-kinase-inhibitors

Several tyrosine kinase inhibitors have been introduced in preclinical models that inhibit IGF1R and INSR activation. This can be possible by targeting binding proteins IGFBPs (specifically IGFBP2 and IGFBP5). These binding proteins having integrin binding site subjected to the activation of integrin-linked kinase. IGFBPs have great affinity to bind with IGFs that were in inactive state, previously. When cancer cells secrete IGFBPs proteases more and more ligands become bioactive and binds to receptor. So, to prevent this kind of receptor activation it is better to target the IGFBPs with inhibitors thus bringing the reduction in neoplastic proliferation.

Concerning the diagnosis strategies it is suggested that clinically IGFs and IGFBPs blood level testing is more efficient to detect the progression of early lesions in carcinogenesis than the old antigen-specific screening methods.

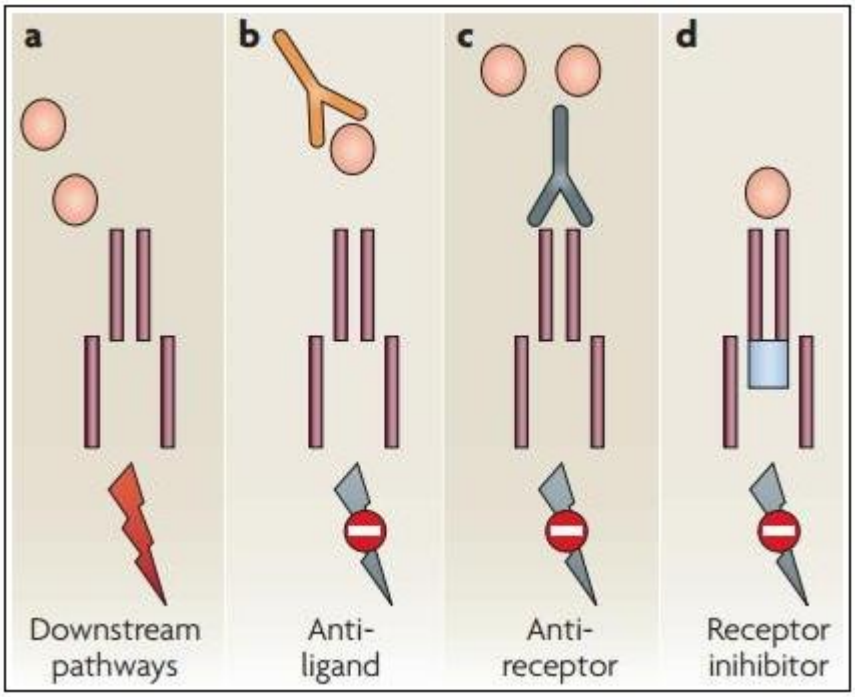


Figure 2.4. Targeting approaches; anti-ligand, anti-receptor and receptor tyrosine kinase inhibition. Inhibiting IGFs binding to their receptors, ligand specific antibodies to lower the ligand concentration in blood, receptor specific antibodies to block IGFs receptors and insulin receptor-IGF1R hybrids (M. Pollak, 2008).

2.9.5. Physical activity

It increases AMPK activation that will stimulate the GLUT4 translocation to surface, is that mean physical activity is promoting cancer as more glucose uptake for cancer cell hyper-metabolism? The answer is no. First of all the physical activity affects normal and cancer cells differently. It can help in cancer and diabetes treatment while promoting the normal cells resistance to drugs or radiations and making the cancer cells more vulnerable. The timing and magnitude of GLUT4 expression are differently controlled by different factors, such as diet and exercise (Calvo et al., 2010). So physical activity is playing a vital role. Obesity is the most probable reason than malnutrition in diabetes and breast cancer (De Pergola & Silvestris, 2013). Exercise has favorable effects on breast cancer cells by changing the levels of INS, IGFs, IGFBPs and inflammatory biomarkers. Physical activity decrease the level of insulin and IGFs. It decreases the level of C-reactive proteins that is high in inflammatory condition and produce interleukins in this response. Physical activity is also beneficial by boosting the cytotoxic activity of natural killer cells. Finally, we can say that exercise ameliorates cancer progression and results in breast cancer patient's survival (Löf, Bergström, & Weiderpass, 2012).

2.9.6. Reduction of GLUT4 expression

As GLUT4 is largely distributed among adipose and muscle tissues it is also present in breast cells. It's expression has been ascertained in triple negative and non-triple negative breast cancer experimental samples (Yang et al., 2015). With the presence of Insulin the P13K/Akt pathways activated that further activate the GLUT 4 transporter on cellular surface. In breast cancer cells the metabolism is very fast with the high level of GLUT 4 expression so that glycolysis is performed at it's efficient. That further generates anabolic metabolites that are subjected to

multiple cellular signaling cascades. So with the down-regulation of GLUT4 expression, the breast cancer cells viability can be impaired. In a research, scientists infected the MCF7 breast cancer cells with GLUT4 shRNA. They checked the cell proliferation rate that was subtled by knocking down the GLUT4. They reported that GLUT4 silenced cells showed reduced ATP production. Moreover these cells revealed low level of lactate thus reshaping the metabolic flux from glycolysis to oxidative phosphorylation and they confirmed this by the analysis of cytochrome oxidase subunit (CO-I) that was highly expressed in GLUT4 silenced cells. As CO-I is an indicator for oxidative phosphorylation. As it is already mentioned above P13K-AKTmTOR signaling cascade regulate the more GLUT4 translocation towards cellular surface and proliferation. So, Akt activation was prevented by down-regulating the GLUT4 and cells were prone to apoptosis. Along this the AMPK activation for autophagy was also certain, this also reduced LC3 cleavage and Sirtuin-I expression that are negative regulators of autophagy.

2.9.7. CoCl2inducedHypoxia and GLUT4

CoCl2 is hypoxia mimetic agent as it block the HIF1α degradation (Cascio et al., 2010; Wang, Wood, &Trayhurn, 2008). HIF1α then activate hypoxia associated genes like VEGF that play a vital role in cancer through angiogenesis. In a study, shRNA approach was used for silencing the GLUT4 gene expression that subsequently increased the levels of active caspase_9 (that regulate apoptosis) and hinder the CoCl2 effect thus compromises the cell viability in hypoxic condition and decreases the rate of cell proliferation and growth.

3. Materials and Methods

3.1. Sampling

Study was approved by ethics committee of Holy Family Hospital as well as ASAB, NUST, Islamabad. Samples were taken from Islamabad and Rawalpindi. Informed consent forms were taken from participants. 5ml blood was withdrawn from each participant. The patients enlisted for the study were categorized into four categories. First category include patients with breast cancer and co-morbidity diabetes. Second category include patients with breast cancer only while third category includes diabetic patients. Fourth category was of controlled subjects that were free of such diseases.

Table 3.1: Categories of study groups.

Group 1 (BC)	Group 2 (DM)	Group 3 (BC+DM)	Group 4
Breast Cancer Patients	Diabetes Mellitus patients	Breast Cancer + Diabetes Mellitus patients	Control group

3.1.1. Subject specificity

Patients diagnosed with Diabetes Mellitus and Breast cancer was selected for the case-control-study. Age factor was considered for all four study groups and human subjects who were in their 40s or more was selected for participation in the study. Patients that were diagnosed with chronic disease conditions related to kidney liver and mental disorders were excluded from the study.

Table 3.2: Sampling criteria of study.

Study Groups	AGE in years	SEX	No	Marital status	No	Duration
BC	23-60 Mostly(30-45)	F	26	Single	1	2-15y
				Married	25	
DM	25-72 Mostly(45-72)	F	5	Single	0	2-10y
				Married	5	
		M	2	Single	0	
				Married	2	
BC+DM	45-65	F	4	Single	0	<5 (3) >10 (1)
				Married	4	
Control	23-65	F	20	Single	3	
				Married	17	
		M	17	Single	4	

				Married	13	

3.2 Primer Designing

GLUT4 and INS Primers were designed using Primer3web software (version 4.0.0). The mRNA sequence was taken from NCBI. Primers were tested for their self-complementarity and other properties by OligoCalc (Oligonucleotide properties calculator). Primers were checked in primer blast and UCSC In-silico PCR for non-specific binding. Both Gene primers were order fromBeta actin was already available in lab as a loading control for determining expression that was tested for self-complementarity and other properties by Oligo Calc.

Table 3.3: Primer sequences for GLUT4 and INS and other properties.

Oligo name	*Sequence (5' to 3')*	*Product size*	*length*	*Tm (°C)*	*GC content*
H *SLC2A4 R*	*CTGTTTTGCCCCTCAGTCATT*	*195*	*21*	*60.6*	*47.62*
H *SLC2A4 F*	*TTCCTTCTATTTGCCGTCCTC*				*47.62*
H INS R	*ACATGCTTCACGAGCCCAGC*	*129*	*20*	*64.5*	*63.42*
H INS F	*TCAGAAGAGGCCATCAAGCA*				*59.01*

3.3 In-silico analysis

STRING and ENRICHR Databases were used to analyze the protein-protein interaction and role of candidate genes in transcription, pathways, ontologies, and diseases. Multiple protein option

was selected then gene ID from NCBI was entered. The species name was chosen to analyze the data.

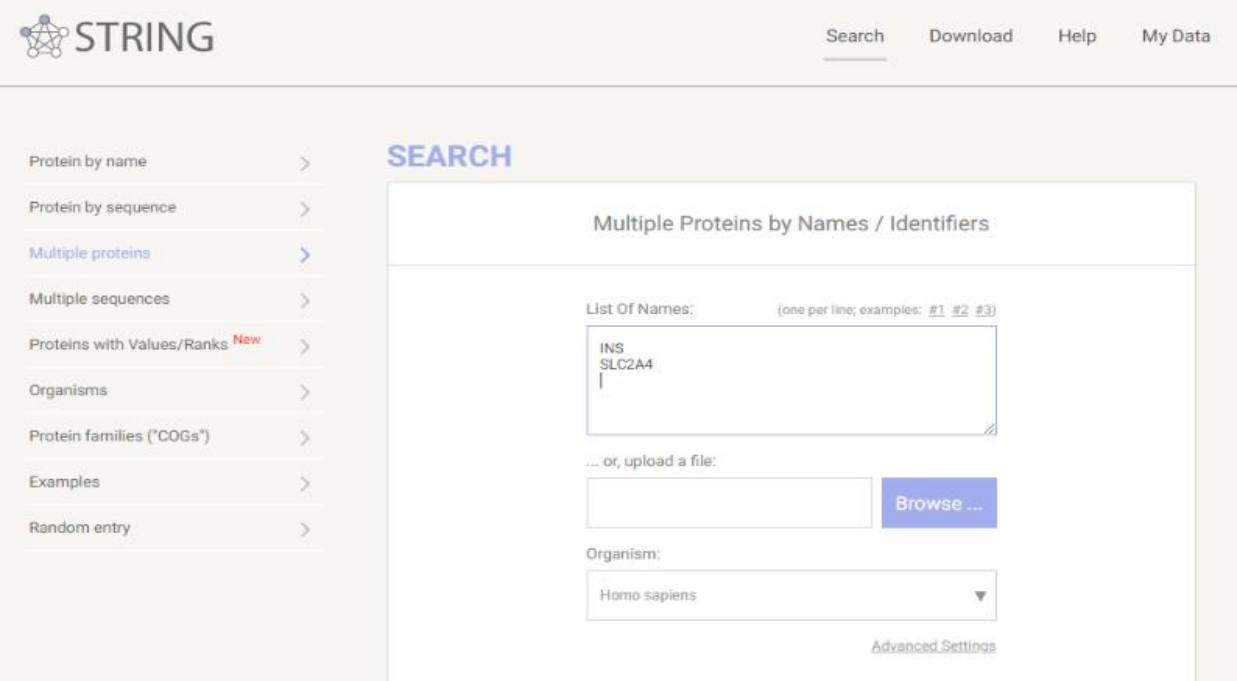


Figure 3.1. Figure showing the initial steps of string analyses of INS and SLC2A4 interaction.

3.4 Expression profiling

3.4.1. RNA extraction

RNA was extracted from blood samples for expression analysis of GLUT4 and INS in diabetic and cancer patients. For this procedure 200ul of blood sample was taken in 1.5ml Eppendorf containing 750ul TRIzol reagent that is used for RNA extraction from cell. Then solution was slightly vortexed to homogenize. The homogenized solution was centrifuged at 12000g for 10 minutes at 4^0C. After that the red/pink intermediate layer was separated from debris and poured into the new and labelled micro centrifuge tube. The tube was then placed in incubator at 25°C for 5 minutes. 5N glacial acetic acid was freshly prepared from which 20ul of glacial acetic acid

was added to the micro tube. Vigorous mixing of solution was done for 15 seconds. Then again incubated for 5 minutes. 200ul of chloroform was added to solution and for better emulsification and obtaining large volume of aqueous phase later in the process the mixture was shaken manually for 15 seconds. The mixture was further left at 25°C for 10 minutes and then two layers were obtained after spinning at 12000g for 15 minutes at 4^{0}C from which upper layer was transferred to a new micro centrifuge tube and 500ul of chilled isopropanol was added and vortexed to precipitate RNA. Room temperature incubation was given for 10 minutes after which mixture was centrifuged at 12000g for 10 minutes at 4^{0}C. The two layers obtained from which the supernatant fluid layer was removed and the bottom pellet was then washed with 1ml of 75% ethanol in DEPC-treated water. This was vortexed very shortly, centrifuged at 12000g (7500rcf) for 5 minutes at 4^{0}C. Again the supernatant obtained was gradually poured out and the pellet remained was air dried. The dried pellet was then put in 25ul of DEPC- treated water.

Finally, that pellet was incubated at 60°C for about 10 to 15 minutes. The RNA obtained was placed in -80°C refrigerator.

3.4.2. Quantitative Analysis

Quantitative analysis of gene expression gives more information about the pathology and evolution of disease than qualitative analysis. For the RNA quantification and purity assessment Thermo Scientific Nano Drop ND-2000 spectrophotometer was used. It is highly sensitive and less time consuming analysis. It measures even minute expression of genes and when the amount of nucleic acid is not enough in the sample. The RNA was taken from -80^{o}C and was immediately transferred to ice. For purity assessment we checked absorbance ratio A260/280 of the RNA sample. As protein absorbs 280nm wave so it determines protein contamination. (The

ratio for pure RNA $A_{260/280}$ **is** ~2.0). In this procedure, 1ul of DEPC treated water was used as a blank and 1ul of RNA in DEPC treated water was used for the measurement.

3.4.3. Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized by using Thermo Scientific Revert Aid Reverse Transcriptase. Purified 1ul RNA sample was used as template in this process. 1ul of oligo DT primers was added to a fresh tube. Then DEPC- treated water was added to bring the volume to12.5ul. Then it was mixed, gently. Then briefly centrifuged. The mixture was heat shocked at 65^0C for 5 minutes followed by chilling on ice, immediately. Again centrifuged the mixture and ice chilled. Added 4 ul of reaction buffer, 20 UNITS that means 0.5 ul of RNase inhibitor, 2ul of10mM dNTP mix, 200U of Revert Aid Reverse Transcriptase to the 1ul volume tube.DEPC-treated water was added to increase the reaction volume to 20ul. Incubated that at 42 degree Celsius for 1 hour. After that heated the mixture at 70 degree Celsius for 10 minutes to end the reaction. Finally, stored the cDNA at -20 degree Celsius.

3.4.4. Semi-quantitative PCR

The cDNA synthesis confirmation was held by adding 2ul Taq polymerase buffer, 2ul Mgcl2, 2ul of 2mM dNTPs mix, 2ul forward and 2ul of reverse primer for beta actin, 0.25ul of cDNA and 0.25ul of Thermo Scientific Taq DNA Polymerase to PCR tube. Nuclease free water was added to make the volume up to 20ul. After a bit spinning the reaction mixture it was loaded to thermal cycler. For beta actin the reaction conditions were 95^0C for 5 minutes for first stage then 35 cycles of denaturation was done at 95^0C for 30 seconds, annealing at 52^0C for 30 seconds and extension at 72^0C for 30 seconds. Final stage: extension was done at 72^0C for 10 minutes and the reaction product was kept at 4^0C.

3.4.5. Gel Electrophoresis

For the amplification confirmation of GLUT4 and INS specific region PCR product was ran in 2% agarose gel. For this 1g of agarose was dissolved in 60 ml of water and then microwaved to dissolve properly. 3ul of Ethidium Bromide (0.5ug/ml) was added into lukewarm mixture to prevent degradation. The gel was formed by 1X TAE buffer that was made from 50X stock solution. The gel was allowed to solidify and after that comb will be removed. Then 5ul of PCR product was loaded into wells along with 3ul of loading dye. The sample was loaded and gel ran for 45min at 70mV. Finally, gel was analyzed on Dolphin-Doc plus gel documentation system. By using Thermo Scientific Gene Ruler 50bp DNA ladder, product size was determined.

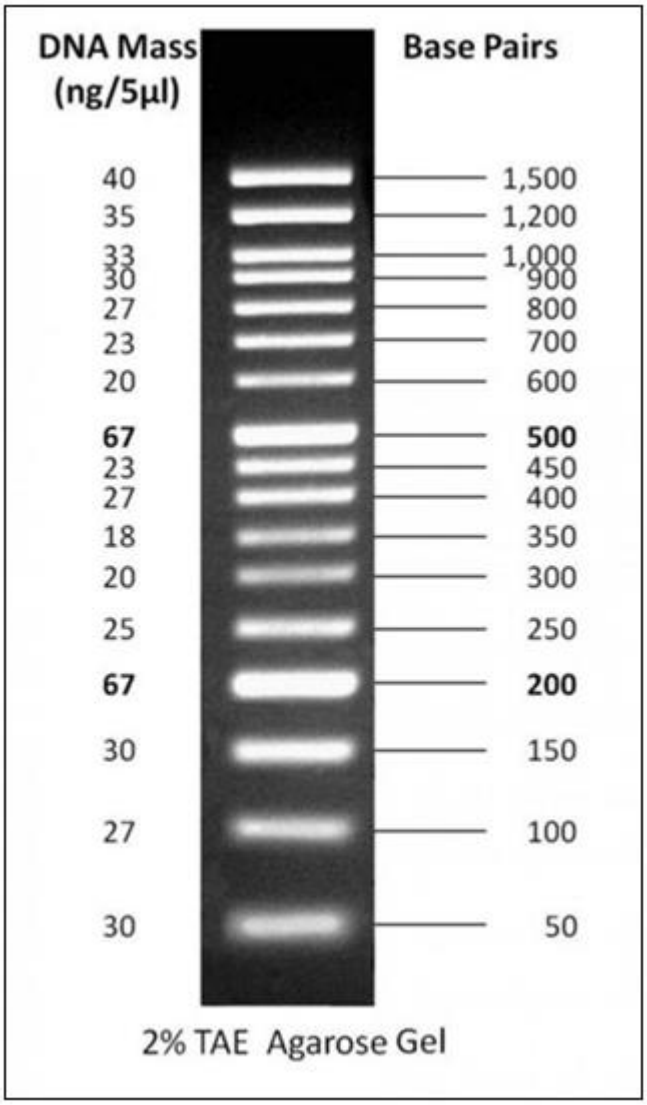


Figure 3.2. 50bp ladder for 2% agarose gel used in gel electrophoresis. It contain bands ranging from 50bp to 1.5kbp length(GOLDBIO, 2020).

3.4.6. Real Time PCR

The relative quantification was done by using real time PCR with the Applied Bios systems 7500 Real time PCR system and fluorescence based SYBER green technology. Quantitative expression of GLUT4 and INS gene was measured by real-time PCR. For normalization of data against a reference gene β-actin was used as internal control. PCR was done in final volume of20ul containing 7.5ul of Thermo Scientific Maxima SYBER green/ ROX qPCR Master-mix

(2X),0.5ul of both forward and reverse primer, 1.5ul of cDNA and 5ul Nuclease free water. The reaction was held in triplicates. Cycling conditions were with initial denaturation at 95^0C for 10 min, 40 cycles of 95^0C for 15 sec and 60.6^0C for 1 min, over a single hold of 1 min at 72^0C and dissociation stage consisting of 95^0C for 15 sec, 60.6^0C for 30 sec and 95^0C for 15 sec. Reaction of every sample was performed in triplicates. Non template control was run in each real time PCR reaction. The PCR product was further ran on 2% agarose gel along with Thermo scientific Gene Ruler of 50bp ladder for amplification of correct sized fragment assurance.

3.4.7. Statistical analysis

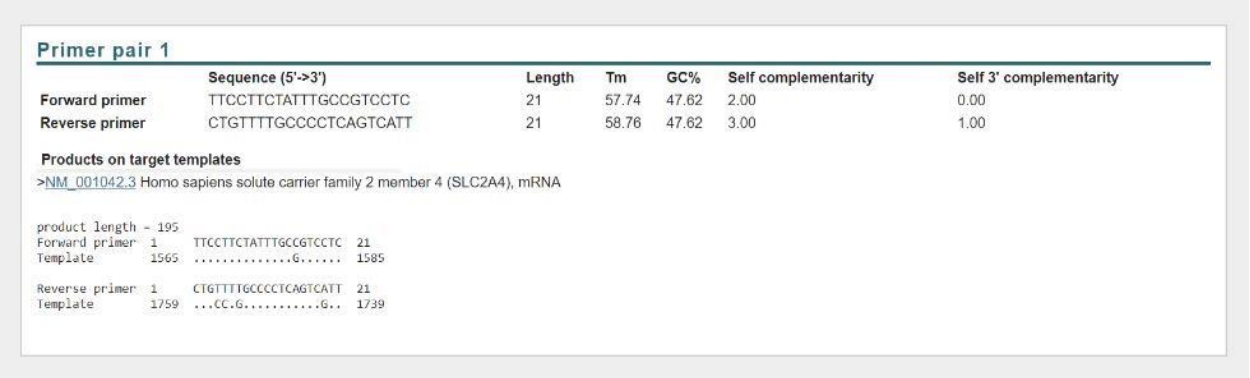
ΔΔCt also called Livak method was used to normalize the data and for this mean Ct was calculated manually and ΔCt was obtained using Excel. After that the fold change was obtained and the graphical representation was done by using Graph Pad Prism.

4. Results

4.1. Primer designing

The primers of SCL2A4 and INS both were designed in Primer3 Plus. Through primer BLAST and UCSC In-Silico PCR the sequence homology of both primers was confirmed.

4.1.1. Primer3Plus result



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCCTTCTATTTGCCGTCCTC	21	57.74	47.62	2.00	0.00
Reverse primer	CTGTTTTGCCCCTCAGTCATT	21	58.76	47.62	3.00	1.00

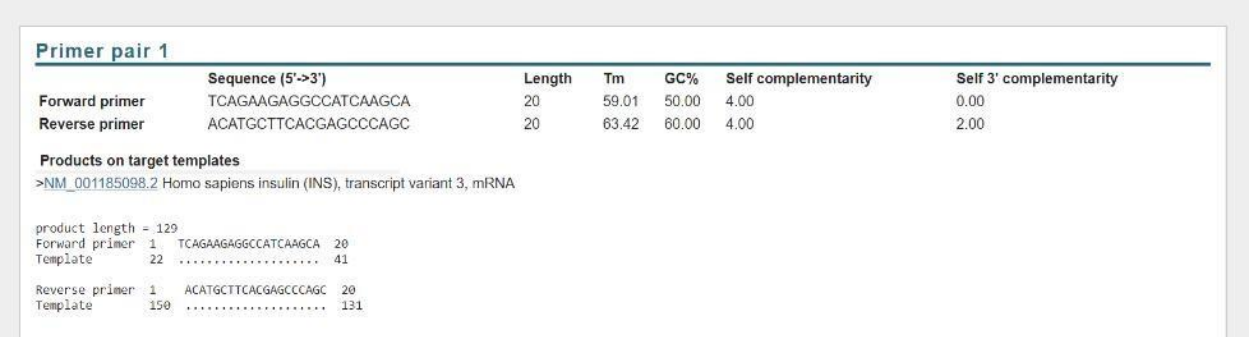
Products on target templates

>NM_001042.3 Homo sapiens solute carrier family 2 member 4 (SLC2A4), mRNA

```
product length = 195
Forward primer  1     TTCCTTCTATTTGCCGTCCTC  21
Template        1565  ..............G......  1585

Reverse primer  1     CTGTTTTGCCCCTCAGTCATT  21
Template        1759  ...CC.G...........G..  1739
```

Figure 4.1. Primer3Plus result of SLC2A4.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCAGAAGAGGCCATCAAGCA	20	59.01	50.00	4.00	0.00
Reverse primer	ACATGCTTCACGAGCCCAGC	20	63.42	60.00	4.00	2.00

Products on target templates

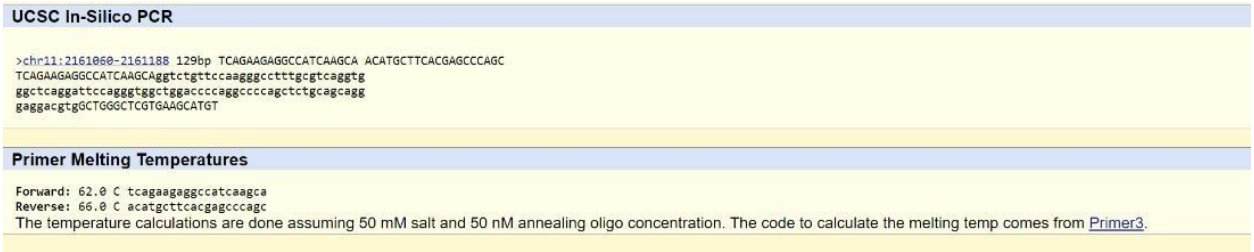
>NM_001185098.2 Homo sapiens insulin (INS), transcript variant 3, mRNA

```
product length = 129
Forward primer  1    TCAGAAGAGGCCATCAAGCA  20
Template        22   ....................  41

Reverse primer  1    ACATGCTTCACGAGCCCAGC  20
Template        150  ....................  131
```

Figure 4.2. Primer3Plus result of INS.

4.1.2 UCSC In-Silico PCR result



UCSC In-Silico PCR

>chr11:2161060-2161188 129bp TCAGAAGAGGCCATCAAGCA ACATGCTTCACGAGCCCAGC
TCAGAAGAGGCCATCAAGCAggtctgttccaaggggcctttgcgtcaggtg
ggctcaggattccaggggtggctggaccccaggccccagctctgcagcagg
gaggacgtgGCTGGGCTCGTGAAGCATGT

Primer Melting Temperatures

Forward: 62.0 C tcagaagaggccatcaagca
Reverse: 66.0 C acatgcttcacgagcccagc
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

Figure 4.3. UCSC In-Silico PCR result of INS.

4.2. String Results

Protein-protein interaction of INS and SLC2A4 and other proteins involved in apoptosis, proliferation, growth regulation, transcription and hypoxia was checked on biological database and web source; STRING. The result showed the link of INS with SLC2A4, IGF1, HIF1α, CMYC, BCL2, VEGF and STAT3. KEGG analysis showed their role in insulin resistance, breast cancer, HIF1α, AMPK, P13K-Akt, FOXO, adipocytokine, prolactin signaling pathway, autophagy, meiosis, longevity and other pathways in cancer. The network showed co-occurrence, co-expression, text mining and protein homology of the selected proteins.

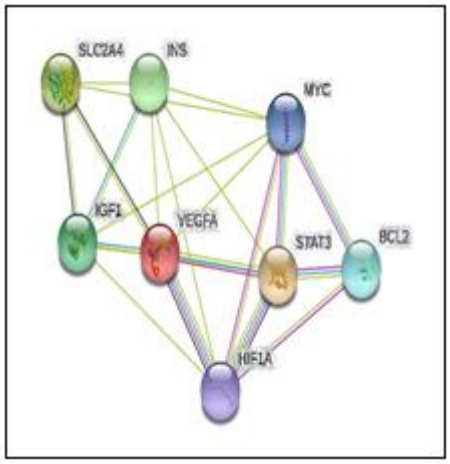


Figure 4.4. The interaction of proteins containing light green line meant for text mining, black for co-expression, light blue for curated database and purple for experimentally determined link. Interacting proteins INS (Insulin), SLC2A4 (solute carrier family 2, facilitated glucose transporter member 4), IGF1 (Insulin-like growth factor 1), VEGF (Vascular endothelial growth factor), STAT3 (Signal transducer and activator of transcription 3), MYC (Proto-Oncogene), BCL 2(*B-cell lymphoma 2*) and HIF1A (Hypoxia-inducible factor 1-alpha).

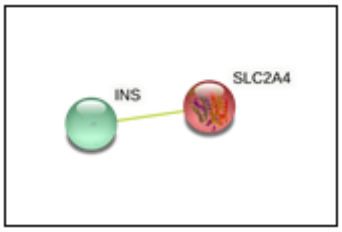


Figure 4.5. Interaction confirmation of INS and SLC2A4 by STRING.

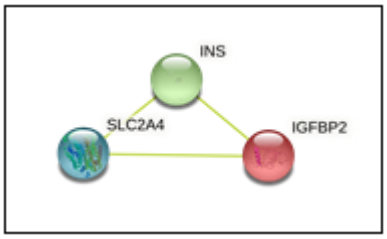


Figure 4.6. Network of INS, IGFBP2 and SLC2A4 through STRING

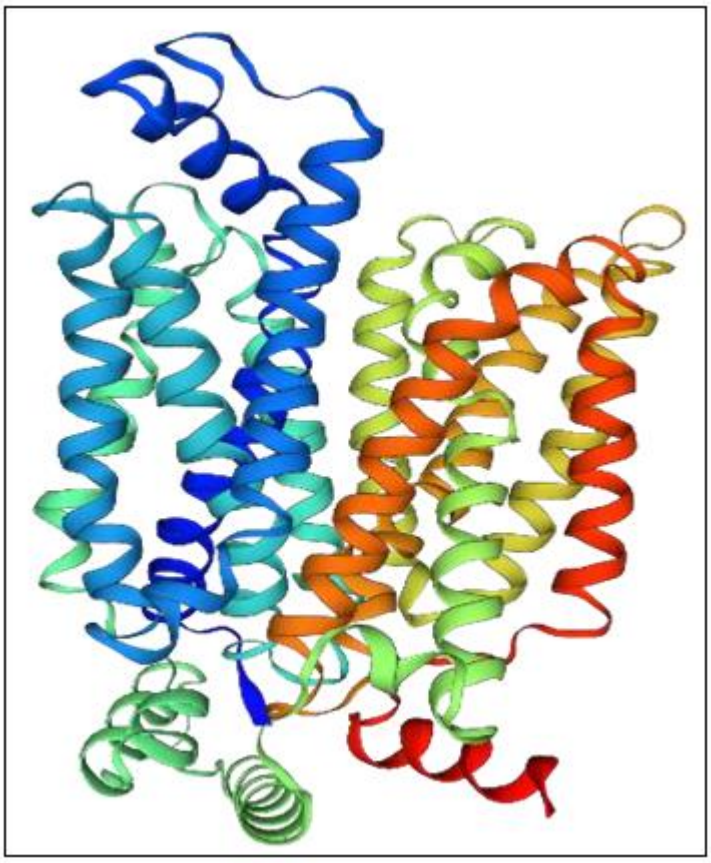


Figure 4.7. Homology model of SLC2A4 generated on STRING. Protein termini are represented by different colors.

4.3. Enrichr results

The pathway enrichment analysis of the two target genes INS and GLUT4 was done by web-tool; Enrichr. According to the year '2019' KEGG human pathway's bar graph representations were downloaded from this search engine. The participation of the curated genes in each pathway was sorted according to the p-value ranking. The finding showed the role of GLUT4 was highest in type 2 diabetes mellitus.

Figure 4.8. The bar graph of SLC2A4 involved pathways; the bars showing the level of enrichment of the gene in each pathway.

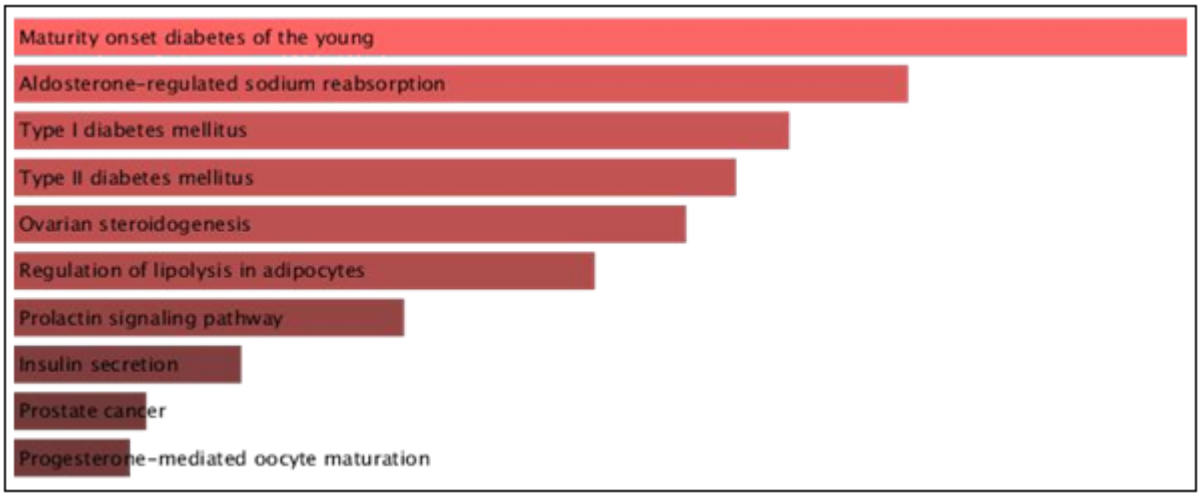


Figure 4.9. The bar graph of INS involved pathways; the bars showing the level of enrichment of the gene in each pathway.

4.4. Gel Electrophoresis Result

Amplification confirmation of GLUT4 and INS specific region PCR products in 2% agarose gel. The 50 BP ladder was opened well at voltage of 70 volts, 400A current for 40minutes. Beta actin was run along the target genes to verify the results. Eight welled combs were used to run the reverse transcriptase PCR processed samples.

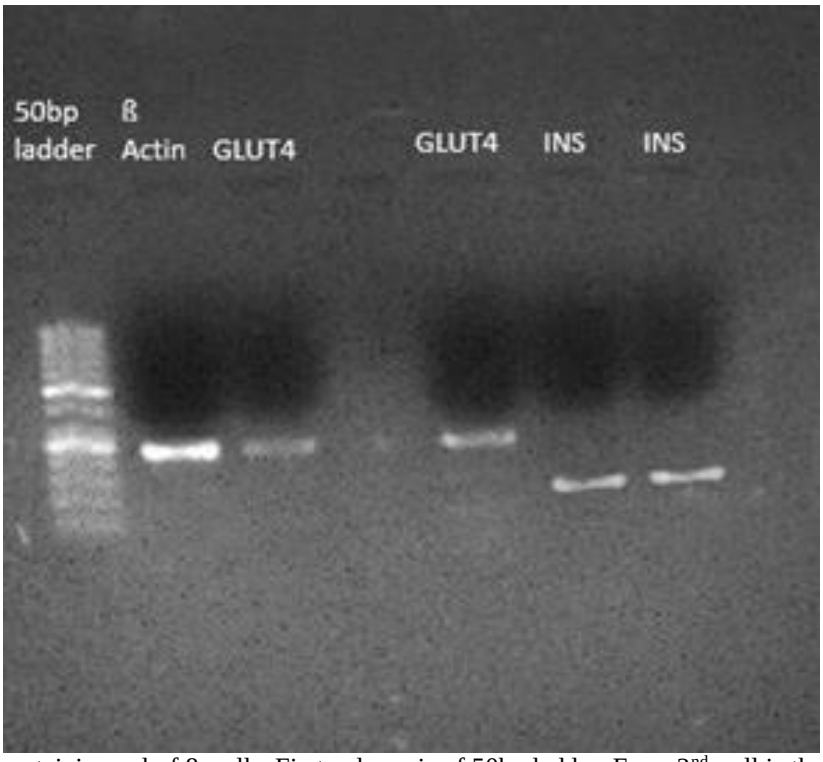


Figure 4.10. Fig containing gel of 8 wells. First column is of 50bp ladder. From 2nd well is the beta actin band of product size–200, from the 3rd and 5th well is GLUT4 band of product size-195 and INS band with product size of 129 is in-front of 6th and 7th well.

4.5. Expression analysis in study groups by Real Time PCR

For expression analysis three breast cancer three diabetic three both diabetic along with breast cancer samples and eight control samples were processed in RT PCR.

ΔΔCT approach was used for normalization against the reference gene that is beta actin and the comparative expressions of INS GLUT4 in all four study groups. The CT average of every sample was calculated for both target genes and a reference gene as the values were in triplicates. Then ΔCT was performed by using the following formula:

$$\Delta Ct = Ct \text{ of target gene} - Ct \text{ of reference gene}$$

After this ΔΔCT was taken,

$$\Delta\Delta CT = \Delta Ct \text{ (Diseased)} - \Delta Ct \text{ (controlled)}$$

This value show the change in the expression of the target genes between the controlled and diseased samples. Finally, the fold change was find through the following equation:

$2^{-\Delta Ct}$ = normalized expression ratio

The standard deviations of the triplicates were calculated with the formula:

$$\sqrt{(S1)^2 + (S2)^2}$$

Where S1 is Standard deviation of target gene and S2 is standard deviation of reference gene.

4.5.1. Comparative expression of GLUT4 in four study groups

Graph Pad Prism was used for the statistical analysis. Bar graphs were generated on the basis of mean fold changes of the genes that were already calculated in Excel. Standard deviation of target genes and beta actin was also considered in presence of triplicates.

Multiple t-test was applied in case of single category, but one way ANOVA was applied for 3 categories of diseases in a graph. All the results were statistically significant with p values less than 0.005. The Y axis in each bar graph belongs to expression of target gene in the study groups that is actually ‘mean fold change’ value.

Table 4.1: The table containing GLUT4 mean fold values and other physiological parameters of patient samples. The average mean fold of GLUT4 in control samples was 1.5.

Diseased Samples	**Glut4 Expression**	**Age Group**	**Duration**
BC	↑	***Around 50y***	***5-10y***
	↑	***Around 45y***	***<5y***
	↑	***Around 35y***	***<5y***
DM	↓↑	***25-30y***	***<5y***
	↑	***Around 46y***	***5-10y***
BC+DM	↑	***Around 45y***	***<5y***
	↑	***Around 54y***	***2y***

4.5.1.1. Comparison of GLUT4 expression in Breast cancer samples to control group.

The diseased samples mean fold change values and average of control samples mean fold values were entered in data. The results showed that GLUT4 expression was comparatively higher in breast cancer samples to the control one. The result was statistically significant with p value of 0.004 in multiple t-test.

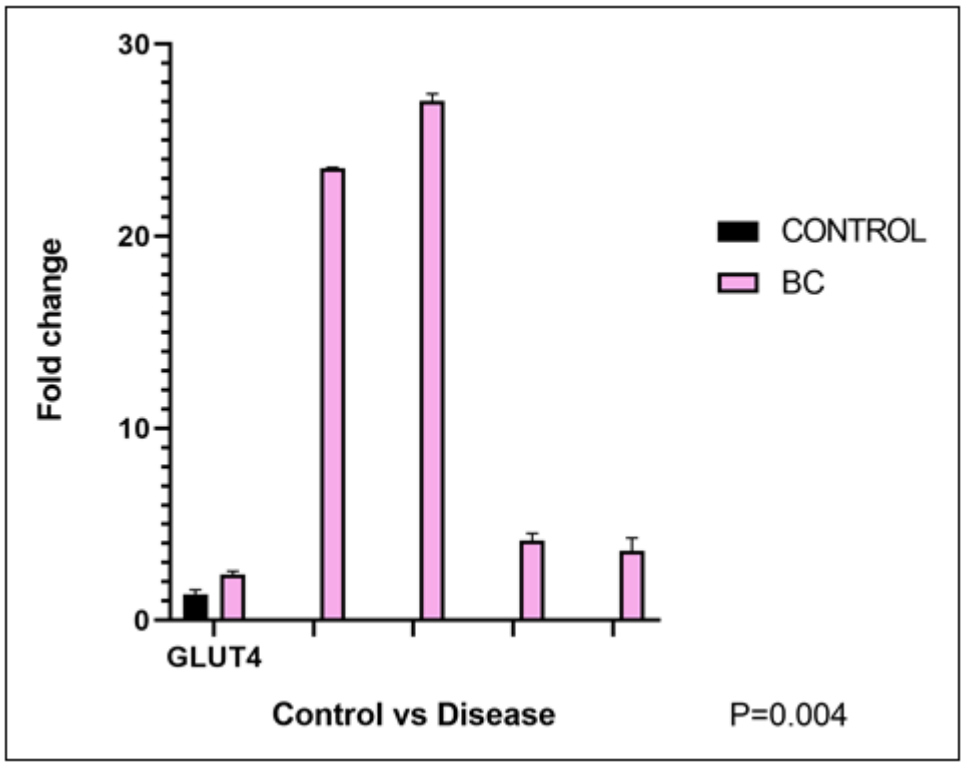


Figure 4.11. The bar graph containing black bar showing control group expression of GLUT4 while pink bars showing expression in breast cancer (BC) samples. Changing expression of target gene belongs to Y axis.

4.5.1.2. Comparison of GLUT4 expression in Diabetes Mellitus samples to control group.

The samples whose mean fold change values were entered in data includes both that showed GLUT4 expression was comparatively lower in diseased samples to the control one and also those that showed higher expression. The reason for this higher expression of some samples will be discussed in 5th chapter. In multiple t test the p value was 0.002.

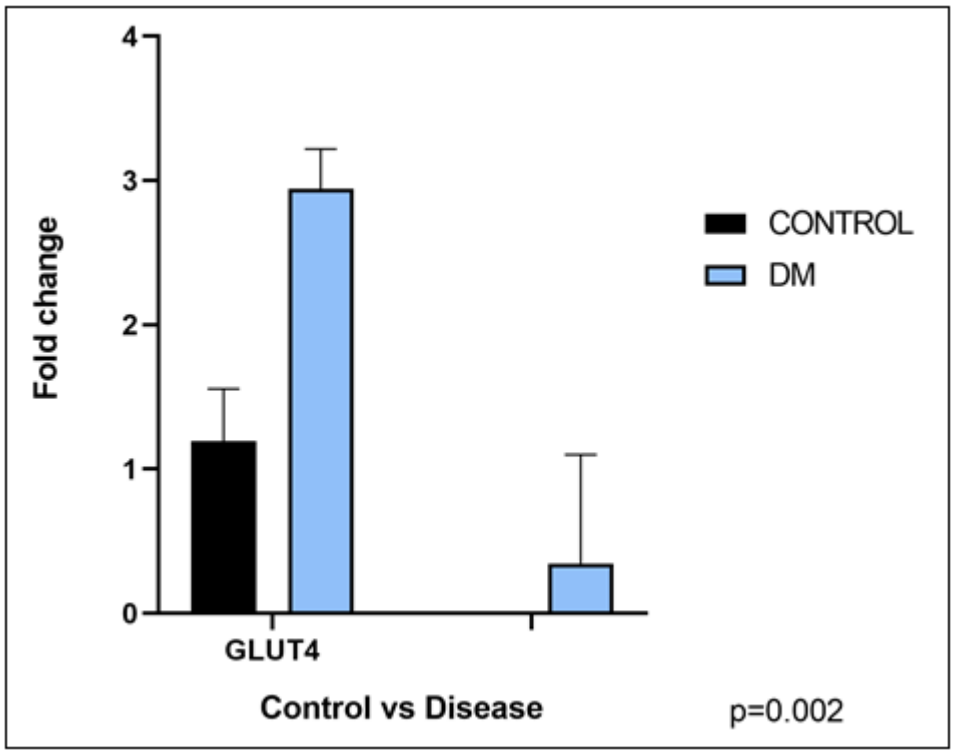


Figure 4.12. The bar graph containing black bar showing control group expression of GLUT4 while blue bars showing expression in Diabetes Mellitus (DM) groups. Changing expression of target gene belongs to Y axis.

4.5.1.3. Comparison of GLUT4 expression in samples with both diseases to control group.

The samples mean fold change values were entered in data. The results showed that GLUT4 expression was comparatively higher in diseased samples to the control one. T-test gave the significant result with p value of 0.004.

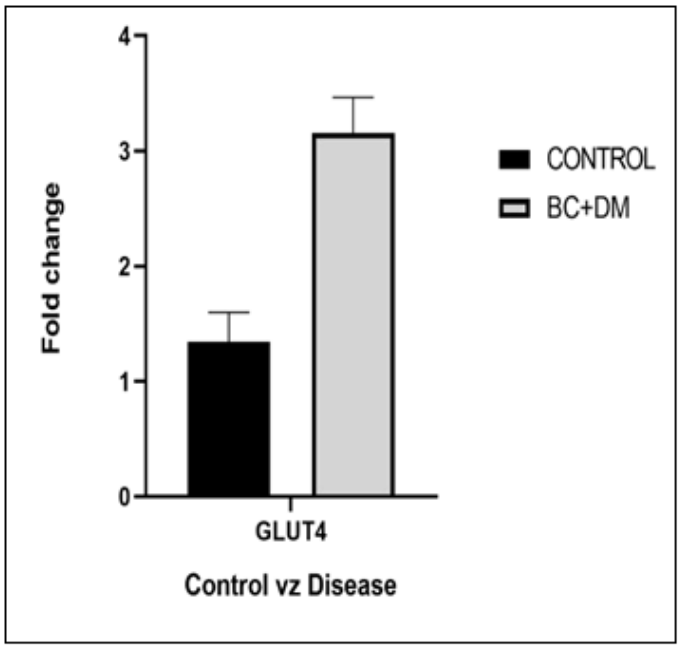


Figure 4.13. The bar graph containing black bar showing control group expression of GLUT4 while grey colored bar showing expression in samples with both Breast cancer (BC) and Diabetes Mellitus (DM) samples. Significant result with p=0.0015.

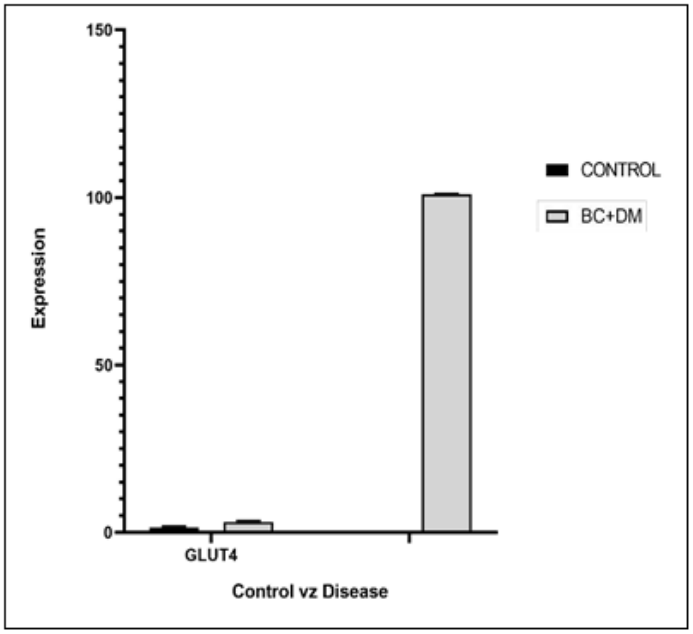


Figure 4.14. Two sample groups in which lower bar is of samples with age above 45y and higher bar of samples with age up to 45y. There is much difference in two diseased sample groups according to some physiological parameters that will be discussed later.

4.5.1.4. Comparison of GLUT4 expression in all four study groups

Average of samples mean fold change values from each study group was selected for the multiple comparisons. One way ANOVA was applied for four categories. The results showed that GLUT4 expression was comparatively higher in breast cancer samples, lower in Diabetic samples and also high in samples with both diabetes and cancer as compared to the control one. Statistical significance proved with p value of 0.004 in the analysis.

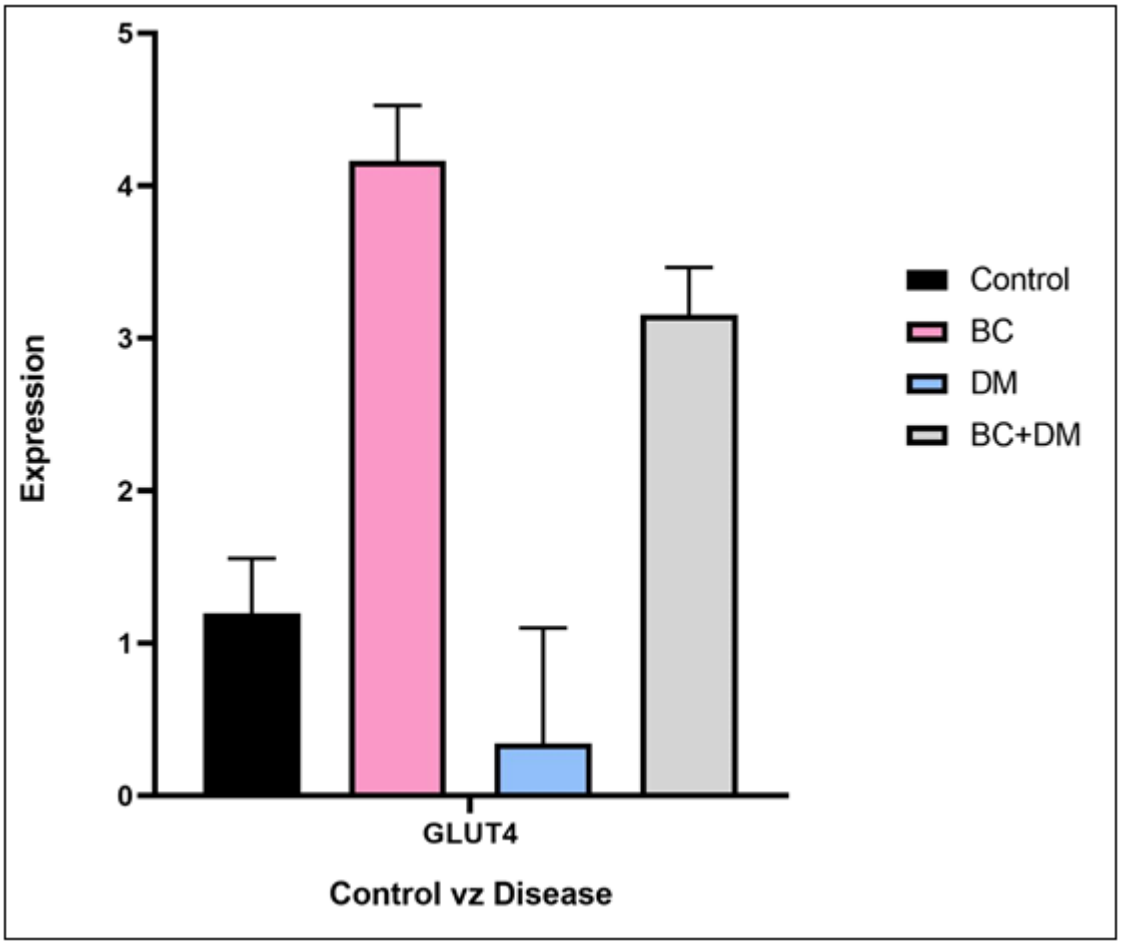


Figure 4.15. Comparison of GLUT4 expression in four study groups that is BC, DM and BC+DM. The differences between study groups are statistically significant, with p value less than 0.0001.

4.5.2. Comparative expression of INS in four study groups

Table 4.2: The table containing INS mean fold values and other physiological parameters of patient samples. The average mean fold of GLUT4 in control samples was 1.1.

Diseased Samples	**INS Expression**	**Age**	**Duration**	**Medication**
BC	↑	***Around 46y***	***<5y***	
	↑	***Around 35y***	***<5y***	
DM	↑	***Around 60y***	***>10y***	**INS**
	↑	***Around 46y***	***5-10y***	**Amaryl MS**
BC+DM	↑	***Around 45y***	***<5y***	
	↑	***Around 54y***	***2y***	

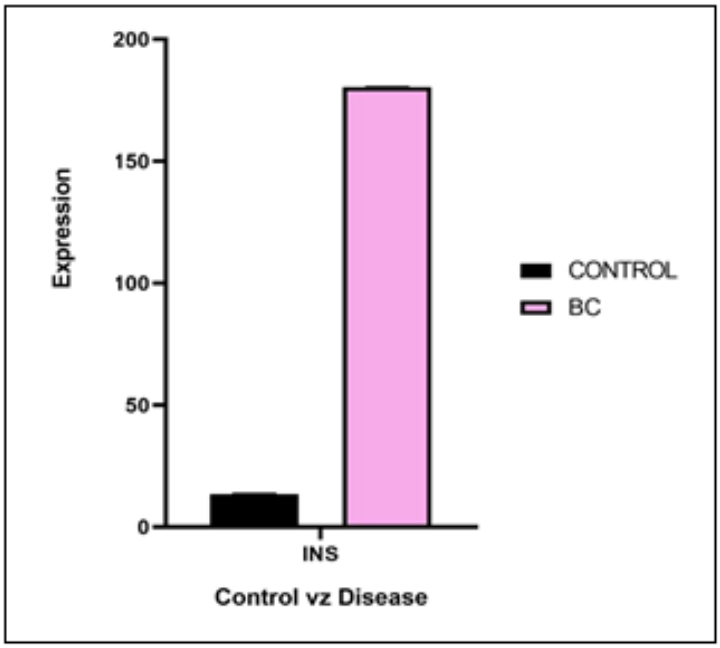


Figure 4.16. Comparative expression of INS in breast cancer (BC) patients vs control group. The mean fold change is showing that INS expression increases in breast cancer patients.

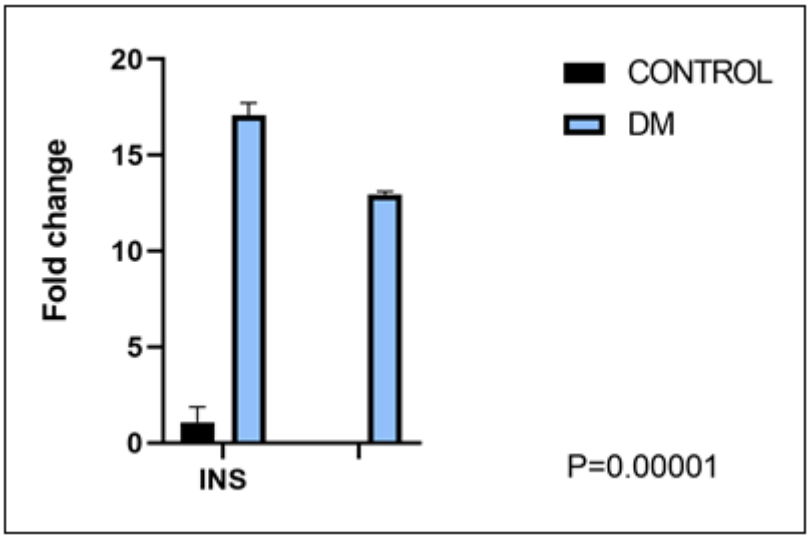


Figure 4.17. Comparative expression of INS in Diabetes Mellitus (DM) samples vs control group. The difference between the groups is statistically significant with p value of 0.00001. The difference between the blue bars height will be discussed later according to some physiological parameters.

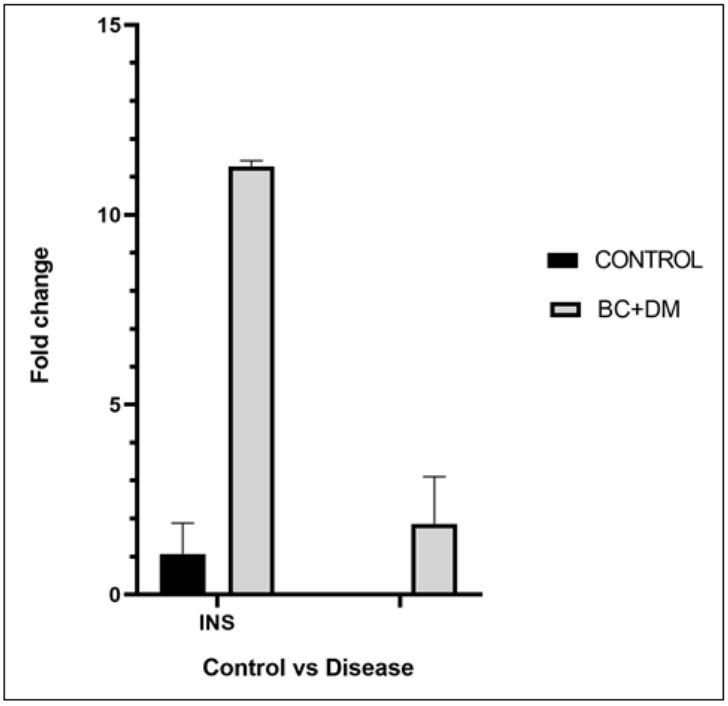


Figure 4.18. The comparison of INS expression in control and patients with BC+DM. The data of samples was entered in graph pad prism sheet for multiple t-test and the result shown significant p value of 0.00003.

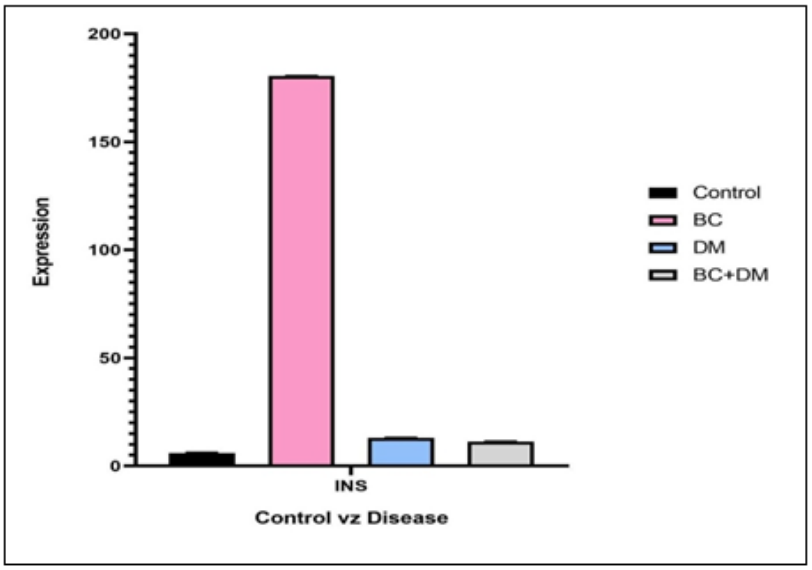


Figure 4.19. Comparison of INS expression in four study groups i.e. BC, DM, BC+DM. The differences between study groups are statistically significant, with p <0.00001.

5. Discussion

The two co-morbidities Type 2 Diabetes Mellitus and Breast cancer prevalence is growing exponentially all over the world. The estimated global prevalence of diabetes was 7.5% (374 million) in 2019 (Saeedi et al., 2019). According to World Health Organization (WHO) the contributors in this sky-kissing rise of such conditions are East Asian countries and North America. In the last few decades, 5.2 million deaths attributed to diabetes and breast cancer is the top leading death causing cancer among females, globally (Ferlay J, 2020).

Aetiological factors are much more alike in these two disorders and includes both genetic as well as environmental factors. The genetic components are fueled by environmental factors like obesity, unhealthy diet (Alcohol, drugs, and tobacco consumption), physical activity, physiological stress (Abrupt emotional and hormonal changes), infections, pollutants and radiations exposure. The emergence of genetic diagnostics and therapeutics has become prominent along with the rise of such non-communicable diseases. So genetic markers are essential for genetic predisposition, diagnosis, and treatments.

The study was planned to investigate the genetic association of GLUT4 and INS to T2DM and breast cancer. The reason behind this selection of INS gene was because of its participation in the main event ‘Hyperinsulinemia’ in such two conditions and where the GLUT4 is insulin dependent glucose transporter and used excessively for cancerous cells growth. It is reported that insulin resistance is concerned with GLUT4 impaired trafficking in T2DM. Beta cells consistently produce insulin to meet the need of cells but this substantial insulin resistance triggers other insulin like growth factors production in blood. These factors lead to the

overgrowth of cells and normal cell cycle shift to cancerous. Now, these cancerous cells will have up regulated GLUT4 expression on cell surface that is induced by IGFs.

In literature it is reported that INS level rise in blood till beta cell destruction occurs in diabetic patients but when the beta cells damage after a long period of stress in pancreas the insulin level falls. Hyperinsulinemia in breast cancer patients brings high level of IGFs that confirms high expression of INS. In the presence of insulin resistance, the GLUT4 that is insulin dependent glucose carrier of cell expressed less in case of diabetes. But in cancer patients the hyper-expression of GLUT4 is known because of its glucose up-taking activity for high metabolizing cancerous cells.

This project explored the role of GLUT4 and INS in four groups to evaluate the correlation between T2DM and breast cancer. Relative expression analysis of four study groups was done by Real Time PCR in our project. The processed samples gave the result in support to previous literature. INS was highly expressing in each study group i.e. breast cancer, Type 2 Diabetes Mellitus and the group with both diseases. But the expression level was varying in different samples according to patient's age, disease duration, stage, and medication they were on. The fold change of GLUT4 was higher in females with age below 45 than those of age above 45, in breast cancer group. But stage factor dominated the age factor. Some patients with age around 35 showed much higher expression than other females with same range of age because those patients were suffering with the stage 4, the metastatic cancer spread beyond the breast while other patients were having stage 1, 2 (fig 4.11).

According to previously conducted studies the GLUT4 expression in DM patients should be low but in case of our results some patients were showing up-regulation. The reason was that

some patients were using ‘Amaryl MSR’ for more than 5years. This medicine is used to boost the glucose uptake by stimulating the cell surface expression of GLUT4. This is done by the interference of Amaryl at a site of the putative insulin resistance defect (Müller, 2000). While some of the patients had some kind of breast diseases in the past. May be something linked with that disease is the reason behind high expression of GLUT4. Varied expression in these patients also observed. The age of some was around 23 year, less than other patients and duration was also comparatively very short so they had higher expression of GLUT4 than other patients. Although some patients were also taking Glucophage but GLUT4 expression was less, may be this medicine is not as effective as Amaryl or may be the reason is just behind the short duration of Glucophage consumption (fig 4.12). GLUT4 expression in group (BC+ DM) was also high according to expectations. The difference among various samples was also relating to age and duration factor. As already mentioned in our study that GLUT4 expression was comparatively higher in patients with lower age 30-45 than patients with age above 45. Some patient’s age was lesser than some other patients of age around 54y and short duration of 2 year (fig 4.14).

The fold change of INS was increasing with the increase of age in breast cancer patients. In diabetic patient of age around 60 year with more than 10-year duration of diabetes INS expression was lower than the patient with age around 46 and duration of less than 10 year (fig 4.17). In patient’s category of BC+DM the gap between the levels of INS expression was due to age gap. Patients with lesser age showed less dysregulation than the older age patients. Moreover, the newly occurred diabetes in some patients giving the reason of higher expression of INS in which pancreatic beta cells were not damaged yet, probably (fig 4.18). Also, the

higher GLUT4 expression observed in younger patients in that glucose is being utilized by the body cell by other means so body isn't over secreting insulin for more glucose uptake.

Diabetic patients are more prone to cancer and cancer patients prone to diabetes. The reason behind this statement has proved in our study through the expression analysis of GLUT4 and INS in these patients. While talking about first half of the statement hyperinsulinemia the main component of diabetes promotes growth factors that will lead to over-growth of cancer cells and proliferation. The second half of the statement that the stressed cancerous cells need high glucose uptake with high expression of GLUT4 more insulin is needed so it will exert stress on beta cells and finally they damage after a certain period thus leading to diabetes. Diabetes before beta cell damage may also occur in cancer patients in which stressed cells deprive normal cells of glucose as in case of some patients (Fig. 4.18) who got diabetic after having breast cancer.

The present study is limited to only blood samples for evaluating the expression of genes. Tissue sampling was planned but could not be done due to shortage of time also out of 80 samples collected we narrowed down the sample processing. There was lack of follow ups and some data concerning patients. What actually happening in signaling pathways remain undetermined.

6. Conclusion and Future prospects

The data results in our study supported that diabetic patients are more prone to cancer and also cancer patients most likely get diabetic. INS and GLUT4 showed significant association with T2DM and breast cancer. In future these genes will provide common biomarkers in such two morbidities for genetic testing and therapies. More confirmation analysis should be done with large sample pool and variety of ethnic groups. Additional research are still needed to clarify the cell signaling pathways and other reasons behind this kind of genetic expressions and thus making such common biomarkers to be functional in health industry.

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