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Functional Role of TET3 Enzyme in Breast Tumorigenesis

M.Sc. Thesis

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Declaration

Here, I declare that my MSc thesis entitled “Functional Role of TET3 Enzyme in Breast Tumorigenesis” is the result of my own research and was written independently with no other sources than quoted.

Sondos M. Odeh

Ramallah, August 2020

Date:

Dedication:

This thesis is wholeheartedly dedicated to my companion, friend, and the person who believes in me the most, my mother. Through every obstacle and through every hardship, she has been there with me to guide me, to mentor me, and to be proud of me. Every proud moment of my life has been and will always be thanks to her and with her.

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Table 1: List of Abbreviations:

%	Percent
°C	Celsius
Ab	Antibody
Amp	Ampicillin
Bp	Base pair
cDNA	Complementary DNA
CpG	Cytosine phosphate guanine
Cy3	Cyanine
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DW	Distilled water
EGF	Epidermal growth factor
FBS	Fetal bovine serum
Hrs	Hours
L	Litter
LB	Lysogeny broth
Min	Minutes
Ml	Milliliter
Ng	Nanogram
PBS	Phosphate buffer saline
RPM	Round per minute
RT	Room temperature
RT-PCR	Real time- polymerase chain reaction
SFM	Serum free media
Tri reagent	Trizol
Mg	Microgram
μL	Microliter
Mm	Micrometer
TSG	Tumor suppressor gene
IF	Immunofluorescent
IHC	Immunohistochemistry
E2	Estrogen
5mc	5-Methylcytosine
5hmc	5-hydroxymethylcytosine
DNMTs	DNA methyltransferase enzymes

TET	Ten Eleven Translocation enzymes
αKG	Alpha Ketoglutarate
DSBH	Double strand beta sheet
HEK	Human Embryonic Kidney cells
NaCl	Sodium chloride
TNBC	Triple negative breast cancer
Uc	Uncut
Nuc	Nuclear
Cyt	Cytoplasm
Tur	Terminus
Kb	kilo base pair
N	Normal
T	Tumor
WT	Wild type
EV	Empty vector
DAPI	4′,6-diamidino-2-phenylindole
PCR	Polymerase chain reaction

Abstract

Carcinogenesis is a multistep process that involves altered gene expression because of both genetic and epigenetic alterations. One of the most studied epigenetic modifications is DNA methylation. Aberrant DNA methylation is linked to carcinogenesis in different cancer types. Progression of such tumors is associated with an imbalance in DNA methylation and demethylation. Previously, methylation was thought to be an irreversible event until the recent discovery of the TET family of enzymes that includes TET1, TET2 and TET3. While TET1 and TET2 roles in breast tumorigenesis were previously studied, little is known about TET3 role in breast tumorigenesis. In the present study, we tried to elucidate the behaviour of TET3 in breast tumorigenesis by overexpressing TET3 cDNA in breast cancer cells that exhibit low TET3 expression. Then, we tested the effect of TET3 overexpression on different cancer phenotypes. Our results showed that TET3 overexpression leads to an increase in cell proliferation as well as survival rate of infected cells when compared to normal cells. Additionally, infected cells showed a higher migration capacity than normal, noninfected cells. Moreover, we tested the expression level and localization of TET3 enzyme in breast cancer samples using IHC. We also tested the expression level of TET3 mRNA using relative qRT-PCR in different breast cancer cell lines under different contexts. We found that invasive breast cancer samples had higher levels of TET3 compared to normal or early stage samples. Additionally, we did qRT-PCR on some target genes that are related to the different cancer hallmarks that we tested in order to find any correlation between the genotype and phenotype and found that TET3 overexpressing cells upregulated some oncogenes while it had a slight or no seen effect on

other genes like tumor suppressor genes. Lastly, and to be able to correlate our findings with clinical cases, we used the Kaplan-Meier (KM) plotter in order to assess the association of TET3 on survival. The obtained results showed that high TET3 expression predicts poor prognosis in breast cancer patients. In conclusion, our results show for the first time that TET3 exhibits an oncogenic behavior.

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Chapter 1: Introduction & Background

- **Cancer**

Cancer is defined as a complex disease that is characterized by rapid and uncontrolled cell proliferation within a tissue. Cancers are generally thought to be genetic diseases of somatic cells that arise as a result of sequential alterations that activate oncogenes and inactivate tumor suppressor genes (Griffiths et al.). These alterations are known as somatic mutations and they can include single-nucleotide substitutions, insertions, deletions, copy number alterations, and structural rearrangements. Patients diagnosed with cancer can have one or a combination of these aberrations (Doyle et al.). For example, in solid tumors such as those derived from the colon, breast, brain, or pancreas, there is an average of 33 to 66 genes that display subtle somatic mutations (Vogelstein et al.). These mutations are expected to lead to alterations in the protein products of these genes. About 95% of these mutations are single-base substitutions (such as C>G), whereas the remainders are deletions or insertions of one or a few bases (such as CTT>CT) (Vogelstein et al.). Of the base substitutions, 90.7% result in missense changes, 7.6% result in nonsense changes, and 1.7% result in alterations of splice sites or untranslated regions immediately adjacent to the start and stop codons (Vogelstein et al.). However, some cancer types display fewer or more than the average of these alterations such as melanomas which display a larger number of these alterations. It is speculated that the large number is due to other factors that may have a role such as ultraviolet light and cigarette smoke, which are known as potent mutagens (Govindan et al.).

The process by which tumors grow and expand is referred to as clonal selection or evolution. This process starts with the acquisition and accumulation of a series of mutations over time in a particular tissue. A normal cell is affected by an induced change that transforms it into a neoplastic state that now has a selective growth advantage compared to the other adjacent normal cells. Proliferation of this neoplastic cell then proceeds either immediately or after a latent period. Then, cells that contain genetic variants are produced due to the genetic instability in the expanding tumor population. One of these cells has an additional selective variant and becomes the precursor, this allows it to proliferate and create a predominant subpopulation. Eventually, the fully developed tumor has a unique, aneuploid karyotype associated with aberrant metabolic behavior and specific antigenic properties, and it also has the capability of continued variation as long as the tumor persists (Nowell; Shackleton et al.).

- **Breast Cancer**

According to the World Health Organization (WHO), breast cancer has the highest morbidity and mortality rates of all cancers in women ("WHO | Breast Cancer"). Breast cancer is characterized by the composition of distinct malignancies that manifest in the mammary glands (Feng et al.). These tumors are composed of a variety of cell types with distinct morphologies and behaviors (Campbell and Polyak).

- **Classification of Breast Cancer**

Classification of breast tumors is difficult due to the fact that there are multiple subtypes of breast tumors. Each of these subtypes has its own patterns of gene expression and associated clinical outcomes. Accordingly, breast cancer is thought to originate from a

single cell that accumulates multiple mutations, allowing it to out-run normal growth controls and proliferate indefinitely (Campbell and Polyak). In Spite of all the different characteristics that tumors have such as size, morphology, antigen expression, and membrane composition as well as behaviors such as proliferation rate, cell-cell interaction, metastatic proclivity, and sensitivity to chemotherapy, all cancers and tumor types share one characteristic in common, which is the variability among the cancer cells within a single tumor. This is referred to as tumor heterogeneity (Campbell and Polyak).

Breast cancer is also classified according to the expression of signaling and growth receptors, which are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Dai, Xiang, et al.). Another molecular classification of breast cancer is according to profile analysis of gene expression. This classifies breast cancer into four major classes, luminal A and B, basal-like, and human epidermal growth factor receptor 2 [HER2] overexpressing. The basal-like tumors are parallel to those that are said to be triple-negative breast cancer (TNBC). TNBC does not express estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor (Montagna et al.).

- **Mutations Leading to Breast Cancer Formation**

The majority of genetic changes found in human breast cancer fall into two categories: gain of function mutations and loss of function mutations. The former occurs in proto oncogenes, which stimulate cell growth, cell divisions, and cell survival, while the latter occurs in tumor suppressor genes that normally help in preventing uncontrolled cellular growth and promote DNA repair and activation of cell cycle checkpoints when necessary (Lee and Muller). Activation of proto-oncogenes to oncogenes is a result of a gain of

function mutation. This mutation may be quantitative, which can cause an increase in the production of an unaltered product, or qualitative, which can lead to the production of a modified product. These alterations may result in the activation of oncogenes leading to the induction of abnormal cell proliferation and therefore tumor development (Brenner et al.). The ErbB2, PI3KCA, MYC, and CCND1 are examples of oncogenes that are frequently deregulated in breast cancer (Lee and Muller). Tumor suppressor genes are known to commonly contribute to the process fidelity of cell cycle replication. They may also act as negative regulators of oncogenes, cell cycle checkpoints, or gene products that supply the appropriate nutrients or components to complete a faithful cell cycle division in the absence of stress. Mutations in tumor suppressor genes are loss-of-function mutations and so occur in both alleles of a gene (Brenner et al.). Among these tumor suppressor genes are the BRCA1 and BRCA2 as well as the P53 family of genes (Lee and Muller).

- **Tumor Formation and Epigenetic Modifications**

Tumorigenesis was initially thought to be a result of pure genetic events that lead to the accumulation of mutations that could eventually cause cancer. However, clear evidence has been evolving for the past two decades suggesting that cancer initiation and progression involves epigenetic alteration (Kanwal and Gupta). Epigenetics is a concept that refers to the modifications that do not occur on the level of the DNA sequence (Reference What is epigenetics?-Genetics Home Reference-NIH). It is the study of heritable changes in gene expression that take place in an independent manner of changes in the primary DNA sequence (Sharma et al.). The majority of these changes are established during differentiation and are stable through multiple cycles of cell division, which allows cells to

have distinct and specific identities while containing the same genetic materials (Sharma et al.).

Epigenetic modifications are responsible for the heritability of the patterns of gene expression. These modifications include methylation of cytosine bases in DNA, posttranslational modifications of histone proteins as well as the positioning of nucleosomes along the DNA, in addition to other types of modifications. The collective system of these modifications is referred to as the epigenome. This provides the cells with a special mechanism in order to be able to regulate which genetic information can be accessed by cell machinery in order to achieve cellular diversity. Failure of the proper maintenance of heritable epigenetic marks can result in inappropriate activation or inhibition of various signaling pathways and lead to disease states such as cancer (Sharma et al.).

Chromatin is composed of basic repeating subunits called Nucleosomes. Each nucleosome is composed of a core, linker DNA, and in most cases, a linker histone (Cutter and Hayes). DNA is wrapped around an octamer structure of histones that contain two copies of four histone proteins; H2B, H2A, H3, and H4, to form a nucleosome. Condensation of the nucleosome to form the chromatin is the basis for chromosome folding (Rivera and Ren). Chromatin structure defines the state in which genetic information in the form of DNA is organized within a cell. The ability of genes to be silenced or activated is greatly influenced by the organization of the genome into a precise compact structure (Sharma et al.). When the chromatin structure is tight i.e. heterochromatin, transcription of genes is most likely silenced. However, when the chromatin structure is relaxed i.e. euchromatin, transcription of these genes is active. This is due to the fact that the structure of chromatin is able to

change the accessibility of transcription factors to promoter regions. The Compaction state of the chromatin structure is highly affected by active modifications in histones body and tail. For example, acetylation of histones by adding an acetyl group via acetyltransferases opens the chromatin structure, which recruits transcription factors to the promoter of target genes. On the other hand, deacetylation by histone deacetylases closes chromatin structure and inhibits expression. Additionally, different studies have shown an association between histone marks and genomic features like promoters and enhancers. For example, H3K4me3 is found to be present in active promoters, while H3K27me3 and H3K9me3 are present in inactive and silenced genes. Also, H3K27Ac indicates the presence of active enhancers and promoters (Rivera and Ren).

- **DNA Methylation**

DNA methylation is another mechanism involved in epigenetic gene expression regulation. DNA methylation is one of the pivotal epigenetic mechanisms that controls cell proliferation, apoptosis, differentiation, cell cycle, and transformation in eukaryotes (Pan et al.). Approximately, eighty percent of the CpG sequences in the human genome show detectable traces of DNA methylation. DNA methylation involves the addition of a methyl group at position 5 of cytosine (Li et al.). This process is catalyzed by DNA methyltransferase enzyme family members; DNMT3a, DNMT3b and DNMT1. Regulation of this mechanism is very important and aberrant DNA methylation can give rise to malignant tumor formation mainly by hyper- or hypomethylation (Pan et al.). For example, DNMT1 knockout in mouse model led to embryonic lethality (Li et al.), while repression of DNMT1 using CRISPR/cas9 in embryonic stem cells led to rapid and massive loss in the DNA methylation which resulted in cell death (Liao et al.). Hypermethylation of DNA

indicates the presence of methylation, which always leads to transcriptional suppression and decreased gene expression. On the other hand, hypomethylation represents a lack of DNA methylation affecting chromosome stability. Normally, global DNA hypomethylation is recognized as an indication of the presence of cancer cells, while promoters that are abnormally methylated may result in the silencing of tumor suppressor genes which eventually influences associated transcriptional pathways resulting in cancer proliferation (Pan et al.).

DNA methylation is associated with a repressed chromatin state and inhibition of gene expression. Modification of cytosine bases is one mechanism by which DNA methylation inhibits gene expression. This inhibition results from disrupting the association of some DNA binding factors with their complementary DNA recognition sequences. Another way is when proteins that recognize methyl-CpG can elicit the repressive potential of methylated DNA (Klose and Bird). Methyl-CpG-binding proteins (MBPs) use transcriptional co-repressor molecules to silence transcription and to modify surrounding chromatin, providing a link between DNA methylation and chromatin remodelling and modification (Klose and Bird). Moreover, the binding of Methyl-CpG-binding proteins to methylated regions recruits' histone deacetylase and other transcriptional repressors to modify chromatin and further repress gene expression (Klose and Bird). Methylation occurs at CpG dinucleotide regions, which are called CpG Islands. These CpG Islands are usually found at the centromeric tandem repeat units and in the promoter area of many genes (Klose and Bird).

- **Examples of DNA Methylation**

Examples of methylation of CpG-rich promoters include the prevention of transcriptional initiation to ensure the silencing of genes on the inactive X chromosome, imprinted genes, and parasitic DNA (Jones and Takai). Moreover, DNA methylation has also been considered integral to fundamental choices, including the suppression of transposable elements, and the establishment and maintenance of stable cellular identities (Bhutani et al.). Additionally, methylation is very important to maintain proper embryogenesis and normal cellular development, cellular differentiation and reprogramming, and aberrations in DNA methylation are associated with many human diseases including cancers. Alteration in DNA methylation patterns is one of the most common events in cancer. Aberrant DNA methylation is considered a hallmark of cancer and it results in part into genetic instability and loss of genomic integrity (Choi and Lee). Cancer is characterized by global hypomethylation and regional hyper methylation of tumor suppressor genes that leads to genomic instability, which has a great role in cancer initiation and progression (Herman; Robertson). Moreover, methylation patterns in cancer cells are different from normal cells, which make it an efficient biomarker for cancer detection and predicting the prognosis of the disease (Mikeska and Craig).

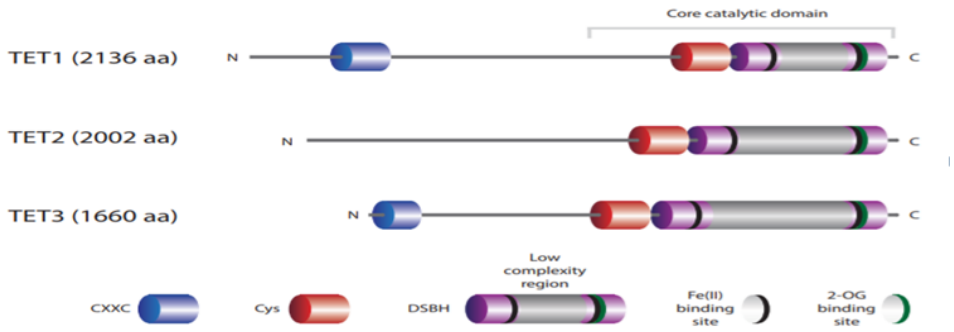
For some time now, DNA methylation has been considered a stable, persistent and heritable mark. This means that methyl groups are added but they are not removed. However, emerging research indicates that transcription factors and related proteins not only protect sequences from methylation but also initiate active DNA demethylation (Sadakierska-Chudy et al.). DNA demethylation is defined as the process of removal of a methyl group from cytosines. This process can be passive or active (Neidhart and Michel). The passive

removal of methylated cytosines is thought to be done by passive dilution by the presence of 5hmc which is an intermediate for the removal of those cytosines. This impairs re-methylation by DNA methyltransferases (DNMTs) when cells divide (Neidhart and Michel). However, the exact active mechanism(s) of DNA demethylation was elusive for many years until the discovery of Ten Eleven Translocation proteins (TETs) (Kohli and Zhang).

- **TET Enzymes**

TET enzymes or Ten-eleven translocation enzymes are a family of enzymes that consists of three members, TET1, TET2 and TET3. Tet enzymes have been found to convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). 5mc plays an important role in gene expression, genomic imprinting, and suppression of transposable elements (Dawlaty et al.). Some studies reported that TET enzymes are important for proper differentiation of embryonic stem cells. In this context TET1,TET2 and TET3 triple knockout mice have aberrant promoter hypermethylation that results in deregulation of developmental gene expression that leads to impairments in the differentiation of ES (Dawlaty et al.). Additionally, it was shown that Tet proteins could generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) from 5mC (Ito et al.). During active demethylation, these 5fC- and 5caC-modified cytosines are rapidly excised by thymine DNA glycosylase (TDG), which are then replaced by unmodified cytosines through base excision repair (BER) mechanisms (Melamed et al.). Moreover, further studies have indicated that TET and the oxidized 5mC derivatives play important roles in various biological and pathological processes, including the regulation of active DNA demethylation, gene transcription, embryonic development, and oncogenesis (Yin and Xu). All TET proteins contain a conserved double-stranded β-

helix (DSBH) domain, a cysteine-rich domain, and binding sites for the cofactors Fe(II) and 2-oxoglutarate (2-OG) that together form the core catalytic region in the C-terminus (Rasmussen and Helin). In addition to their catalytic domain, TET1 and TET3 have an N-terminal CXXC zinc finger domain that can bind DNA **Fig.1**. (Rasmussen and Helin; Zhao and Chen). It was shown that overexpression of TET1 catalytic domain causes global DNA demethylation, while overexpression of full-length TET1 didn't lead to the same effect, which reveals that the CXXC domain of TET1 is important for targeting of CpG islands. Moreover, TET1 CXXC domain was found to bind unmethylated CpG sequences which indicates that TET1 is mainly involved in maintaining a hypomethylation signal (C. Jin et al.). Moreover, the localization of TET1 to the heterochromatin is determined through the guidance of Methyl-CpG binding domain protein 1 (Mbd1) through its CXXC domain (Zhang et al.). TET3, CXXC domain mutagenesis revealed that this domain has higher affinity for 5caC and the other derivatives of the cytosine (S.-G. Jin, Zhang, Dunwell, Harter, Wu, Johnson, Li, Liu, Szabó, Lu, G.-L. Xu, et al.). Although TET2 doesn't have CXXC domain (**Scheme 1)**, it still has the ability to bind DNA through the help of IDAX, which has the same structure as the CXXC domain (Ko et al.). Tet1, Tet2, and Tet3 were identified as mammalian homologues of the trypanosome proteins JBP1 and JBP2, which oxidize the 5-methyl group of thymine, and experimentally demonstrated that Tet1 has the capacity to catalyze the conversion of 5mC to 5hmC (Zhao and Chen). Shortly afterwards, Tet2 and Tet3 were also shown to have 5mC hydroxylase activity . Subsequent studies revealed that Tet proteins can further catalyze the oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are two less abundant bases (Zhao and Chen).



Scheme 1: Domain structure of TET proteins. The C-terminal core catalytic domain shared by all TET enzymes consists of the DSBH domain, a cysteine rich (Cys) domain, and binding sites for the Fe(II) and 2-OG cofactors. The DSBH domain contains a large low-complexity region of unknown function. TET1 and TET3 have an N-terminal CXXC domain that can bind directly to DNA and facilitate recruitment to genomic target sites (Rasmussen and Helin)..

5-hydroxymethylcytosine (5hmC) is a constituent of nuclear DNA and is present in many tissues and cell types. It is enriched in embryonic stem cells and Purkinje neurons. 5hmC in the mammalian genome depends on pre-existing 5-methylcytosine (5mC) (Hashimoto et al.). The highest levels of 5hmc were found to be concentrated in brain tissue, which is due to the overexpression of TET1 in brain which is very important for the demethylation of very essential genes in the nervous system like fibroblast growth factor 1 FGF1 and brain-derived neurotrophic factor BDNF (Guo et al.). 5hmc decrease is associated with tumor development and progression. Thus, 5hmc has been identified as a biomarker that is markedly reduced in human breast, liver, lung, pancreatic, colon, prostate, brain, and myeloid cancers (Gambichler et al.). Another role for 5hmC has been proposed to act as a potential intermediate in active DNA demethylation via the base excision repair pathway (Hashimoto et al.).

All the members of the Tet family of proteins possess similar functions in terms of their demethylation activity. However, they differ in their domain architecture, tissue specificity, and expression levels (S.-G. Jin, Zhang, Dunwell, Harter, Wu, Johnson, Li, Liu, Szabó, Lu,

G.-L. Xu, et al.). It was found that mRNA levels of Tet1 and Tet2 are abundant in ESCs and primordial germ cells while mRNA levels of Tet3 are substantially expressed in oocytes and zygotes (S.-G. Jin, Zhang, Dunwell, Harter, Wu, Johnson, Li, Liu, Szabó, Lu, G.-L. Xu, et al.; Melamed et al.).

- **TET Enzymes and Cancer**

In cancer, different studies have shown that TET enzymes play a very important role in tumorigenesis (Yin and Xu). For example, in TET1 knockout mice, the liver shows a low level of 5hmc, which indicates that the loss of 5hmc during tumorigenesis is, in part, due to the downregulation of TET1 (Thomson et al.). In another study which compared the level of TET1, TET2, and TET3 in cervical cancer (CC) tissue to normal cervical tissue samples, the level of TET1, TET2 and TET3 expression was significantly decreased in cervical cancer patients compared to normal subjects (Bronowicka-Kłys et al.). Recently, in a study conducted on gastric cancer samples, immunohistochemistry results showed decreased levels of 5hmc in gastric cancer tissues compared to the adjacent normal tissues. This reduction was associated with low expression of the TET1 enzyme (Wang et al.). TET2 loss of function in different types of cancer was found to result from mutations or epigenetic silencing and was shown to be the most common genetic alteration among hematological malignancies (Scourzic et al.). However in parathyroid carcinoma, TET2 expression was significantly reduced in cancer tissue compared to normal tissue as a result of heavy TET2 promoter methylation (Barazeghi et al.).

- **TET3**

As for TET3, many studies have shown that the role of TET3 is different from one cancer type to another. For example, TET3 was found to inhibit epithelial-mesenchymal transition in ovarian cancer by demethylating the tumor suppressor microRNA mir-30d which directly targets TGF-β, which is one of EMT inducers. Thus, it was shown that down regulation of TET3 leads to the activation of TGF-β1-induced EMT and progression of ovarian cancer (Ye et al.). TET3 was also found to be downregulated in metastatic breast cancer cell lines compared to non-tumorigenic ones. Also, TET3 was found to inhibit the proliferation of breast cancer cells with the help of BRAC1 to co-repress EZH2 gene which promotes the tumorigenesis and metastasis of breast cancer (Wang et al.). In hypoxia in breast cancer, HIF1α was found to induce the transcription of TET1 and TET3 (Wu et al.). However, another study that was performed on ovarian cancer patients has found that those who presented with higher levels of TET3 had significantly shorter survival time than those with a lower level of TET3 (Cao et al.).

TET3 is the predominantly expressed TET enzyme in oocytes and zygotes, as well as the high expression in neurons (Melamed et al.). Although many studies have shown that the role of TET3 is different from one cancer type to another, little or no information is available regarding the role of Tet3 in breast tumorigenesis. Therefore, this study comes to elucidate the potential role that Tet3 plays in breast cancer and whether it acts as a tumor suppressor gene or an oncogene.

Study Aims and Objectives

TET3 is the third member of the TET family of enzymes that is least studied, therefore, this study aims at investigating the expression pattern and functional role of TET3 in breast cancer.Additionally, this study aims at elucidating the effect of TET3 overexpression in breast cancer cell lines under different cellular conditions in order to understand the behavior of this enzyme in different cancer hallmarks.Moreover, this study tries to determine the expression patterns and localization of TET3 in different human breast cancer tissue samples and stages using immunihistochemistry as well as try to correlate between the obtained results and different clinical cases.

Chapter 2: Materials and Methods

Table 2: List of Materials Used

#	Material	Manufacturer
1	MDEM/F12 media	Biological industries
2	RPMI (1640) media	Gibco Thermofisher
3	Horse serum	Biological industry
4	Fetal bovine serum	Gibco Thermofisher
5	Hydrocortisone	Sigma
6	Insulin	Sigma
7	Cholera toxin	Sigma
8	Glutamine	Biological industries
9	Penicillin/streptomycin	Biological industries
10	Dimethyl Sulfoxide	Sigma
11	PBS	Biological industries
12	Skim milk	Sigma
13	Anti TET3 ab PA5-34431,	Thermofisher

14	ECL	Thermofisher
15	GAG-pol plasmid	Addgene
16	VSV-G plasmid	Addgene
17	Mirus TransLTi	TransfectionExperts, MirusBio
18	Ampicillin	Sigma
19	Puromycin	Sigma
20	Maxi prep kit	Invitrogen
21	Mini prep kit	Macherey-Nagel
22	Paraformaldehyde	Electron Microscopy Siences
23	XTT kit	Biological industries
24	Anti-rabbit horse raddish peroxidase conjugated Ab	Bethyl
25	Isopropanol biological gradient	Sigma
26	Ethanol biological gradient	Sigma
27	Chloroform biological gradient	Sigma
28	qScript™cDNA synthesis kit	Quanta Biosciences

29	SYBR® Green	Applied Biosystems
30	TRIZOL	Sigma
31	Agarose	Hy-labs
32	100bp DNA ladder	Genedirex
33	DNase1 kit	Biolabs
34	Polyacrylamide	Biological industries
35	GoTaq® Green Master Mix	Promega
36	Triton X-100	Sigma
37	Goat serum	Biological industries
38	Anti-TET1 N-terminus GTX125888 Ab	Gene TEX
39	Cy3	Bethyl
40	DAPI	Sigma
41	Xylene	LOBA CHEMIE
42	Ethanol	Biolabs
43	Citrate buffer	Sigma
44	Hydrogen peroxidase	Sigma

45	CAS	Invetrogen
46	Tween	Sigma
47	Mayer’s hematoxylin	BioGnost
48	FH-TET3-pEF	Addgene
49	PSF-LENTI-CMV	Sigma
50	Kpn I	Biolabs
51	Xba I	Biolabs
52	PagI	Thermofisher
53	Gel/PCR extraction	Hy-Lab
54	1 Kb DNA ladder	Thermo-fisher
55	T4 DNA ligase	Biolabs
56	DH5α	Agilent Technologies
57	BamHI	Biolabs
58	0.22 μm filters	JET BIOFIL
59	Methanol	Sigma
60	Coomassie blue	BioRad
61	Chamber slide	Lab-Tek

62	DNA extraction	Geneaid
63	Protease inhibitors	Sigma
64	HEPES	Biological industries
65	KCl	Biological industries
66	EDTA	Sigma
67	DTT	Thermo Scientific
68	Nonidet P-40	Sigma
69	Tris base	Sigma
70	NaCl	pubChem
71	Glycerol	Sigma

Table 3: List of Equipment and Tools Used

No	Equipment or tool	Company	Industrial country
1	Inverted microscope	Olympus ck40-SLP	Japan
2	Biological hood (HERA guard)	Heraeus	Germany
3	Biofuge Stratos Reconditioned	Heraeus 75005289R	Germany
4	Biofuge Fresco	Heraeus 75005521	Germany
5	Hera cell 150 CO2 Incubator	Heraeus	Germany

6	Labofuge 200 centrifuge	Heraeus	Germany
7	Autovortex SA6	Stuart Scientific	U.K
8	Water Bath Orbital Shaking	Grant OLS200	U.K
9	Water Bath	Grant LTD6G	U.K
10	Ultracentrifuge	BECKMAN COULTER optima LE80H	U.S.A
11	SPIN-micropipette site	Nano Spinreact	china
12	Digital dry bath	Labnet	U.S.A
13	Elisa reader	BioTek EL-X800	U.S.A
14	Analytical Balance	METLER TOLEDO AB104	Switzerland
15	Autoclave	HIRAYAMA HV-110	U.S.A
16	RT-PCR machine	(Applied Bio-systems 7500 FAST Real Time PCR	Singafora
17	PCR machine	Applied Biosystem #9902	Singapore
18	SD semi-dry transfer cell	BioRad	U.S.A
19	G:BOX Chemi XX6 Gel Imaging System	Geneflow	UK

Methodology

1. Cells Culture

To culture breast cancer cell lines including MCF7, MDA MB231, T47D, HCC70, Sum 149, BT 549 cells, and Human Embryonic Kidney (HEK 293T), RPMI media (Gibco-Thermofisher) was prepared to contain 10% FBS (Gibco-Thermofisher), 1% glutamine, and 1% penicillin/streptomycin (Biological Industries).

MCF10A cells were cultured in DMEM/F12 media (Biological industries) that was prepared to contain 5% horse serum (Biological industries), 1% glutamine, 1% Penicillin/Streptomycin (Biological industries), 20 ng/mL EGF, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, and 100 ng/mL cholera toxin (Sigma Aldrich).

Upon culturing, all cells were placed in an incubator at 37 °C with 5% CO2.

2. Cells Freezing

Freezing media was prepared with 70% growth media, 20% fetal bovine serum (Gibco-Thermofisher), and 10% DMSO (Sigma).

To freeze cells, cells were detached from the culture plate by adding 1ml of trypsin, collected in freezing media and divided into aliquots in cryotubes. Then, tubes were stored at -80 °C and then transferred after a period of two weeks to liquid nitrogen.

3. Cells Passage

To pass cells, old media was aspirated, and then 1 ml of trypsin-EDTA was added to each plate and then a small amount of the added trypsin was aspirated. Then, cells were incubated in a CO2 incubator at 37 °C until the cells completely detached. Finally, cells

were mixed with the fresh media and a certain amount of cells was passed and incubated in a CO2 incubator at 37 °C.

4.Cells Thawing

To reinstate frozen cells from liquid nitrogen, cryotubes that contain cells were transferred immediately to a 37 °C water bath from liquid nitrogen. Upon thawing, cells were transferred to a new conical tube that contains 5 ml fresh medium. Lastly, the cells were resuspended in fresh media and cultured on tissue culture plates.

5.Cell Count

In 6-well plates, a number of $3x10^4$ cells were seededin triplicates. First, cells were trypsinized and collected into 15 ml conical tubes, they were then centrifuged at 1600 rpm for 10 min. Next, cells were resuspended in 1 mL media after the removal of the supernatant. Finally, and over the course of 4 days at about the same time, 10 µl of the homogenous supernatant was counted using counting chamber slides.

6. XTT Cell Proliferation Assay

In a 96-well plate, a number of $2x10^3$ cells were cultured in triplicates for 24 hours. The experiment was carried over the course of 4 days. Each day, the XTT kit reagents were prepared by mixing 50 µl of the XTT reagent and 1 µl of the activation reagent per well. On day 0, the time of adding the reagent mixture until the time of noticing a change of color in the media was recorded to serve as a template for the rest of the days. That is, everyday for the next 3 days, the XTT reagent mixture was added and the results were assessed at the same time as day 0. Finally, everyday the results were interpreted via the ELISA reader (BioTek EL-X800).

7. Wound Healing Assay

In 12-well plates, a number of 2.5 x10^5 cells were cultured in triplicates. After reaching 100% confluency, the cell monolayer was scratched using the 10-µl plastic pipette tip. After the floating cells were removed, the same area of the plate was photographed over multiple period of times using a camera attached to an Inverted Microscope (Olympus, CK-40)

8. Colony Forming Assay/Survival Assay

a number of 400 cells were cultured in triplicates in 6 well-plates. Each 3-4 days, the media was changed until cell colonies were visible by naked eye. Then the media was removed and the wells were washed with PBSX1 (Biological industries). PBS was then aspirated and wells were left to dry. Cells were fixed with absolute methanol (Sigma) after drying for about 15 min at RT. Then, wells were left to dry and then stained using crystal violet (Bio-Rad) for about 15 min. Lastly, stain was removed and wells were washed using tap water.

9. RNA Extraction, cDNA synthesis and qRT-PCR

RNA was extracted using the Trizol reagent (Sigma Aldrich).First, tissue culture plates were placed on ice and media was removed. Then, 0.5 ml of the trizol reagent was added to the plate and incubated on ice for 5 minutes. Cells were then scraped from the plate and collected into cold eppendorf tubes. After that, 200 ml of cold chloroform were added and vortexed on ice where tubes were left to incubate for 15 minutes. Following that, tubes were placed in a pre-cooled centrifuge for 15 minutes at 1200 rpm at 4 degrees celsius. Then the supernatant layer was transferred into cold tubes. Next, 500 ml of cold isopropanol were added to the tubes. Everything was mixed and placed on ice for 15

minutes to incubate. Tubes were then placed in the cold centrifuge for 15 minutes at 1200 rpm at 4 degrees celsius. Then, the supernatant was discarded leaving a small white pellet at the bottom of the tube. After that, 1500 ml of 70% ethanol were added and tubes were placed back in the centrifuge for 10 minutes at 1200 rpm and 4 degrees celsius. Finally, ethanol was removed and the pellet was dissolved in about 30 µl of DEPC-treated water and placed in a hot dry bath for 10 minutes at 60 degrees celsius.

For cDNA synthesis, DNase I treatment kit (biolabs) was used to avoid DNA contamination. cDNA was synthesized using Q-Script cDNA synthesis kit according to manufacturer's instructions (Quanta Biosciences). Relative quantitative real-time PCR (qRT-PCR) was done using Applied Biosystems® 7500 Real-Time PCR machine using power SYBR Green Mix (Applied Biosystems). The sequence of primers that was used to detect the presence of *TET3* mRNA is F.P_5'- ccaagagtctgctggacaca-3' (forward primer), R.P_5'- ccaggaacaaccaaaggaga-3' (reverse primer). To measure the expression of ectopic TET3 in cell clones we used HA-F.P_5'-GAGGATACCCCTACGACGTG-3' (forward primer that targets HA tag) and *TET3*-R.P_5'-AAGCTCCCTACCATCACCTG-3' (reverse primer that targets TET3). To test the effect of *TET3* overexpression on the expression of cancer hallmarks related genes, we used the primers listed in appendix 3.

10. Immunohistochemistry (IHC)

Tumor sections of 5 µm were deparaffinized and rehydrated with Xylene (LOBA CHEMIE) 3 times for 5 min, then 100 % ethanol (BioLab) 3 times for 2 min, 95 % ethanol 2 times for 2 min, 80 % ethanol for 3 min, and then transferred into distilled water (DW) for 5 min. After that, tissues were denatured for 4 min in a pressure cooker in a

citratebuffer (Sodium citrate dehydrate and Citric acid, both Sigma-Aldrich) 0.01 mol/L, pH 6.0. Then slides were cooled to RT in a cold water bath, and then washed and incubated with DW for 5 min. Afterwards slides were blocked by 3% Hydrogen peroxidase (Sigma-Aldrich), washed and incubated with DW for 5 min. After that, slides were blocked again with CAS® block (Invetrogen,00-8120) reagent for 15 min. Then the sections were incubated with anti-TET3 Abs (TET3 PA5-34431, Thermofisher) overnight at 4 °C according to manufacturer's recommendations. Next, slides were washed 3 times for 5 min each with X1 TTBS (100 ml TBS X10, 900 mL DW, and 0.5 mL Tween, Sigma-Aldrich). Then, slides were incubated with secondary antibodies conjugated to HRP (Bethyl, A120-101P). To visualize the stained tissue, slides were incubated with HRP chromogen substrate (Impress HRP reagent kit, vector laboratories). Finally, slides were counterstained with Mayer's hematoxylin (BioGnost). All samples were obtained from Augusta Victoria Hospital. All patients signed the Augusta Victoria Hospital IRB form.

11. Bioinformatics

TET3 mRNA sequence was retrieved from gene bank https://www.ncbi.nlm.nih.gov/website using the accession number NM_001287491.1. All primers used in this thesis were designed using the primer 3 software http://primer3.ut.ee/. For virtual Plasmid digestion and cloning NEB cutter software http://nc2.neb.com/NEBcutter2/ was used.

12. *TET3* gene cloning

12.1. Digestion of FH-TET3-pEF and PSF-LENTI – LENTIVIRUS

FH-TET3-pEF (Addgene, Plasmid #49446) was used as a template. The viral vector PSF-LENTI - LENTIVIRUS PUROMYCIN CMV PLASMID was our destination vector (Sigma-Aldrich, OGS269). An amount of 20 µg of both FH-TET3-pEF and PSF-LENTI were digested with Kpn I (BioLab, R0142S) and Xba I (BioLab, R0145S). PagI (BspH1) (Thermo Fisher, ER1281) was used to digest the FH-TET3-pEF vector backbone (Appendix 2). Digestion conditions are shown in appendix 5. Then, digestion products were run on 0.7 % agarose (hy-labs). Specific bands were purified from the gel using Gel/PCR extraction (Hy-Lab, EX-GP 200) kit.

12.2. Ligation

To clone the TET3 gene with the viral vector PSF-Lenti, different ligation molar ratios (1:3, 1:5, and 1:10) were used. The molar ratios were calculated using the online calculator http://www.insilico.uni-duesseldorf.de. Ligation was performed according to T4 DNA ligase (BioLabs, M0202S) protocol.

12.3. Transformation

The chemically competent DH5α bacteria (Agilent Technologies Cat# 210518) was transferred from -80 °C and directly placed and incubated on ice for 30 min. Then, 50 µl of DH5α was mixed with 5 µL of ligation products. After the incubation period was over,

bacterial tubes were quickly transferred and incubated at 42 °C for 1 min. Then, tubes were directly returned to ice for 5 min, and then transferred and incubated at RT for 5 min. Transformed DH5α cells were grown in 1mL LB liquid with shaking for 2 hrs at 30 °C, then centrifuged at 8000 rpm for 1 min. Then, most of the supernatant was removed and cells were resuspended in the residual media left. Finally, transformed DH5α cells were grown on Ampicillin (Sigma-Aldrich) LB agar plates and incubated at 30 °C for 24 hrs.

12.4. Digestion of mini-prep products

Digestion was done using BamHI enzyme (Biolabs, R01363) that cuts one time in both PSF-Lenti and TET3 mRNA sequence (appendix 1) in order to test which colony has TET3-PSF-Lenti.

12.5. Plasmid preparation (Maxi-perp)

After the bacteria was grown in 0.5 L LB media containing antibiotic selection markers at 30 °C overnight with shaking, cells were collected and Maxi-prep was done using HiPure Plasmid Maxiprep kit (Invitrogen, K210006) according to manufacturer instructions.

12.6. Lentivirus preparation

Lentivirus particles were generated by three plasmid expression system, in which HEK 293T cells were co-transfected with the following three vectors: packaging GAG- pol (Addgene), envelope pCMV-VSV-G (Addgene), and TET3-PSF Lenti or PSF lenti vector. A day before the transfection, HEK-293T cells were plated to reach 60% confluency. A day after, cells were fed with fresh medium and transfected using MIRUS (TransfectionExperts, MIR2300) transfection reagent. An amount of 2.2 µg packaging GAG-Pol, 1.2 µg Envelop

VSVG, and 5 µg of each TET3-PSF-lenti and PSF-lenti vector were transferred to tubes that contain 21 µl MIRUS mixed with 2 ml serum free medium. 15 min after incubation at RT, the mixture was added to the cell culture media. 24 hrs after transfection, cell culture media was replaced with fresh media. On the second and third days after transfection, cell culture media that contain the viral particles was collected and centrifuged for 10 min at 53000 rpm in order to get rid of cellular debris. Finally, the collected virus-containing media were centrifuged at 40000 rpm for 2 hrs using BECKMAN COULTER (OptimaTM LE-80K Ultracentrifuge). After that, most of the media was removed and the viral particles were then resuspended and filtered using 0.45 µm filters (JET BIOFIL).

12.7. Infection and selection

A number of $0.5x10^6$ MDA MB231 cells were infected with 0.75 mL of viral particles suspension. Cells were incubated with viral particles for two days in a CO2 incubator at 37 °C. Next, media-containing viruses was removed, and replaced with fresh media for 24 hrs. The infection process was repeated twice. To select for clones, cells were grown in media that contained 1 µg/mL of puromycin (Sigma) until the control un-infected cells completely died.

13. Kaplan-Meier (KM) plotter database

The Kaplan Meier plotter (http://kmplot.com/analysis) is based on an online database and is capable of assessing the association of genes on survival in four types of cancer samples including breast cancer, ovarian cancer, lung cancer and gastric cancer. Clinical data included gender, age, histology, stage, grade, TP53 mutation status and applied chemotherapy for all patients. (Cao et al.).

Chapter 3: Results

1. TET3 expression in breast cancer cell lines

In order to learn about TET3 expression patterns in breast cancer, we tested TET3 mRNA expression in different breast cancer cell lines using qRT_PCR. As shown in Fig. 1, the luminal cell line MCF7 has the highest expression level as compared to other cell lines. However, for our study, the cell line of choice was MDA MB231 since it shows the lowest levels of TET3 expression, which allowed us to manipulate it and overexpress it as desired.

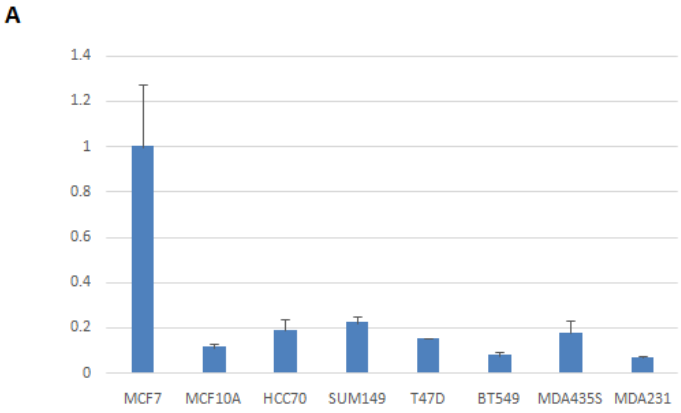


Figure 1: Expression pattern of different TET enzymes in breast cancer cell lines. mRNA expression of TET3 in different breast cancer cell lines was measured using qRT PCR. Results were measured relative to MCF7 cell line. Bars represent standard error of the mean.

2. TET3 gene cloning in Lentiviral vector

a.Isolation of *TET3* fragment and digestion of the lentiviral destination vector:

In order to study the TET3 function in breast cancer, we first cloned *TET3* into a lentiviral vector. To do so, we excised out *TET3* mRNA from FH-TET3-pEF plasmid by using KpnI and XbaI enzymes. However, because the TET3 fragment (6200 bp)

size is close to the size of the backbone (5469 bp), PagI enzyme was used to cut the backbone into smaller fragments to make it easy to isolate the desired TET3 fragment from the gene **Fig. 2.** After cutting *TET3* mRNA using KpnI and XbaI enzymes, the same restriction enzymes were used to digest our destination lentiviral vector. Following that, the products were ligated as described in the materials and methods section. To confirm successful *TET3* mRNA cloning in a lentiviral vector, we prepared minipreps from several bacterial colonies transformed with the ligation product. Then, the minipreps were digested using the BamHI enzyme that cuts one time in both PSF-Lenti and *TET3* mRNA sequence (appendix 4). Gel electrophoresis analysis of the digested products revealed that all colonies that we analyzed were positive and carried the TET3-PSF lentiviral vector. These results confirm the successful cloning of *TET3* cDNA in PSF-lentiviral vector **Fig. 3**. In order to obtain a large amount of TET3-PSF-Lenti to be further used for viral particle preparation and cell infection, we did maxi-prep plasmid isolation. In order to further confirm the success of cloning, we did conventional PCR on the viral vector using *TET3* specific primers. A positive PCR product was obtained from both our positive control sample as well as from our cloned vector (data not shown). Finally, to show that the cloning process didn't result in mutations in *TET3* cDNA, we sequenced the plasmid using *TET3* specific primers. The sequencing results showed that indeed *TET3* is cloned into the vector and that no mutations are found in the cloned *TET3* cDNA (Appendix 2). Altogether, these results confirm that *TET3* was successfully cloned into our viral vector and that the cloned *TET* mRNA is mutation free.

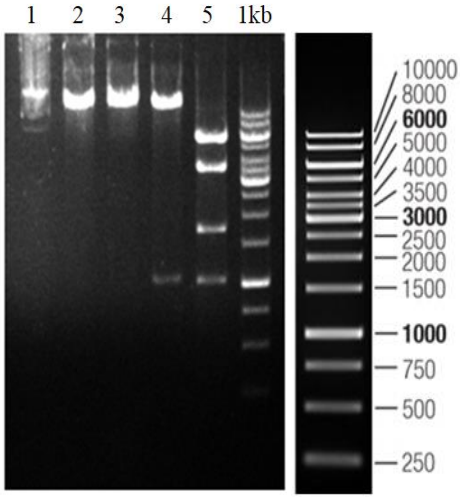


Figure 2: Digestion of FH-TET3-pEF and PSF-lentiviral vectors. Lane1: FH-TET3-pEF uncut, Lane2: FH-TET3-pEF cut with KpnI, Lane3: FH-TET3-pEF cut with XbaI, Lane4: FH-TET3-pEF cut with PagI, Lane5: FH-TET3-pEF cut with KpnI, XbaI, and PagI, Lane6: 1 Kp ladder**.**

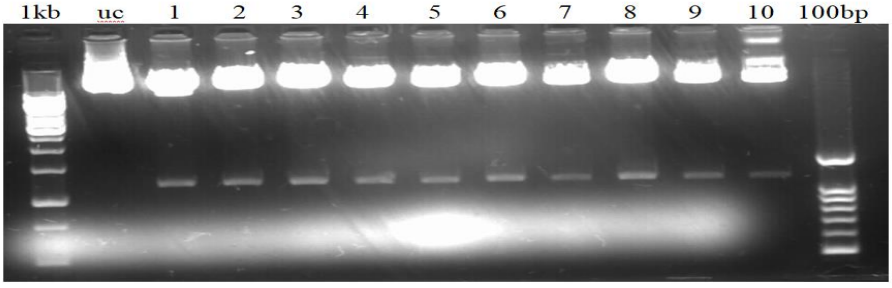


Figure 3: Testing for successful *TET3* cloning by analysing cloning minipreps. Agarose gel photograph showing minipreps digested with BamHI enzyme (Lanes 1-10), Lane1 uc: TET3-PSF-lenti uncut miniprep control. 1Kb: 1Kb DNA ladder, lane 13: 100 bp ladder.

b. Generation of TET3 overexpressing MDA MB231 cell

In order to study the function of TET3 enzyme in breast cancer, we infected breast cancer cell line MDA MB231 with either viral particles expressing HA-tagged *TET3* (TET3-PSF-Lenti) or empty viral vector control (PSF-Lenti). Two days later, we added puromycin to select for TET3 positive cells. The same was done for control cell selection. After one week of selection, only control non-infected cells died, while the control empty vector and TET3 overexpressing cells were resistant to puromycin selection. To ensure TET3 overexpression

in our clones, we did qRT-PCR on RNA extracted from both the control and TET3 overexpressing cells. We first did qRT-PCR using TET3 specific primers and indeed, our results from this experiment proved that we have about 10 fold *TET3* overexpression in our clones compared to control clones (**Fig. 4A**). To further verify TET3 overexpression and to ensure that the overexpression that we got with *TET3* specific primers is not transient and didn't result from the cloning and selection process, we used a primer in which the forward primer targets the HA tag while the reverse one targets TET3. The results confirmed overexpression of TET3 in our clones (**Fig. 4B**).

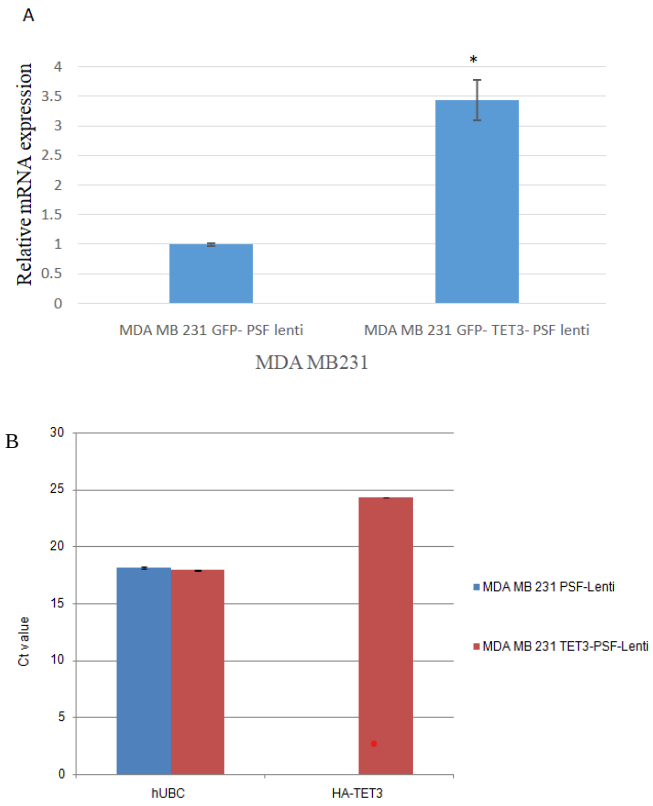


Figure 4: Generation and validation of TET3 clones. A. qRT-PCR on mRNA extracted from either MDA MB231 infected with *TET3* (TET3-PSF-lenti) or MDA MB231 infected with empty vector (PSF-lenti) using either *TET3* specific primers (**A**) or primer pair where the forward primer targets HA-tag while the reverse primer targets *TET3* (**B**). Results are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA levels in MDA MB231 control cells infected with empty vector. Bars represent SEM. * indicates that p-value is <0.05.

3. Effect of TET3 overexpression on MDA MB231 cell phenotypes

Cancer development and progression is considered a multistep process that leads to the transformation of normal tissues to tumorous tissues that possess a neoplastic state. This multistep process gives the transformed cells the capability to survive and become malignant by acquiring different biological hallmarks (Hanahan & Weinberg, 2011). These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011). In order to elucidate the effects of TET3 on these hallmarks, we have tested TET3 overexpression in different experiments.

We first tested the effect of TET3 overexpression on cell proliferation. To do so, we performed the cell count assay and monitored cell proliferation by comparing the growth rate of TET3 overexpressing cells to the growth rate of control cells. Our results show that TET3 overexpression increases cell proliferation as shown in **Fig. 5**.

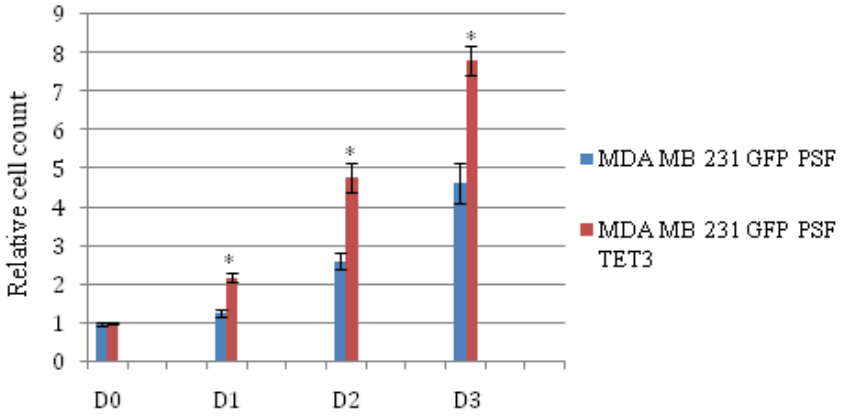


Figure 5: Effect of TET3 overexpression on MDA MB231 cell phenotypes. Representative graph showing the relative proliferation rate of TET3 overexpressing compared to control cells using cell count assay. Cell growth was monitored over three days. * indicates P value <0.05.

Motility and cell migration is another studied hallmark of cancer. In order to see the effect of TET3 overexpression on this hallmark, we used the wound healing assay or also known as migration assay. The obtained results show that TET3 overexpressing cells induced cell migration to the wounded area **(Fig. 6)**. Over time, the distance of the wound of TET3 overexpressing cells was noticeably narrowing down compared to that at the same interval of the not TET3-overexpressing cells as seen in Fig.6.A.

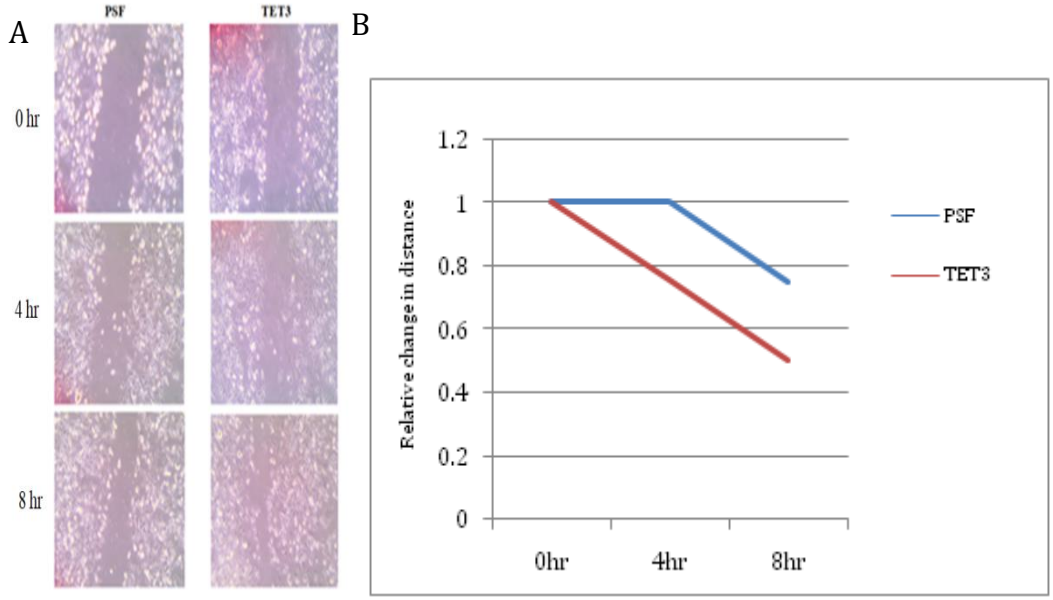


Figure 6: Behavior of TET3 overexpressing cells in motility and cell migration. Representative images showing the migration capability of TET3 overexpressing cells in comparison to control cells using wound healing assay **(A).** Numerical representation of the relative change in the wound's distance measured over time **(B)**. Cell growth was monitored after 4 hrs & 8 hrs.

Another hallmark that we studied that characterizes cancer cells growth is cell autonomy and survival independence on cell-cell communication. The principle of this experiment is based on the ability of a single cell to divide and form a colony. So, to find out the effect of the manipulated TET3 on cell survival, we performed cell survival assay by seeding and culturing a low number of cells (400 cells) on a big surface area. The number of colonies that the TET3 overexpressing cells formed was slightly higher than that of the control cells.

Thus, when compared to control cells, TET3 cells showed a slightly higher cell survival index. **(Fig. 7).**

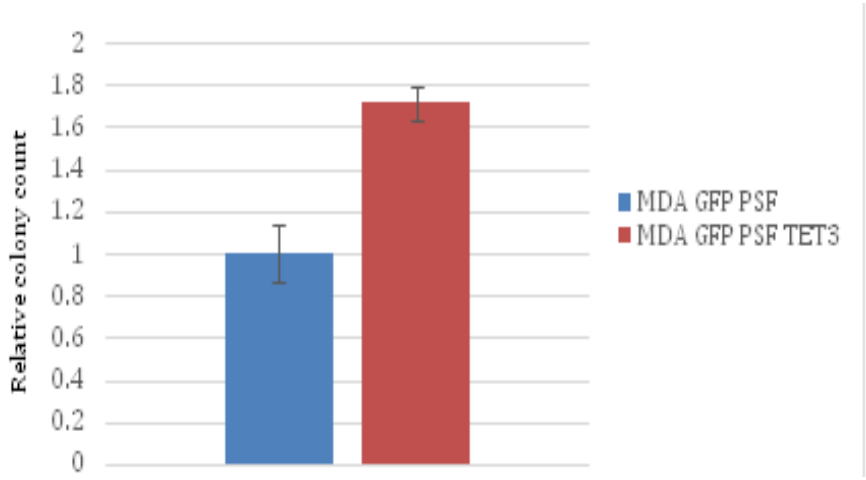


Figure 7: Effect of TET3 overexpressing cell survival. Representative statistical analysis of the survival rate of TET3 overexpressing cells in comparison to control cells using cell survival assay.

4. TET 3 enzyme expression in breast cancer tissue samples.

To determine the prevalence of altered TET3 level in breast cancer in Palestinian patients, we examined it's expression in different types of human breast cancer samples by IHC using antisera against TET3 enzyme. Distinct TET3 expression levels and localization were seen when comparing tumors from different patients (**Fig. 8A**). In addition, we noticed that the expression level of TET3 correlates with the degree of aggressiveness of tumors. As shown in **Fig. 8B**, TET3 expression level is higher in invasive tumors compared to DCIS and normal samples.

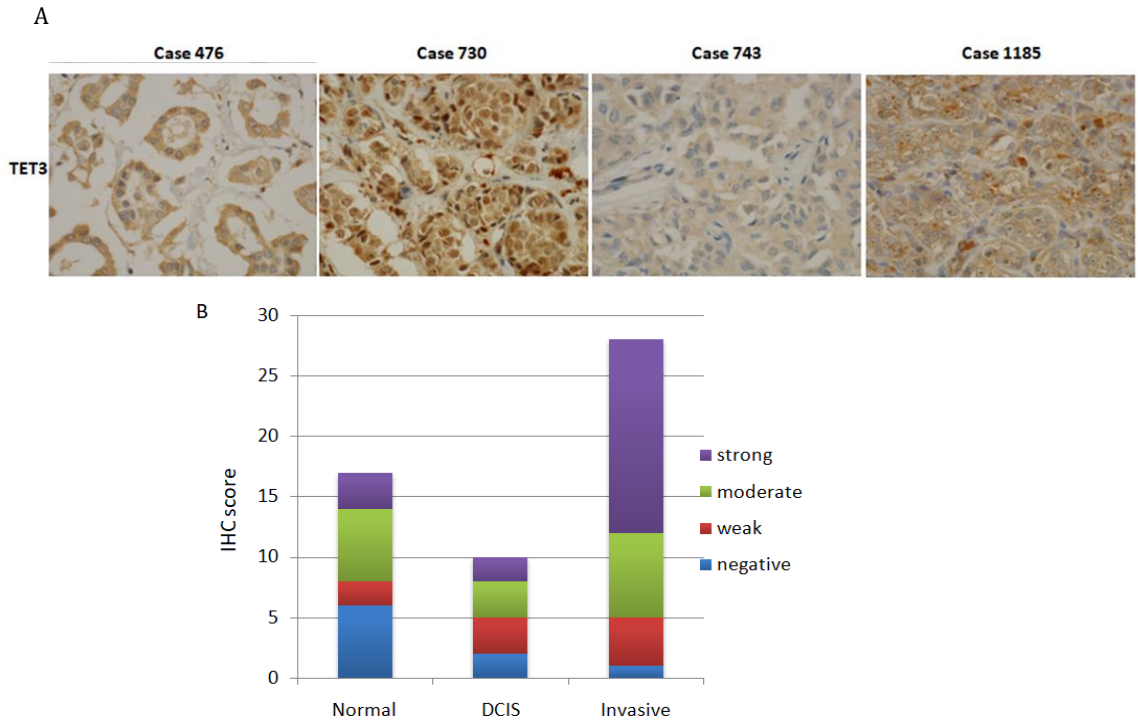


Figure 8: TET 3 enzyme expression in breast cancer tissue samples. (A) IHC staining showing different expression level and pattern ranging from moderate cytoplasmic level (case 476), strong mixed cytoplasmic and nuclear (case 730), weak cytoplasmic (case 743) and mixed cytoplasmic and prominent nucleolar (case 1185) staining **(B).**

5. High TET3 expression predicts poor prognosis in breast cancer

In order to show the correlation between TET3 expression and breast cancer patient survival, we used the Kaplan-Meier plotter online database. Results showed that patients with higher TET3 level had significantly shorter survival time than those with a lower TET3 level **(Fig. 9 A-E).** All patients expressing high levels of TET3 mRNA showed poorer prognosis and less months to live compared to those with lower levels of TET3. **Fig. 9F** represents the survival curve for breast cancer patients with high and low expression of TET3 levels at the level of protein. The cohort with high levels of TET3 were expected to

live for about 17 months while those expressing lower levels were expected to live for 48 months.

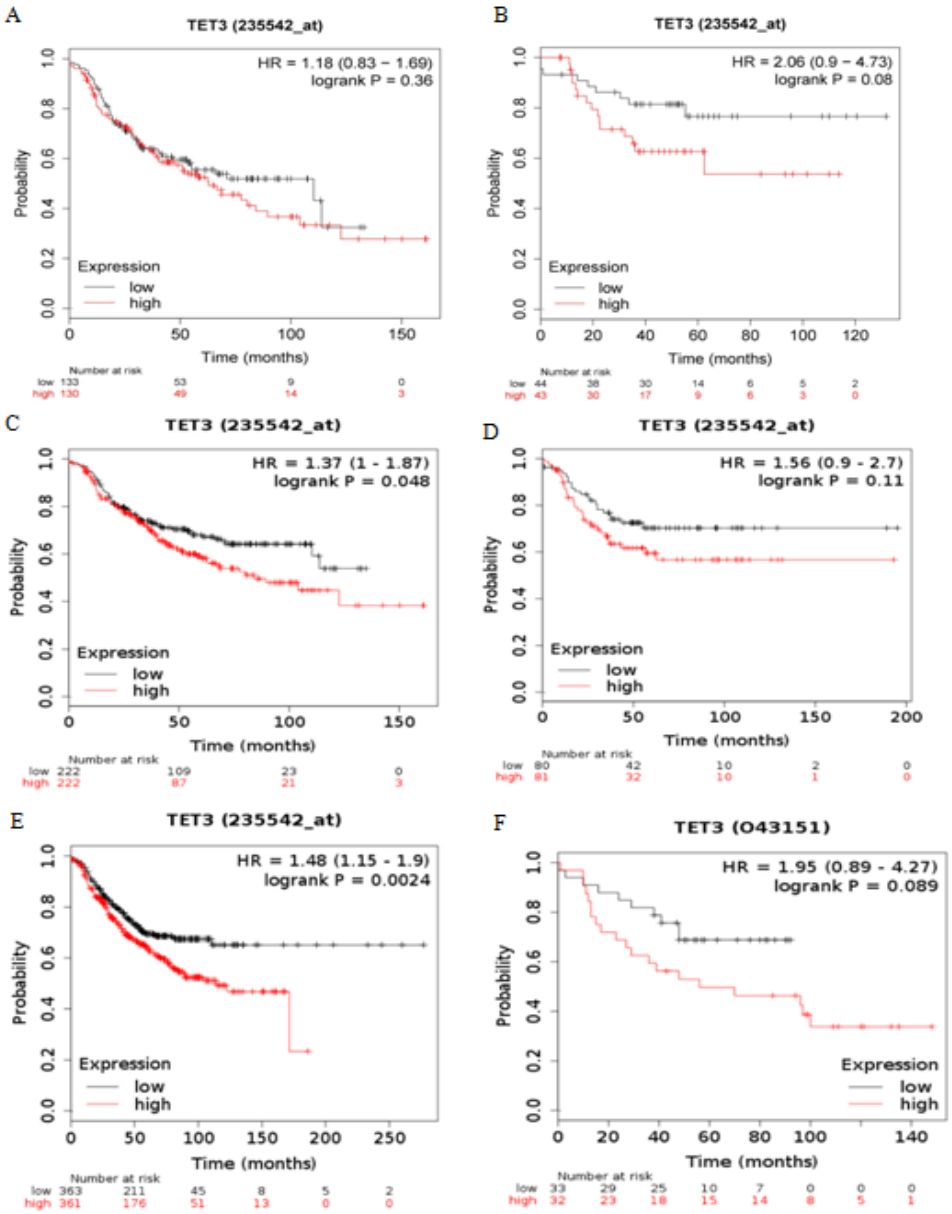


Figure 9: High TET3 expression predicts poor prognosis in breast cancer. The prognostic value of TET3 at mRNA level was estimated by Kaplan-Meier plotter analysis tool **(A-E)**. **(A)** +lymph node and grade 3 (n=369). **(B)** TNBC and grade 3 (n=138). **(C)** grade 3 (n=903). **(D)** TNBC (n=255). **(E)** +lymph node (n=1113). **F** Represents the result of TET3 overexpression on the protein level. Accessed on 22/2/2020. For each outcome, analysis was restricted to different subtypes.

6. Effect of *TET3* overexpression on other genes like Oncogenes and TSGs

In order to try to explain the results that we obtained from the phenotypes experiments that are related to TET3 overexpression at the molecular level, we tested the effect of TET3 manipulation on the expression level of different genes that are linked to the tested cancer hallmarks. We tested the expression of both oncogenes and tumor suppressor genes in MDA MB231 cells infected with either empty vector or TET3 lentiviral vector. The list of the tested genes included *AKT 1, Cyclin B1, IDH1, Nanog, PCDH7, SLIT2, Snail1,* and *Wnt-5A*. Some genes didn't show any significant change in gene expression (SNAIL and WNT5A), while the expression of others was either induced (SLIT2 and IDH1) or reduced upon TET3 overexpression (AKT).

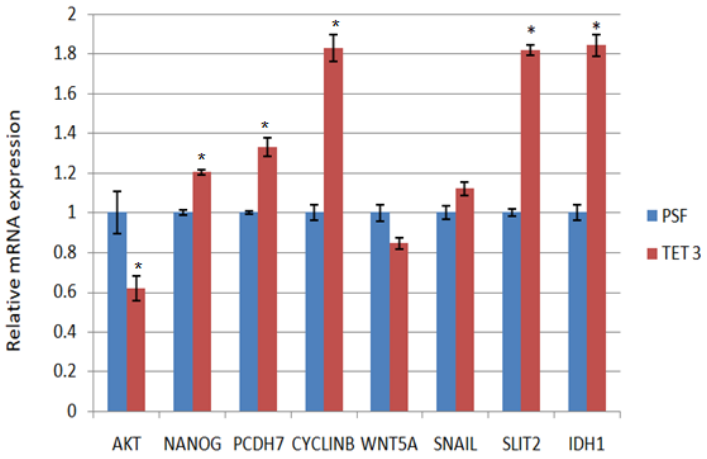


Figure 10: Effect of *TET3* overexpression on other genes like Oncogenes and TSGs. Relative qRT-PCR results showing the expression level of different TET3 target genes in MDA MB231 cell line. mRNA levels are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA level of each gene in MDA MB231 infected with EV. Bars represent SEM. * indicates that p-value is <0.05. PSF: PSF-Lentiviral, TET3: TET3-PSF-Lentiviral.

Chapter 4: Discussion

TET family of enzymes is a newly discovered family that is known for its capability of demethylating genes and actively reverting a normal methylation state. These enzymes were found to be differentially regulated in different types of cancer, including breast cancer (Yang et al.). Cancer is characterized by aberrant gene expression, particularly, aberrant methylation patterns (Yang et al.). These patterns are maintained through different mechanisms, one of which is the demethylation of CpG rich sequences by TET enzymes. Studies that target elucidating the expression and function of TET enzymes are abundant, however, TET3 is the least studied among the family. Therefore, in this study, we try to find more about the expression pattern and function of TET3 in order to have a generalized picture of how these enzymes affect the state of methylation in cancer.

We first tested the expression levels of TET3 in different breast cancer cell lines in order to choose the suitable target cells to work with for the course of our study. Our results show that the expression of TET3 is low in some breast cancer cell lines (MDA MB231, MCF10A for example) while high levels were seen in other cell lines (MCF7). Due to the very low TET3 expression in it, the cell line of choice was the MDA MB231 in order to be able to manipulate the levels of overexpression as desired. MDA MB231 cells showed the lowest level of TET3 expression even though this cell line is a highly aggressive one (Welsh). This could be due to many different reasons. One explanation for this could be that the environment created by cancer cells inside the body to survive is different than that artificially created to mimic the actual environment these cells grow and proliferate within. Another reason for this differential expression between cell lines is explained by the type of

the tumor (basal, luminal, HER2 overexpressing, or normal-like). For example, basal types of breast cancer are likely to be of grade 3 (Dai, Li, et al.). Moreover, recent studies suggest that TET3 has three different isoforms (S.-G. Jin, Zhang, Dunwell, Harter, Wu, Johnson, Li, Liu, Szabó, Lu, G.-L. Xu, et al.). This finding could also explain the differential expression of TET3 in different tissue samples as well as in different cell lines. To address this in future studies; we suggest doing PCR analysis with primers that target different exons in different cell lines of the same cell of origin. And in this regard, we noticed that when comparing MDA MB231 (basal cell line) TET3 expression to MCF10A cells (basal cell line), we didn't see the drastic difference in TET3 expression between these two basal cell lines as compared to the big difference in the expression of TET3 upon comparing a luminal cell line (MCF7) to a basal cell line (MDA MB231).

When we tested the behaviour of the manipulated MDA MBA231 cells through the different functional assays that target three cancer hallmarks (cell proliferation, motility and cell migration, cell survival) we saw that those cells showed a higher proliferation rate, a higher induction rate of motility and cell migration, as well as a higher survival rate compared to the control unmanipulated cells. In order to try to explain these results, we tested the effect of TET3 overexpression on target genes that are related to the studied phenotypes as well as others. These genes are either TSGs or Oncogenes. We tested the effect of TET3 overexpression on two tumor suppressor genes, PCDH and SLIT2, and on six oncogenes, *AKT 1, Cyclin B1, IDH1, Nanog, Snail1,* and *Wnt-5A.* Our results show that these genes were differentially affected. Some oncogenes showed an increase in expression such as Cyclin B1, which is a cell cycle regulator that is overexpressed in many cancer

types (Dong et al.), and IDHI, which is a key enzyme that functions in cellular metabolism (Molenaar et al.). However, contrary to what we expected regarding TSGs, which are expected to be reduced, PCDH and SLIT2 were slightly elevated. This indicates that there are other factors that need to be further studied that play a role in regulating the expression of other genes. In breast cancer, SLIT2 was shown to suppress breast carcinogenesis and progression by attenuating β-catenin signaling pathway (Prasad et al.). These results indicate that overexpression of TET3 has an effect on some oncogenes by elevating their expression levels, leading to the conclusion that TET3 is an oncogene. However, recent studies are emerging indicating that TET3 has different isoforms (S.-G. Jin, Zhang, Dunwell, Harter, Wu, Johnson, Li, Liu, Szabó, Lu, G. Xu, et al.), which means that the role or roles of TET3 need to be further elucidated.

To further validate the obtained conclusion of oncogenic behaviour of TET3, our results from IHC on different samples including a DCIS (Ductal Carcinoma In Situ), which is considered a very early stage of breast cancer, differed from those taken from a more aggressive state. DCIS showed lower levels of TET3 expression while the invasive type of cells showed moderate to stronger expression of TET3 suggesting that TET3 in this case is an oncogene. Additionally, IHC staining showed that different breast cancer tissue samples exhibited different expression patterns of TET3 enzyme ranging from moderate cytoplasmic levels to strong mixed cytoplasmic and nuclear indicating that there are other factors affecting the role of TET3 in different cellular contexts.

Furthermore, and to correlate our results with clinical outcomes, we did Kaplan-Mieyer analysis. Also here, our results support our findings from the cell culture model, which

indicate that TET3 is an oncogene. Almost all patient samples studied with the Kaplan-Mieyer online tool predicted that patients overexpressing TET3 in their breast tissue showed poor prognosis compared to patients expressing low levels of TET3. To support our findings, Cao et al. did a bioinformatics integrative analysis using the Kaplan-Meier plotter online database. They also found that increased TET3 expression predicts unfavorable prognosis in ovarian cancer patients (Cao et al.).

DNA hypomethylation is an epigenetic marker that is present in many tumors. However, studying the effects of this alteration has been rarely approached due to the fact that hypomethylation was demonstrated to occur at high levels in repeated DNA sequences which contain approximately 56% of CpG content (Ehrlich; Ross et al.). Recent studies have shown that DNA hypomethylation is important in cancer (Cao et al.). High resolution genome-wide studies confirm that DNA hypomethylation is usually the almost constant companion to hypermethylation of the genome in cancer in different sequences (Ehrlich). Given that the function of TET3 enzyme is to actively demethylate methylated CpG rich sequences resulting in a state of hypomethylation, our results are in agreement with the fact that TET3 possesses an oncogenic behavior that correlated with hypomethylation observed in cancer. This was noticed from the performed experiments on different cancer hallmarks: cell proliferation, cell migration, and cell survival. The yielded results from our experiments support the theory that TET3 is an oncogene that is overexpressed in invasive breast cancer tissue samples.

Different studies have shown that high levels of TET3 are expressed in invasive cancer cells. In a study that analyzed TET3 gene expression levels between normal and ovarian

cancer tissues in four distinct ovarian cancer datasets, it was found that TET3 mRNA expression levels were significantly higher in ovarian cancer samples compared to normal tissue samples (Cao et al.). On the other hand, some studies show that TET3 is affected by the binding of different domains or subunits of other proteins. In their study, Wang et al. showed that the expression of TET3 was increased by the co-expression with BRCA1, TET3 could bind to the promoter region of EZH2 and negatively regulate EZH2 transcription (Wang et al) Additionally, other studies have shown that silencing of TET3 induced EMT in melanoma samples (Wang et al). This further proves the theory that the role of TET3 is not the same in all tissues or cancer types and that there are indeed other factors taking a role.(Gong et al.)

Overall, our results demonstrate that TET3 is most likely an oncogene. However, and with all the emerging data regarding the TET family of enzymes in general, and TET3 in particular, further analysis is needed to elucidate the molecular mechanism behind TET3 oncogenic function both by itself and in relation to other effectors.

Conclusion:

This study aimed at elucidating the function of TET3 in breast tumorigenesis through overexpression of this enzyme. Our results show that TET3 has an oncogenic behavior that is seen in different cellular contexts. For future studies, we suggest cloning the different isoforms of TET3 in order to have a wider and generalized vision of the function and behavior of TET3 in breast cancer. We will also check the function of this enzyme by knocking out the different isoforms of TET3 and test its effect on different cellular phenotypes.

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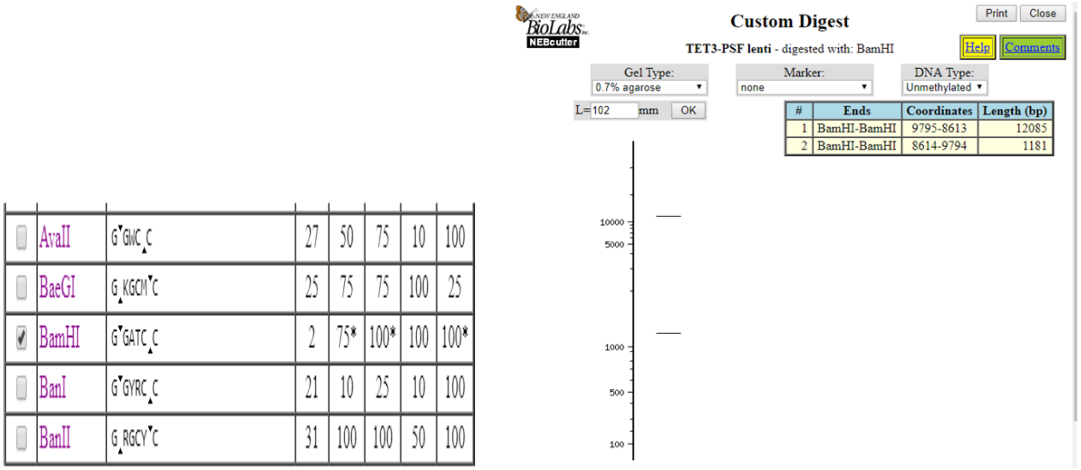
Appendices:

Appendix 1:



☐	AvaII	G^GWC_C	27	50	75	10	100
☐	BaeGI	G_KGCM^C	25	75	75	100	25
☑	BamHI	G^GATC_C	2	75*	100*	100	100*
☐	BanI	G^GYRC_C	21	10	25	10	100
☐	BanII	G_RGCY^C	31	100	100	50	100

Figure 11: Digestion of TET3-PSF-Lenti with BamHI.A. Bioinformatic result from NEB cutter for TET3-PSF-Lenti digested with BamHI enzyme. **B**. Bioinformatic gel picture for TET3-PSF-Lenti digested with BamHI enzyme.

Appendix 2:

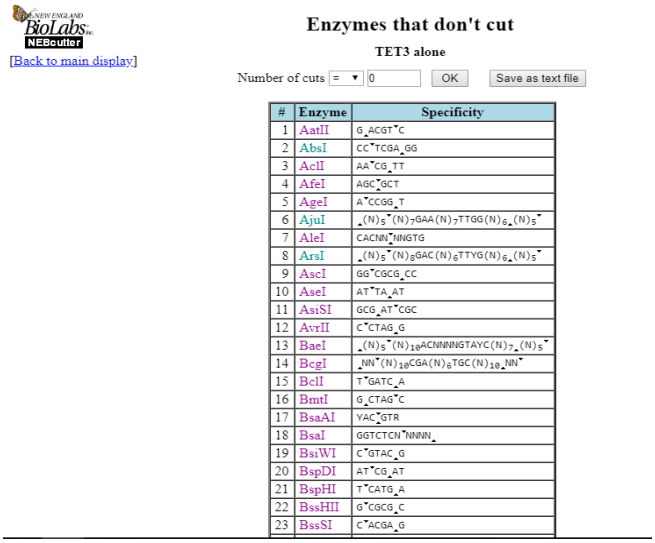
Figure 12: sequencing of ***TET3* cloned in PSF-Lenti viral plasmid.** Sequence alignment of cloned *TET3* against reference sequence using Blast web-available software.

Appendix 3:

Table 4: **list of designed primers for target genes.**

No	Gene	Primers	Reference
1	*AKT1*	F.P_5'- cacaaacgaggggagtacat -3' R.P-5'- tgcgccacagagaagttg-3'	NM_005163.2
2	*Cyclin B1*	F.P_5'- gtcaccaggaactcgaaaat -3' R.P-5'-ttaccaatgtccccaagagc-3'	NM_031966.3
3	*IDH 1*	F.P_5'- ctacatagctatgatttaggc -3' R.P-5'- ctcaaccctcttctcatcagg-3'	NM_001282386.1
4	*Nanog*	F.P_5'- acggagactgtctctcctct-3' R.P-5'- tttgcgacactcttctctgc-3'	NM_024865.3
5	*PCDH7*	F.P_5'-atggaaaatgattcaaggcctc-3' R.P_5'-aggctggctcttcttcctct-3'	NM_001173523.1
6	*SLIT2*	F.P_5'-gctatacaggcttgatctcagtg-3' R.P_5'-ctgaatgccccatcttcaat-3'	NM_004787.4
7	*Snail1*	F.P_5'- acactggcgagaagccctt -3' R.P-5'- gcctggcactggtacttctt -3'	NM_005985.3
8	*Wnt5A*	F.P_5'- atgaagaagtccattggaat -3' R.P-5'- ctgggcgaaggagaaaaata -3'	NM_003392.4
9	*hUBC*	F.P 5'- gtcgcagttcttgtttgtgg-3' R.P 5'-gatggtgtcactgggctcaa-3'	NM_021009.6

Appendix 4:



NEBcutter

[Back to main display]

Enzymes that don't cut

TET3 alone

Number of cuts = 0 OK Save as text file

#	Enzyme	Specificity
1	AatII	G_ACGT^C
2	AbsI	CC^TCGA_GG
3	AclI	AA^CG_TT
4	AfeI	AGC^GCT
5	AgeI	A^CCGG_T
6	AjuI	_(N)5^(N)7GAA(N)7TTGG(N)6_(N)5^
7	AleI	CACNN^NNGTG
8	ArsI	_(N)5^(N)8GAC(N)6TTYG(N)6_(N)5^
9	AscI	GG^CGCG_CC
10	AseI	AT^TA_AT
11	AsiSI	GCG_AT^CGC
12	AvrII	C^CTAG_G
13	BaeI	_(N)5^(N)10ACNNNNGTAYC(N)7_(N)5^
14	BcgI	_NN^(N)10CGA(N)6TGC(N)10_NN^
15	BclI	T^GATC_A
16	BmtI	G_CTAG^C
17	BsaAI	YAC^GTR
18	BsaI	GGTCTCN^NNNN_
19	BsiWI	C^GTAC_G
20	BspDI	AT^CG_AT
21	BspHI	T^CATG_A
22	BssHII	G^CGCG_C
23	BssSI	C^ACGA_G

Figure 13: Bioinformatics and design of FH-TET3-pEF. Bioinformatics result from NEB cutter for HA-*TET3* mRNA coding sequence digested with BspHI/PagI enzyme.

Appendix 5:

Table 5: Digestion volumes and conditions

	FH-TET3-pEF (µL)	PSF- Lenti (µL)	
20 µg template	**36.5**	**30**	**First digestion, incubated at 37 °C for 3 hrs**
KpnI enzyme	**3**	**3**	
Buffer 1.1	**5**	**5**	
Ultra-Pure H_2O	**25.5**	**32**	
XbaI enzyme	**3**	**--**	**Second digestion, incubated at 37 °C for 3 hrs**
Cut smart buffer	**5**	**5**	

PagI enzyme	**3**	**--**	**Third digestion, PagI incubated at 37 °C for 3 hrs**
Buffer O	**5**	**--**	

الملخص

في سرطان الثدي TET3 الدور الوظيفي لإنزيم

تكون السرطان هو عملية متعددة المراحل تنطوي على اعتلال في التعبير الجيني بسبب كل من التغييرات الجينية والفوق جينية. واحدة من التعديلات الجينية الأكثر دراسة هو اضافة مجموعة ميثيل الى سلسلة الحمض النووي. يرتبط اضافة مجموعة الميثيل الى سلسلة الحمض النووي بشكل غير منتظم الى تحفيز الاصابة بأنواع السرطان المختلفة. ويرتبط تطور مثل هذه الأورام مع عدم التوازن في اضافة مجموعة الميثيل الى الحمض النووي وإزالة هذه المجموعة . في السابق ، كان الاعتقاد السائد ان اضافة مجموعة الميثيل حدث خلوي لا رجعة فيه حتى الاكتشاف الأخير لعائلة TET من الإنزيمات التي تشمل TET1 و TET2 و TET3. بينما تمت دراسة أدوار TET1 و TET2 في الأورام السرطانية في الثدي ، لا يُعرف الكثير عن دور TET3 في الأورام السرطانية للثدي.في هذه الدراسة ، حاولنا توضيح سلوك TET3 في تكوين أورام الثدي عن طريق الإفراط في التعبير عن cDNA TET3 في خلايا سرطان الثدي التي تظهر تعبيرًا منخفضًا من TET3. بعد ذلك، اختبرنا تأثير الإفراط في التعبير عن TET3 على أنماط ظاهرية مختلفة من السرطان. أظهرت نتائجنا أن الإفراط في التعبير عن TET3 يؤدي إلى زيادة تكاثر الخلايا وكذلك معدل بقاء الخلايا المصابة مقارنة بالخلايا الطبيعية. بالإضافة إلى ذلك، أظهرت الخلايا المصابة قدرة هجرة أعلى من الخلايا العادية غير المصابة. علاوة على ذلك، قمنا باختبار مستوى التعبير وتوطين إنزيم TET3 في عينات سرطان الثدي باستخدام IHC. اختبرنا أيضًا مستوى التعبير عن TET3 mRNA باستخدام qRT-PCR النسبي في خطوط خلايا سرطان الثدي المختلفة في سياقات مختلفة. وجدنا أن عينات سرطان الثدي الغازية كانت تحتوي على مستويات أعلى من TET3 مقارنة بعينات المرحلة العادية أو

المبكرة. بالإضافة إلى ذلك ، قمنا بعمل qRT-PCR على بعض الجينات المستهدفة التي ترتبط بسمات السرطان المختلفة التي اختبرناها من أجل العثور على أي ارتباط بين النمط الجيني والنمط الظاهري ووجدنا أن الخلايا المفرطة في التعبير TET3 نظمت بعض الجينات الورمية بينما كان طفيفًا أو لم تتم رؤيته التأثير على الجينات الأخرى مثل الجينات الكابتة للورم. أخيرًا، ولكي نكون قادرين على ربط نتائجنا بالحالات السريرية ، استخدمنا مخطط(Kaplan-Meier (KM من أجل تقييم ارتباط TET3 بالبقاء على قيد الحياة. أظهرت النتائج التي تم الحصول عليها أن التعبير العالي لـ TET3 ينبئ بتقدم لدى مرضى سرطان الثدي. في الختام، تظهر نتائجنا لأول مرة أن TET3 يُظهر سلوكًا مُسببًا للورم.