Type 1 Diabetes Mellitus: Cellular and Molecular Pathophysiology at A Glance

Bahar Saberzadeh-Ardestani, M.Sc.^{1#}, Razieh Karamzadeh, Ph.D.^{1#}, Mohsen Basiri, Ph.D.¹, Ensiyeh

Hajizadeh-Saffar, Ph.D.¹, Aisan Farhadi, M.Sc.¹, A.M. James Shapiro, Ph.D.², Yaser Tahamtani,

Ph.D.^{1*}, Hossein Baharvand, Ph.D.^{1, 3*}

1. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

2. Clinical Islet Transplant Program and Department of Surgery, University of Alberta, Edmonton, AB, Canada 3. Department of Developmental Biology, University of Science and Culture, Tehran, Iran

#The first two authors equally contributed to this article.

*Corresponding Address: P.O.Box: 16635-141, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran Emails: yasertahamtani@royaninstitute.org, baharvand@royaninstitute.org

Received: 23/Sep/2017, Accepted: 10/Dec/2017

Keywords: Diabetes Complication, Environment, Etiology, Genetic, Type 1 Diabetes Mellitus

Cell Journal (Yakhteh), Vol 20, No 3, Oct-Dec (Autumn) 2018, Pages: 294-301_

Citation: Saberzadeh-Ardestani B, Karamzadeh R, Basiri M, Hajizadeh-Saffar E, Farhadi A, Shapiro AMJ, Tahamtani Y, Baharvand H. Type 1 diabetes mellitus: cellular and molecular pathophysiology at a glance. Cell J. 2018; 20(3): 294-301. doi: 10.22074/cellj.2018.5513.

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results from beta-cell destruction in pancreatic islets. Although it may occur at any age, T1DM most typically presents in adolescence with a peak onset around puberty. The incidence of T1DM is equal in both sexes during childhood, but males more commonly present with this disease in early adult life (1). Although previously most prevalent in Europeans, it is becoming more common in other ethnic groups. The International Diabetes Federation (IDF) 2015 Atlas has estimated that 415 million people worldwide have diabetes. This number is predicted to increase to 642 million by 2040 (2). T1DM comprises 5-10% of all causes of diabetes and is one of the most frequent autoimmune diseases of early life. The incidence of T1DM is escalating in all populations. It has been predicted that the incidence of T1DM in the under 5-year-old age group will increase two-fold in less than 20 years in Europe (3).

Although the precise causes of T1DM remain unknown, it is clear that both genetic and environmental factors play a role. The genetic region most strongly linked to T1DM is the human leukocyte antigen (HLA) locus (4). However, not all diabetes-related genetic factors are related to the immune system since genes associated with insulin production or beta-cell function have also been identified.

Environmental factors are important in T1DM to the extent that monozygotic (identical) twins with identical genomes may have different health fates due to exposure to different environmental factors (4). In contrast to the tremendous amount of data about the role of genetic factors in T1DM pathogenesis, there is much less information about the role of environmental factors. Because of the complexity of environmental parameters, their mechanisms of action are mostly unknown (5).

Over the past decades new treatments such as islet cell transplantation and generating insulin producing cells from stem cell have been investigated (6-8). However, in order to discover new therapeutic approaches for T1DM, it is necessary to understand the pathophysiology of T1DM and the mechanisms of its complications. This review summarizes some of the most important genetic and environmental etiologies of T1DM and their known mechanisms of action (Fig.1) and also presents T1DM-related chronic complications at the cellular and molecular levels (Fig.2).



Fig.1: Genetic, immunologic, and environmental etiologies of type 1 diabetes mellitus (T1DM). The outer circle shows some of the most important environmental etiologies of T1DM and the inner circle presents some of the most important genetic etiologies. The central circle demonstrates each genetic or environmental factor's known mechanisms of action. The left lower part of the circle shows the dsRNA virus, TEN/AIPS, GLIS3, CTSH, PTPN2 and HLA class 1 mechanism of action at the cellular level in the pancreas microenvironment, which leads to either necrosis or apoptosis of islet beta-cells. The upper part of the circle shows CTLA4, IL10, IL2, IL2RA, BACH2, and viral mechanisms of action in the lymph node. The right lower part of the circle shows PTPN22, HLA class2, and insulin mechanisms of actions which take place in the thymus.

AIP3; Actin interacting protein 3, CTLA4; Cytotoxic T-lymphocyte associated protein 4, CTSH; Cathepsin H, GLIS3; GLIS family zinc finger 3, HLA; Human leukocyte antigen, IFIH1; Interferon induced with helicase C domain 1, IL; Interleukin, IL2RA; Interleukin 2 receptor subunit alpha, INS; Insulin, JNK; c-Jun N-terminal kinase, MAVS; Mitochondrial antiviral-signaling, PTPN2; Protein tyrosine phosphatase non-receptor type 2, PTPN22; Protein tyrosine phosphatase non-receptor type 22, BACH2; BTB domain and CNC homolog 2, Tc; Cytotoxic T cell, Th; Helper T cell, NK; Natural killer cell, Treg; Regulatory T cell, DC; Dendritic cell, SP T cell; Single positive T cell, TCR; T cell receptor, and NF+kB; Nuclear factor kappa-light-chain-enhancer of activated B cells.

Type 1 diabetes mellitus pathophysiology

T1DM develops through elicitation of the immune system against beta-cell antigens and initiation of proinflammatory responses. After antigen presenting cells (APCs) present beta-cell antigens to the immune system, chronic immunological responses occur due to inefficient regulation of immunological reactions, which leads to destruction of beta-cells. Beta-cell death via virus directed or physiological mechanisms induces release of antigens and initiation of immune responses against other betacells. Usually dendritic cells (DCs) uptake these antigens and present them to T cells. An auto-immune response is only possible if autoreactive T cells have escaped thymic negative selection. Autoreactive T cells, activated by DCs, stimulate autoreactive cytotoxic T and B cells. Finally, effector mechanism of beta-cell destruction require the collective cooperation of DCs, macrophages, T, B, and natural killer (NK) cells (9).



Fig.2: Chronic complications of type 1 diabetes mellitus (T1DM). T1DM-related chronic complications are divided into two groups based on their pathogenesis: macrovascular and microvascular. The right half of the circle shows the pathogenesis of macrovascular complications [activation of protein C kinase and direct effect of AGEs] and the list of cardiovascular complications. The left half of the circle shows the pathogenesis of microvascular complications (indirect effect of AGEs] and defects in polyol metabolism) and the list of related complications. LDL; Low density lipoprotein, AGE; Advanced glycation end product, ECM; Extracellular matrix, VEGF; Vascular endothelial growth factor, GSH; Glutathione,

and NADPH; Nicotinamide adenine dinucleotide phosphate.

Essential role of environmental factors in type 1 diabetes mellitus

There are numerous environmental factors proposed to be important for development of T1DM. Some of the most cited environmental factors include reduction in gut microbiota, obesity, early introduction to fruit or cow milk during childhood, gluten, toxins, lack of vitamins, and viruses (5, 10, 11). As well, there are organs such as pancreas which take part in the pathophysiology of T1DM (Fig.1). For example, the effect of dsRNA viruses on pancreatic beta-cells and the relationship between these cells and the immune system is shown in the lower left quadrant of Figure 1. Lymph nodes and related mechanisms are presented in the upper right quadrant.

Gut microbiota reduction

Aconfrontation between immune cells and gut microbiota during early childhood activates immunoregulatory mechanisms which control autoimmune reactions-a phenomenon known as the "hygiene hypothesis". Tolllike receptor (TLR) 4, stimulating lipopolysaccharide (LPS), and other bacterial products that have contact with the immune system are reported as suppressors of autoimmunity (12). Therefore, a reduction in gut microbiota can lead to loss of control by the immune system, which is followed by immune cell activities against cells of the self, and finally lead to diabetes (13).

Obesity

Weight gain is another environmental issue in diabetes

that results in a higher beta-cell load and increasing insulin resistance (14). The accelerator hypothesis identifies constitution, insulin resistance, and autoimmunity as accelerators of beta-cell destruction through apoptosis. However, none of the mentioned accelerators leads to diabetes without obesity (5, 15). Higher weight gain in infants has been described as a risk factor for T1DM later in childhood (16).

Early fruit induction

Studies show that early introduction to fruit is associated with an increase in autoimmunity to betacells (17-19). This association may suggest an abnormal immune response to solid food antigens in the immature gut immune system in children with HLA susceptibility to diabetes, and can take part in T1DM pathogenesis (18). Furthermore, the "overload hypothesis" suggests that environmental exposures of food may overstimulate beta-cells, thus increasing their autoimmune-mediated destruction (20). Therefore, early introduction to fruit can lead to beta-cell autoimmune-mediated destruction.

Early bovine milk induction

Virtanen et al. (17) have shown that consumption of high amounts of milk products increases the risk of autoimmunity against beta-cells in young children with HLA susceptibility to diabetes. This increase may be the result of insulin autoantibody, because of the crossreactivity between bovine and human insulin (5). Studies show that children who lack the ability to develop *www.SID.ir* oral tolerance to bovine insulin are at risk for beta-cell autoimmunity. Therefore, reaction of the immune system to bovine insulin may lead to antibodies which attack human insulin in these children (5, 21).

Gluten

The introduction of gluten-containing foods (e.g., cereal) in diets of children younger than 3 months is associated with a significant increase in islet autoantibody production (22). Diabetic patients with human leukocyte antigenantigen D related (HLA-DR) allele have boosted T-cell reactivity to gluten derived polypeptides. This response has been characterized by IFN- γ and IL-17 secretion. Intestinal inflammation and T-cell activation induced by gluten could participate in the development of beta-cell autoimmunity (23).

Toxins

Early exposure to toxins (e.g., Streptomyces-infected root vegetables) can cause an abnormal processing of proinsulin and endoplasmic reticulum stress in betacells of the pancreas. Exposure of the immune system to abnormal proinsulin from beta-cells may activate autoimmunity mechanisms during early life (24).

Lack of vitamin D

Epidemiological analyses present strong evidence that vitamin D decreases the risk of diabetes (25). Vitamin D can directly modify T- and B-cell functions. Vitamin D receptor (VDR) agonists induce regulatory T (Treg) cells by stimulating tolerance (26). VDR agonists stop differentiation and maturation of DCs, downregulate expression of co-stimulatory molecules such as CD40, CD80 and CD86, and reduce production of interleukin 12 (IL-12). On the other hand, VDR agonists facilitate IL-10 production (27). All such mechanisms may lead to an immunosuppressive effect.

Viruses

Viruses are the most researched of the mentioned environmental factors (5, 28). A variety of studies have proposed that certain viruses are linked with progression of T1DM in animal models. Human studies further showed a similar role for enteroviruses (29, 30). Viruses may lead to T1DM by at least two possible mechanisms: i. A direct cytolytic effect on beta-cells (e.g., dsRNA virus as seen in the upper left section of Figure 1) or ii. Indirect triggering of a diabetes-associated autoimmune process against beta-cells which finally leads to beta-cell destruction (e.g., viruses as seen in the upper right section of Figure 1). The latter effects of viruses are attributed to the structural similarity between some viral structures and beta-cell antigens. Persistent virus infections may also be associated with induction of autoimmunity against betacells. Enteroviruses, rotaviruses, cytomegalovirus, mumps virus, rubella virus, Ljungan virus, and retroviruses may be implicated in the pathogenesis of T1DM (31).

Genetic factors

Genetic studies propose a considerable heritability (more than 80%) for T1DM (32). Thus far, genome-wide association studies (GWAS) and meta-analyses have identified almost 60 genes which contribute to the genetic susceptibility to T1DM (33). These genes are expressed in different cells of the immune system or pancreatic beta-cells, which reflect the autoimmune nature of the disease. In addition to risk prediction and heritability, these genes are considered valuable clues to molecular mechanisms of T1DM. Although detailed mechanisms of T1DM are mostly unknown, here we briefly describe some mechanisms for the genetic pathogenesis of T1DM by focusing on genes with recognized mechanisms.

Impaired central immune self-tolerance

Autoimmune diseases such as T1DM are caused by failure of self-tolerance mechanisms. Genetic factors of the genomic locus of HLA are considered to account for almost half of the genetic risk of T1DM (34, 35). Therefore, genetic factors of T1DM can be categorized into HLA and non-HLA factors in terms of their impact on genetic risk of the disease. Most associations between T1DM and the HLA locus pertain to HLA class II genes. These genes are expressed in APCs such as DCs, macrophages and the thymus epithelium. In the thymus epithelium, HLA class II is responsible for presentation of self-antigens which leads to development of T cell self-tolerance. Inefficient HLA class II alleles involved in interacting and presenting insulin in thymic epithelium are relatively associated with T1DM (36). This may permit insulinreactive T cells to escape negative selection. Lack of insulin expression in the thymus may also hamper negative selection. Polymorphisms which impair insulin gene expression in the thymus, but not beta-cells, are associated with T1DM (37, 38).

Polymorphisms in protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) gene which encodes lymphocytespecific tyrosine phosphatase (LYP) can also affect immune self-tolerance. LYP is a negative regulator of T cell receptor (TCR) signaling and a hyperactive LYP encoded by the PTPN22 risk variant that can inhibit TCR signaling during negative selection (39).

Impaired immune regulation and reactivity

Pathways and genes involved in progression and regulation of the immune response may also contribute to the development of autoimmunity in T1DM. For instance, it is proposed that polymorphisms in HLA class I genes contribute to progression of the autoimmune response in the later stages of beta-cell destruction. This hypothesis is supported by findings that a HLA class I risk variant can bind to T1DM autoantigens including proinsulin epitopes (40, 41).

An association exists between polymorphisms in cytotoxic-Tlymphocyte-associatedprotein4gene(*CTLA4*) and T1DM (42). CTLA4 plays an immunoregulatory role *www.SID.ir*

in effector T cells by suppressing the T cell response (43). CTLA4 is crucial for proper repressive function of Tregs in mice (44). Consistent with this idea, research has shown an association between a CTLA4 susceptibility variant and the frequency of Tregs in humans (45). These and other evidences suggest that CTLA4 dampens the immune response through both effector and Treg cells (46); hence, its T1DM risk variants may hamper either or both of these mechanisms. BTB and CNC homology 1 gene (*BACH2*) expresses a transcription factor that regulates Treg activity. The T1DM risk associated variant of *BACH2* causes abnormal Tregs which can stimulate autoimmunity due to ineffective regulatory control on inflammatory responses (47).

Cytokine signals between the cells of the immune system may be influenced by a genetic background. Different IL and IL receptor genes such as IL10, IL2, and IL2RA (codes for the α subunit of the IL2 receptor) are among the genetic risk factors of T1DM (48). These cytokines usually have multiple functions in the immune system; however, the net effect of their polymorphisms may demine their impact in T1DM autoimmunity. For instance, *IL2RA* is required for both regulatory and effector T cells. The Tregs express this gene constitutively, while effector T cells only express it after their activation. A variant of IL2RA with higher expression has been shown to have a protective association with T1DM (49). Polymorphisms in interferon induced with the helicase C domain 1 gene (*IFIH1*) may provide an example for interaction between genetic and environment factors of T1DM. IFIH1 is involved in evoking the immune response against RNA viruses. IFIH1 variants with reduced expression have a protective association with T1DM (50).

Beta-cell dysfunction and vulnerability

A number of genes linked to diabetes are involved in beta-cell functions (51). Immune destruction of betacells is mediated by an extrinsic apoptotic pathway that involves FAS-mediated T cell interaction (52) along with proinflammatory cytokines such as IL-1 β and interferon gamma (IFN-7) (53). Beta-cell sensitivity to these death signals can be influenced by the genetic background. For example, BACH2 is not only involved in regulation of the immune response, but also inhibits BIM activation and JNK1 phosphorylation via beta-cell response to proapoptotic signals. BACH2 has a crosstalk with another diabetes candidate gene PTPN2, which is an inhibitor of proapoptotic protein c-Jun N-terminal kinase 1 (JNK1) (54). The above mentioned apoptotic pathway is targeted with other T1DM genes such as CTSH (55) and GLIS3 (56). TNFAIP3, another T1DM gene, has been shown to provide a negative feedback loop for the proapoptotic activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (57, 58). Since nitric oxide and FAS-mediated pathways are downstream of NF- κ B in beta-cells (58), impaired TNFAIP3 function may influence these inflammatory and apoptotic mechanisms.

Most mechanisms that underlie the progression of T1DM by genetic factors remain to be determined. However, the above examples show how the genetic background can contribute to T1DM pathogenesis. Further functional analyses of these genes may shed light on the molecular mechanisms behind T1DM onset and progression.

Complications

The two major classes of late complications attributed to T1DM, microvascular and macrovascular, affect the heart, limbs, nervous system, eyes, and kidneys (Fig.2). The right half of the circle presents macrovascular complications whereas the left half shows microvascular complications. The pathogenesis of macrovascular complications is demonstrated by the role played by large vessels, the extracellular matrix (ECM), and cells in the right half of the figure. Intracellular mechanisms of neurological and lower extremity complications are shown in a neuron cell at the lower left quadrant of the circle. Finally, the upper left quadrant of the circle shows related mechanisms of ophthalmologic and renal complications.

Macrovascular complications of type 1 diabetes mellitus

Macrovascular complications comprise a group of large blood vessel diseases that occur in diabetic patients. In comparison with non-diabetics, the risk of cardiovascular disease in diabetic patients is four times higher. Coronary artery, cerebrovascular, and peripheral vascular diseases are categorized as macrovascular complications. Hemodynamic (blood pressure), metabolic (lipids and glucose), and genetic factors can increase the risk of these complications. Hyperglycemia is a major biochemical factor that increases the probability of cardiovascular disease. In addition, hypertension can increase the risk of diabetic related macrovascular complications such as coronary artery disease and stroke. Risk of hypertension in T1DM patients is 30% higher than non-diabetics. Oxidative stress plays an important role in hypertension related damage to vascular endothelial cells and cardiac hypertrophy. Optimal blood glucose and hypertension control in diabetics are effective ways to reduce the risk of macrovascular complications (59, 60).

Microvascular complication of type 1 diabetes mellitus

Damage to small vessels (capillaries) during high blood glucose levels can cause microvascular complications in tissues where glucose uptake is independent of insulin such as with neurons, the kidneys, and retina. Hyperglycemia, as the most important risk factor in diabetics, can cause neuropathy, nephropathy, and retinopathy by different mechanisms. Some of these mechanisms are more important in specific complications. Here, we classify microvascular complications into three categories–retinopathy, neuropathy, and nephropathy (60).

Retinopathy

Diabetes related damage to the macula, retina, or both can cause visual problems and blindness. The probability of retinopathy as a common diabetic complication is closely related to the duration of diabetes. Up to 50% of T1DM patients are at risk for retinopathy. Microvascular changes in diabetics as a result of hyperglycemia such as small vessel basement membrane thickening and increase in endothelial cell permeability can cause ophthalmological and renal complications (61).

Neuropathy

Damage from hyperglycemia to peripheral nerves, including sensory, autonomic, and motor neurons, can cause neuropathy. Hyperglycemia, disease duration, and genetic factors can increase the risk of this complication. Peripheral neuropathy can be characterized by axonal thickening, axonal loss, loss of microfilaments, neural demyelination, and neural death (61).

Nephropathy

Diabetic nephropathy is characterized by loss of glomerular filtration rate, albuminuria (>300 mg/day), and damage to glumeruli. Diabetic nephropathy can be seen in about 30-40% of diabetics. Hyperglycemia, hypertension, and hyperlipidemia are the main metabolic risk factors that increase kidney disease by several known metabolic pathways (61).

Pathophysiology of macro-and microvascular complications

Several mechanisms have a role in the pathogenesis of micro- and macrovascular complications. We classify these mechanisms into the following four categories (61).

Direct effect of advanced glycation end products

During long-standing hyperglycemia in diabetics, glucose forms covalent bonds with proteins through a non-enzymatic reaction between the free amino group of an amino acid and the carbonyl group of reducing sugars. This process leads to formation of advanced glycation end products (AGEs). Glycation disrupts molecular conformation and alters protein function. AGEs have crucial role in diabetes related cardiovascular and renal complications (62). AGEs can bind to intracellular and extracellular proteins and alter tissue functions. Binding of AGES to ECM proteins creates anchoring sites for proteins such as albumin, collagen, and elastin that leads to ECM thickening and atherosclerosis. Interactions of AGEs with ECM can impair matrix-cell and matrixmatrix interactions. This can induce cell death, cell differentiation, and cell migration. In cardiomyocytes, interaction of AGEs with intracellular proteins such as Ryanodine can disrupt Ca²⁺ homeostasis and induce the risk of heart related complications. Diabetic patients with cardiovascular disease have higher than normal serum AGEs. The high level of AGEs in serum can be used as a biomarker for cardiovascular diseases (63).

Indirect effect of advanced glycation end products

Binding of AGE to the cell's surface receptor leads to activation of multiple signaling pathways inside the cells and different responses of endothelial cells, smooth muscle cells, macrophages, and T cells. Activation of nicotinamide adenine dinucleotide phosphate (NADPH) and the MAPK pathway in response to AGE interaction with cell surface receptors can induce reactive oxygen species (ROS) production and NFkB activation, respectively. ROS has pivotal roles in diabetes related cardiovascular and ophthalmological complications. Transcription activation of multiple genes such as IL-6, tumor necrosis alpha (TNF- α), and vascular endothelial growth factor (VEGF) by NFkB can increase inflammation and arthrosclerosis (63). In different cell types, an increase in pre-clotting activity occurs in response to AGE interactions with cell surface receptors. In addition to pre-clotting activity cytokine production in T cells and macrophages, there is an increase in the dividing rate in smooth muscles and stimulation of ECM secretion by these cells can be seen during AGEs interactions with their related receptors (64).

Activation of protein kinase C

Diacylglycerol (DAG) accumulation in cells as a result of hyperglycemia can induce protein kinase C (PKC) activation. PKC is a type of serine/threonine kinase that has multiple isoforms. Different isoforms of this enzyme can be activated in various tissues to induce different complications. Hyperglycemia can induce β and δ isoform activation in vascular cells (65). The DAG-PKC pathway can induce cardiovascular complication by multiple ways such as ECM synthesis, angiogenesis and change of vascular permeability by VEGF production, cytokine activation, and cell growth. PKC β overexpression in transgenic mice can cause cardiomyopathy. In addition to activation of ROS and inflammation in cardiomyocytes in response to PKC activation, PKC can induce insulin resistance by phosphorylation of serine/threonine residues in cardiomyocytes. Disruption of insulin metabolism in cardiac cells can induce heart related complications (66).

Defects in polyol metabolism

In hyperglycemia, disruption of normal glucose metabolism leads to activation of the polyol pathway. Polyol pathway activation can cause peripheral nerve damage and increase the risk of lower limb amputation, or neuropathy (67). In hyperglycemia, there is a decrease in the level of glutathione (GSH) which is a precursor of NADPH. Decreased NADPH causes less production of fructose from sorbitol. The polyol pathway in neurons can cause cell death by osmotic damage and ROS production. In addition to the polyol pathway, PKC, AGEs, and hexosamine pathways have important roles in diabetes related neuropathy. These pathways can induce ROS and inflammation in neurons. Among the all above mentioned mechanisms, PKC activation and direct/indirect effects of AGE play a role in vascularization which has a critical role in numerous T1DM complications (68).

However, environmental factors are not the only pathogenic source of complications. Genetic factors can also affect this process. GWAS have important roles in discovering diabetes complication related genes and pathways. Identification of complication specific genetic variants can facilitate improvement of new and targeted therapeutic methods for each specific diabetes related complication. Different genetic variants have been discovered for diabetes complications. Diabetic vascular complication is good example that clarifies the role of genetic factors, environmental factors, and their interactions in disease progression. Polymorphism in lipid related metabolism genes such as APOE, APOB, APOC, CETP, and PON increase the risk of macrovascular complications in diabetic patients compared to healthy individuals. In addition to diabetic related cardiovascular complications, the role of genetic factors in diabetic related retinopathy, nephropathy, and neuropathy have been studied. VEGFA (encodes vascular endothelial growth factor A), and AKR1B1 (encodes aldose reductase, one of the polyol pathway enzymes) are the two best studied genes that play a role in diabetic related retinopathy. A study of the diabetics genome revealed that 11 single nucleotide polymorphism (SNPs) in different chromosomes could increase the risk of nephropathy. SNPs on chromosomes 7p, 9p, and 11p that are located near CPVL, FRMD3, and CARS play important roles in induction of nephropathy risk (69, 70).

Conclusion

This review has discussed T1DM pathogenesis, the role of primary genetic and environmental factors in this process, and the mechanisms of complications. However, much remains to be understood. Therefore, research efforts to elucidate the underlying mechanisms of T1DM can provide further therapeutic options for T1DM treatment.

Acknowledgments

The study was financially supported by grants from Royan Institute, Iran National Science Foundation (INSF), and Iran Science Elites Federation. We express our appreciation to all members of the Beta-cell and Diabetes Program at Royan Institute for their helpful deliberations and consultation during this work. The authors declare that there is no conflict of interest associated with this manuscript.

Author's Contributions

B.S.-A, R.K.; Drafted the review and prepared the figures. M.B.; Revised the section "genetic factors". E.H.-S.; Revised the "complications" section. A.F.; Contributed in drafting and revising the "complication"

section of the manuscript. A.M.J.S.; Revised the primary draft of the manuscript. Y.T., H.B.; Were responsible for overall supervision and finalizing the manuscript.

References

- Karvonen M, Pitkaniemi M, Pitkaniemi J, Kohtamaki K, Tajima N, Tuomilehto J. Sex difference in the incidence of insulin-dependent diabetes mellitus: an analysis of the recent epidemiological data. World Health Organization DIAMOND Project Group. Diabetes Metab Rev. 1997; 13(4): 275-291.
- Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract. 2017; 128: 40-50.
- Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G, Group ES. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet. 2009; 373(9680): 2027-2033.
- Steck AK, Rewers MJ. Genetics of type 1 diabetes. Clin Chem. 2011; 57(2): 176-185.
- Knip M, Simell O. Environmental triggers of type 1 diabetes. Cold Spring Harb Perspect Med. 2012; 2(7): a007690.
- Khosravi-Maharlooei M, Hajizadeh-Saffar E, Tahamtani Y, Basiri M, Montazeri L, Khalooghi K, et al. Therapy of endocrine disease: islet transplantation for type 1 diabetes: so close and yet so far away. Eur J Endocrinol. 2015; 173(5): R165-R183.
- Montazeri L, Hojjati-Emami S, Bonakdar S, Tahamtani Y, Hajizadeh-Saffar E, Noori-Keshtkar M, et al. Improvement of islet engrafts by enhanced angiogenesis and microparticle-mediated oxygenation. Biomaterials. 2016; 89: 157-165.
- Baharvand H, Jafary H, Massumi M, Ashtiani SK. Generation of insulin-secreting cells from human embryonic stem cells. Dev Growth Differ. 2006; 48(5): 323-332.
- 9. Wallberg M, Cooke A. Immune mechanisms in type 1 diabetes. Trends Immunol. 2013; 34(12): 583-591.
- Adamczak DM, Nowak JK, Frydrychowicz M, Kaczmarek M, Sikora J. The role of Toll-like receptors and vitamin D in diabetes mellitus type 1--a review. Scand J Immunol. 2014; 80(2): 75-84.
- Norris JM, Barriga K, Klingensmith G, Hoffman M, Eisenbarth GS, Erlich HA, et al. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. JAMA. 2003; 290(13): 1713-1720.
- Itoh A, Ridgway WM. Targeting innate immunity to downmodulate adaptive immunity and reverse type 1 diabetes. Immunotargets Ther. 2017; 6: 31-38.
- Kondrashova A, Hyoty H. Role of viruses and other microbes in the pathogenesis of type 1 diabetes. Int Rev Immunol. 2014; 33(4): 284-295.
- 14. Hindmarsh PC, Matthews DR, Silvio LD, Kurtz AB, Brook CG. Relation between height velocity and fasting insulin concentrations. Arch Dis Child. 1988; 63(6): 665-666.
- Wilkin TJ. The accelerator hypothesis: weight gain as the missing link between type I and type II diabetes. Diabetologia. 2001; 44(7): 914-922.
- Knip M, Veijola R, Virtanen SM, Hyoty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes. 2005; 54 Suppl 2: S125-136.
- Virtanen SM, Nevalainen J, Kronberg-Kippila C, Ahonen S, Tapanainen H, Uusitalo L, et al. Food consumption and advanced beta cell autoimmunity in young children with HLA-conferred susceptibility to type 1 diabetes: a nested case-control design. Am J Clin Nutr. 2012; 95(2): 471-478.
- Frederiksen B, Kroehl M, Lamb MM, Seifert J, Barriga K, Eisenbarth GS, et al. Infant exposures and development of type 1 diabetes mellitus: the diabetes autoimmunity study in the young (daisy). JAMA Pediatr. 2013; 167(9): 808-815.
- Virtanen SM, Kenward MG, Erkkola M, Kautiainen S, Kronberg-Kippila C, Hakulinen T, et al. Age at introduction of new foods and advanced beta cell autoimmunity in young children with HLA-conferred susceptibility to type 1 diabetes. Diabetologia. 2006; 49(7): 1512-1521.
- Dahlquist G. Can we slow the rising incidence of childhood-onset autoimmune diabetes? The overload hypothesis. Diabetologia. 2006; 49(1): 20-24.
- 21. Vaarala O, Knip M, Paronen J, Hamalainen AM, Muona P, Vaatainen M, et al. Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes.

- Diabetes. 1999; 48(7): 1389-1394. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early 22. infant feeding and risk of developing type 1 diabetes-associated autoantibodies. JAMA. 2003; 290(13): 1721-1728.
- Mojibian M, Chakir H, Lefebvre DE, Crookshank JA, Sonier B, 23. Keely E, et al. Diabetes-specific HLA-DR-restricted proinflammatory T-cell response to wheat polypeptides in tissue transglutaminase antibody-negative patients with type 1 diabetes. Diabetes. 2009; 58(8): 1789-1796.
- Hettiarachchi KD, Zimmet PZ, Myers MA. Dietary toxins, endoplas-24. mic reticulum (ER) stress and diabetes. Curr Diabetes Rev. 2008; 4(2): 146-156.
- Grant WB. Epidemiology of disease risks in relation to vitamin D 25. insufficiency. Prog Biophys Mol Biol. 2006; 92(1): 65-79.
- 26. Adorini L. Intervention in autoimmunity: the potential of vitamin D receptor agonists. Cell Immunol. 2005; 233(2): 115-124.
- 27. Penna G, Adorini L. 1 Alpha, 25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol. 2000; 164(5): 2405-2411.
- 28. von Herrath M. Can we learn from viruses how to prevent type 1 diabetes?: the role of viral infections in the pathogenesis of type 1 diabetes and the development of novel combination therapies. Diabetes. 2009; 58(1): 2-11.
- 29. Hyoty H, Taylor KW. The role of viruses in human diabetes. Diabetologia. 2002; 45(10): 1353-1361.
- 30. Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ. 2011; 342: d35.
- Knip M, Siljander H. Autoimmune mechanisms in type 1 diabetes. 31. Autoimmun Rev. 2008; 7(7): 550-557.
- 32. Groop L, Pociot F. Genetics of diabetes - are we missing the genes or the disease? Mol Cell Endocrinol. 2014; 382(1): 726-739.
- 33. Bakay M, Pandey R, Hakonarson H. Genes involved in type 1 diabetes: an update. Genes. 2013; 4(3): 499-521.
- 34. Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P, et al. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. Diabetes. 2008; 57(4): 1084-1092.
- Noble JA, Valdes AM, Varney MD, Carlson JA, Moonsamy P, Fear 35. AL, et al. HLA class I and genetic susceptibility to type 1 diabetes: results from the type 1 diabetes genetics consortium. Diabetes. 2010; 59(11): 2972-2979.
- Zhou Z, Jensen PE. Structural characteristics of HLA-DQ that may 36. impact DM editing and susceptibility to type-1 diabetes. Front Immunol. 2013; 4: 262.
- 37. Durinovic-Bello I, Jelinek E, Schlosser M, Eiermann T, Boehm BO, Karges W, et al. Class III alleles at the insulin VNTR polymorphism are associated with regulatory T-Cell responses to proinsulin epitopes in HLA-DR4, DQ8 individuals. Diabetes. 2005; 54 Suppl 2: S18-S24
- Pugliese A. The insulin gene in type 1 diabetes. IUBMB Life. 2005; 38. 57(7): 463-468.
- Bottini N, Vang T, Cucca F, Mustelin T. Role of PTPN22 in type 1 39. diabetes and other autoimmune diseases. Semin Immunol. 2006; 18(4): 207-213.
- Skowera A, Ellis RJ, Varela-Calviño R, Arif S, Huang GC, Van-40 Krinks C, et al. CTLs are targeted to kill β cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest. 2008; 118(10): 3390-3402
- 41. Velthuis JH, Unger WW, Abreu JR, Duinkerken G, Franken K, Peakman M, et al. simultaneous detection of circulating autoreactive CD8+ T-cells specific for different islet cell-associated epitopes using combinatorial mhc multimers. Diabetes. 2010; 59(7): 1721-1730.
- 42. Wang J, Liu L, Ma J, Sun F, Zhao Z, Gu M. Common variants on cytotoxic T lymphocyte antigen-4 polymorphisms contributes to type 1 diabetes susceptibility: evidence based on 58 studies. PLoS One. 2014; 9(1): e85982
- 43. Lu Y, Schneider H, Rudd CE. Murine regulatory T cells differ from conventional T cells in resisting the CTLA-4 reversal of TCR stopsignal. Blood. 2012; 120(23): 4560-4570.
- Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fe-44. hervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008; 322(5899): 271-275.
- 45. Atabani SF, Thio CL, Divanovic S, Trompette A, Belkaid Y, Thomas DL, et al. Association of CTLA4 polymorphism with regulatory T cell frequency. Eur J Immunol. 2005; 35(7): 2157-2162.
- 46. Walker LSK. Treg and CTLA-4: Two intertwining pathways to im-

mune tolerance. J Autoimmun. 2013; 45: 49-57.

- 47. Roychoudhuri R, Hirahara K, Mousavi K, Clever D, Klebanoff CA, Bonelli M, et al. BACH2 represses effector programs to stabilize Treg-mediated immune homeostasis. Nature. 2013; 498(7455): 506-510.
- 48. Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Erlich Ha, et al. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet. 2009; 41(6): 703-707.
- Qu H-Q, Verlaan DJ, Ge B, Lu Y, Lam KCL, Grabs R, et al. A 49 cis-acting regulatory variant in the il2ra locus. J Immunol. 2009; 183(8): 5158-5162.
- Downes K, Pekalski M, Angus KL, Hardy M, Nutland S, Smyth DJ, 50. et al. Reduced expression of ifih1 is protective for type 1 diabetes. PLoS One. 2010; 5(9): e12646.
- 51. Santin I, Eizirik DL. Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and β -cell apoptosis. Diabetes Obes Metab. 2013; 15 Suppl 3: 71-81.
- Reddy S, Ross JM. Fas and fas ligand immunoexpression in pan-52. creatic islets of nod mice during spontaneous and cyclophosphamide-accelerated diabetes. Ann N Y Acad Sci. 2003; 1005: 166-169.
- Wachlin G, Augstein P, Schröder D, Kuttler B, Klöting I, Heinke P, 53. et al. IL-1 β , IFN- γ and TNF- α increase vulnerability of pancreatic beta cells to autoimmune destruction. J Autoimmun. 2003; 20(4): 303-312.
- Marroquí L, Santin I, Dos Santos RS, Marselli L, Marchetti P, Eizirik 54. DL. BACH2, a candidate risk gene for type 1 diabetes, regulates apoptosis in pancreatic β-cells via JNK1 modulation and crosstalk with the candidate gene PTPN2. Diabetes. 2014; 63(7): 2516-2527.
- 55. Floyel T, Brorsson C, Nielsen LB, Miani M, Bang-Berthelsen CH, Friedrichsen M, et al. CTSH regulates β-cell function and disease progression in newly diagnosed type 1 diabetes patients. Proc Natl Acad Sci USA. 2014; 111(28): 10305-10310.
- Nogueira TC, Paula FM, Villate O, Colli ML, Moura RF, Cunha Da, 56. et al. GLIS3, a susceptibility gene for type 1 and type 2 diabetes, modulates pancreatic beta cell apoptosis via regulation of a splice variant of the BH3-Only protein bim. PLoS Genetics. 2013; 9(5): e1003532.
- Elsby LM, Orozco G, Denton J, Worthington J, Ray DW, Donn RP. 57. Functional evaluation of TNFAIP3 (A20) in rheumatoid arthritis. Clin Exp Rheumatol. 2010; 28(5): 708-714.
- Liuwantara D, Elliot M, Smith MW, Yam AO, Walters SN, Marino 58. E, et al. Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes. 2006; 55(9): 2491-2501
- 59. Barry S, Jones RE. Management of hypertension in diabetes. Diabetics Spectrum. 2006; 19(1): 25-31.
- 60. Forbes JM, Cooper ME. Mechnisms of diabetes complications. Physiol Rev. 2013; 93(1): 137-188.
- 61. Vithian K, Hurel S. Microvascular complications: pathophysiology and management. Clin Med (Lond). 2010; 10(5): 505-509.
- Hu H, Jiang H, Ren H, Hu X, Wang X, Han C. AGEs and chronic 62. subclinical inflammation in diabetes: disorders of immune system. Diabetes Metab Res Rev. 2015; 31(2): 127-137.
- 63 Hegab Z, Gibbons S, Neyses L, Mamas MA. Role of advanced glycation end products in cardiovascular disease. World J Cardiol. 2012; 4(4): 90-102.
- Vlassara H, Uribarri J. Advanced glycation end products (AGE) and 64. diabetes: cause, effect, or both? Curr Diab Rep. 2014; 14(1): 453.
- 65. Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. JAMA. 2002; 288(20): 2579-2588
- Kolter T, Uphues I, Eckel J. Molecular analysis of insulin resistance 66. in isolated ventricular cardiomyocytes of obese Zucker rats. Am J Physiol. 1997; 273(1 Pt 1): E59-E67.
- 67. Vinik AI, Holland MT, Le Beau JM, Liuzzi FJ, Stansberry KB, Colen LB. Diabetic neuropathies. Diabetes Care. 1992; 15(12): 1926-1975
- Chawla D, Bansal S, Banerjee BD, Madhu SV, Kalra OP, Tripathi 68. AK. Role of advanced glycation end product (AGE)-induced receptor (RAGE) expression in diabetic vascular complications. Microvasc Res. 2014; 95: 1-6.
- Ahlqvist E, van Zuydam NR, Groop LC, McCarthy MI. The genetics 69. of diabetic complications. Nat Rev Nephrol. 2015; 11(5): 277-287.
- 70. Tang ZH, Fang Z, Zhou L. Human genetics of diabetic vascular complications. J Genet. 2013; 92(3): 677-694.

Breast Cancer Heterogeneity: A focus on Epigenetics and In Vitro 3D Model Systems

Suresh Palamadai Krishnan, Ph.D.*

Department of Biomedical Sciences, School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu

*Corresponding Address: Department of Biomedical Sciences, School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu

Email: p.k.suresh@vit.ac.in

Received: 15/Aug/2017, Accepted: 30/Nov/2017

Abstract -

Breast cancer (BC) is a widely prevalent form of neoplasia in women with fairly alarming mortality statistics. This aspect may be attributed, in part, to the current spatial and temporal heterogeneity-based limitations in therapies with possible recurrence of this tumour at primary and/or secondary sites. Such an extensive phenotypic heterogeneity in breast cancer is unlikely to be adequately or completely comprehended by an immuno-histopathology-based classification alone. This finding has warranted research and development in the area of microarray-based methods (i.e. transcriptomic and proteomic chips) for an improved molecular classification of this complex and heterogeneous tumour. Further, since epigenetics can also be an important determinant in terms of diagnosis, prognosis and therapy, this review provides an insight into the molecular portrait of BC in genetic terms. Specifically, the roles of characteristic DNA and histone-based modifications as well as mi-RNA-based alterations have been discussed with specific examples. Also, their involvement in epithelial mesenchymal transition (EMT) processes in cancer stem cells (CSCs) has been outlined. Last but not least, the salient aspects and the advantages of *ex vivo/in vitro* 3D model systems in recapitulating several aspects of BC tumour (particularly the architecture as well as the apico-basal polarity) are mentioned. This review hopes to provide not only an improved and updated understanding of the epigenetics of breast cancer, but to also elaborate on tumour model development/refinement, biomarker evaluation, drug resistance and test of individual drugs or drug combinations and drug delivery systems.

Keywords: Breast Cancer, Epigenetics, Heterogeneity, In Vitro

Cell Journal(Yakhteh), Vol 20, No 3, Oct-Dec (Autumn) 2018, Pages: 302-311 ____

Citation: Suresh PK. Breast cancer heterogeneity: a focus on epigenetics and in vitro 3D model systems. Cell J. 2018; 20(3): 302-311. doi: 10.22074/ cellj.2018.5442.

Introduction

The most common site for the development of neoplasia in women is the breast. The mortality rate for those afflicted with this disease has been reported to be 25% (1). This statistic is alarming, despite the many advances in diagnosis and treatment. One of the major challenges in improving patient stratification, accurate prognosis and therapy optimisation is the complex, heterogeneous nature of breast cancer (BC) tumours (2). In this regard, the availability of public resources such as the cancer genome atlas (TCGA) provides an unprecedented opportunity to identify the molecular factors that would aid stratifying patients as responders versus those that are indolent to therapy. Also, improvements can be made in prognosis-based markers as well as in identifying markers for optimal therapeutic response.

The TCGA repository of cancer-related molecular information was compiled following data generated at several omics levels including whole exome sequencingbased mutational spectra and DNA copy number changes, transcriptomic expression patterns (expression data) and reverse-phase protein array (RRPA)-related alterations (3).

BC-related heterogeneity has been attributed, at least in part, to potentially reversible epigenetic alterations in the methylome and miRNA expression. Hence, a thorough characterization and analysis of the various epigenetic players *ex vivo/ in vivo* would provide an updated review of these molecules in human breast cancer tumours.

However, a considerable amount of BC epigenetic data has been obtained from several model systems including *in vitro* assays. Hence, systematic cataloguing and updating of results obtained from *in vitro* models would serve as an extra piece of important information, provided the results are concordant with observations in humans or widely accepted *in vivo* xenograft animal models as well as transgenic animal systems. Furthermore, it is widely accepted that one of the major causes of treatment resistance, aggressive behaviour, disease severity, and rate of progression and relapse is due to cancer stem cells (CSCs) (4).

Some or all of these cells may survive the currently employed treatment methods including chemo- and radiotherapy. This thus shows the need to evaluate and possibly refine existing model systems that can mirror, at least in part, the spatial and temporal epigenetic heterogeneity in breast CSCs. However, heterogeneity in the vasculature as well as the role of stromal factors is also an important determinant in mimicking the tumour in the context of its microenvironment. Hence, this review is to also provide an update with regards to the existing 3D model systems.

This approach has been substantiated by several reports demonstrating that these systems are better alternatives to the 2D environment, wherein a monolayer of cells is cultured in artificial conditions.

Molecular features of breast cancer

Ductal and lobular carcinoma

Based on the shape, structure and site of origin, breast tumours are broadly classified as invasive ductal carcinoma, not otherwise specified (IDC, NOS-more common) and ductal carcinoma in situ-ductal carcinoma in situ (DCIS). These tumours may be known as ductal or lobular based on the site of their origin. The term DCIS is a generic term that refers to the non-invasive, abnormal cell growth that is localized to the ducts and lobules and may become malignant. The IDCs refer to cancers that have infiltrated into the extracellular matrix region through the wall of the duct (2).

A more recent and elaborate classification of BC has, however, changed IDC, NOS (2003) to invasive carcinoma of no special type (NST). The other types of BCs are of mixed types including invasive lobular carcinoma, tubular carcinoma and invasive cribriform carcinoma, carcinomas with medullary features, metaplastic carcinoma, carcinomas with apocrine differentiation, adenoid cystic carcinoma, mucinous carcinomas and carcinomas with signet-ring cell differentiation, invasive mucinous carcinoma, carcinomas with neuroendocrine features, invasive papillary and micropapillary carcinoma, secretory carcinoma, oncocytic carcinoma, polymorphous carcinoma, sebaceous carcinoma of the breast, lipid-rich carcinoma, glycogen-rich clear cell carcinoma, acinic cell carcinoma, microinvasive carcinoma and inflammatory carcinoma (5).

Despite the similarities in the 5- and 10-year survival rates, the invasive lobular carcinoma (ILC) category of cancers (ER +ve, PR +ve and a HER2 +ve subset) is more likely to metastasize and bilateral variants are more frequent. Since the clinical course and the underlying biology are different, histology-based studies may not be sufficient for the accurate classification/sub-stratification of the five subtypes of ILC. Further, the non-IDC and non-ILC cancers (not listed above) include those that are comedo as well as the mucinous A (paucicellular) and B (hypercellular) subtypes. As is evident from the classification mentioned above, the challenges posed during BC therapy can be attributed largely to the complex and heterogeneous nature of the tumour (6).

In this context, the availability of RNA sequencing methods has provided the opportunity for a better classification based on the tumour transcriptome and for also identifying molecular mechanisms that may contribute to the observed variability in BC (e.g. exon skipping and promoter switching) (7).

To circumvent this problem, molecular classification now differentiates ductal and lobular carcinomas into ER+/luminal (luminal A/luminal B), basal-like, Erb-B2+ and normal breast. These results were obtained following whole-transcriptomic microarray analysis of 40 breast tumours. These results were correlated with those obtained from 11 cultured cell lines. The genes selected were those showing a similar expression pattern in the same individual, while differences were observed among patients.

Reproducibility of the results (independent and repeated sampling) as well as the strong correlation of the quantitative expression data between a primary and a metastatic tumour further added to the strength of the evidence. Hence, experimental designs of this nature (by analysing more genes) are warranted to refine the molecular signature and make it more representative of breast tumour variability (8).

A similar hierarchical clustering approach was later used to stratify 115 malignant breast tumours using 534 "intrinsic" genes. This approach was replicated independently and confirmed the earlier findings and sub-stratified BC tumours into basal-like, two luminallike, normal-like and ERB-B2 over-expressing cancers. This approach was further validated by similar substratification results reported in another two independent studies based on samples obtained from different patient populations. The selected patient groups tested were not only different in terms of age distribution, stage of the tumours, the microarray technology platforms utilised were also different. Nevertheless, variation was observed in gene clusters in the different populations studied. This level of variation may be due to the small number of genes studied, methodology-based variations and statistical testing issues. Another line of evidence for breast tumours being distinct entities was the increased formation of basal type BCs in individuals positive for BRCA1. In terms of clinical outcome, the basal and ERBB2+ subtypes are the most severe in terms of the period of time between primary tumour formation and metastasis, while the luminal B subtype is an intermediate between the two (9).

These results underscore the distinctiveness of the basal subtype and also the need to further classify the other subtypes in molecular terms, likely leading to a better diagnosis and prognosis. The luminal B subtype has been further characterized based on mutational analysis as well as methylation-based studies. *TP53*, *FOXO3* and *PIK3CA* genes were found to be frequently mutated. Other genes were found to be overexpressed, possibly due to copy number alterations (CNAs). Next-generation sequencing of another cluster of tumours have showed that *KCNB2*, *UTRN* (6q24) and *MDN1* (6q15) were mutated often in this subtype (10).

The luminal A category tumours have been associated with specific mutations in *GATA3*, *PIK3CA* and *MAP3K1*. Furthermore, the existence of two categories of HER-enriched BC subtypes were identified accordingly (HER1, pHER1; HER2, pHER2) (3). This study added more information to the molecular portrait of these subtypes, which was originally described by Perou et al. (8), and also provided evidence for heterogeneity and plasticity within rather than between subtypes.

A more recent paper has classified DCIS into two groups, namely DCIS-C1 and DCIS-C2. This classification was www.SID.ir based on integrated pathway-based modelling analysis of RNA-sequence data. DCIS-C1 was classified as being representative of the more aggressive, highly proliferative, basal-like or ERBB2 subtype with characteristic features of a phenotype similar to that of Tregs. This subtype should be contrasted with DCIS-C2, in which tumours display a low to moderate proliferation capabilities. Furthermore, the DCIS-C2 tumour subtype was ER/PR double positive and luminal-like. In the context of epigenetic factors, IncRNA HOTAIR was upregulated, while the SOX family of tumour suppressor genes, particularly SOX10, SOX11 and SOX15 as well HOXA5 was silenced (11). In the case of ILCs, the five major histological subtypes were classified into two categories based on their molecular architecture. The immune-related subtype showed an increase in the expression of PD-L1, PD-1 and CTLA-4 at the transcript level and exhibited a greater susceptibility to DNA-damaging agents in certain cell lines. The hormone-related subtype, was associated with epithelial mesenchymal transition (EMT) transitions, chromosomal gain (1q and 8q) and loss (11q) in addition to increase in the expression level (mRNA and the protein) of PGR, *ESR1*, *GATA3* and *FN1*) (12).

Triple negative cancer molecular phenotype

The basal subtype of BC lacks ER, PR and HER2, and comprises approximately 16% of all BCs. This subtype is thus known as triple negative BC (TNBC), a phenotype that is recalcitrant to conventional therapies with an approximate 20% response rate.

TNBC has a very poor prognosis with the medial survival being only 1 year and the relapse rate is about 30%. Hence, there is an imperative need to further stratify the TNBC subtype. Such an approach may provide a better understanding of the mechanisms involved in TNBC development as well as possibly introducing a better model for the development and/or refinement of ethno-based drugs (13).

Another triple negative subtype with a poor prognosis is known to be in the claudin-low category. This subtype of cells showed expression of EMT marker genes associated with immune response and stem cell-like features (14). Furthermore, these cells represent invasive ductal carcinomas with an excessive differentiation of medullary and metaplastic features apart from a low or absent expression of luminal differentiation markers. Interestingly, cell lines and animal models are available that mimic this subtype. These tools have received a lot of attention especially since the claudin-low sub-type closely mimics the BC stem cell and is an attractive epigenetic therapeutic target (15).

In a gene expression analysis, 21 BC data sets comprising 587 TNBC cases were analysed, leading to the identification of six subtypes in addition to the identification of suitable model cell lines (for drug testing). The two basal-like subtypes (BL1 and BL2) had a higher induction of cell cycle and DNA damage response genes. The other subtype was immune-modulatory (IM), while the mesenchymal (M) and mesenchymal stem-like (MSL) subtypes had a molecular profile which resembled that of EMT transition. The luminal androgen receptor (LAR) subtype was linked to AR-mediated signalling and patients under this category included those that had a lower relapse-free survival rate (13).

The heterogeneity seen in the tumours *in vivo* is mirrored largely in the commercially available BC cell lines. In cell line studies (MCF-7 versus MCF-7 derived cell lines), noncoding RNAs and possible differential splicing were identified apart from the presence of a cluster of genes whose expression was correlated to steroid-based drug response/lack of response (16). It is known that tumour heterogeneity (intra and inter tumoural) can be due to genetic and epigenetic factors, with these factors affecting differentiation and cell death (17).

In specific, it has been shown that EMT processes, regulated transcriptionally and epigenetically, occur preferentially in BC cells with the basal-like phenotype. For instance, increased expression of markers that can be used to flag an EMT process (cadherin-11; N-cadherin; smooth muscle actin; vimentin) were observed. Also, an increased expression of ECM remodelling and invasion-related proteins (fascin, laminin and SPARC) was also reported. Last but not least, classical epithelial markers (E-cadherin and cytokeratins) were also shown to be down-regulated (18).

This final aspect should be contrasted with an earlier report wherein high expression of membrane E-cadherin was linked to invasive carcinomas (with a pathology similar to that of a mixed ductal and lobular cancer), while its lack of expression was linked to lobular carcinoma. This study thus suggested E-cadherin membrane expression as a marker to distinguish between these two major histological subtypes of cancer (19).

Epigenetic processes and breast cancer

DNA hypo/hyper-methylation, histone acetylases and deacetylases, methyltransferases and demethylases are epigenetic changes considered to be important in BC. Micro-RNA-based alterations are also involved in terms of regulating epigenetic processes in BC.

Of note, PRC2 complexes are, in general, associated with gene repression, while PRC1 complexes are related to gene activation. Specifically, the PRC2 complex proteins like Suz12, EED and Ezh2 have been known to mediate gene silencing by the trimethylation of lysine 27 in the H3 histone proteins. The general concept is that euchromatisation ("open form") around the promoter region would favour accessibility of the transcription factors for the initiation of transcription. Heterochromatisation, however promotes a more repressed chromatin state and this alteration may thus be linked with the silencing of gene expression. Epigenetic regulation has been reported *www.SID.ir* in diverse BC-related events including plasticity and EMT induction (20). The following section highlight the importance of key epigenetic events associated with neoplastic transformation of the human breast as well as BC stem cells.

EZH2, EED, SUZ12 and tumourigenesis

The three proteins (EZH2, EED and SUZ12) are part of the PRC2 complex that is associated with gene silencing. Murine cell-based data as well as results from primary human tumours has provided evidence for the activation of Ezh2. This activation has been linked to certain upstream events involved in the pRb/E2F pathway. Deregulation of certain tumour suppressor genes (e.g., p16) results in the activation of the cyclindependent kinases (CDK4 and CDK6).

This activation, in turn, hyper-phosphorylates pRb, thereby causing the release of E2F transcription factors to increased Ezh2 expression. EZH2 is known to methylate H3K27Me3 and H3K9Me3 (21), possibly in the regions upstream of tumour suppressor genes. This methylation thus provides a possible mechanistic link to gene silencing since HPC2, a protein member of the PRC1 complex, can be recruited to the PRC2 complex, thereby facilitating gene silencing.

Involvement of H3K9Me3and H3K20

It has been reported that desmocollin 3 (a cell-cell adhesion molecule in the family of cadherins) is downregulated in certain BC specimens and is linked to aberrations in cytosine methylation in its promoter elements (22). MASPIN (a protease inhibitor associated with growth and metastasis in nude mice) may be inhibited by an epigenetic mechanism involving G9a (methyl transferase trimethylating H3K9Me3 at certain promoter regions), wherein cytosine methylation and hetero-chromatisation at the promoter region may account for its observed decreased expression which is associated with poor prognosis. Hence, G9a-mediated epigenetic regulation of MASPIN and other target genes may be considered as an important epigenetic factor (23).

Snail, one of the important transcription factors associated with the induction of EMT in primary tumour cells, it thought to recruit G9a. This protein is associated with *in vivo* recurrence of tumours and also a predictor of a decrease in relapse-free survival. The methylation of lysine 9 (H3K9Me2) in the region surrounding the E-cadherin promoter may contribute to the decreased expression of E-cadherin (24).

A later study has further shown that Snail also recruits Suv39H1 (suppressor of variegation 3-9 homolog 1), a methyl transferase forming H3K9Me3 in the vicinity of the E-cadherin promoter, thus down-regulating its expression (25).

Furthermore, it has been shown that there is a correlative increase in the DNA methylation status (discussed in

detail below) flanking the same gene, thereby providing evidence of a link between this type of epigenetic phenomena with that of key changes in histone proteins.

This mechanism of Snail-mediated E-cadherin silencing is reiterated again in a more recent paper, in which certain histone methylation enzymes (PRC2, Suv39H1 and G9a) are recruited to contribute to the hetero-chromatisation in the vicinity of the E-cadherin promoter (H3K9Me3). The gene silencing then occurs due to the concomitant DNA hyper-methylation of the CpG islands. The DNA and histone-based epigenetic changes act in a coordinated manner to mediate EMT processes.

The following sequence of events occurs during the Snail-mediated choreography of multiple epigenetic events, culminating in the down-regulation of E-cadherin expression and in turn EMT processes. LSD1 (demethylase)/HDAC (deacetylase) is recruited to the E-cadherin promoter by Snail via its SNAG domain. This demethylase is involved in removing methyl groups from H3K4Me2/3, while the deacetylase removes acetyl groups from H3/H4. This demethylation event is thought to promote Snail-mediated recruitment of G9a and Suv39H1 on the E-cadherin promoter. HDAC may also be involved in aiding the interaction of Snail with PRC2, thereby contributing to the binding of the latter to the promoter elements. The sequential methylation of H3K9Me to H3K9Me2 and H3K9Me3 is mediated by G9a (interactions with the C-terminal domain of Snail) and Suv39H1 (via the SNAG domain of Snail) respectively. These two methyl transferases are also involved in the final step of E-cadherin down-regulation in recruiting DNA methyltransferases (DNMT) for hyper-methylationmediated silencing of E-cadherin, which is a significant event in EMT (26).

There are other reports where G9a has been shown to act differentially in a cell type-specific manner. GATA3 forms a complex with G9a and NURD (MTA3) and silences ZEB2 (an important transcription factor involved in EMT induction). With BC progression, GATA3, G9a and MTA3 are down-regulated and ZEB2 is up-regulated. This upregulation, in turn contributes to the decrease in the expression levels of G9a and MTA3 via the G9a/ NURD (MTA1) complex (27).

GATA3, a transcription factor involved in the mesenchymal epithelial transition (MET), plays a pivotal role in E-cadherin silencing via its transactivation domain. This event is mediated by the GATA-3-induced chromatin architecture changes (local histone modification and nucleosome eviction) or other changes that do not result in an accessible chromatin. Such MET requires binding as well as recruitment of BRG1, an ATPase of the SWI/SNF family of chromatin remodelers (28).

The SET8 (also known as PR-Set7/9, SETD8 or KMT5A) is a histone methyl transferase (HMT) with a SET domain. Its recruitment is mediated by TWIST (another transcription factor involved in the induction of EMT). This HMT, in turn, mono-methylates H4K20 and suppresses E-cadherin expression, while a similar methylation event in the *www.SID.tr*

N-cadherin promoter activates it (29).

This apparently paradoxical finding can be explained based on the type of dimerisation of TWIST. In specific, homo-dimerization of TWIST leads to the activation of N-cadherin, however, hetero-dimerization of this transcription factor with Mi2/NuRD, MTA2, RbAp46, Mi2 and HDAC2 proteins leads to suppression by the formed complexes. This TWIST-mediated recruitment of the aforesaid complex proteins, to the promoter of E-cadherin, represses this gene, which is apart from direct binding of the transcription factor to the E-cadherin promoter (29, 30).

Involvement of H3K27Me3

A hydrolase inhibitor, 3-Deazaneplanocin A (DZNep), can cause the levels of ado-homocysteine to be elevated. This elevation, in turn, results in the decrease in the levels of EZH2, SUZ12 and EED. Hence, there is an inhibition of H3K27 and not H3K9 methylation marks, which leads to a reversible reactivation of certain genes. Significantly, activation of an effector of apoptosis (FBXO32) may account for DZNep-induced apoptosis in BC cells. This molecular baiss provides us an elegant approach for modulating epigenetic proteins that may possibly aid in selective activation of apoptosis in BC cells (31).

An increase in EZH2 is associated with increased invasiveness, increased proliferation rate and increased aggressiveness behaviour. It is also considered to be a marker for a pre-cancerous lesion in a tissue that is histologically normal (32).

In both cases, EZH2-mediated trimethylation of H3K27Me3 leads to local heterochromatisation as well as DNMT1-mediated gene silencing. However, the final outcome may to some extent be dependent on the types of genes that are silenced (e.g. those that contribute to the neoplastic phenotype or those with pro-apoptotic function). Although Snail is involved in recruiting G9a to the H3K9 site, it has been reported that the activation of Snail is mediated by the removal of the repressive H3K27Me3 marks. These methylation marks are removed by the de-methylating action of KDM6B (also known as JMJD3–part of the Jumoji family) (33).

The section below outlines the links between hypermethylation of the CpG islands in the promoter region and silencing of the gene while bearing in mind that multiple studies have reported DNMT1 and SNAIL1 to be involved in the repression of E-cadherin expression.

H3K27 acetylation

The p300-mediated acetylation (H3K27Ac mark), in addition to the repressive H3K27Me3 mark, is maintained by DUSP4, a phosphatase targeting threonine/serine and tyrosine residues. Knockdown of this phosphatase, as well as DUSP6, enhances stem cell formation, while down-regulation of DUSP1 decreases stem cell formation (CD44^{hi}/CD24^{lo}/EpCAM+breast CSCs). However, DUSP6 overexpression has been observed in HER2+ BCs. In this context, it is pertinent to point out that there are inhibitory and activating phosphorylation marks (1834 active and 89 inhibitory marks) that regulate the activity of the p300 HAT enzyme which can activate certain genes involved in the formation of euchromatin (34).

DNA hypo/hypermethylation

Hypomethylation and the possible consequent constitutive expression of the JAK/STAT pathway has been reported in CD44+/CD24 low putative BC stem cells. Also, increased expression of several genes associated with this pathway has been shown in the mammosphere model (35).

Hypermethylation of cytosines in the regulatory region of the E-cadherin gene has been observed in an E-cadherin-negative BC cell line. Interestingly, treatment with a demethylating agent returns its expression (both transcript and protein) to normal levels, thereby providing evidence for this epigenetic modification being responsible for promoter inhibition. Furthermore, the expression of a reporter gene under the control of the E-cadherin promoter provided fairly definitive evidence of the presence of the transcriptional machinery in the E-cadherin-negative BC cells. In a later study, it was again demonstrated that E-cadherin methylation correlated with fibroblastlike morphology in BC cell lines. This phenotype also correlated with the expression of genes associated with EMT transition including TGF- β -related genes and the genes involved in CDH1 regulation (ZFHX1B and SNAI2 but not SNAI1 and TWIST) (36).

Specifically, epigenetic regulation in the form of DNA hyper-methylation and the consequent inactivation of the Wnt pathway (an important mitogenic cell signalling pathway) has been known to be involved in BC development. In this regard, Dickkopf2 (an endogenous inhibitor of Wnt signalling) can arrest cells in G0 and G1, and induce apoptosis. This study was undertaken on 10 BC cell lines, 98 primary tumours and 21 normal breast tissues (37).

Another study has reported that Dickkopf3 inhibited the canonical Wnt/ β catenin pathway, thereby leading up to β -catenin migrating from the nucleus to the cytoplasm and the membrane. This mechanism along with the presence of reduced levels of active β -catenin can further activate the non-canonical JNK signalling. DKK3 was shown to inhibit BC cell migration due to a reversal of EMT and a decrease in stem cell markers (38). Recruitment of DNMT1 by δ EF1 (ZEB1) may be associated with a decrease in E-cadherin expression due to hyper-methylation. This transcription factor, along with SIP1/ZEB2, is associated with the repression of E-cadherin expression, which is an important marker of the EMT phenotype characteristic of CSCs (39).

The ZEB1 transcription factor has been shown to be regulated by the asymmetric dimethylation of arginine 3 of histone H4 by PRMT1 (an arginine methyltransferase) at its promoter. This modification has been linked to EMT induction as well as senescence (40). While the concept of hyper-methylation and gene silencing is widely accepted, it is necessary to link this epigenetic change with alterations in microRNA (miRNA; small RNA molecules that are involved in gene regulation either by the degradation of target mRNA or by the inhibition of gene expression at the translational level) expression in tumours (41) with functional assays. Adoption of a battery of widely accepted assays can provide definitive or corroborative evidence for the involvement of certain miRNA in BC. The section below aims to provide an overview of the importance of miRNA in BC development.

miRNA and breast cancer

Hypermethylation at the miR-200c-141 locus and a concomitant increase in EMT features in an *in vitro* cellular model provided evidence for the simultaneous occurrences of these intermediate phenotypes. Specifically, the transcription factors ZEB1 and ZEB2 were up-regulated under these circumstances and may contribute to an increase in invasiveness and tumourigenicity (42). A negative correlation has been reported for the methylation status of the two promoters (P1 and P2) regulating the miR-200b gene. These results were observed in 8 out of 9 cell lines and *in vitro* reporter gene assays.

These results were substantiated in clinical samples, with hypermethylation at P1 linked to metastasis to the lymph nodes, while P2 showed association with loss of ER or PR. Results of this kind provide us a sound basis for validating and emphasizing the role of promoter hypermethylation at miRNA promoters as possible biomarkers for BC (43). For example, miR-18b, miR-103, miR-107 and miR-652 may be good predictors of an increased probability of tumour recurrence and reduced patient survival, and also serve as markers of prognosis in TNBC patients (44). Increase in the miR-30 expression has been linked to an increase in apoptosis possibly via its effects in down-regulating AVEN, an anti-apoptotic protein, in BT-IC cells grown under non-attachment conditions (45).

A systematic step-wise experimental design involving a combination of microarray analysis, artificial neural network (ANN)-based data-mining, real-time PCR and correlation analysis with clinico-pathological features was followed. Seventy six differentially expressed miRNAs were identified by microarray analysis based on total RNA of blood samples from women with luminalA BC. The ANN-based strategy enabled the selection of 10 miRNAs (miR19b, miR-29a, miR-93, miR-181a, miR-182, miR-223, miR-301a, miR-423-5p, miR-486-5 and miR-652) for follow-up. Of these, four of them may have biomarker potential, since they were down-regulated in affected women. In addition, the combined signature of three of these (miR-29a, miR-181a and miR-652) may discern tumours from controls (46).

While the increased expression of ZEB1/2 transcription factors, mediated by the down-regulation of miR200, has been reiterated in basal-like BC, distal BC metastasis has been associated with an increase of this miRNA family. This implies a possible role for them in the establishment of these cancer cells at a site distal from its origin, which may possibly be due to a feed-forward loop-mediated repression of ZEB 1/2 by miR200 (47).

It has been reported that the transcription of *PTPN6* and miR200c/141 are tightly linked together under a wide variety of physiological conditions. The regulation of miR200c/141 involves by-passing the expression of PTPN6 (SHP1) either by the use of an alternative polyadenylation signal or by a later termination of the transcription of *PTPN6* gene. The alternative mechanism may be based on DNA looping where the transcriptional machinery of both genes physically interact, providing an opportunity for a common epigenetic regulation (48).

Moreover, miRNA regulate the behaviour of BC stem cells (BCSCs) (49). For instance, a miRNA signature has been developed that can predict the prognosis of BC in hormone receptor +ve, HER –ve BC patients. It can also classify these patients into high and low risk groups. This signature (based on miR-21, miR-30c, miR-181a, miR-181c, miR-125b, miR-7, miR-200a, miR-181a, miR-22 and miR-200c expression levels) also correlated with distant relapse-free survival (49). Irrespective of the miRNA profile, targeting genes linked to the CSC phenotype may currently be the approach of choice in epigenetic-based therapeutics.

Epigenetics and breast cancer stem cells

Side-population BC stem cells expressing membrane-bound drug efflux transporters and other markers have been shown to contribute to the observed recalcitrance of the tumour to the drug as well as tumour recurrence. Also, these cells divided rapidly and exhibited a relatively high frequency of survival. BCSCs have been associated with the different stages of the multi-step process of BC including invasion and metastasis (50).

A unifying model based on the presence of BC stem cells as well as the clonal evolution model has been proposed (51). This final model, based on the inherent plasticity of stem cells, includes the hierarchical aspects being different at different times and different regions of the tumour. This variability can be attributed to the internal and external pressures affecting the survival of the tumours. However, the heterogeneity associated with BC is also mirrored in the variable surface marker profile of BCSCs. This classification was done by comparing CSCs in the special histological type category with those observed in the non-special type category.

Specifically, the CD44^{+/high}, CD24^{+/low} cancer cells belong to the low grade, luminal subtype. The high grade (basal-like, claudin-low) subtype CSCs of the medullary, metaplastic cancer category exhibit the CD44+/CD24-/low/ALDH1+ CSC phenotype. This classification is important for diagnosis, prognosis and therapy (including the development of novel drug molecules) (51). Such markers may be used for cell isolation, monitoring treatment efficacy and diagnosis/ prognosis.

Methylation marks and cancer stem cells

There is a strong association between DNA hypermethylation and histone methylation-mediated loss of tumour suppressor gene expression. There is also an association between DNA hyper-methylation and histone deacetylation. KDM5B, a histone demethylase, acts on H3K4Me3 and its over-expression can repress cell proliferation, adhesion and migration. This demethylase acts in concert with NuRD and HDAC1 in contributing towards repression of genes associated with cell proliferation (52).

DNMT1 expression has been associated with hypermethylation and suppression of ISL1 expression in mammary tumours as well as in BCSCs. Hence, down-regulation of DNMT1 and ISL1 may lead to a decrease in the population of stem cells. This axis may be useful for drug development (53). EMT transition (e.g., loss of DNA hypermethylation-mediated silencing of E-cadherin and activation of N-cadherin) and the epigenetic links to the loss of this stem cell feature is reiterated here to underscore its importance and its possible reversibility. However, such changes have to be measured at the population level rather than at the single cell level.

JARID1B (a H3K4 demethylase) expression was shown to be amplified in luminal breast tumours and is associated with an expression profile that is characteristic of this subset of cancers. High activity of this enzyme is also linked to poor outcome in patients (54).

Epithelial mesenchymal transition, cell signalling and stem cells

It is known that EMT transition has been consistently associated with CSCs in various cancers including BC. In all these cancers, epigenetic events may modulate the CSC phenotype and a number of examples with respect to this aspect are provided below.

Apart from part/E2F and Ezh2, other signalling pathways such as Wnt/ β -catenin and RANK/RANKL have been associated with EMT induction in CSCs. Specifically, increase in RANK/RANKL signaling has been shown to increase the population of CD44+/ CD24- stem cells, and induce EMT and stemness in human mammary epithelial cells, thus being involved in

BC tumour initiation, progression and metastasis (55).

Studies have shown that Nodal signalling is associated with the aggressive features in BC. The endogenous negative regulator of Nodal (Lefty1-a regulatory protein normally sequestered in the hESC microenvironment) is not expressed in cancer cells (56), thereby providing a plausible mechanism for Lefty1-mediated epigenetic silencing-mediated uncontrolled growth of cancer cells (57).

The position-dependent effect of GATA-3 has been demonstrated by the observation that GATA-3 can alter the open versus closed state of chromatin at certain loci. At other positions, the sliding of the nucleosomes may not be associated with the formation of accessible chromatin. In addition, removal of the transactivating domain of GATA-3 can affect the reprogramming of chromatin without altering its binding ability (28). This may thus affect the formation of the MET phenotype.

Despite the inherent complexities in mimicking the reported tumour heterogeneity, the ability to capture molecular changes in vitro has led to the development and/or refinement of 3D model systems. Such 3D model systems can help in validating the results obtained in terms of testing the apoptotic/anti-oxidant potential of ethno-derived biomolecules in 2D systems (58, 59). This type of analysis will enable us to better understand the strengths and limitations of the existing model systems and the rationale behind their development before validating novel findings in the classical xenograft/patient-derived xenograft model systems. This seems vital since it is widely accepted that the 2D environment does not fully recapitulate the complex interactions and the heterotypic signalling necessary to develop an adequate model system for mechanism-based research and drug testing. A catalogue of the important 3D BC model systems is presented in a tabular format below (Table 1). Also the major findings are indicated in terms of their ability to mimick, at least in part, the heterogeneity observed in vivo.

Refinement of 3D models should take into account the heterogeneity in the vasculature and the role of the stroma (heterotypic signalling) as well as other spatial and temporal variation in the breast tumour. Accordingly, knowledge gleaned from research in the area of patient-derived xenografts would be extremely useful in terms of better understanding the mechanisms involved in BC patho-physiology as well as possibly providing a molecular basis for the often observed increase in drug resistance. Mounting evidence has shown that xenografts have a strong potential since they mimic the *in vivo* pathology of the primary tumour in terms of heterogeneity; behaviour and metastatic properties even after serial passaging.

Table 1: 3D breast cancer model systems			
Sl. No.	Details of the 3D model development	Key findings	Reference
1	Chamber slide well-initially coated with 100% Matrigel. After solidification (1mm in thickness), MCF-10A dispersed cells were plated on this gel. Medium had hormones, growth factors and 2% Matrigel. The assay medium was altered every 4 days. Cells grow and form clusters after 5-6 days in 3D culture, and subsequently form acini	Able to recapitulate and mimic many aspects pertaining to the architecture of the mammary gland (growth arrest and polarized acini)	(60)
2	 A. 3D "embedded" assay-cells cultured by embedding in IrECM (soluble extract derived from the EHS mouse sarcoma cells) B. The 3D 'on-top' assay-cells are cultured on a dilute solution of IrECM. This cell suspension is placed gently on top of a thin IrECM gel 	Formation of polarized, growth arrested acinus-like colonies-better mimics than 2D cultures-amenable to downstream processing of the molecules extracted from these cells cultured in 3D	(61)
3	The 3D 'on-top' assay to produce cells with different morphologies, namely round mass, grape-like and stellate	"Signal transduction regulation" was found to be different in terms of gene expression profiles* of cells grown in 2D versus 3D. Also, "Enzyme Regulator Activity" was also close to being statistically significant (in term of differences in the gene expression profile of the two systems.	(62)
		*It is expected that the differences would be greater since regulation can also occur post-transcriptionally (in the context of the gene expression).	
4	5% Matrigel [™] drip was compared with 3D Matrigel [™] drip with sECM and 5% ECM in terms of apico basal polarity. Also, it was examined whether collagen IV and/or laminin 111 is required for apical polarity.	5% Matrigel ^{1M} drip with collagen IV is sufficient and necessary for establishing apico-basal polarity-a fundamental prerequisite for better understanding factors contributing to apical polarity loss (multilayer of cells and lack of basal positioning of the nuclei).	(63)
5	3D spheroid developed using SKBR-3 cells in a well pre-coated with HEMA (indicating the importance of the substratum)	HER2 homodimer formation favoured-signaling diverted from the PI3K/Akt pathway to the ERK 1/2, MAPK pathway. Homodimer a better target for trastuzumab. Phosphorylated PAK2 is part of the survival pathway since this protein is not inactivated by trastuzumab.	(64)
6	A co-culture model-3 major cell types- normal and malignant breast; luminal cells, myoepithelia cells and fibroblasts from the stroma (for the 1st time)	Organization into structures that reproduced features seen in the normal as well as that of the DCIS breast-homing of myo-epithelial cells around the luminal population-basement membrane disrupted; β 4-integrin lost (as in DCIS <i>in vivo</i>) -importance of the tumour associated fibroblast; disrupted the co-unit organization	(65)
7	This type of model mimics the structural and functional aspects of normal and malignant breast cancer tissues. MCF-10A cells were suspended in a collagen gel. These cells formed both acinar and tubular structures. The gel should be detached well from the cell culture plate. Cell contraction should occur in the suspension stage.	Collagen organization as well as biomechanical factors (cell-collagen interactions) is important for formation, elongation and branching of ducts.	(66)
8	Microscale cavities were created in the type I collage gel mould. This was done using certain posts with a defined geometry and spacing. Epithelial cells were seeded into these cavities and another layer of collagen was placed over the cells.	Depending on the shape of the cavities, hollow tissues were formed. Morphogenesis was observed after 1-3 days of culture. This experimental design can be extended to study interactions between luminal epithelial and myoepithelial cells.	(67)
9	Patient-derived mammary epithelial cells (reduction mammoplasty-cell suspension triturated, washed and depleted of fibroblasts) were used for the 3D culture using a hydrogel with defined components (collagen I, hyaluronan, fibronectin and laminin).	Under these defined experimental, serum-free conditions, the cells were converted into a morphological complex structure mimicking the native breast tissue (in terms of a central lumen, formation of lipid droplets, similar ductal morphology and branching). This branching commenced from a cluster of cells that expressed putative mammary stem cell markers	(68)
10	Myo-epithelial and luminal cells (from reduction mammoplasty) were combined in a collagen gel matrix. The structure formed was a physiologically relevant surrogate of the <i>in vivo</i> bilayer structure. Furthermore, induction of HER2 expression selectively in the luminal compartment may lead to the filling of the luminal cavity	This experimental design demonstrates the importance of the collagen matrix as well as the roles of the two cell types. This 3D model mimics, at least in part, DCIS. Hence, this model may be used as a testing tool for drugs/biopharmaceuticals targeting HER2	(69)
11	MCF-7 cells were cultured under 3D conditions using calcium alginate hydrogel. The proliferation rate correlated with the elastic modulus of the gel.	Under 3D conditions, the cells formed spheroids with their conformation similar to what is observed <i>in vivo</i> . The maximal proliferation rate was measured after 2 weeks for the softest hydrogel (E=150-200 kPa). This approach may be used as a tool to develop a more relevant model for <i>in vitro</i> cancer studies.	(70)

Conclusion

The use of cutting-edge molecular tools has provided a better molecular portrait (based on genetic and epigenetic features) of BC that correlate with several variables including biological characteristics, diversity, clinical course and patient outcome. Comparative analyses of the epigenetic molecules, including those related to CSCs and EMT processes, in different tissues may facilitate the development and/or refinement of the existing signatures. This approach may not only aid the development and/or validation of the existing 3D model systems that better resemble the tumour phenotype, but it may also validate cell line-based and patient-derived xenografts. This would eventually lead to an improved understanding of the underlying mechanism and a better predictive power in terms of biomarker development, clinical course, response to drugs and drug combinations (from natural or synthetic sources) in addition to elucidating an epigenetic basis for the acquisition and maintenance of drug resistance.

Acknowledgements

The author thanks Vellore Institute of Technology for its constant encouragement and infrastructural support including unlimited Internet connectivity. Also, the author thanks the Institute for creating the scientific ambience. The author thanks the students and research scholars for their support. The author expresses his gratitude to DST (SERB) (SB/SO/HS-157-2013) for creating an invigorating research environment by funding a project on drug delivery systems for which the author is the principal investigator. There is no conflict of interest in this study.

Author's Contributions

The author has reviewed the literature and provided an overview of the topic based on the existing body of information in the relevant databases. The author has subsequently compiled the manuscript and edited the same for technical content.

References

- Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and mortality and epidemiology of breast cancer in the world. Asian Pac J Cancer Prev. 2016; 17(S3): 43-46.
- Makki J. Diversity of breast carcinoma: histological subtypes and clinical relevance. Clin Med Insights Pathol. 2015; 8: 23-31.
- Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012; 490(7418): 61-70.
- Suresh P.K. A letter in response to "Cancer stem cells: emerging actors in both basic and clinical cancer research" Turk J Biol (2014) 38: ©TÜBITAK-doi:10.3906/biy-1406-93 Cancer stem cells (CSCs): targets and strategies for intervention. Turkish J Biol. 2015; 39: 517-521.
- Sinn HP, Kreipe H. A brief overview of the WHO classification of breast tumors, 4th edition, focusing on issues and updates from the 3rd edition. Breast Care (Basel). 2013; 8(2): 149-154.
- Bertos NR, Park M. Breast cancer-one term, many entities? J Clin Invest. 2011; 121(10): 3789-3796.
- Eswaran J, Horvath A, Godbole S, Reddy SD, Mudvari P, Ohshiro K, et al. RNA sequencing of cancer reveals novel splicing alterations. Sci Rep. 2013; 3: 1689.
- Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000; 406(6797): 747-752.

- Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA. 2003; 100(14): 8418-8423.
- Cornen S, Guille A, Adélaïde J, Addou-Klouche L, Finetti P, Saade MR, et al. Candidate luminal B breast cancer genes identified by genome, gene expression and DNA methylation profiling. PLoS One. 2014; 9(1): e81843.
- Abba MC, Gong T, Lu Y, Lee J, Zhong Y, Lacunza E, et al. A molecular portrait of high-grade ductal carcinoma in situ. Cancer Res. 2015; 75(18): 3980-3990.
- Michaut M, Chin SF, Majewski I, Severson TM, Bismeijer T, de Koning L, et al. Integration of genomic, transcriptomic and proteomic data identifies two biologically distinct subtypes of invasive lobular breast cancer. Sci Rep. 2016; 6: 18517.
- Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 2011; 121(7): 2750-2767.
- Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010; 12(5): R68.
- Prat A, Adamo B, Cheang MC, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triplenegative breast cancer. Oncologist. 2013; 18(2): 123-133.
- Callari M, Guffanti A, Soldà G, Merlino G, Fina E, Brini E, et al. In-depth characterization of breast cancer tumor-promoting cell transcriptome by RNA sequencing and microarrays. Oncotarget. 2016; 7(1): 976-994.
- Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, et al. Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer Res. 2014; 34(3): 1071-1077.
- Sarrió D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res. 2008; 68(4): 989-997.
- Acs G, Lawton TJ, Rebbeck TR, LiVolsi VA, Zhang, PJ. Differential expression of E-cadherin in lobular and ductal neoplasms of the breast and its biologic and diagnostic implications. Am J Clin Pathol. 2001; 115(1): 85-98.
- Basse C, Arock M. The increasing roles of epigenetics in breast cancer: implications for pathogenicity, biomarkers, prevention and treatment. Int J Cancer. 2015; 137(12): 2785-2794.
- Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J. 2003; 22(20): 5323-5335.
- Oshiro MM, Kim CJ, Wozniak RJ, Junk DJ, Muñoz-Rodríguez JL, Burr JA, et al. Epigenetic silencing of DSC3 is a common event in human breast cancer. Breast Cancer Res. 2005; 7(5): R669-R680.
- Maass N, Hojo T, Zhang M, Sager R, Jonat W, Nagasaki K. Maspina novel protease inhibitor with tumor-suppressing activity in breast cancer. Acta Oncol. 2000; 39(8): 931-934.
- Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell. 2005; 8(3): 197-209.
- Dong C, Wu Y, Wang Y, Wang C, Kang T, Rychahou PG, et al. Interaction with Suv39H1 is Critical for Snail-mediated E- cadherin Repression in Breast Cancer. Oncogene. 2013; 32(11): 1351-1362.
- Lin Y, Dong C, Zhou BP. Epigenetic regulation of EMT: the Snail story. Curr Pharm Des. 2014; 20(11): 1698-1705.
- Si W, Huang W, Zheng Y, Yang Y, Liu X, Shan L, et al. Dysfunction of the reciprocal feedback loop between GATA3- and ZEB2-nucleated repression programs contributes to breast cancer metastasis. Cancer Cell. 2015; 27(6): 822-836.
- Takaku M, Grimm SA, Shimbo T, Perera L, Menafra R, Stunnenberg, HG, et al. GATA3-dependent cellular reprogramming requires activation-domain dependent recruitment of a chromatin remodeler. Genome Biol. 2016; 17: 36.
- 29. Yang F, Sun L, Li Q, Han X, Lei L, Zhang H, et al. SET8 promotes epithelial-mesenchymal transition and confers TWIST dual transcriptional activities. EMBO J. 2012; 31(1): 110-123.
- Fu J, Qin L, He T, Qin J, Hong J, Wong J, et al. The TWIST/Mi2/ NuRD protein complex and its essential role in cancer metastasis. Cell Res. 2011; 21(2): 275-289.
- Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 2007; 21(9): 1050-1063.

- Ding L, Erdmann C, Chinnaiyan, AM, Merajver SD, Kleer CG. Identification of EZH2 as a molecular marker for a precancerous state in morphologically normal breast tissues. Cancer Res. 2006; 66(8): 4095-4099.
- Ramadoss S, Chen X, Wang CY. Histone demethylase KDM6B promotes epithelial-mesenchymal transition. J Biol Chem. 2012; 287(53): 44508-44517.
- Boulding T, Wu F, McCuaig R, Dunn J, Sutton CR, Hardy K, et al. Differential roles for DUSP family members in epithelial-to-mesenchymal transition and cancer stem cell regulation in breast cancer. PLoS One. 2016; 11(2): e0148065.
- Hernandez-Vargas H, Ouzounova M, Le Calvez-Kelm F, Lambert MP, McKay-Chopin S, Tavtigian SV, et al. Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. Epigenetics. 2011; 6(4): 428-439.
- Lombaerts M, van Wezel T, Philippo K, Dierssen JW, Zimmerman RM, Oosting J, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-tomesenchymal transition in breast cancer cell lines. Br J Cancer. 2006; 94(5): 661-671.
- Mu J, Hui T, Shao B, Li L, Du Z, Lu L, et al. Dickkopf-related protein 2 induces G0 / G1 arrest and apoptosis through suppressing Wnt / β-catenin signaling and is frequently methylated in breast cancer. Oncotarget. 2017; 8(24): 39443-39459.
- Xiang T, Li L, Yin X, Zhong L, Peng W, Qiu Z, et al. Epigenetic silencing of the WNT antagonist Dickkopf 3 disrupts normal Wnt/ beta-catenin signalling and apoptosis regulation in breast cancer cells. J Cell Mol Med. 2013; 17(10): 1236-1246.
- Fukagawa A, Ishii H, Miyazawa K, Saitoh M. δEF1 associates with DNMT1 and maintains DNA methylation of the E-cadherin promoter in breast cancer cells. Cancer Med. 2015; 4(1): 125-135.
- Gao Y, Zhao Y, Zhang J, Lu Y, Liu X, Geng P, et al. The dual function of PRMT1 in modulating epithelial-mesenchymal transition and cellular senescence in breast cancer cells through regulation of ZEB1. Sci Rep. 2016; 6: 19874.
- 41. Macfarlane LA, Murphy PR. MicroRNA: biogenesis, function and role in cancer. Curr Genomics. 2010; 11(7): 537-561.
- Neves R, Scheel C, Weinhold S, Honisch E, Iwaniuk KM, Trompeter HI, et al. Role of DNA methylation in miR-200c/141 cluster silencing in invasive breast cancer cells. BMC Res Notes. 2010; 3: 219.
- 43. Wee EJ, Peters K, Nair SS, Hulf T, Stein S, Wagner S, et al. Mapping the regulatory sequences controlling 93 breast cancer-associated miRNA genes leads to the identification of two functional promoters of the Hsa-mir-200b cluster, methylation of which is associated with metastasis or hormone receptor status in advanced breast cancer. Oncogene. 2012; 31(38): 4182-4195.
- Kleivi Sahlberg K, Bottai G, Naume B, Burwinkel B, Calin GA, Børresen-Dale AL, et al. A serum MicroRNA signature predicts tumor relapse and survival in triple-negative breast cancer patients. Clin Cancer Res. 2015; 21(5): 1207-1214.
- Ouzounova M, Vuong T, Ancey PB, Ferrand M, Durand G, Le-Calvez Kelm F, et al. MicroRNA miR-30 family regulates non-attachment growth of breast cancer cells. BMC Genomics. 2013; 14: 139.
- McDermott AM, Miller N, Wall D, Martyn LM, Ball G, Sweeney KJ, et al. Identification and validation of oncologic miRNA biomarkers for luminal A-like breast cancer. PLoS One. 2014; 9(1): e87032.
 Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spad-
- Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 2008; 9(6): 582-589.
- Batista L, Bourachot B, Mateescu B, Reyal F, Mechta-Grigoriou F. Regulation of miR-200c/141 expression by intergenic DNA-looping and transcriptional read-through. Nat Commun. 2016; 7: 8959.
- Gong C, Tan W, Chen K, You N, Zhu S, Liang G, et al. Prognostic value of a BCSC-associated microRNA signature in hormone receptor-positive HER2-negative breast cancer. EBioMedicine. 2016: 11: 199-209.
- 50. Wang M, Wang Y, Zhong J. Side population cells and drug resistance in breast cancer. Mol Med Rep. 2015; 11(6): 4297-4302.
- Shah M. Allegrucci C. Keeping an open mind: Highlights and controversies of the breast cancer stem cell theory. Breast Cancer

(Dove Med Press). 2012; 4: 155-166.

- Klein BJ, Piao L, Xi Y, Rincon-Arano H, Rothbart SB, Peng D, et al. The histone-H3K4-specific demethylase KDM5B binds to its substrate and product through distinct PHD fingers. Cell Rep. 2014; 6(2): 325-335.
- Pathania R, Ramachandran S, Elangovan S, Padia R, Yang P, Cinghu S, et al. DNMT1 is essential for mammary and cancer stem cell maintenance and tumorigenesis. Nat Commun. 2015; 6: 6910.
- Yamamoto S, Wu Z, Russnes HG, Takagi S, Peluffo G, Vaske C, et al. JARID1B is a luminal lineage-driving oncogene in breast cancer. Cancer Cell. 2014; 25(6): 762-777.
- Palafox M, Ferrer I, Pellegrini P, Vila S, Hernandez-Ortega S, Urruticoechea A, et al. RANK induces epithelial-mesenchymal transition and stemness in human mammary epithelial cells and promotes tumorigenesis and metastasis. Cancer Res. 2012; 72(11): 2879-2888.
- Postovit LM, Margaryan NV, Seftor EA, Kirschmann DA, Lipavsky A, Wheaton WW, et al. Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. Proc Natl Acad Sci USA. 2008; 105(11): 4329-4334.
- Kalyan A, Carneiro BA, Chandra S, Kaplan J, Chae YK, Matsangou M, et al. Nodal signaling as a developmental therapeutics target in oncology. Mol Cancer Ther. 2017; 16(5): 787-792.
 George VC, Kumar DR, Suresh PK, Kumar RA. Antioxidant, DNA
- George VC, Kumar DR, Suresh PK, Kumar RA. Antioxidant, DNA protective efficacy and HPLC analysis of Annona muricata (soursop) extracts. J Food Sci Technol. 2015; 52(4): 2328-2335.
- Naveen Kumar DR, George VC, Suresh PK, Kumar RA. Acceleration of pro-caspase-3 maturation and cell migration inhibition in human breast cancer cells by phytoconstituents of Rheum emodi rhizome extracts. EXCLI J. 2013; 12: 462-478.
- Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in threedimensional basement membrane cultures. Methods. 2003; 30(3): 256-268.
- 61. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods. 2007; 4(4): 359-365.
- Kenny PA, Lee GY, Myers CA, Neve RM, Jeremy R, Spellman PT, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol. 2007; 1(1): 84-96.
- Plachot C, Chaboub LS, Adissu HA, Wang L, Urazaev A, Sturgis J, et al. Factors necessary to produce basoapical polarity in human glandular epithelium formed in conventional and high-throughput three-dimensional culture: example of the breast epithelium. BMC Biol. 2009; 7: 77.
- Pickl M, Ries CH. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. Oncogene. 2009; 28(3): 461-468.
- 65. Holliday DL, Brouilette KT, Markert A, Gordon LA, Jones JL. Novel multicellular organotypic models of normal and malignant breast: tools for dissecting the role of the microenvironment in breast cancer progression. Breast Cancer Res. 2009; 11(1): R3.
- Dhimolea E, Maffini MV, Soto AM, Sonnenschein, C. The role of collagen reorganization on mammary epithelial morphogenesis in a 3D culture model. Biomaterials. 2010; 31(13): 3622-3630.
- Nelson CM, Inman JL, Bissell, MJ. Three-dimensional lithographically defined organotypic tissue arrays for quantitative analysis of morphogenesis and neoplastic progression. Nat Protoc. 2008; 3(4): 674-678.
- Sokol ES, Miller DH, Breggia A, Spencer KC, Arendt LM, Gupta, PB. Growth of human breast tissues from patient cells in 3D hydrogel scaffolds. Breast Cancer Res. 2016; 18(1): 19.
- Čarter EP, Gopsill JA, Gomm JJ, Jones JL, Grose, RP. A 3D in vitro model of the human breast duct: a method to unravel myoepithelial-luminal interactions in the progression of breast cancer. Breast Cancer Res. 2017; 19(1): 50.
 Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R, Scaglione S.
- Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R, Scaglione S. Microenvironment complexity and matrix stiffness regulate breast cancer cell activity in a 3D in vitro model. Sci Rep. 2016; 6: 35367.