UNIVERSITY OF PISA

BIOS Research Doctorate School in BIOmolecular Science

PROGRAM IN EXPERIMENTAL AND MOLECULAR ONCOLOGY

XXIII CYCLE (2008-2010)

Research Doctorate Thesis

The Pathogenic Role of the Human Mammary Tumor Virus (HMTV) in Human Breast Cancer

Supervisor

Prof. Generoso Bevilacqua

Candidate

Dr. Mohammad Al Hamad

Tutor

Dr. Chiara Maria Mazzanti

Dedication

To my father, mother, brothers and sisters for their love and support.

To my lovely wife for her support, kindness and patience, and to my children Sara and Omar.

Acknowledgements

I would like to express my deep and sincere gratitude to my supervisor Professor Generoso Bevilacqua for the opportunity to do exciting research in the field of Molecular Oncology. His guidance and support were invaluable.

This dissertation would not be finished without the support, encouragement and help of many people who I appreciate.

My tutor Dr. Chiara Maria Mazzanti whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. Dr. katia Zavaglia for her valuable advice and friendly help. Dr. Giovanni Fanelli for his help in selecting the samples. I would like to thank Ms Maria Pia for her help. I want to thank also my colleagues Mazhar, Francesca, Sara, Alesandro, Claudia, and Evana for the scientific discussions that taught me many things during my research.

I am very grateful to Professor Mauro Pistello for collaboration and the fruitful scientific discussion that significantly influences my thesis.

I would like to express my love and gratitude to my beloved families; for their understanding & endless love, through the duration of my studies.

Mohammad Al zoubi

Table of Contents

ABSTRACT **9**

INTRODUCTION: **11**

RISK FACTORS OF BREAST CANCER 11

Age *11*

Reproductive factors *11*

Hormonal status *12*

Genetics *13*

Virus etiology of mammary tumor *13*

Methods used in the past for viral detection *14*

MOUSE MAMMARY TUMOR VIRUS 19

History: *19*

TAXONOMY AND CLASSIFICATION: 19

PROPERTIES OF THE VIRION: 20

VIRAL GENOME 21

Super antigen *22*

MMTV life cycle *23*

Recombination of exogenous and endogenous MMTV *25*

MMTV and Tumorigenesis *26*

Replication cycle of MMTV *28*

HUMAN ENDOGENOUS RETROVIRUS: 32

Classification *33*

The biological function of HERV *34*
HERV-K family *35*

MATERIALS AND METHODS **36**

Specimens *36*
Histological criteria *38*
LASER MICRODISSECTION 41
DNA extraction from microdissected tissue *42*
DNA EXTRACTION FROM PARAFFIN EMBEDDED TISSUE 43
RNA EXTRACTION FROM CELL CULTURE 44
cDNA synthesis: *45*
DNA AMPLIFICATION SUITABILITY 46
HMTV SEQUENCE DETECTION 47
SEQUENCING OF HMTV *ENV* PCR FRAGMENTS 49
HMTV Real Time PCR assay *50*
Amplification of the probe (2.7 kb env-LTR) *52*
Labeling the probe *53*
CHROMOGEN IN SITU HYBRIDIZATION (CISH) 54
TISSUE CULTURE 56
Collecting medium *56*
Primary culture medium: *56*
Complete culture medium *56*
Antibiotic solution (ABC-PBSA) *56*
Day 1 protocol *57*
Day 2 protocol *58*
Subculture *58*

Removal of fibroblastic cells from the tissue culture *59*
Cryopreservation the cells *60*
STATISTICAL ANALYSIS 61
RESULTS 62
HMTV frequency in phases of breast cancer progression *62*
HMTV viral load with RT-PCR *66*
CHROMOGEN IN SITU HYBRIDIZATION (CISH) 69
The frequency of HMTV in metastatic lymph nodes *72*
The frequency of HMTV in primary and metastatic breast cancer *73*
The frequency of HMTV in primary breast cancers and their lymph nodes *75*
TISSUE CULTURE 78
Primary culture of human breast cancer *78*
Presence of HMTV in primary culture of human breast cancer *80*
DISCUSSION 82
REFERENCES: 89

Table of figures and tables

Figure 1 Structure of proviral sequences in human breast cancer. . 17
Figure *2* Electron microscopy of MMTV. 18
Figure 3: The structure of MMTV 21
Figure 4: MMTV life cycle 25
Figure 5: Histological types, and the number of breast cases 37
Figure 6: The cases number of breast cancer and their lymph nodes 38
Figure 7: Breast cancer progression 40
Figure 8: Laser capture microdissector 41
Figure 9: Laser capture microdissection of breast cancer 42
Figure 10: Fluorescent capillary electrophoresis 49
Figure 11: Rotor gene real time PCR 52
Figure 12: CISH protocol 55
Figure 13*:* The frequency of HMTV env sequence in normal breast cells and all phases of breast cancer progression. 63
Figure 14: Gel electrophoresis of HMTV env sequence 66
Figure 15: RT-PCR of HMTV env sequence of 2 cases harbor normal cells, DCIS and IDC 68
Figure 16: RT-PCR of 7 cases harbor DCIS and IDC 69
Figure 17: CISH analysis of HMTV env 71
Figure 18: The frequency of HMTV in primary breast cancers and their metastatic lymph nodes. 74

Figure 19: The frequency of HMTV in primary breast cancers and their metastatic lymph nodes 76
Figure 20: The frequency of HMTV in primary breast cancers and their non metastatic lymph nodes. 77
Figure 21: Primary culture of human breast cancer.....79
Figure 22 : Quantification of viral RNA in cell lysates of human breast cells in the presence and absence of dexamethasone 81

Table 1: Primer sequences..... 47
Table 2: Number of cases tested with different histological types and number of HMTV env positive cases found in all phases of breast cancer progression. …….…….64
Table 3: The presence of HMTV in different breast cancer progression cases……...……. 73

ABSTRACT

A viral etiology of human breast cancer has been postulated for decades after the identification of MMTV (Murine Mammary Tumor Virus). The detection of HMTV (Human Mammary Tumor Virus) *env* exogenous sequences in 30-40% of invasive breast carcinoma increased the interest towards this hypothesis. To look for HMTV *env* exogenous sequences during cancer progression could contribute to a better understanding of their role in breast cancer. This work analyzes the presence of HMTV *env* exogenous sequences in the first phases of carcinogenesis, i.e. the pre-invasive, as well as in metastatic lesions.

Formalin fixed and paraffin embedded samples were utilized: 20 Usual type Ductal Hyperplasia (UDH), 22 Atypical Ductal Hyperplasia (AH), 49 Ductal Carcinoma In Situ (DCIS), 20 Infiltrative Ductal Carcinoma (IDC), 26 Normal Epithelial Cells (NEC) collateral to DCIS or IDC, i.e. present in the same histological section, 22 primary breast cancers and their respective non metastatic lymph nodes, 23 primary breast cancers and their metastatic lymph nodes.

As a negative control we used reductive mammoplasties, thyroid and colon carcinoma, and blood of healthy donors. All samples were laser microdissected. Fluorescent nested - PCR was used to detect the presence of HMTV *env* exogenous sequences. Generated fluorescent amplicons were sized on an automatic DNA sequencer. DNA extracted from tissues of 9 patients was analyzed by quantitative RT-PCR. Moreover, we created primary cell line of human breast cancer that was positive for HMTV *env* exogenous sequences, and then we treated the cells with 10^6 M Dexamethasone. HMTV *env* exogenous sequences were found in 19% of NEC collateral to DCIS or IDC, 27% of AH, 80% of DCIS, 35% of IDC, primary breast cancer cases that do not develop metastasis 50%, their respective lymphocytes 36%, primary breast cancers that develop metastasis 69.5%, and their metastatic cancer in lymph nodes 68.4%. Controls results were negative. RT-PCR and CISH confirmed these results. The expression of HMTV in primary breast cancer cell line was started to increase after 16 days of treatment with Dexamethasone. These data could contribute to understanding the meaning of the presence of HMTV in breast carcinogenesis.

INTRODUCTION:

Risk factors of breast cancer

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women[1]. Despite the fact that it represents the most frequent cancer in women and that it is largely studied all over the world since many decades, the etiology of breast carcinoma is largely unknown. However, the strongest risk factors for breast cancer include age, familial and reproductive factors, lifestyle and hormonal factors have also been linked to breast cancer risk, as well as the virus but the data is inconsistent and inconclusive.

Age

Aging is considered as one of the greatest risk factors for the development of new breast cancer. Breast cancer incidence is strongly associated with an increase of age, with estimated 64% of women over the age of 55 at the time of breast cancer diagnosis[2].

Reproductive factors

Breast cancer risk increases in women who experience late age menopause, increasing age at first pregnancy, and low parity[3].

Menopause: Although the risk of breast cancer incidence increases with age, postmenopausal women have a lower risk of developing breast cancer than do premenopausal women of the same age. Each 1 year delay in the onset of menopause is associated with 3% increase risk of breast cancer[4].

Age at menarche: later age at menarche (age at 15 or more) is associated with reduction in risk of breast cancer compared to early age (at 12 or less). 1 year delay in the onset of menarche is associated with 5% reduction in the risk of developing breast cancer in later life [5].

Parity: increasing parity is associated with decreased risk for breast cancer compared with nulliparity. Each birth reduces the relative risk of breast cancer by an average of 7%. This reduction in the risk per birth is greater in young women (before age 20 years) compared to older ages[6].

Hormonal status

Breast cancer risk has been extensively reported in women with exposure to exogenous sex steroids such as oral contraceptives (OC) and postmenopausal hormone replacement therapy (HRT).

The hormonal effect of OC on breast cancer is complex. They often cause protective anovulatory cycles, but the mixture of progesterone and estrogen may also stimulate the mitotic activity on the breast[7]. One case control study found a relative risk of 1.4 in women who took oral contraceptives for long than 12 years compared with non users. These results are consistent with the earlier meta analysis results, which had shown no difference in risk after 10 or more years of discontinuing of oral –contraceptive use [8].

Hormone replacement therapy increase the risk of breast cancer in current users with increasing substantial differences between the effect of estrogen only and estrogen /progesterone preparations[9].

Genetics

Women with a family history of breast cancer are at high risk of developing the disease that estimated to be 5% of breast cancer cases. The number of breast cancer genes is not yet known. However two autosomal dominant genes, BRCA1, BRCA2, which are located on chromosome 17and 13 respectively, have been account for most of the cases of familial breast cancer. The lifetime risk of developing breast cancer for BRCA1 and BRCA2 mutation carriers is 80-85%[10]. According to a combined analysis of 22 studies, the average cumulative risk in BRCA1- mutation carried by age 70 was 65%, and 45% in BRCA2-mutation carriers [11].

Virus etiology of mammary tumor

The etiological role of the Murine Mammary Tumor Virus (MMTV) in the development of tumors of the mammary gland in mice is demonstrated since a long time [12]. It is interesting to note that much of what is known about the pathogenesis of human breast carcinoma was learned by the experimental model of the MMTV-induced mouse mammary tumors [13, 14].

In particular, the concept of cancer progression and the recognition of the so called preinvasive lesions as morphological steps of its development are based on this murine model[15]. Moreover, the promotional role of estrogens was built on the observations conducted in mice [16, 17].

These strong similarities between the human and the murine disease represented the reason for the quest of a possible viral etiology of breast carcinoma in women since more than half century: MMTV viral antigens were found in human breast tumors [18] ,MMTV particles were described in human cells and milk [19, 20], MMTV sequences were found in humans [21].Unfortunately, these data were never considered conclusive mainly because of their scarce reproducibility. Moreover, the less sensitive and specific techniques that used at that time are considered the main reason of the variability of results.

Methods used in the past for viral detection

In the '70s, most of the experiments were based on immunohistochemical techniques. The envelope protein of MMTV (MMTV gp-52) was studied in human breast tumor, and in human breast cancer cell line T47D [18], but there was conflicting results, that may be due to low sensitive technique used.

The electron microscopy results were also conflicting. Some laboratories report the presence of morphologically related retrovirus particles, called RVLPs (Retrovirus-Like Particles) in samples of human milk [22],and in macrophages of breast cancers tissues as well as in human breast cancer cell line T47D, after stimulation with estradiol, followed by stimulation with progesterone [23]. Other researchers considered those particles as cellular debris and have no correlation with the onset of breast cancer[24].

A subsequent wave of interest came in the early '80s, when some laboratories reported the presence of reverse transcriptase in samples of breast cancer or in the serum of patients. An essential characteristic of retroviruses is to encode a DNA-dependent RNA polymerase (called reverse transcriptase) that retrotrascripe the viral RNA into a double helix of DNA. The enzyme reverse transcriptase is therefore considered a marker of retroviruses and can be used as an indicator of their presence[25]. Again, there are conflicting results obtained [20, 26], the variability in the results obtained by different working groups due to the low sensitivity of the techniques used.

The same authors conclude that if the human variant of MMTV exists, it must be present in amount so low be detectable with the techniques used. In addition, the methods discussed do not allow a clear discrimination between the endogenous retroviral sequences and the exogenous virus. In subsequent years it has been shown that certain endogenous sequences (type HERV-K) are capable of producing complete viral particles, related to MMTV[27, 28].

The molecular biology techniques significantly contributed in detecting the MMTV sequences in human breast cancer.

Sequences homologous to MMTV were first shown in human DNA by using hybridization experiments [21, 29], but there a doubt that these sequences due to presence of endogenous retrovirus.

In 1995, Wang et al [30] selected a region of 660 bp of the MMTV *envelope* gene (MMTV *env*), with a homology of only 16% to

HERVK10, the prototype human endogenous retrovirus and highly similar to MMTV. Later other authors, by using MMTV-specific primers located in the 660 bp region, identified a MMTV *envelope* gene-like sequence (MMTV *env* like) in 38% of a series of human infiltrating breast carcinoma, these sequences were 90–98% homologous to the MMTV *env*,. On the other hand, MMTV *env* like was found in only 2% of normal human breast samples.

Several other groups were able to confirm these data, [31-33], whereas negative results were published too [34, 35].This discrepancy could be consequence of differences in technical procedures, of tissue heterogeneity, and of the fact that *env* sequences are present in few copies.

More in favor of the exogenous origin of these env-like sequences relate to the amplification of whole proviral structure from human breast carcinomas **(**figure: 1**)** as shown in the total homology from the carcinomas was 95% to MMTV in 9.9 Kb and 57%to HERV-K10 in 3.5Kb. Homology to the endogenous retrovirus was seen primarily in the pol gene, which is known to be conserved among different retroviruses and partially in the gag gene[36].

In 2006 Zammarchi et al [37]from this laboratory designed a rigorous methodological approach able to overcome these difficulties, based on the association of a laser microdissection procedure and a highly sensitive fluorescent nested PCR. The MMTV *env*-like sequence was detected in 33% of a series of human breast carcinoma, whereas normal breast tissues and other types of human tumors resulted negative.

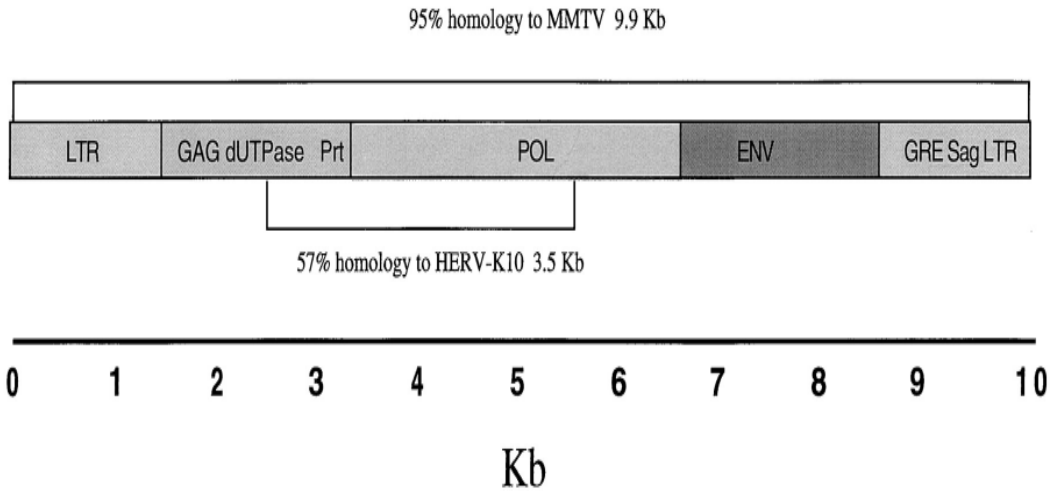

Figure 1 Structure of proviral sequences in human breast cancer showing genes, ORFs, and sequence homology to MMTV and HERV-K10.

Subsequently, in 2007, Pogo et al. isolated the HMTV particles from ascitic and pleural fluid of patient with metastatic breast cancer whose primary tumor where positive for env sequence and expressing env protein. They demonstrated the presence of retroviral particles electron microscopy technique. The virus particles in culture medium showed retroviruses of type B, with size between 100 and 110 nm, a spherical shape, an eccentric dense core and the presence of mature or immature capsid [38] (Figure: 2).

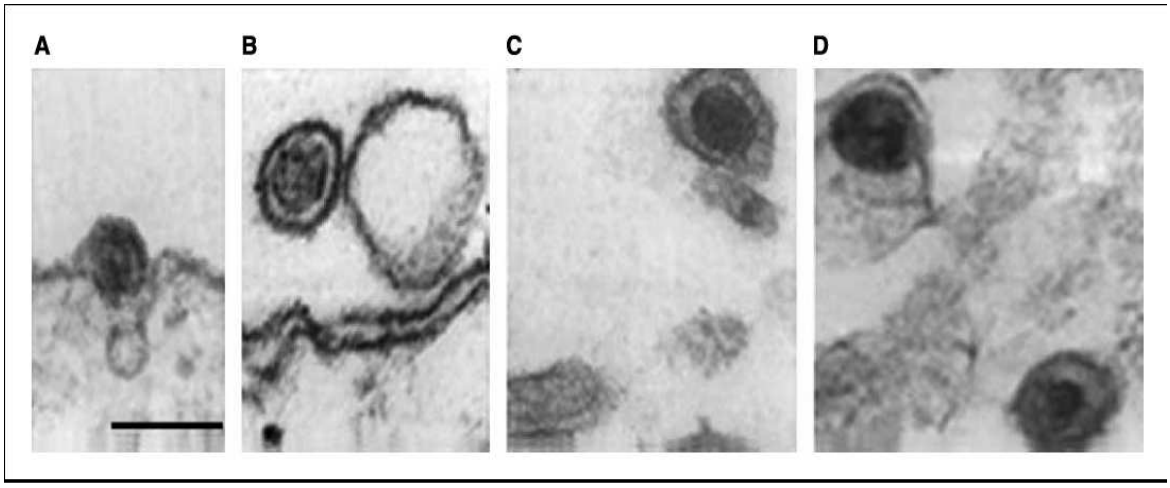

Figure *2* Electron microscopy of MSSM3 cell associates and PF viral particles. A and B, selected examples of cell-associated viral particles. C and D, selected examples of viral particles in PFs. Note that the nucleoids are condensed to varying degrees. Original magnification,130,000 (bar, 0.2 Am).

Today the idea that a virus could be involved in the etiology of human breast carcinoma receives much more attention that in the past. Several papers go towards this direction, even if in general they restrict their research to demonstrate viral particles and proteins in tumor cells [38, 39].

MMTV, if really involved in breast human carcinogenesis, could act as a promoting agent or as an etiological/pathogenetical cofactor linked to some steps of cancer progression, such as infiltration or metastasis. To individuate where during cancer progression MMTV exogenous sequences appear could be useful to unveil their role in human breast cancer.

Mouse mammary tumor virus

History:

Mouse mammary tumor virus (MMTV) was first reported in 1933 by the Jackson Memorial Laboratory and by Korteweg in 1934 as an extrachromosomal influence on the incidence of breast cancer in inbred mouse strains[40]. Subsequently, in 1936 Bittner showed that a cancerous agent, which he called “milk factor”, could be transmitted by cancerous mother young mice while nursing [41]. In 1966 it was proven that Bittner’s milk factor was virus that remained dormant during the early stage of life in mouse but produce cancer in the middle age when the sexual hormones were in right conditions[42].

Taxonomy and Classification:

MMTV is a prototype species of the genus B retaretrovirus in the family Retroviridae. These viruses previously were referred to as type B retroviruses based on their appearance by electron microscopy (a characteristic acentric core within particles of c. 100 nm).

Multiple double-stranded DNA copies are found in the chromosomal DNA of most commonly used laboratory strains of mice (called integrated or endogenous proviruses)[43].

These endogenous proviruses presumably represent viral insertions into chromosomal DNA of germline cells and are referred to as Mtv followed by an Arabic number, for example, Mtv8.

Most endogenous Mtvs have defects in one or more genes and, therefore these proviruses often fail to produce infectious virus [44] . Currently, MMTV is classified with other betaretroviruses including Mason–Pfizer monkey virus (MPMV), Jaagsiekte sheep retrovirus (JSRV), and human endogenous retrovirus type-K (HERV-K) [45].

Properties of the Virion:

The mature MMTV virion is100nm in diameter, contain a single-stranded positive-sense RNA, which exists as a dimer, and is encapsidated as a helical ribonucleoprotein (RNP) by the nucleocapsid (NC) protein; reverse transcriptase (RT) and integrase (IN) are closely associated with the RNP.

The RNP is surrounded by an icosahedral shell composed of capsid (CA) protein.

MMTV capsids are bound via the matrix (**MA**) protein to the **viral envelope**, a portion of the cellular plasma membrane that has been modified by the insertion of the surface (**SU**) and transmembrane (**TM**) proteins [46] (figure: 3).

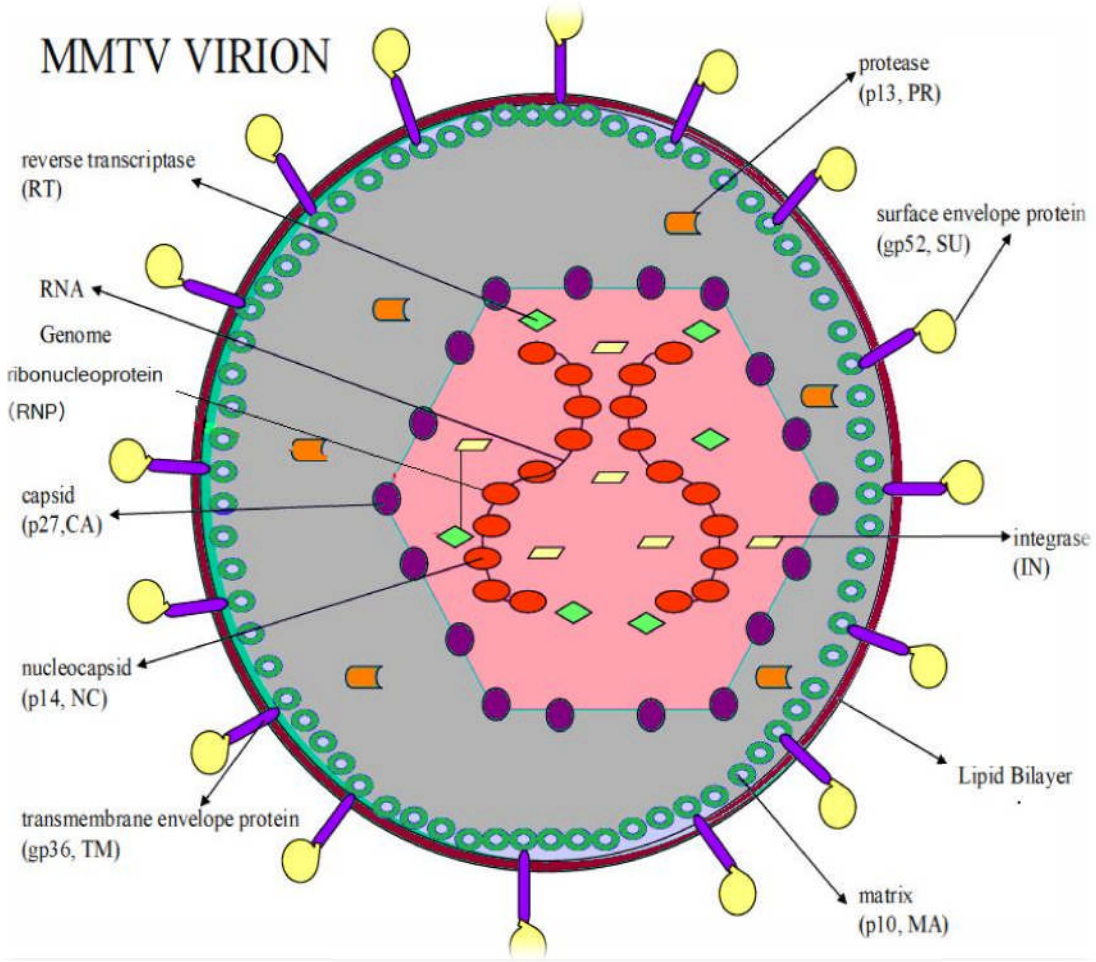

Figure 3: The structure of MMTV

Viral genome

The viral RNA is bound at either end by a short direct repeat (R) of 15 bp. The R regions are adjacent to a regions of approximately 120 and 1200 bp, respectively, present at the 5 (U5) or 3 (U3). U5: A

unique, non-coding region which is the first part of genome to be reversed transcribed, forming 3' of the provirus genome.

U3: A unique, non-coding region which forms the 5' of the provirus after reverse transcription contains the promoter elements responsible for transcription of the provirus.

A cellular tRNA (tRNA3Lys) is bound through 18 bp of complementarity to each copy of the viral RNA at the primer-binding site (PBS) located just downstream of U5.

The first splicing donor (SD) site precedes the group-specific antigen (gag) region that encodes a Gag precursor. The virus also encodes two other precursor polypeptides, gag-pro, gag-pro-pol from genomic RNA. Gag -pro encodes the gag proteins, a dUTPase (DU), and the viral protease (PR). Whereas the gag-pro-pol protein encodes RT, including a ribonuclease H (RNase H) activity and IN. and finally the env region that encodes the envelope protein which compose of surface (SU) and transmembrane protein (TM).

Super antigen

The long terminal repeat (LTR) of MMTV harbor an Open Reading Frame (ORF) that encodes a superantigen (Sag), essential for its life cycle [47]. The superantigens are different from normal antigens for their ability to stimulate a greater amount of T cells, this property derives from their ability to bind to all T cells expressing a particular Vβ chain of T-cell receptor (TCR), and not only to the groove formed by the α and β chains, as do antigens conventionally. In this way, all T cells that express the chain

Vβ, have the ability to recognize and be stimulated by superantigens, which are presented as exogenous antigens [48]. Another characteristic of superantigens is to be present only in the context of major histocompatibility complex molecules (MHC) class II [49]. MMTV require functional MMTV sag to establish infection of the mammary gland of mice. Laboratory experiments have shown that exogenous virus with impaired function of Sag, are not infectious [50]. MMTV also needs a functional immune system (B cells, T cells) to complete its infectious cycle[51].

MMTV life cycle

The virus can be transmitted vertically (endogenous virus) when embryonic germline cells are infected [52]but it is usually transmitted horizontally through maternal milk (called exogenous virus or milk-borne virus)[12]. MMTV particles ingested by newborn mice in maternal milk that cross the epithelium cells through M cells in small intestine. The virion initially encounters and infects dendritic cells and B cells located in Peyer's patches of gastrointestinal tract[53]. Following virus expression, superantigen (sag) protein is produced and presented on B cells by MHC II. The Sag binds both MHC II on B cells and the Vβ of the T cell receptor making it a potent T cell stimulus [54]. The stimulated T cell through sag release cytokines, which cause proliferation of many B cells that subsequently, also become infected with MMTV [55].

These infected lymphoid cells become a reservoir of MMTV that will preserve MMTV infectivity prior to the onset of puberty in mice

when a source of susceptible and dividing mammary cells becomes available [50].

MMTV infection increases during lactation, where high virion production ensures that large amounts of viral particles will increase the probability of newborn offspring infection [56].

The chronic infection of mammary cells induces the formation of malignant tumors, mammary adenocarcinomas. Infected T-cells rarely become tumorigenic; though, when T-lymphocytes are transformed, T-cell lymphomas do occur [57] (figure: 4).

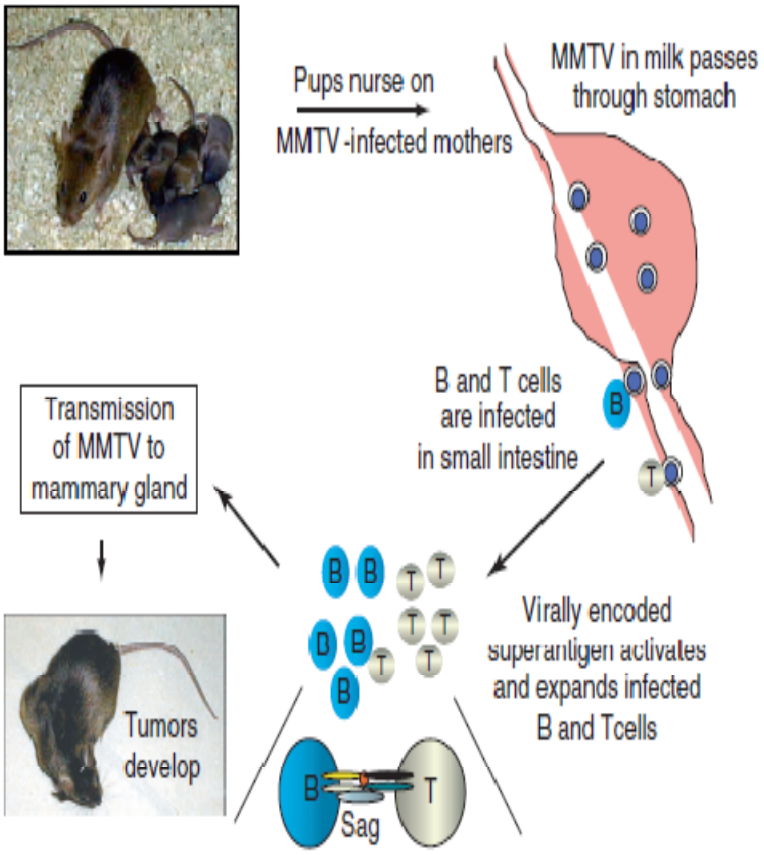

Figure 4: MMTV life cycle

Recombination of exogenous and endogenous MMTV

The coexpression of exogenous and endogenous RNA molecules in vivo results in viral recombination. Recombination between exogenous and endogenous genomes occurs during cDNA synthesis and is responsible for the generation of variants virus with new biological characteristics. This recombination occurs because the

RNA-dependent DNA polymerase, reverse transcriptase, can switch templates during replication[58].

When an exogenous MMTV infected cells where the endogenous Mtv are highly expressed, there is a 'high possibility of recombination between two viral forms. This event generates new viruses that have the ability to infect different strains of mice than the parental forms.

Exogenous MMTV infects epithelial cells of mammary gland, endogenous Mtv which is highly expressed in this tissue may be co-packaged and result is a recombinant virus. If the hypervariable region of the exogenous MMTV is different from that of the endogenous Mtv, the recombinant viral particle is capable infecting murine strain different than the original particles of exogenous MMTV [59].

MMTV and Tumorigenesis

MMTV does not encode any oncogene, and mammary tumorigenesis is therefore takes place after proviral insertion near specific cellular proto-oncogene, activating them in a process called insertional mutagenesis [60]. Retroviral integration is not sequence-specific, therefore the greater the amount of virus, the greater probability that the proviral DNA integrates near a proto-oncogene. In fact there has been a considerable correlation between viral load in breast milk and the incidence of breast cancer in a given mouse strain[61]. Once a provirus become integrated into the host genome, expression of proviral DNA is regulated by specific sequences within the

LTR that cause increased viral transcription in response to glucocorticoid receptor/steroid hormone complexes. The most important steroid hormone that increased the transcription of MMTV during pregnancy is the progesterone [62]. Analysis of mouse mammary tumors induced by MMTV has shown an alteration in six genes [63]. These genes belong to different family members of fibroblast growth factor (FGF) and members of the family Wnt gene. They are: Int-1/Wnt-1, Int-2/Fgf-3, Wnt-3, Hst/k-Fgf/Fgf-4, Wnt-10b and FGF-8.

An interesting feature common to these genes is that they are all involved in the short-range cellular communication, many of them, in fact, encode secreted proteins[64]. The activation of the expression of these genes, caused by MMTV is mainly due to the presence of enhancer sequences in the 5 'LTR (Long Terminal Repeat) of the genome of MMTV. These regions act on promoters of adjacent genes, located up to 20 kb from the LTR. The MMTV provirus integrates generally outside the region coding of the proto-oncogene and only rarely within it[65].

The Wnt genes, members of a family consist of structurally related genes which encode secreted signaling proteins. These proteins have been implicated in oncogenesis and in several developmental processes, including morphology development of the tissue during embryonic and adulthood stage[66].

The other common target for the integration of MMTV is the gene family of fibroblast growth factor (FGF). The proteins encoded by these genes are potent angiogenic factors in vivo, and

ivolve in cell differentiation [67]. Three of the eight members of the FGF family, namely, Int-2 (FGF-3), FGF-4 and FGF-8, are activated by the insertion of proviral MMTV. As in the case of genes Wnt-1 and Wnt-3, the three FGF genes are not normally expressed in the mammary gland, but are found only or in embryonic life or in adult tissues other than breast [68, 69]. It has been shown that the onset of murine breast cancer is associated with activation of targeted genes (int-2 (Fgf-3*),* hst (Fgf-4)*, and* Fgf-8) by MMTV proviral insertion. These observations suggest therefore that different genes cooperate in tumorigenesis [70].

Replication cycle of MMTV

MMTV replication cycle begin when the viral surface glycoprotein ,gp 52 bind to tranferrin receptor (tfr1) that expressed in many rodent cells. Human tfr1 is failed to allow viral entry, however use of MMTV bound to the trf 1 enters the cell via endocytosis of clathrin-coated pits, and receptors are recycled to the cell surface where they allow entry of additional MMTV particles into previously infected cells.

Following entry and partial uncoating in the cytoplasm, the virally encoded RT is activated. PBS, the primer binding sequence of viral RNA is complementary to the 3' nucleotides of the host tRNA. The host tRNA, lys-3 acts as the DNA primer for the reverse transcriptase to synthesize minus-strand proviral DNA.

The initial product of reverse transcription is an RNA-DNA complex. The RNase H digests the RNA strand, and allows the synthe-

sis of double-stranded provirus[71]. However, the product of reverse transcription is different from the starting template so that the U5 and U3 sequences present uniquely in viral RNA are duplicated to give longer repeats at each end of the provirus (LTRs).

Because nuclear entry of the preintegration complex containing the provirus is thought to require nuclear envelope breakdown during mitosis, it is generally believed that MMTV must infect dividing cells. Once the preintegration complex enters the nucleus the viral integrase randomly inserts the provirus into the host genome[72]. Integrase introduces an asymmetric cut 2 bp (cytosine, and adenine) from the linear ends of the provirus as well as an asymmetric break exactly 6 bp apart on opposite DNA strands of host DNA. Proviral integrations are not site-specific and may occur at transcriptionally active sites. Following the joining reaction, the repair of virus–cell junctions by cellular enzymes generates a 6 bp direct repeat of cell DNA that flanks the viral LTRs[73].

The integrated MMTV LTR is a stretch of DNA (1195 base pairs in length) at the 5’ end of the viral genome that is responsible for the expression of the virus in mammary epithelial cells and for the activation of cellular proto oncogenes.

Transcription is initiated from the standard promoter in the 5' LTR starting at the U3/R junction and terminating at the R/U5 junction. Transcription of the MMTV LTR produces full- length mRNA, spliced mRNAs, as well The spliced mRNAs are the *env* mRNAs and the *sag* mRNAs [74, 75] .MMTV also produces a doubly spliced mRNA that encodes the RNA export protein, Rem[76]. In

most cell types, the levels of gag-pol and env mRNAs greatly exceed sag and rem mRNAs.

The viral mRNAs are translated into the viral proteins in the cytoplasm using the host cellular machinery. The *gag* mRNA is translated into the precursor protein Pr77, a Gag polyprotein. Proteolytic processing of Pr 77 by the viral protease produces the structural proteins: the matrix protein p10 or MA; the capsid protein p27 or CA; the nucleocapsid protein p14 or NC, and some smaller proteins p21, p3, and p8 that have no known function [77, 78]. Though, p21 is cleaved into a few smaller proteins[79].

Capsid protein forms the structural shell of the core of the virion, which enclose the ribonucleoprotein comple that contains the genomic RNA. The capsid protein appears to be important in the assembly of immature capsid [80].

The nucleocapsid protein has a sequence that includes many basic amino acids and a cysteine-histidine box that is similar to a zinc finger domain seen in DNA-binding proteins, which explain its packaging role of the viral RNA in the cytoplasm[81].

The *gag-pro* mRNA is translated as a precursor protein Pr 110, a Gag-Pro polyprotein. Within Pr 110, the NC sequence of the *gag* gene and the first part of the *pro* gene comprise a *dut* gene, which encodes DU a dUTPase. DU prevents misincorporation of uracil and mutation of newly synthesized proviruses in nondividing cells[82].

The C terminus end of the *pro* gene codes for p13 or PR the viral protease. PR is responsible for the cleavage events that produce the

mature virion proteins, MA, p21, p3, p8, CA, and NC, the functions of p21, p3, and p8 are currently unknown[83]. The precursor protein Pr160, a Gag-Pro-Pol polyprotein encodes a viral reverse transcriptase with RNase H activity and an integrase[84].

The env mRNA is translated into a precursor protein, pr73 env on membrane bounded polyribosomes. This precursor is modified by glycosylation in both the endoplasmic reticulum and the Golgi, where a cellular protease cleaves the Env poly protein to produce gp52, SU a viral surface envelope protein and gp36, TM a viral transmembrane envelope protein. SU and TM remain attached to each other via disulfide bonds[85]. They travel to the host plasma membrane in vesicles and are then incorporated into the lipid envelope of the mature B type virion when the immature A particle buds from the host cell[86].

The sag mRNA is translated into a precursor protein Pr 48, SAg, a superantigen protein. This protein is glycosylated and cleaved in the Golgi by protease to generate SAg protein[87]. Sag is associated with major histocompatibility complex (MHC) class II protein at the surface of antigen-presenting cell. Sag is essential activation of certain T cell subsets containing Vβ TcR [88].

MMTV utilizes a polyprotein strategy for viral assembly. This process insures that none of the individual proteins: matrix, capsid, and nucleocapsid, are individually targeted to the membrane. The order of the proteins in the polyproteins corresponds to their relative location in the virion from the outside of the virus to the inside of the virus.

MMTV polyproteins assemble into a capsid shell or immature A particle in the cytoplasm. The formation of the A particle occurs prior to its transport to the plasma membrane[86],then the immature A particles bud from the tips of long filamentous actin projections as mature virions [89]. The viral envelope proteins are transported to the plasma membrane via the host secretory pathway. The A particles associate with the cell membrane, they acquire a viral lipid envelope with viral envelope proteins as the virus buds from the host cell. [90].

The budding process of MMTV seems to involve the host actin cytoskeleton. The immature viral A particles have been observed at the tips of long filamentous projections [86].In addition, cytochalasin D, which disrupts the actin cytoskeleton, reduced the number of mature viral B particles released into the supernatant by 80% [91]Thus budding of MMTV appears to be an actin-dependent process. Infected human breast cells with MMTV initiated the formation of filamentous projections with virus particles at their tips. These filopodial projections emerged from the cell surface of infected live cells when viral production was hormonally induced with dexamethasone[92].

Human endogenous retrovirus:

Human endogenous retroviral (HERV) elements comprise 8% of human DNA [93], and are likely to be derived from ancient viral infections during evolution but their biological relevance is largely unknown.

It is thought that exogenous infection allowing HERVs sequences to be inserted into the genome of germ line cells, where they have been replicated along with the host's cellular genes following a Mendelian pattern [94] and[95]. Integration of endogenous retroviruses into the human germ line is thought to have occurred 2 to about 70 million years ago depending on the individual retroviruses and were introduced by mechanisms involving reverse transcription. Most of human endogenous retroviruses (HERVs) are defective, due to multiple mutations or deletions, and are therefore none of them is capable of encoding complete viral particles and cause infection [96]. Although many HERVs are transcriptional active, they are not able to produce functional proteins. The high number of copies present in the human genome is attributed to repeated cycles of infection and retrotransposition [97].

Classification

The known HERVs families have been grouped into classes. HERV class I shows clustering phylgenetically with Gammaretrovirus, those clustering with the betaretrovirus are class II, and class III HERV clustering with spumaviruses [98].

HERV families that reside in human genome are estimated to be between 30 and 50[99], among them various so-called HERV-K families. The letter K indicates that a primer binding site specific for lysine-tRNA was used to prime reverse transcription. In total, 10 HERV-K families have been defined based on sequence similarities, and they have been named HERV-K(HML-1) to HERV-

K(HML-10) (for human MMTV [mouse mammary tumor virus]-like) because of some sequence relationship to the mouse mammary tumor virus [100].

The biological function of HERV

The distribution of endogenous retroviral sequences, scattered throughout the genome of mammals, suggests that they have important biological functions and therefore have been conserved during evolution. Using reverse transcription and subsequent insertion of their genome into the host DNA, these sequences regulate the plasticity of genome, accelerating the evolution of new genes and altering the transcription of existing genes [101].

Their biological function is still unknown and there are many assumptions that, to date, are proposed. In analogy with animal model, it has been hypothesized that these endogenous retroviral sequences are protected in respect of their endogenous counterparts. In the case of MMTV, for example, the expression of endogenous Sag coding sequences during the formation of the immune system, induces clonal deletion of all T cells reactive to that particular Sag and at the same time, makes it immune to the forms of MMTV exogenous coding Sag with the same specificity to T cells [50, 102].

There are several experimental evidences that show the possibility of recombination between exogenous viruses and endogenous retroviral sequences. It has been identified a variant of MMTV, highly tumorigenic, resulting from recombination between sequences

derived from endogenous Mtv, and a strain of exogenous MMTV [59].

HERV-K family

Unlike the majority of HERV, the family of HERV-K has genes an open reading frame (ORF) for essential genes of retroviruses (gag, pol and env) as well as its ability to synthesize all essential retroviral proteins [103, 104]. This family was originally identified for its high homology with the Mouse Mammary Tumor Virus (hence the nomenclature further HML, human endogenous MMTV-like). The HERV-K are present in the human genome with about 30-50 proviral copies and approximately 10,000 LTRS solitary, arising, probably by homologous recombination between LTRS, followed by excision of the viral genome intermediate[102].

HERV-K families have been classified into six subgroups (HML by HML-1 to-6), based on sequence homology in the conserved region of the pol gene, encoding the enzyme reverse transcriptase [105, 106]. HERV-K10 has been sequenced and showed to have a complete provirus of 9 Kb, and contains ORFs for all retroviral genes, but showed to have a stop codon in the env gene and a frameshift in the gene gag[107].

MATERIALS AND METHODS

Specimens

All tissues, formalin fixed and paraffin embedded, were collected and archived (2005-2009) at the Division of Surgical, Molecular and Ultrastructural Pathology, University of Pisa.

We have collected 91 paraffin embedded tissue cases of ductal carcinoma insitu (DCIS) and invasive ductal carcinoma (IDC), as well as usual ductal hyperplasia (UDH) Atypical ductal hyperplasia (ADH). 49 of samples were DCIS, 20 samples of them have also IDC. It was possible to collect 26 samples of normal epithelial cells (NEC) collateral to DCIS or IDC (*c*-NEC) (figure: 5).

We have collected 22 samples of primary breast cancer and their respective non metastatic lymph nodes in order to study the HMTV status in the primary tumor and their respective non metastatic lymph nodes. Moreover, we have collected also 23 samples of primary breast cancer and their respective metastatic lymph nodes to compare the HMTV status in non metastatic compared to metastatic lymph nodes (figure: 6). As negative control were used: 20 NEC from reduction mammoplasty (*nc*-NEC); 6 papillary thyroid carcinoma; 6 colon adenocarcinoma, and DNA extracted from 6 healthy blood donors. DNA of a mouse infected by MMTV was the positive control. Of the DCIS, 15 were poorly differentiated, 33 moderately differentiated and 1 well differentiated.

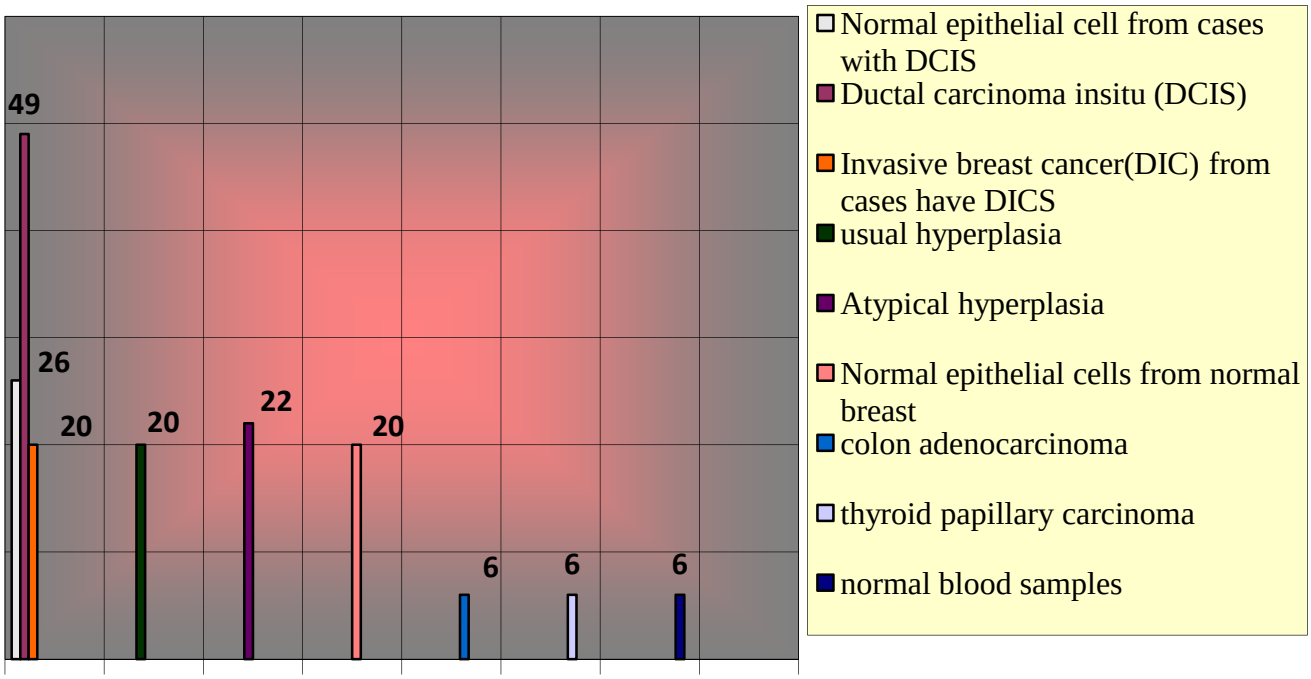

Figure 5: The histological types, and the number of breast cases (benign and malignant) and negative controls.

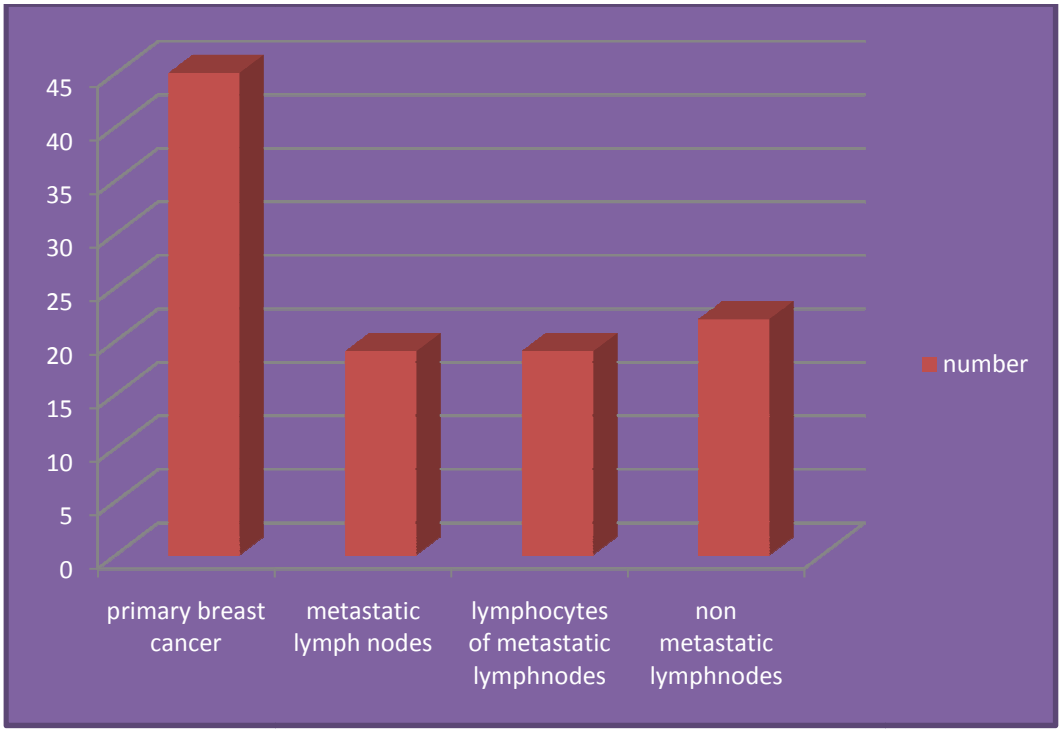

Figure 6: The Cases number of breast cancer and their lymph nodes.

Histological criteria

The adjective “collateral” is used to indicate: a) NEC present in the same histological section hosting a preinvasive lesion or an infiltrating carcinoma and b) preinvasive lesions present in the same histological section hosting an infiltrating carcinoma.

Usual ductal hyperplasia (UDH) indicates a benign ductal epithelial proliferative lesion characterized by secondary lumens and streaming of the central proliferative cells. A typical ductal hyperplasia (ADH) has cytological and architectural features of low-grade

DCIS present in 1 or more ducts or in an aggregate not exceeding 0.2 cm. DCIS can be of high, intermediate, or low grade: a) pleomorphic large cells with abundant mitoses and with variable architecture, often solid and with a central necrotic debris; b) uniform cells, with small nuclei and frequently with a cribriform or micropapillary configuration; c) the neoplastic nuclei show pleomorphism of a degree between high and low grade DCIS, (figure: 7).

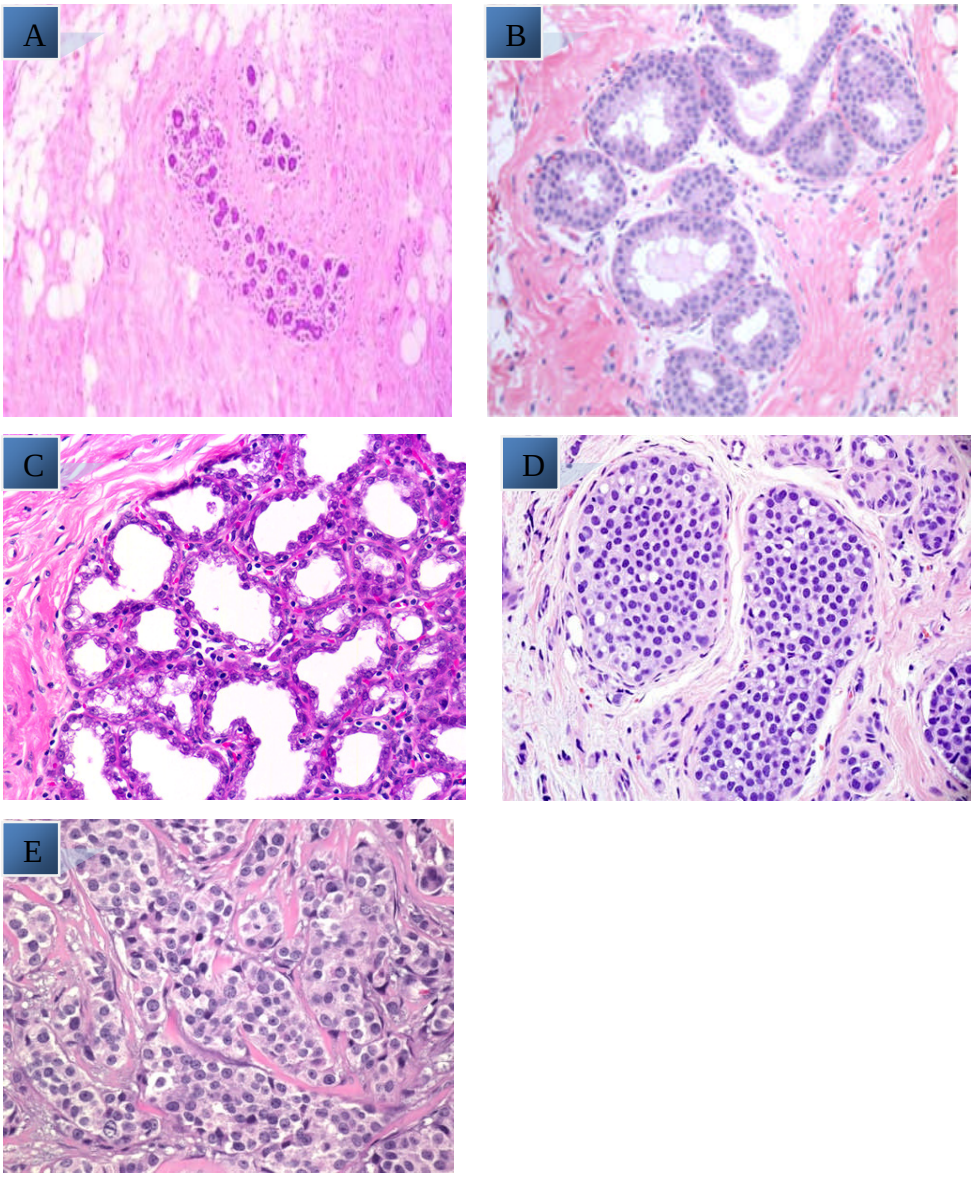

Figure 7: Breast cancer progression A, normal breast; in B, usual hyperplasia C, Atypical hyperplasia D, ductal carcinoma in situ, E infiltrating ductal carcinoma

Laser microdissection

A Leica ASLMD automatic laser microdissector was used to select the epithelial cell population to be studied (Figure: 8). Three µm thick sections were cut from each case using each time a new microtome blade, and applied on microdissecting slide, followed by staining with haematoxylin and eosin, then obtaining a total of 10,000–15,000 cells (figure:9). Stromal and inflammatory cells were carefully excluded**.** Due to the long experience of the laboratory with this method, no difficulty was found in selecting areas of interest.

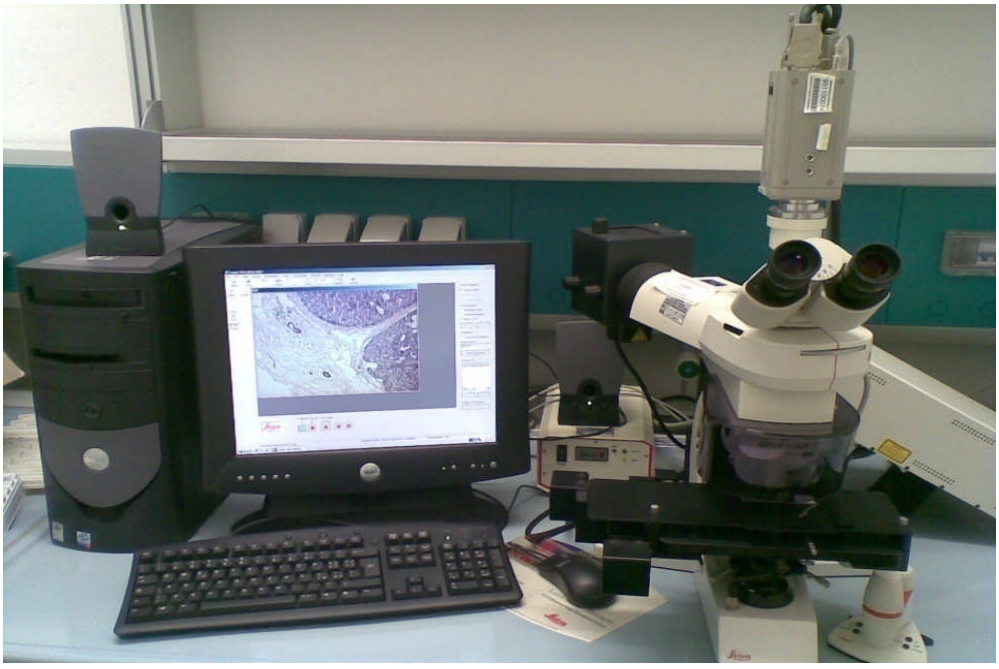

Figure 8: Laser capture microdissector

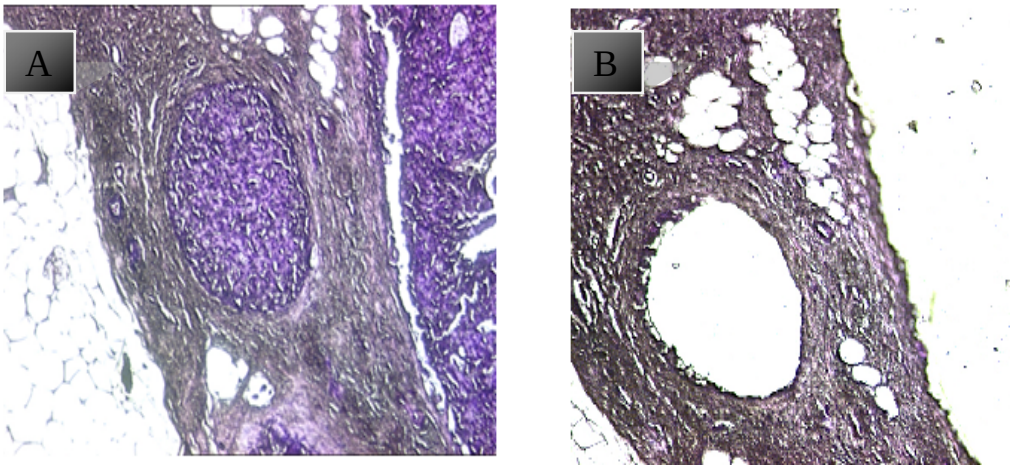

Figure 9: Laser capture microdissection, A) before applying microdissection, B) after applying microdissection

DNA extraction from microdissected tissue

Microdissected Cells are suspended in buffer containing 10 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1% Tween 20, and 0.1 mg/ml proteinase K, pH 8.0, and incubated overnight at 56°C. Higher concentrations of proteinase K have been reported to improve the quality of DNA recovered from fixed tissue sections. The sample is subsequently heated at 95°C for 10 minutes to inactivate the proteinase K and the DNA is ready for PCR analysis. To avoid cross-contamination, blank DNA samples (lysis buffer with proteinase K) were processed in parallel with the tissue samples. The quality and amount of extracted DNA was evaluated by agarose gel electrophoresis and UV spectrophotometry (NanoDrop, Celbio).

DNA extraction from paraffin embedded tissue

We have used the QIAamp DNA FFPE Tissue Kit for purification of DNA from FFPE tissue sections.

1. Cut 2 sections of 10 µm thick, using sterile forceps and blade for each sample, place it in sterile 1.5ml eppendorf tube
2. Add 180 µl Buffer ATL, and 20 µl proteinase K, and mix by vortexing.
3. Incubate overnight at 56^{o}C
4. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
5. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl of ethanol (96–100%), and mix again thoroughly by vortexing.
6. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
7. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2 ml collection tube), and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
8. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min, then discard the collection tube containing the flow-through.

9. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min, then discard the collection tube containing the flow-through.
10. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely.
11. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 50 µl Buffer ATE to the center of the membrane.
12. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

RNA extraction from cell culture

We have obtained cell pellet from primary tissue culture of breast cancer after the first subculture for RNA extraction in order to study the gene expression of the HMTV. RNA extraction was performed using Using Qiagen's RNA Mini Kit.

1. Harvest cells according to standard tissue culture procedures to obtain a cell pellet, divide the pellet into two separate 15 ml tubes.
2. To the cell pellet in each tube, add 600ul of Buffer RLT containing 6 µl B-Mercaptoethanol.

3. Vortex the pellet to disrupt it as much as possible, then transfer the lysate to QiaShredder columns, and Centrifuge at maximum speed for 2 minutes to fully homogenize the cells.
4. Add 600ul of 70% Ethanol to each tube of the homogenized cells and mix well, Pipet 600ul of the sample in each tube onto RNeasy mini columns.
5. Centrifuge at 11,000rpm for 30 seconds. Discard the flow.
6. Add 700ul of Buffer RW1 to the columns, centrifuge at 11,000rpm for 30 seconds then discard the flow.
7. Add 500ul of buffer RPE to the columns, Centrifuge at 11,000rpm for 30 seconds, and then discard the flow.
8. Add another 500ul of Buffer RPE to the columns, centrifuge at 11,000rpm for 2 minutes then discard the flow.
9. Transfer the RNeasy columns into fresh 1.5mL nucleic acid free tubes.
10. Add 30ul of RNase free water directly onto the columns, centrifuge at 11,000rpm for 1 minute.
11. Quantitate the RNA on the nanodrop, keep it at -70 ^{o}C.

cDNA synthesis:

We have used fermantas cDNA synthesis kit to synthesis cDNA using RNA extracted from cell culture as a template.

1. Add 0.5-1µg of RNA into sterile nuclease-free tube on ice.
2. Add also 1µl hexamer primer
3. Make the total volume 12µl by adding nuclease-free water.

4. Add the following in the indicated order
    - 4µl of 5x reaction buffer
    - 1 µl of Ribolock 10 RNase inhibitor (20u/ µl)
    - 2 µl of mM dNTP mix
    - 1 µl of RevertAID M-MuLV reverse transcriptase (200u/ µl).
5. Mix gently and centrifuge, then incubate for 5min at 25 ^{o}C. followed by 60 minutes at 42 ^{o}C.
6. Terminate the reaction by heating at 70 ^{o}C for 5 minutes.

The synthesized cDNA can be used immediately or store at -20 ^{o}C for less than a week. For longer storage, -70 ^{o}C is recommended.

DNA amplification suitability

DNA was checked for the absence of PCR inhibitors by amplifying HERV-K10, β actin provirus target templates. Primers used for targete amplifications are in table: 1. Cycle conditions were: 1 cycle, 94 °C, 10 min; 30 cycles, 94 °C, 30 s; 50 °C, 30 s; 72°C, 45 s; final extension, 72 °C, 7 min in 30 µl reaction mixture containing 1× PCR Buffer (500 mM KCl, 150 mM Tris-HCl, pH 8.0), 1.5 mM $MgCl^2$, 200 µM each deoxynucleotidetriphosphates, 0.5 µM of forward and reverse primer and 2.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). DNA samples were considered free of PCR inhibitors if *HERV-K10, β actin* amplicons were clearly visible on a 1.8% agarose gel.

PRIMERS	SEQUENCE (3′-5′)
HERV-K10 5′	CCACGGGTAAATTTACA
HERV-K10 3′	TATTTTTGGTCTTGGGGTAG
β-actin 5′	CTTCTGCCGTTTTCCGTAGG
β- actin 3′	TGGGATGGGGAGTCTGTTCA
ENV 5′	GATGGTATGAAGCAGGATGG
ENV 3′	CCTTTTCTCTATATCTATTAG
ENV3′- nested	AAGGGTAAGTAACACGGGTCATGTA
ENV5L	CCAGATCGCCTTTAAGAAGG
ENVLTR 3	CGAACAGACACAAACACACG

Table 1: Primer sequences

HMTV sequence detection

Fluorescent nested-PCR was used to detect the presence of the HMTV env-like sequence. Generated fluorescent amplicons were sized on an automatic DNA sequencer. The pairs of primer were designed on the bases of the sequence available in GenBank (Accession No. AF243039). Slight changes were introduced in the procedure previously used by ourselves [37] to adapt it to paraffin embedded tissues. They consisted mainly in decreasing the length of the amplicons to a maximum of 250bp. The outer primers yield a 248 bp fragment from nucleotide [nt] positions 231–480 of MMTV-like env, the inner primers yield a 202 bp fragment (nt positions 231 and 431). Sequences of the outer primers for the first PCR were: Forward: 5′gatggtatgaagcaggatgg3′ and Reverse: 5′ cctcttttctctatatctattag3′ and for the nested PCR the forward primer sequence was

the same as the above while the reverse was Reverse Nested-5′aagggtaagtaacacaggcagatgta3′. Both PCRs were carried out in 50 μl containing 1× standard PCR buffer, 1.5 mm MgCl2, 200 μm each dNTP, 0.5 m unlabelled reverse primer (MWG Biotech), 0.5 μm 6-FAM-labelled forward primer (Applied Biosystems, Milan, Italy) and 2.5 U AmpliTaq Gold (Applied Biosystems). Input target template was 500 ng genomic DNA in the first round PCR and 2 μl of first round PCR in the second, respectively. Amplification profile was as follows: 1 cycle 94 ◦C, 10 min; 40 (first round) and 30 (second round) cycles at 94 °C, 45 s; 58 °C, 45 s; 72 °C, 60 s; final extension, 72 °C, 7 min. To prevent PCR contamination, water controls and negative DNA samples were included every five samples in each run. Fluorescent amplicons were analysed by capillary electrophoresis and appeared as peaks in an electropherogram (figure: 10). Amplicon size was extrapolated from a molecular size ladder re-suspended in PCR buffer and run in parallel. Briefly, 3 μl PCR products from both amplification rounds were mixed with 0.5 μl ROXlabelled size standard (Gene Scan 400 HD ROX; Applied Biosystems) and 11.5 μl formamide (Hi-Di Formamide, Applied Biosystems). After denaturation at 95 °C for 3 min, samples were loaded onto an ABI PRISM 3100 automatic genetic analyser and analysed using GENESCAN software, version 3.1 (Applied Biosystems).

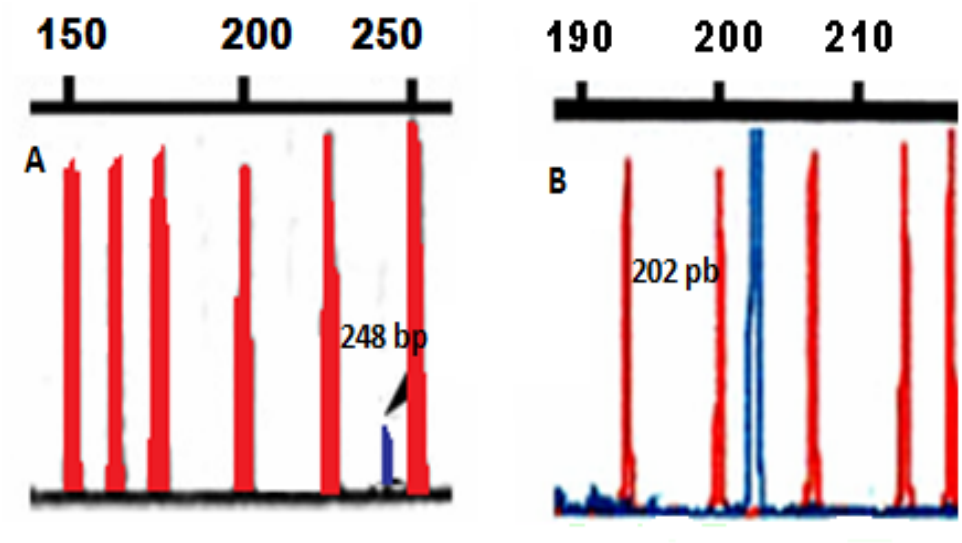

Figure 10: Fluorescent capillary electrophoresis, A: first PCR, B: nested PCR

Sequencing of HMTV *env* PCR fragments

HMTV *env* PCR fragments were purified using Multi Screen PCR Plates (Millipore) following this protocol:

- Dilute PCR by adding 70 µl of water. Mix gently by pipeting up and down 3-5 times.
- Transfer diluted reactions into the bottom of the plate wells.
- Place the plate on the vacuum manifold.
- Set the vacuum to 23-25" Hg.
- Apply vacuum until the solution has been completely removed from the wells.
- Shut off the vacuum source and blot the excess liquid from the bottom of the plate.
- Add 100 µl of water and shake for 2 minutes on a micro-

plate shaker.

- Apply vacuum until the solution has been completely removed from the wells.
- Shut off the vacuum source and blot the excess liquid from the bottom of the plate.
- Resuspend the purified sequencing products in 25 µl of water by shaking for 10 minutes on a microplate shaker.
- Transfer the purified sequencing products to an appropriate plate

Then we performed Cycle Sequencing reaction using Big-Dye Terminator kit v3.1 (Applied Biosystems) and 2,5 pmol/µl of each primer, for a final volume of 20 µl. We used these conditions: 96 °C using the QIAquick Gel Extraction kit (Qiagen) and sequenced on an ABI PRISM 3130XL (Applied Biosystems). Each sequence was aligned by using BLAST search function (http://blast.ncbi.nlm.nih.gov) to MMTV sequences deposited in GenBank.

HMTV Real Time PCR assay

Extracted DNA from 9 patients was analysed by quantitative RT-PCR: in 2 patients NEC, DCIS and IDC were available, whereas in the other 7 cases DCIS and IDC only. Briefly, 25µl PCR reaction mixture was prepared using 2X Master Mix (MesaGreeen qPCR, Eurogentec, San Diego, CA), 0.5 µl of HMTV primer (0.3uM) for the ENV region (nested PCR 202bp). The viral load was determined as the mean of triplicate sample values. GAPDH which is a single copy gene was used as an internal reference control. Four ten-fold

dilutions ($1X10^1$–10^4 copies/ml) of genomic human DNA (GAPDH gene) and of HMTV env amplicons were included as standard curves on each run. HMTV *env*-like and corresponding GAPDH. A HMTV positive control DNA extracted from a mouse known to be infected with the virus and a template negative control (PCR mix only) were also included in each PCR run. PCR were run on the Rotor-Gene Q from Qiagen (figure 11). The standard curves were plotted by the Rotor-Gene software detector system printing the Ct values against each known concentration of standards (HMTV or GAPDH sequence) and MMTV copy number per DNA sample was calculated by the detector.

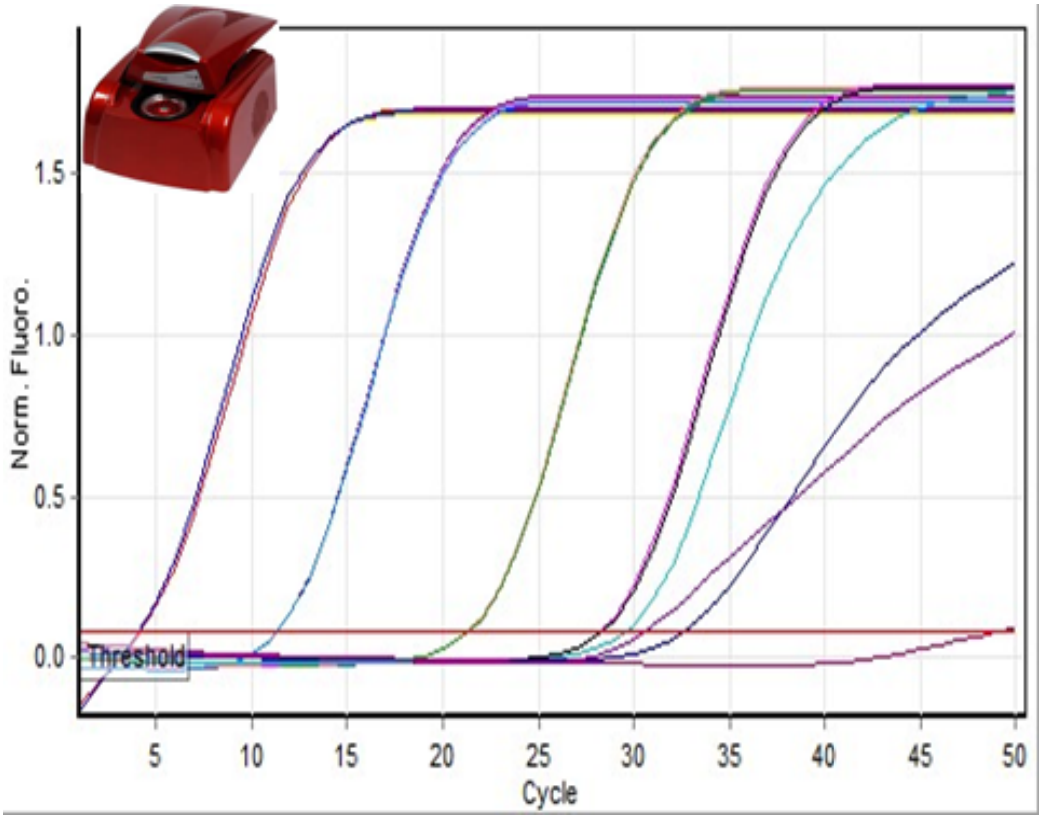

Figure 11: Rotor gene real time PCR

Amplification of the probe (2.7 kb env-LTR)

We have prepared homemade probe of HMTV env-LTR 2.7kb. Amplification of 2.7kb env-LTR was achieved using takara EX taq kit, the 50 µl reaction contains 3 units of enzyme, 5 µl 10xPCR buffer, 2 µl of 50 mM mgcl2, 8 µl of 2.5 mM dNTP, 0.2µm of each primer (5L, LTR3) [36], 1µg of mouse DNA positive for HMTV was incubated at 94 for 2 minutes, 94 for5 seconds 68 for 5minutes for 40 cycles.

Labeling the probe

The band at 2.7kb was cut and purified using centri-sep kit (applied biosystem), then sequenced to make sure it's the true target.

Labeling probe was done using DIG-NICK translation mix kit (Roche) as the following:

1. Add 1 µg the purified PCR product to sterile, double distilled water and end up with a final volume of 16 µl.
2. Add 4 µl DIG-Nick Translation Mix, mix and centrifuge briefly.
3. Incubate for 90 min at 15°C, and then place the reaction on ice.
4. Take a 3 µl aliquot per 20 µl reaction volume from the reaction, add gel loading buffer, denature it at 95°C for 3 min, put it on ice for 3 min and run the sample on an agarose minigel along with a DNA molecular weight marker. The probe should range between 200 and 500 nucleotides in length.
5. If necessary reincubate the reaction at 15°C and check the fragment size again.
6. When correct probe length is achieved stop the reaction by adding 1 µl 0.5 M EDTA (pH 8.0) per 20 µl reaction volume and heating to 65°C for 10 min.

Chromogen in situ hybridization (CISH)

Sections were cut at 4 μm thicknesses, mounted onto positively charged microscope slides, and left to dry overnight at 37°C. Sections were then deparaffinized and rehydrated. Antigen retrieval was achieved by heat retrieval using water bath (DAKO). The slides were placed in Coplin jars containing enough 0.01 M sodium citrate solution (pH 6.0) to cover the sections, and then incubate in water bath at 80°C for 30 minutes. In order to expose the nuclear components, proteinase K was applied on the sections for 10 minutes at 37°C. The hybridization solution is prepared just before applying, for each slide make up to 10 μl of the solution of the probe (50 ng) in hybridization solution (2x SSC, 50% formamide, 10% dextran sulfate). The section then dehydrated using absolute ethanol, followed by adding up to 10μl of probe with hybridization solution on tissue, then apply cover slip over the probe solution and avoid bubbles formation, after that seal the edge of cover slip with cement material. Denaturation and hybridization were achieved by incubate the slide for 5minutes at 80°C and 16 hours at 37 °C respectively using thermobrite (ABBOTT hybridizer). The day after get the slide out of the hybridizer, remove the cover slip and wash with 0.5 x SSC at 75^{o}C for 30 seconds, followed by incubation in 2x SSC at room temperature. the results were detected by applying anti-digoxigenin-AP conjugate for 30 minutes at 37 °C, that produce a characteristic nuclear black stain after 5-20 minutes of applying NBT/BCIP substrate (figure: 12).

also prepared. The positive control slides were prepared from a case known to be positive for HMTV env in PCR study. The negative control slides were prepared from the same tissue block, but incubated with hybridization buffer instead of the HMTV env probe.

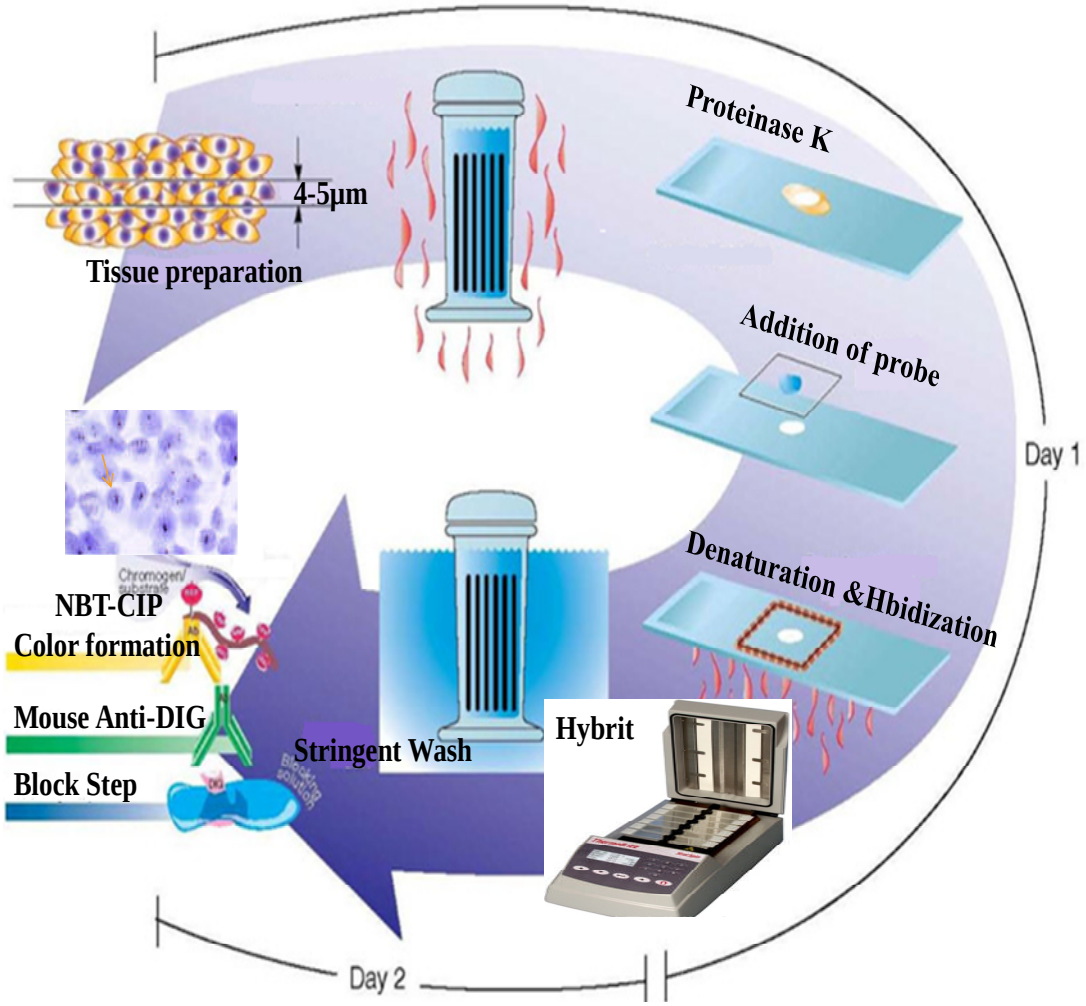

Figure 12: CISH protocol

Tissue culture

We have prepared the media needed for primary breast cancer tissue cultures as the following:

Collecting medium

DMEM F12 with 200U/ml penicillin, 200 µg/ml streptomycin, 5 µg/ml fungizone.

Primary culture medium:

DMEM F12 supplemented with

1. 0.5µg/ml hydrocortisone
2. 5µg/ml insulin
3. 20 ng/ml EGFR
4. 200 µg/ml cholera toxin
5. 0.004M/ml bovine pituitary extract
6. B27 2%

Complete culture medium

DMEM F 12 containing 10% heat-inactivated fetal bovine serum albumin (HIFBS).

Antibiotic solution (ABC-PBSA)

1. Phosphate buffered saline (PBSA)
2. 200 U/ml penicillin
3. 200 µg/ml streptomycin
4. 5µg/ml fungizone

Day 1 protocol

1. Preparing the hood by ensuring that it's clear and swabbing it by 70% alcohol.
2. Brings the reagents and materials necessary for the procedure, swab bottles with 70% alcohol and place items required immediately in the hood.
3. Obtain breast tumor biopsies from pathology and transport to the laboratory in colleting medium. If the tissue cannot be processed immediately (recommended),it may be stored refrigerated for up to 24 h without significant loss of viability
4. Wash tissue extensively with phosphate buffered saline lacking Ca2+ and Mg2+ (PBSA) supplemented with 200U/ml penicillin, 200 µg/ml streptomycin, 5 µg/ml fungizone (ABS-PBSA)
5. Minced finely by using crossed scalpels in a sterile Petri dish.
6. Wash twice in ABS-PBSA.
7. Disaggregate for 18-20 h in 200 U/ml collagenase A (made up in complete culture medium) in a 37C incubator (either in multiwell plate, or Petri dish)
8. Remove tissue from incubator, and shake vigorously by hand. This will break up any remaining large clumps of partially digested tissue
9. If there still undigested tissue re incubate the tissue with fresh medium and collagenase 200U/ml.

Day 2 protocol

1. Transfer the Petri dish contents to 50 ml sterile centrifuge tube, close the cap and wrap it with parafilm.
2. Spin at1200g for 5 minutes.
3. Culture the pellet in the primary cultured medium using primary culture flask (blue cap); incubate the flask at 37^{o}C and 5% Co2.
4. The day after change the medium to get rid of unattached cells in case of attached conditions, after that change the medium every 2 days.
5. For floating cell culture, incubate the cells in

Subculture

The need to subculture implies that cells in the primary culture have increased to become 70-80% confluent.

1. Take the culture flask to the sterile working area under the hood, then remove and discard the medium
2. Add PBSA/EDTA prewash (0.2ml/cm^2) to the side of the flask opposite the cells to avoid dislodging cells.
3. Add trypsin (0.1ml/cm^2) to the side of the side of the flasks opposites the cells. Turn the flask over and lay them down. Ensure that the monolayer is completely covered.
4. Incubate at room temperature (5-10 min), with the flasks lying flat, until the cells round up; when the flask is tilted the

monolayer should slide down the surface. Don't leave the flask longer than necessary.

5. Add complete culture medium (0.1-0.2ml/ cm^2), and dispersed the cells by repeated pipetting over the surface bearing the monolayer.
6. Finally, pipette the cell suspension up and down for a few times, with the tip of the pipette resting on the bottom corner of the flask, taking care not to create foam.
7. Count the cells with hemocytometer; dilute the cell suspensions to appropriate seeding concentration, by adding appropriate volume of cell suspension to a premeasured volume of medium in a culture flask, and incubate the flasks at 37^oC and 5% Co2.

Removal of fibroblastic cells from the tissue culture

We have used Anti-fibroblast microbeads form MACS to get rid of the fibroblast from tissue culture.

1. Trypsinized the cells as mentioned above, and determine the cell number.
2. Centrifuge cells at 300xg for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 80 µl of buffer per 10^7 total cells.

4. Add 20 µl of Anti- fibroblast Microbeads per 10^7 total cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2-8^{o}C).
6. Washing cells by adding 1-2 ml of buffer 10^7 cells and centrifuge cells at 300xg for 10 minutes. Aspirate super-natant completely.
7. Resuspend in 500 µl buffer.
8. Prepare the magnetic field and the adaptor, then apply the column and prime it with PBS.
9. Apply a sterile collecting tube under the applied column, then add the labelled cells to be separated to the column and collect the negatively selected cells (fibroblast con-fined in the column)
10. The negatively selected cells (no fibroblast) then sub cultured.

Cryopreservation the cells

Grow the culture to late log phase; prepare a high concentration of cell suspension by:

1. Trypsinize monolayer and resuspend cells in medium at 1-10x10^{10}/ml
2. Add cryoprotectant, dimethyl sulfoxide (DMSO) to 10% v/v
3. Divide cells in a prelabelled polypropylene plastic ampoules
4. Clip the ampoules on to an aluminium cane, insert in a card-board tube and transfer to a -70 ^{o}C.

Statistical analysis

One way Anova test was used to statistically evaluate the differences in viral copy numbers between the early and later stages of tumour progression in the same patient.

RESULTS

HMTV frequency in phases of breast cancer progression

The presence and the frequency of HMTV *env* sequence in normal breast and in all phases of breast cancer progression are described below and reported in Table 2 and in Figure 13.

Normal epithelial cells: a) exogenous HMTV *env* sequences were found in 19, 2% (5 out of 26) of NEC collateral to DCIS or IDC. b) the 20 cases of normal breast samples obtained by reductive mammoplasty were result all negative (*c* and *nc* stand for collateral and non collateral respectively, Figure.13)

Ductal epithelial hyperplasia: a) the 20 cases of UDH resulted all negative. b) exogenous HMTV *env* sequences were found in 27% (6 out of 22) samples of AH.

Breast carcinoma: a) exogenous HMTV *env* sequences were found in 79.6% (39 out of 49) of samples of DCIS. The presence of the virus was not related to the presence of the preinvasive lesion in the same histological section hosting an infiltrating carcinoma. b) 35% (7 out of 20) of samples of IDC resulted positive.

Controls: all the 6 cases of infiltrating papillary thyroid carcinoma, the 6 cases of colon adenocarcinoma and the 6 cases of DNA from blood of healthy donors were negative.

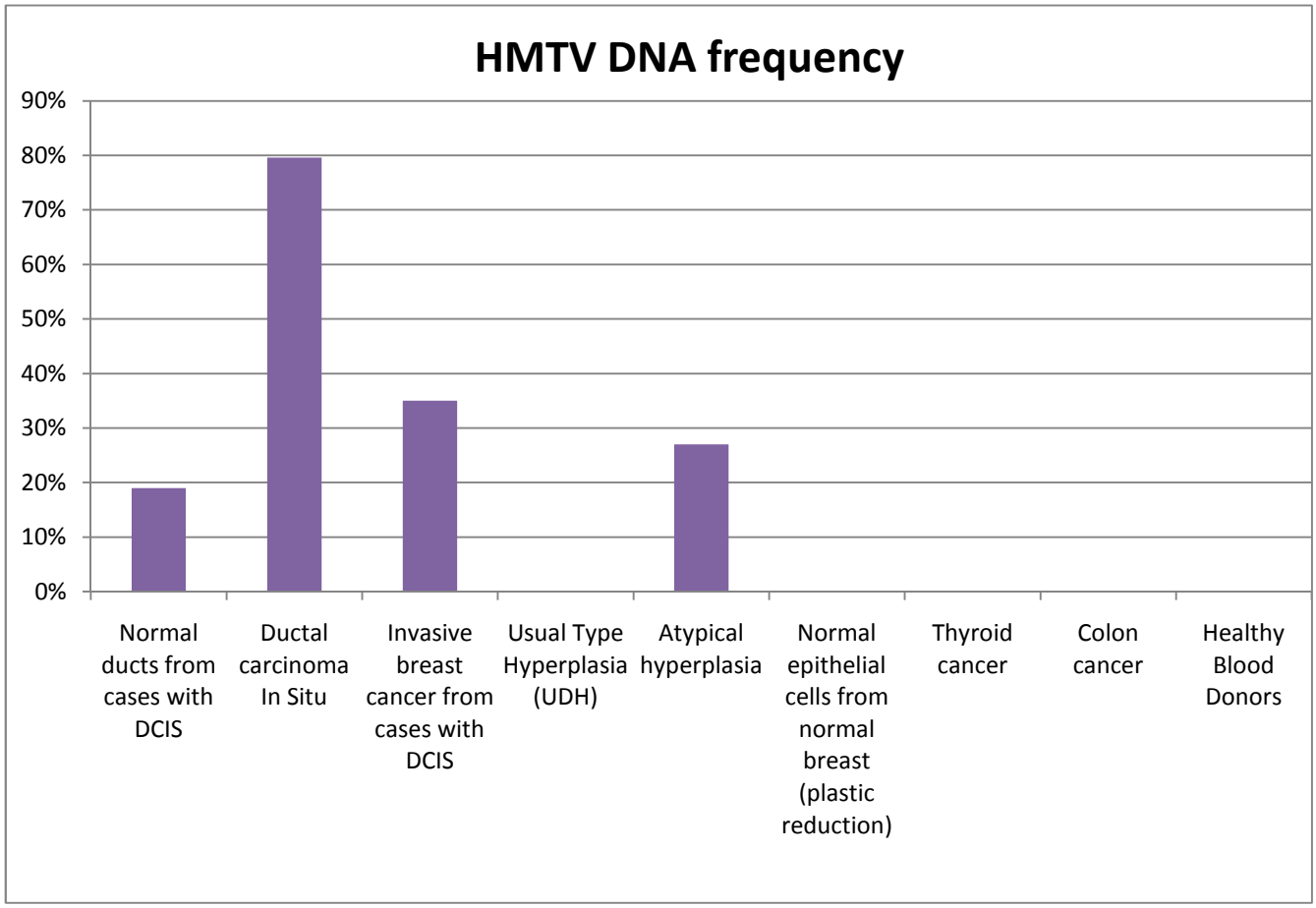

Figure 13*:* The frequency of HMTV env sequence in normal breast cells and all phases of breast cancer progression.

Tissue	Cases	HMTV env positivity	%
Ductal Carcinoma In Situ	**49**	**39**	79.6
Invasive Ductal Carcinoma from cases with DCIS	**20**	**7**	35
Normal ducts from cases with DCIS	**26**	**5**	19
Usual Type Hyperplasia	**20**	**0**	0
Atypical Hyperplasia	**22**	**6**	27
Normal ducts from plastic breast reductions	**20**	**0**	0
Papillary Thyroid carcinoma	**6**	**0**	0
Colon Adenocarcinoma	**6**	**0**	0
Healthy blood donors	6	0	0

Table 2: Number of cases tested with different histological types and number of HMTV env positive cases found in all phases of breast cancer progression.

Different histological structures obtained from the same patient: in 14 patients with DCIS positive for HMTV *env*-like sequences, normal breast and IDC were also available; in one case all the three samples resulted positive (case 1) , in 4 cases both DCIS and IDC were positive (cases 6-9), whereas the normal structures negative; in 3 cases normal structures and DCIS were positive, and IDC negative (cases 2-4); in 6 cases only DCIS were positive (cases 12-17). In the 6 patients with DCIS negative for HMTV *env*-like sequences in which normal structures and/or IDC were also available also the last two resulted negative (cases 41-46). Of four cases of positive DCIS in which IDC were available, the last one was positive in 2 and negative in 2 (cases 10-11 and 26-27).

In 9 cases of positive DCIS in which only normal gland structures were also available, 8 of the last ones resulted negative and 1 positive (cases 18-25 and 5). In the 16 cases with only DCIS available, 13 were found positive and 3 negative (cases 28-40 and 47-49).

Sequence detection

To determine whether the amplified fragments were indeed homologous to HMTV, the 248 bp amplicons that showed clear band on agarose gel (Figure: 14) were sequenced, aligned to the corresponding region of the prototype MMTV sequence and MMTV (GenBank Accession Nos AY152 721 and AF243039, respectively). Multiple nucleotide alignment showed 97% homology to both MMTV and HTMV (Human MTV) env sequences. Finally, no significant hits were found when the 2 sequences were compared to the human genome sequences available in GenBank, indicating that

these amplicons were not of human genomic or endogenous retrovirus origin.

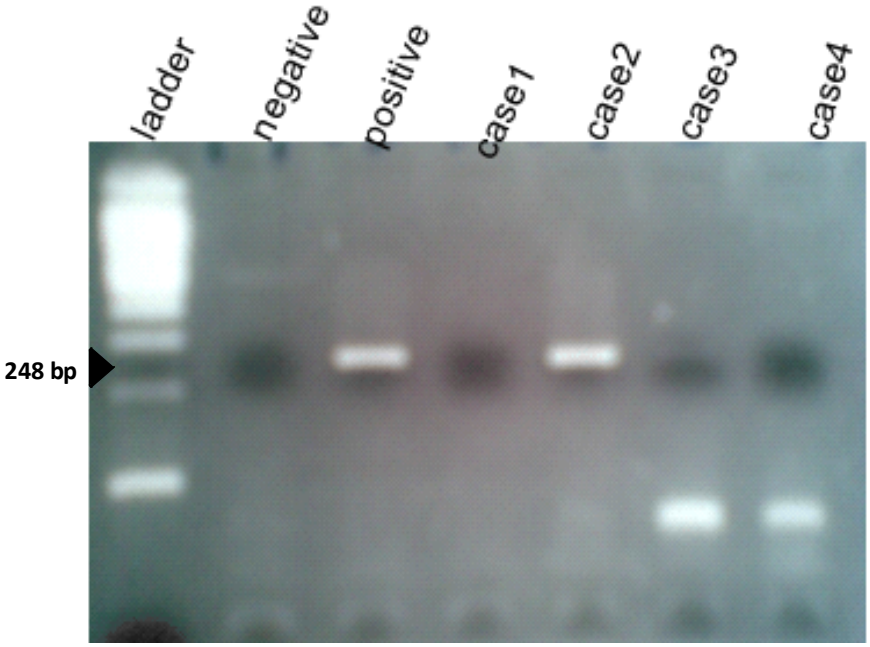

Figure 14: Amplification of 248 bp HMTV env gene by PCR, 2% agarose gel electrophoresis.

HMTV viral load with RT-PCR

Real time PCR was conducted on DNA extracted from DCIS and IDC from 7 different patients. GAPDH was used as the internal gene reference control, so each bar represents the ratio between HMTV DNA copy number and GAPDH DNA copy number (Figure 15). Figure 16 shows also the average of the HMTV/GAPDH DNA copy number ratio (A) for DCIS and IDC of 7 samples. There was a

dramatic decrease in HMTV copy number between the two stages with a statistical significance of p-value= 0.01. RT PCR was conducted also on DNA extracted from NEC, DCIS and IDC from two different patients (Figure15). In each patient PCR was positive in NEC and DCIS, whereas negative in IDC. In both patients there is an increase in HMTV DNA copy number after the progression from normal epithelium to DCIS, while there is a complete loss of the virus in the invasive stage.

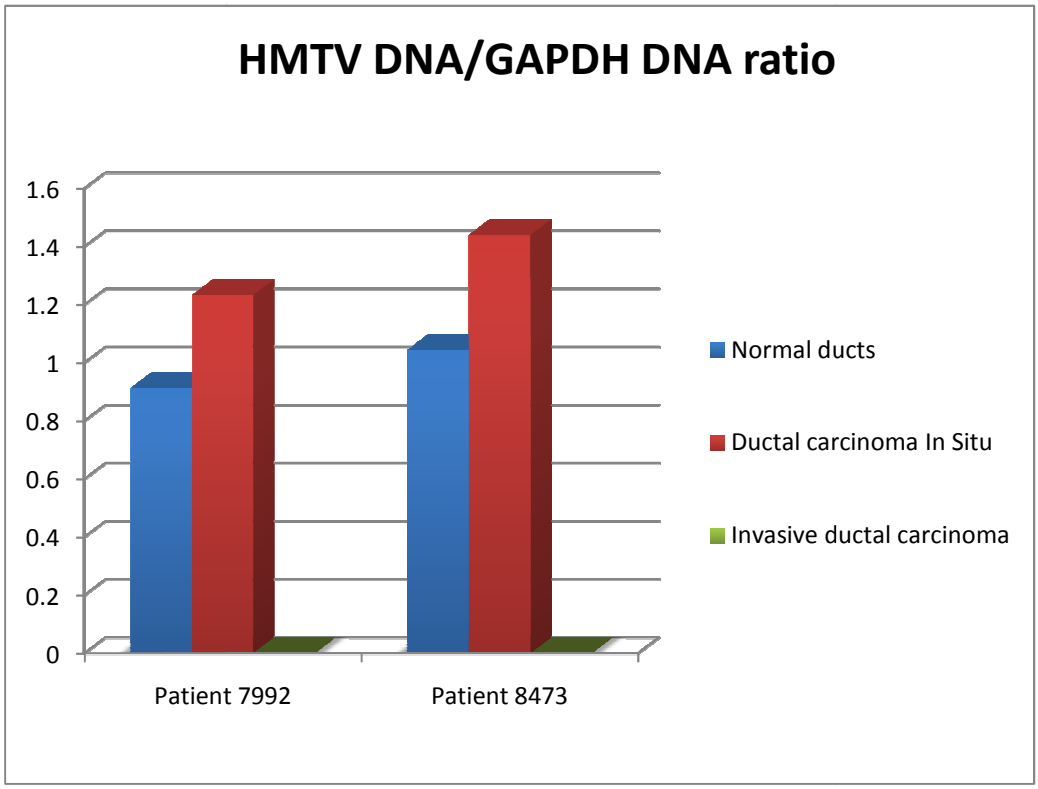

Figure 15: RT-PCR conducted on genomic DNA extracted from cells of different nature in two different patients: normal cells collateral to carcinoma, DCIS and IDC three histological stages (normal ducts, DCIS and IDC). Bars represent the relative HMTV DNA copy number to GAPDH DNA copy number used as an internal gene reference control.

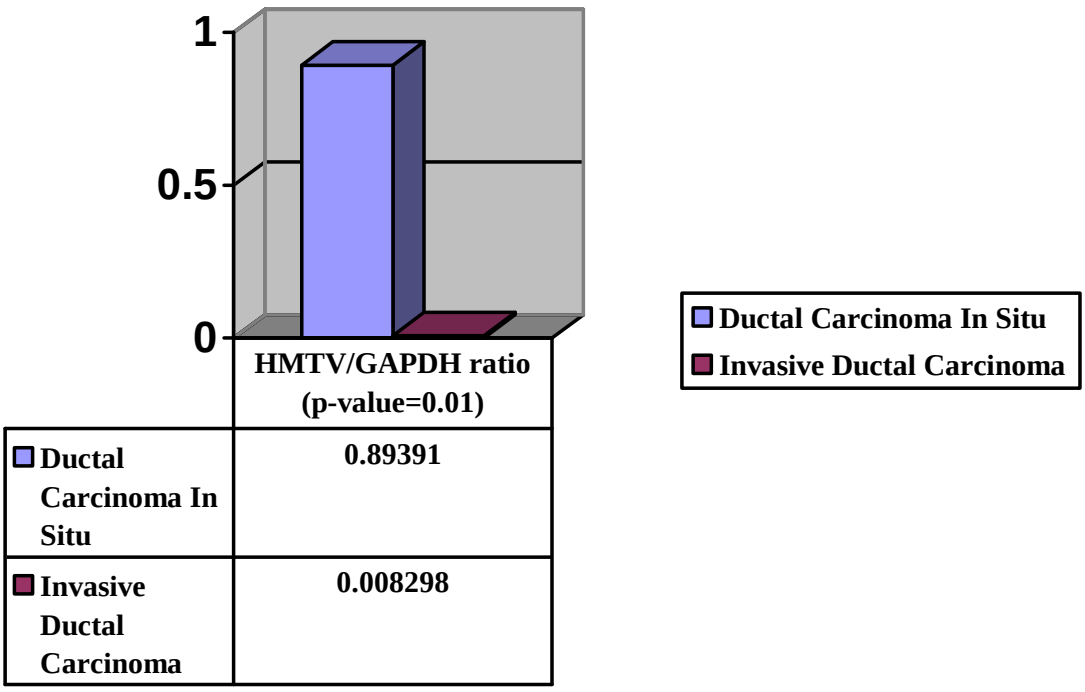

Figure 16: RT-PCR conducted on genomic DNA extracted from seven samples of two histological stages of cancer, DCIS and IDC. Bars represent an average of all HMTV DNA / GAPDH DNA ratios. Difference is highly significant, with a p-value of 0.01.

Chromogen in situ hybridization (CISH)

We have designed homemade probe to investigate the presence of HMTV sequence in the tumor cells under the light microscope.

The idea behind doing CISH on our samples mainly DCIS and IDC is a further confirmation of our results. CISH is very specific tech-

niques that detect the viral sequence in the nucleus of infected cells as coloured dots.

Fluorescent nested PCR and Real time PCR have showed higher expression of HMTV sequences in DCIS compared to IDC. These results are further confirmed by CISH that showed higher number of infected cells with HMTV in DCIS compared to IDC (figure 17).

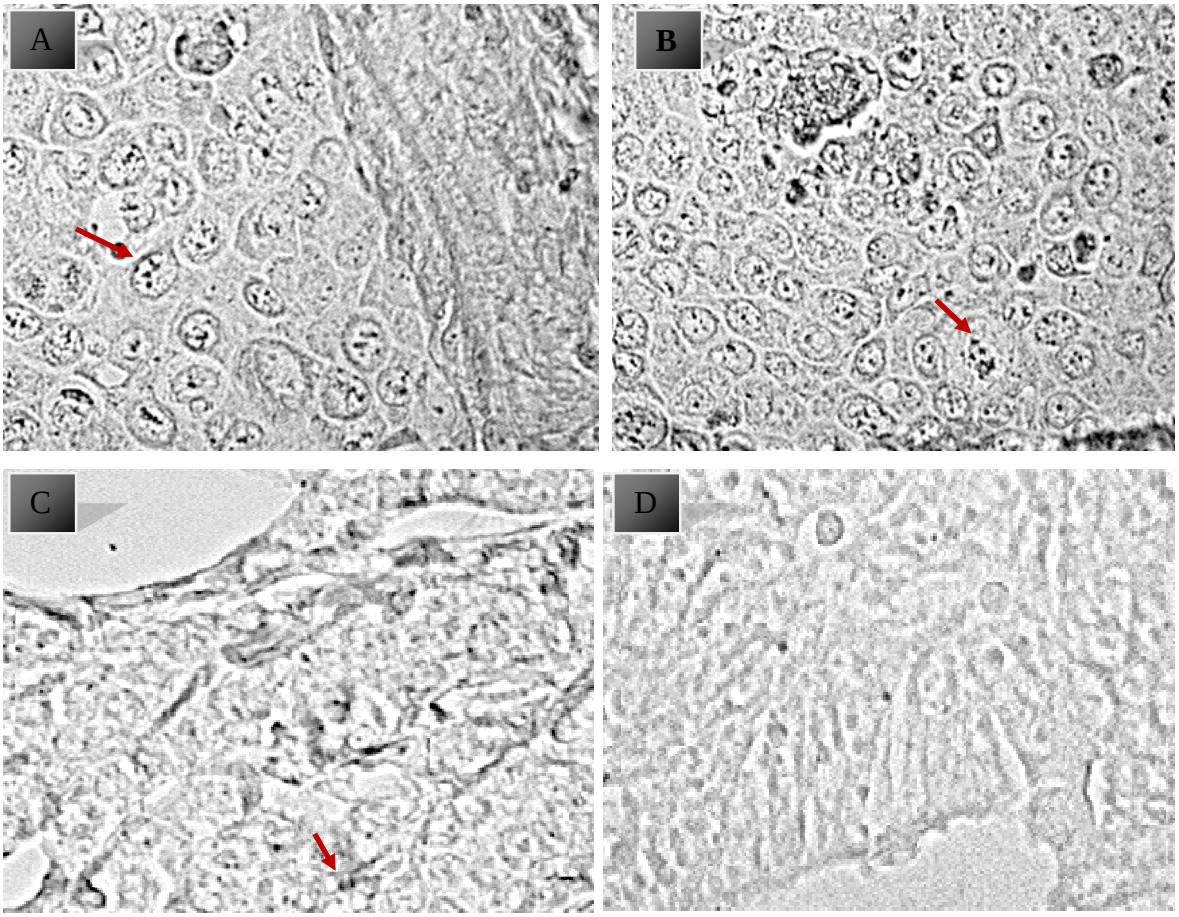

Figure 17: CISH analysis of HMTV of paraffin embedded tissue; A) positive signals in cells of DCIS, arrow indicates a positive signal in one cell; B DCIS with multiple positive cells; C) invasive ductal carcinoma that shows less positive cells; D is adenocarcinoma of colon as a negative control

The frequency of HMTV in metastatic lymph nodes

The presence and the frequency of exogenous HMTV *env*-like sequence in primary breast tumor, metastatic lymph nodes, and their lymphocytes as well as in non metastatic lymph nodes are described below and reported in Table 3.

Primary breast cancer (DCIS+ IDC) that do not develop metastasis expressed exogenous HMTV env sequences in 11 out of 22 cases (50%),whereas primary breast cancer that develop metastasis sowed exogenous HMTV env positivity in 16 out of 23 cases (69.5%). Furthermore we have also studied the presence of exogenous HMTV in metastatic breast cancer cells which microdissected from lymph nodes in order to compare the exogenous HMTV env status in primary and their respective metastatic breast cancer cells. The result was very similar as we detected the exogenous HMTV env in 11 out of 19 cases (68.4%) compared to 69.5% in their primary breast cancer. In a try to study the HMTV status in lymphocytes we have microdissected lymphocytes from lymph nodes which invaded by primary breast cancer cells, and the exogenous HMTV env was detected in 11 out of 19 (57.8%) comparing to 36% (8 out of 22) in lymphocytes from lymph node that do not develop any metastasis.

Tissue	Cases	HMTV env positivity	%
Primary breast cancer (DCIS+ IDC) that do not develop metastasis	22	11	**50**
Primary breast cancer(DCIS+ IDC) that develop metastasis	23	16	69.5
Metastatic breast tumor in lymph nodes	**19**	**13**	68.4
Lymphocytes of metastatic lymph nodes	**19**	**11**	57.8
Non metastatic lymph nodes	22	8	36

Table 3: Number of HMTV positivity in different breast cancer progressions.

The frequency of HMTV in primary and metastatic breast cancer

The frequency of exogenous HMTV env sequence in metastasis of breast cancer compared to their primary tumor was investigated for a possible correlation between the presences of exogenous HMTV env sequence in the primary and their respective metastatic breast cancer (figure 18). We have detected the exogenous HMTV env

sequence in both primary and their respective metastatic breast cancer cells in 13 out 23 cases. However, 3 cases of metastatic breast cancer were negative for exogenous HMTV env sequence, whereas their respective primary breast tumor were positive. On the other hand, 2 cases of metastatic breast cancer but not their respective primary tumor was positive for exogenous HMTV env sequence.

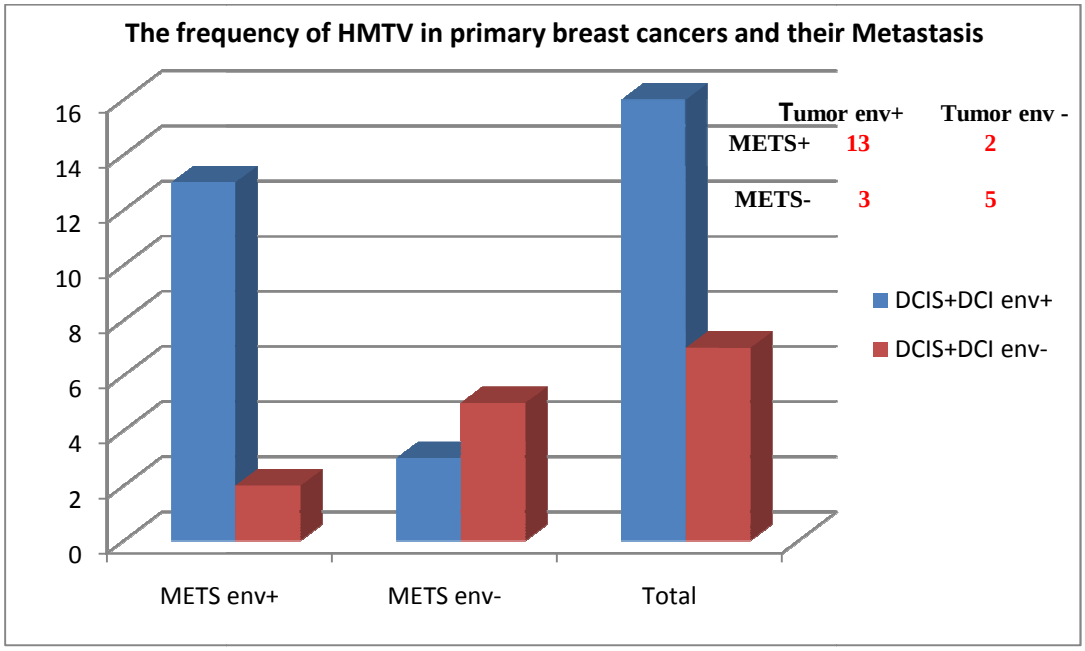

Figure 18: The frequency of HMTV env in primary breast cancers and their metastatic lymph nodes.

The frequency of HMTV in primary breast cancers and their lymph nodes

The presence and the frequency of exogenous HMTV *env*-like sequence in primary breast cancer and in their non metastatic lymph nodes, as well as in the primary breast cancer and in the lymphocytes of their metastatic lymph nodes are described below and reported in Figure 19, 20.

The exogenous HMTV env sequence was detected in 10 out of 19 (52.6%) cases of primary breast cancer and in the lymphocytes of their metastatic lymph nodes. It has been shown that only two cases were positive for exogenous HMTV env sequence of the primary breast cancer cells but negative in the lymphocytes of their metastatic lymph nodes, while only one case harbor the viral sequence in the lymphocytes of their metastatic lymph nodes but not in their respective primary tumor (figure 18). On the other hand, the presence of exogenous HMTV env sequence was detected in 5 out 22 of cases (22.7%) of primary breast cancer and the lymphocytes of their respective lymph nodes. Exogenous HMTV env was detected in 8 cases of the primary breast cancer but not in the lymphocytes of their metastatic lymph nodes, while 3 cases showed positivity in the non metastatic lymph nodes, but not in the primary breast cancer (figure 20).

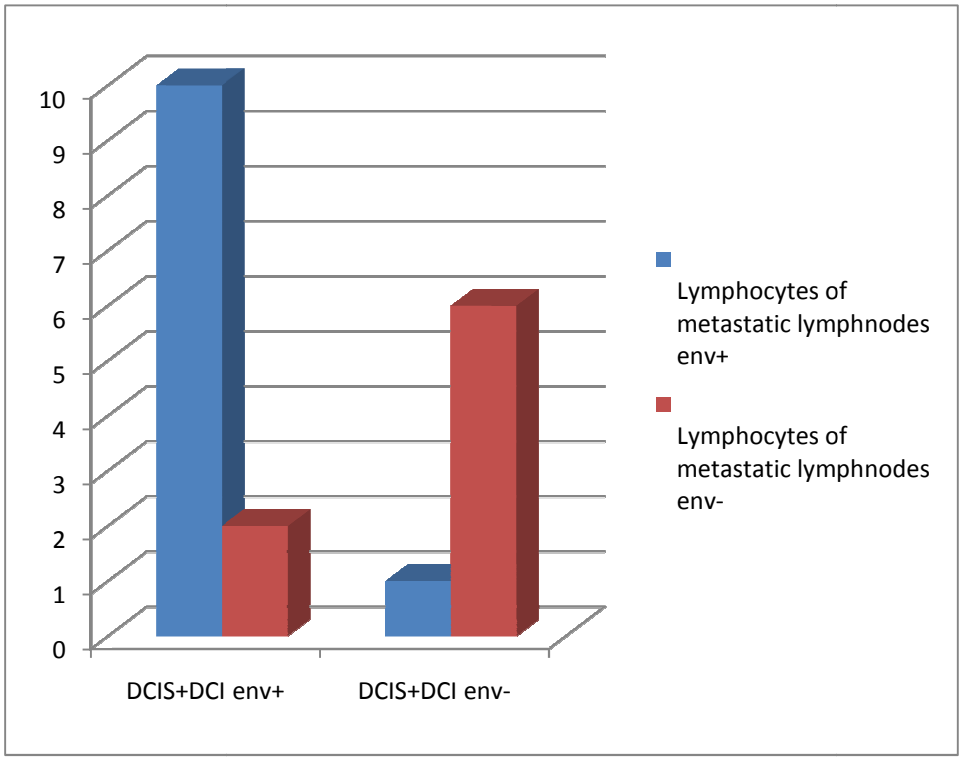

Figure 19: The frequency of HMTV in primary breast cancers and their metastatic lymph nodes

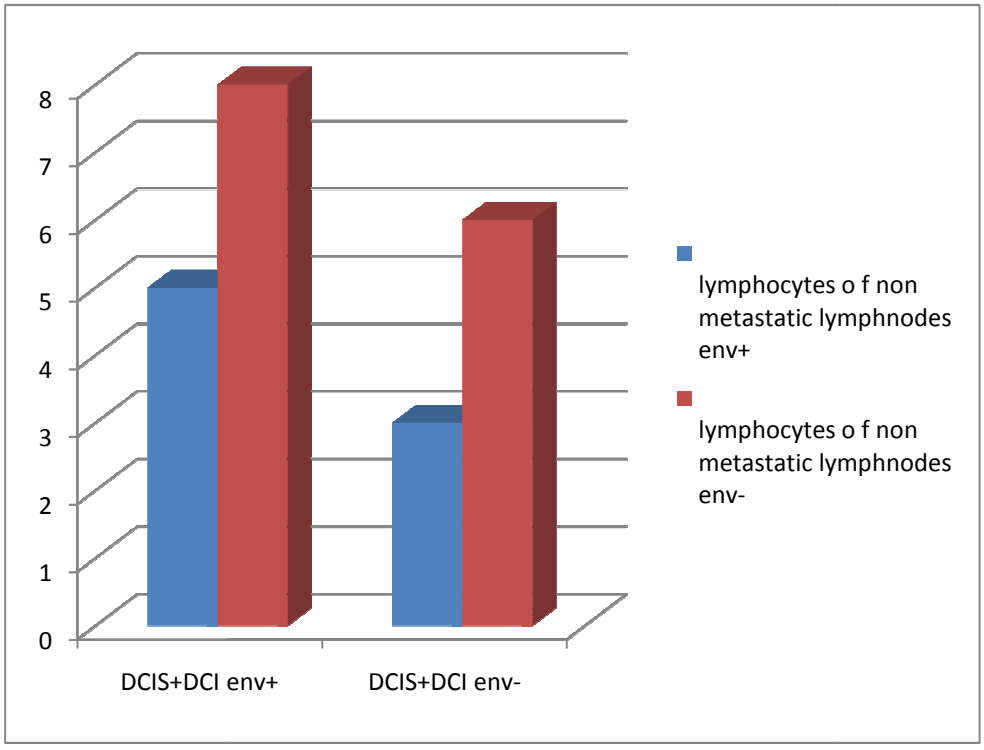

Figure 20: The frequency of HMTV in primary breast cancers and their non metastatic lymph nodes.

Tissue culture

Primary culture of human breast cancer

We have created primary cell line that has been grown for more than a month. Breast cancer tissue was subjected to mechanical disruption followed by enzymatic treatment that resulted in the release of breast organoids (clumps of tissue). Further enzymatic digestion of the organoids yielded single cells that were seeded in low-attachment plates (6 wells plate) in media devoid of serum (see Materials and Methods). Floating spheroids, termed mammospheres, were observed within 5–7 days (figure 20). Cells obtained by the enzymatic dissociation of mammospheres at each passage were further seeded under culture conditions that allow cell attachment using primary cell culture flasks (figure 21). New additives to media such as B27 sustain the growth of mammary tumor cells for long period (more than a month).

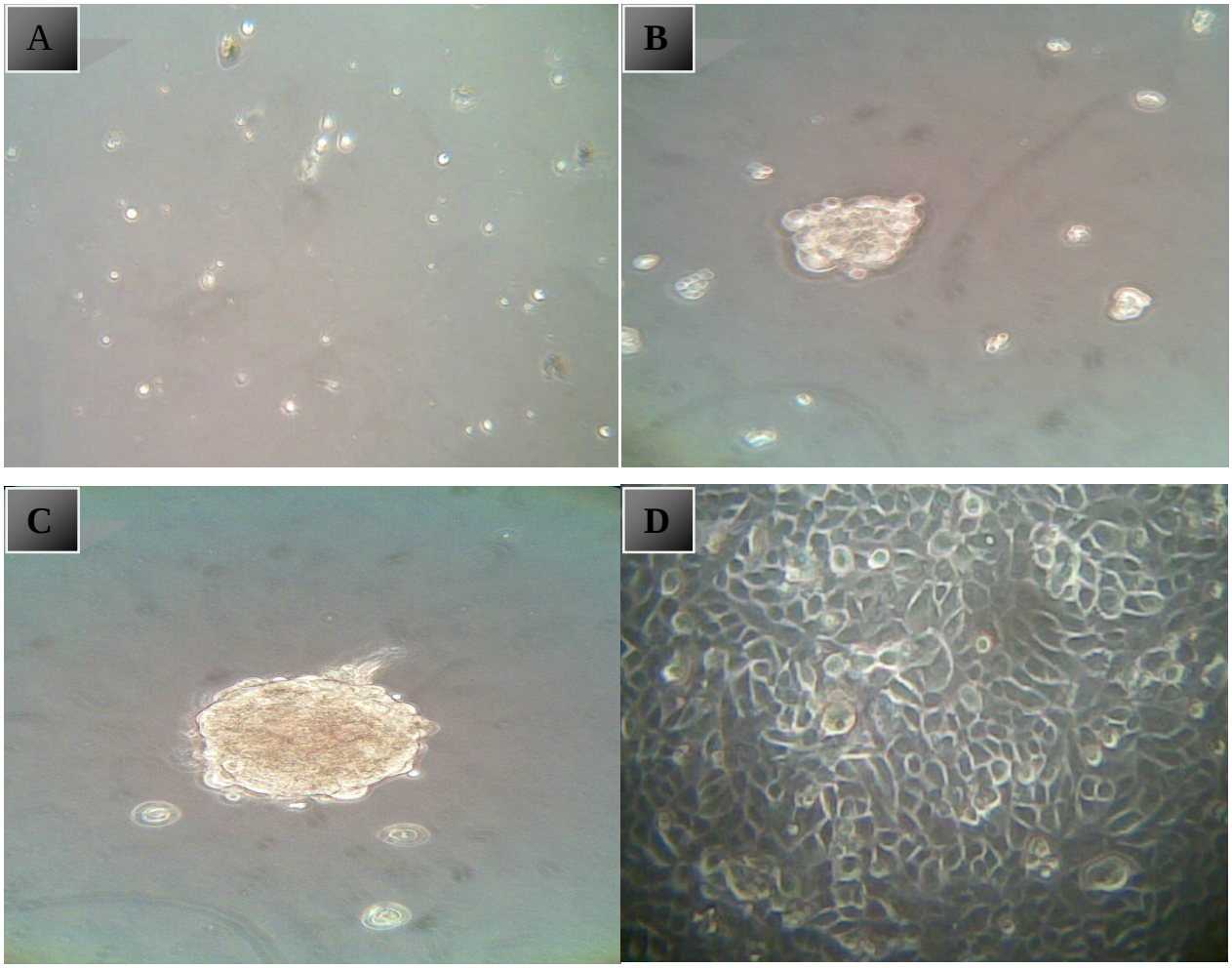

Figure 21: primary culture of invasive breast carcinoma: A) floating cells after enzymatic disaggregation of organoids material; B) aggregates of cell (mammospheres) after 5 days of culturing; C) mammospheres after 7 days of culturing under floating conditions; D) primary breast cancer cells after 5 days of culturing under adherent conditions.

Presence of HMTV in primary culture of human breast cancer

We have checked the primary breast cancer cell line that we have created for the presence of exogenous HMTV env sequence. Exogenous HMTV env sequence was detected in both DNA and cDNA extracted from cells of primary culture that was monitored by a fluorescent nested PCR assay which targeted 248 bp of env sequence.

Infected cells were treated with 10^6 M dexamethasone (DEX) a potent synthetic member of the glucocorticoid class of steroid drug that increase spreading of MMTV in cultured human breast cells[92].

Two weeks after applying of DEX, the DNA from these cells was subjected to the HMTV-specific PCR analysis. Stronger PCR signals were detected at later cultivation time points (Figure 22).

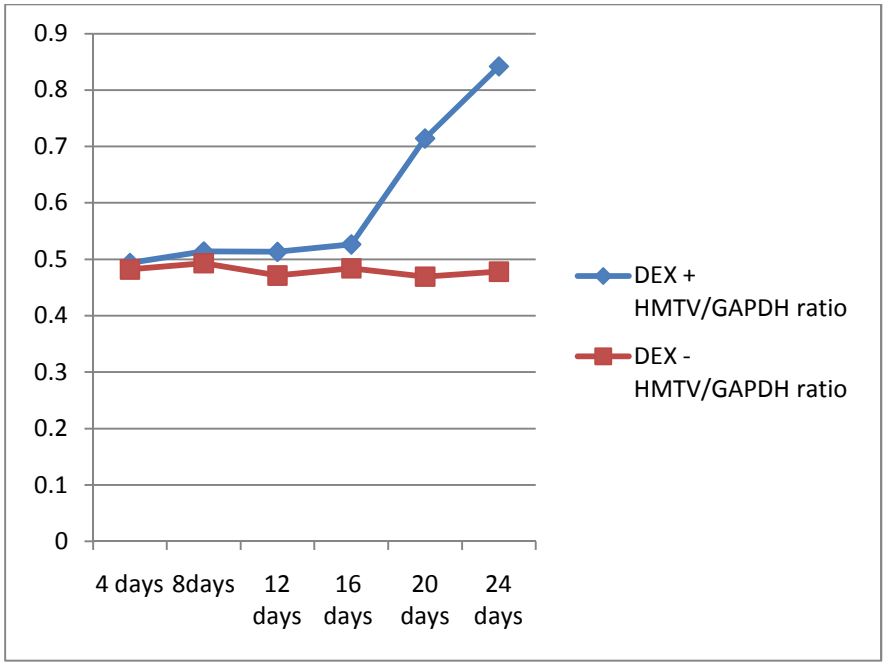

Figure 22 Quantification of viral RNA in cell lysates of human breast cells in the presence and absence of 10^{6} M DEX.

DISCUSSION

Breast cancer evolves from atypical ductal hyperplasia, to carcinoma *in situ*, then invasive carcinoma, and finally distant metastasis, which is usually the cause of death [108, 109]

The etiology of the sporadic breast carcinoma is unknown, with the exception of the rare radiation induced neoplasms [110]. Since many years the promoting role of estrogens is well established, but a sound evidence of their possible causative action has not been reached yet [111]

The molecular approach to breast cancer pathogenesis clearly demonstrates that the classic distinction in ductal and lobular carcinoma is giving place to a new terminology based on bio-molecular characteristics [112-114].The provisory molecular classification in use is clarifying that the group of “ductal carcinoma”, accounting for about 80% of all types of breast carcinoma, contains an unpredictable number of different molecular subtypes. This molecular heterogeneity could be consequence of different causative agents or of different transforming pathways caused by the same agent. In synthesis: no etiological factor has been identified for sporadic breast cancer; it is possible that different factors exist; it is also possible that one or a few factors can give origin to the many molecular pathways of breast carcinoma by using different transforming mechanisms.

A possible viral etiology is receiving today more consideration than in the past, mainly because the demonstration of the causative

role of HPV for the carcinoma of the cervix [115] has opened this new frontier, but also because of the not rare papers demonstrating the presence of exogenous MMTV sequences in infiltrating human tumours[37-39] . The amount of information towards a possible existence of a human MTV accumulated during several decades is impressive: a) viral particles in milk and in tumor tissues[19, 20, 38, 116] b) reverse transcriptase in milk[117], c) MMTV antigens in serum and in tumors [118], d) epidemiological observation that human breast cancer incidence is higher in geographic areas where *Mus domesticus* is the most prevalent mouse species[119], d) association between breast cancer and lymphoma[120], e) occurrence of breast cancer in husbands and wives [121, 122], and f) finally the presence of exogenous MMTV sequences in a percentage of 30-40% of human breast carcinomas[30, 37]. Interestingly, a very recent paper shows a higher expression of Wnt1 protein in human DCIS and IDC positive for MMTV [123].

Unfortunately, none of these data, alone or combined, is sufficient to demonstrate that the most common cancer in women is given by MMTV. On the other hand, there is no element clearly against this hypothesis, whereas quantity and quality of data encourage proceeding in exploring this possibility.

For what concerns MMTV *env*-like, it seems clear that some negative results were consequence of the low quantity of *env* sequences or of technical and methodological problems. A recent paper from this laboratory demonstrates that a correct approach can avoid these limitations [37]; in particular, a laser microdissection procedure to

obtain a pure and enriched population of tumor cells and a highly sensitive FN-PCR to have limited steps in DNA analysis. On the basis of all the data present in the literature, it is reasonable to conclude that the presence of exogenous sequences of MMTV in infiltrating human breast carcinoma is real and that the percentage of positive cases is between 30 and 40%.

If MMTV *env* like is involved in human breast carcinogenesis, it could play an early, an intermediate or a late role. On other words, it could behave as a transforming agent, an etiological cofactor, and a pathogenetic agent. Establishing when exogenous viral sequences appear during the natural history of the disease would represent an interesting contribution to answer the previous question. This work investigates for the first time the presence of HMTV *env* in preinvasive lesions, as well as in infiltrating carcinomas and in normal tissues. HMTV exogenous viral sequences are absent in UDH, whereas present in 27% of AH, in 79.6% of DCIS and in 35% of IDC. Normal epithelium collateral to DCIS is positive in 19% of cases; whereas it is always negative when obtained from healthy women undergo reduction mammoplasty.

These data seem well in line with the model of progression usually accepted for breast cancer: normal epithelium, UDH, AH, DCIS, IDC [124-129]. AH and DCIS are considered obliged precursor of IDC; AH and low grade DCIS are now included in the same category of DIN1, Ductal Intraepithelial Neoplasia. *Usual type epithelial hyperplasia* represents a common finding in human breast, being expression of a hormonal de-rearrangement. In facts, it is con-

sidered not related to cancerogenesis, as shown by its substantial polyclonality and absence of chromosomal alterations and also by the absence of dysregulation in adhesion and extracellular matrix pathways. However, there are a small proportion of cases which are monoclonal (3%), with the occurrence of LOH up to 15% of cases, with few chromosomal aberrations. *Normal epithelium* can show molecular alterations similar to those occurring in cancer, mainly in areas close to an invasive carcinoma, as expected by the hypothesis of "field cancerogenesis" occurring in breast as in other organs. In summary, the few epithelial cells transformed by the unknown cancerogenetic agent in a normal looking breast glandular structure, under the proliferative stimulus of estrogens can give origin to small neoplastic clones in a small number of UDH, which is a very frequent benign alteration. The subsequent expansion of the neoplastic clones, with the accumulation of genomic aberrations, gives origin to AH, where nuclear atypia is easily evident.

Exogenous HMTV *env* sequences are present in normal epithelium close to IDC in a small percentage of cases and have not been found in normal epithelium of normal breast tissue obtained by reduction mammoplasty and in UDH not associated to IDC. Their frequency is well represented in AH, then increases dramatically in IDC and goes back to a however well consistent value in IDC, similar to that of AH.

The presence of HMTV *env* not only in IDC, but also in normal breast, AH and DCIS demonstrates that these exogenous sequences represent an event bound to early steps of cancerogenesis. The per-

centage of 35% of positivity in IDC is expected on the basis of previous data. The absence of positivity in normal breast not associated to cancer, the low number of positive cases in normal glandular structures close to IDC and the absence of positivity in UDH are in line with the field cancerogenesis model and with the biology of UDH. The only apparently controversial result is the very high frequency of HMTV *env* sequences in DCIS, of about 80%, with a percentage of less than half in the two lesions immediately upstream and downstream, AH and IDC.

However, on the basis of the high level of technological accuracy and the robust controls, there is no reason to doubt it. A possible explanation for the strong decrease in positive cases moving from DCIS to IDC can be that HMTV *env* sequences are relevant for transformation, but less important for the transition DCIS-IDC. Moreover, HMTV *env* sequences could be lost as consequence of the high level of chromosomal rearrangement characterizing IDC. A selective depletion of tumor cells expressing HMTV antigens can also be assumed, as consequence of their identification and elimination by the immune system. These data are supported by results obtained by RT-PCR, demonstrating that the HMTV *env* sequence copy number is quite small and that there is a dramatic reduction moving from DCIS to IDC. In addition, the copy number increases from normal cells to DCIS. Furthermore, we have used another supportive technique that further confirmed the results obtained by PCR techniques; CISH, demonstrating that HMTV env sequence is integrated in breast cancer cells but not in adenocarcinoma of colon

or normal cells of the breast, and showed higher number of DCIS cells harbouring HMTV *env* sequence comparing to IDC.

Furthermore, a secondary but not negligible result of this study is the demonstration that HMTV *env* sequence can be easily detected in formalin fixed and paraffin embedded tissues.

Exogenous HMTV *env* sequences are detected in 50% of primary breast cancer (DCIS and IDC) cases that do not developed metastatic tumor. On the other hand, the primary tumor that developed metastases were HMTV positive in 69.5%, and 68.4% in their corresponding metastatic tumor. A possible explanation for the higher number of HMTV positive cases in metastatic tumor is that HMTV first integrated the tumorigenic cells (cancer stem cell) , some of them escape the immune system and develop metastasis. Moreover, HMTV was positive in two cases of metastatic breast cancer and negative in their primary cancer, this probably due to the fact that the immune system negatively selected the virally infected tumor cells, while some of them escape the immune system and develop metastasis. The presence of HMTV env sequence in the lymphocytes of metastatic and non metastatic breast cancer may be considered as HMTV reservoir and rout of transmission.

HMTV viral particles were isolated from peritoneal fluid of patient with metastatic breast cancer whose primary breast cancer was positive to HMTV env sequences[38]. We have succeeded in creating primary breast cancer cell line that was positive for HMTV env sequence. Moreover, the expression of HMTV was increased after

treating the cells with Dexamethasone, that has been shown to infect human mammary cell line after incubated with MMTV positive murine cell line [92]. The prospective study of this project will focus on tissue culture and isolates the viral particles, as well as studying the transforming ability of the virus.

In conclusion, exogenous HMTV env sequences are easily identifiable in the early steps of breast cancer progression, suggesting their possible role in the transformation process, at least as etiological co-factor. On the other hand, the strong reduction of positive cases moving from DCIS to IDC suggests that they are not relevant for the progression of the disease. Our data are sufficient to support the hypothesis the HMTV env exogenous sequences are related to sporadic breast cancer progression.

REFERENCES:

1. Gonzalez-Angulo, A.M., F. Morales-Vasquez, and G.N. Hortobagyi, *Overview of resistance to systemic therapy in patients with breast cancer.* Adv Exp Med Biol, 2007. **608**: p. 1-22.
2. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
3. Key, T.J., P.K. Verkasalo, and E. Banks, *Epidemiology of breast cancer.* Lancet Oncol, 2001. **2**(3): p. 133-40.
4. *Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer.* Lancet, 1997. **350**(9084): p. 1047-59.
5. Hunter, D.J., et al., *Non-dietary factors as risk factors for breast cancer, and as effect modifiers of the association of fat intake and risk of breast cancer.* Cancer Causes Control, 1997. **8**(1): p. 49-56.
6. Steiner, E., D. Klubert, and D. Knutson, *Assessing breast cancer risk in women.* Am Fam Physician, 2008. **78**(12): p. 1361-6.

7. Clamp, A., S. Danson, and M. Clemons, *Hormonal and genetic risk factors for breast cancer.* Surgeon, 2003. **1**(1): p. 23-31.
8. *Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer.* Lancet, 1996. **347**(9017): p. 1713-27.
9. Beral, V., *Breast cancer and hormone-replacement therapy in the Million Women Study*. Lancet, 2003. **362**(9382): p. 419-27.
10. Emery, J., A. Lucassen, and M. Murphy, *Common hereditary cancers and implications for primary care.* Lancet, 2001. **358**(9275): p. 56-63.
11. Antoniou, A., et al., *Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies.* Am J Hum Genet, 2003. **72**(5): p. 1117-30.
12. Bittner, J.J., *Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence in Mice.* Science, 1936. **84**(2172): p. 162.
13. Ross, S.R., *MMTV infectious cycle and the contribution of virus-encoded proteins to transformation of mammary*

tissue. J Mammary Gland Biol Neoplasia, 2008. **13**(3): p. 299-307.

14. Cardiff, R.D. and N. Kenney, *Mouse mammary tumor biology: a short history.* Adv Cancer Res, 2007. **98**: p. 53-116.
15. Medina, D., *Premalignant and malignant mammary lesions induced by MMTV and chemical carcinogens.* J Mammary Gland Biol Neoplasia, 2008. **13**(3): p. 271-7.
16. Medina, D. and K.B. DeOme, *Effects of various oncogenic agents on tumor-producing capabilities of series D BALB-c mammary nodule outgrowth lines.* J Natl Cancer Inst, 1970. **45**(2): p. 353-63.
17. Fernandez, S.V. and J. Russo, *Estrogen and xenoestrogens in breast cancer.* Toxicol Pathol, 2010. **38**(1): p. 110-22.
18. Mesa-Tejada, R., et al., *Immunohistochemical detection of a cross-reacting virus antigen in mouse mammary tumors and human breast carcinomas.* J Histochem Cytochem, 1978. **26**(7): p. 532-41.
19. Moore, D.H., et al., *Type B particles in human milk.* Tex Rep Biol Med, 1969. **27**(4): p. 1027-39.
20. Al-Sumidaie, A.M., et al., *Particles with properties of retroviruses in monocytes from patients with breast cancer.* Lancet, 1988. **1**(8575-6): p. 5-9.
21. Callahan, R., et al., *Detection and cloning of human DNA sequences related to the mouse mammary tumor virus genome.* Proc Natl Acad Sci U S A, 1982. **79**(18): p. 5503-7.

22. Schlom, J., et al., *Quantitation of RNA tumor viruses and viruslike particles in human milk by hybridization to polyadenylic acid sequences.* Science, 1973. **179**(74): p. 696-8.
23. Keydar, I., et al., *Properties of retrovirus-like particles produced by a human breast carcinoma cell line: immunological relationship with mouse mammary tumor virus proteins.* Proc Natl Acad Sci U S A, 1984. **81**(13): p. 4188-92.
24. Strauchen, J.A., et al., *Search for retrovirus-like particles in human breast cancer cells in culture.* Cancer Res, 1980. **40**(11): p. 3880-5.
25. Burns, J.C., et al., *Polymerase activity in lymphocyte culture supernatants from patients with Kawasaki disease.* Nature, 1986. **323**(6091): p. 814-6.
26. Kahl, L.P., et al., *An evaluation of the putative human mammary tumour retrovirus associated with peripheral blood monocytes.* Br J Cancer, 1991. **63**(4): p. 534-40.
27. Boller, K., et al., *Evidence that HERV-K is the endogenous retrovirus sequence that codes for the human teratocarcinoma-derived retrovirus HTDV.* Virology, 1993. **196**(1): p. 349-53.
28. Muster, T., et al., *An endogenous retrovirus derived from human melanoma cells.* Cancer Res, 2003. **63**(24): p. 8735-41.

29. May, F.E. and B.R. Westley, *Characterization of sequences related to the mouse mammary tumor virus that are specific to MCF-7 breast cancer cells.* Cancer Res, 1989. **49**(14): p. 3879-83.
30. Wang, Y., et al., *Detection of mammary tumor virus env gene-like sequences in human breast cancer.* Cancer Res, 1995. **55**(22): p. 5173-9.
31. Etkind, P., et al., *Mouse mammary tumor virus-like ENV gene sequences in human breast tumors and in a lymphoma of a breast cancer patient.* Clin Cancer Res, 2000. **6**(4): p. 1273-8.
32. Ford, C.E., et al., *Progression from normal breast pathology to breast cancer is associated with increasing prevalence of mouse mammary tumor virus-like sequences in men and women.* Cancer Res, 2004. **64**(14): p. 4755-9.
33. Lawson, J.S., et al., *Presence of mouse mammary tumour-like virus gene sequences may be associated with morphology of specific human breast cancer.* J Clin Pathol, 2006. **59**(12): p. 1287-92.
34. Witt, A., et al., *The mouse mammary tumor virus-like env gene sequence is not detectable in breast cancer tissue of Austrian patients.* Oncol Rep, 2003. **10**(4): p. 1025-9.
35. Bindra, A., et al., *Search for DNA of exogenous mouse mammary tumor virus-related virus in human breast cancer samples.* J Gen Virol, 2007. **88**(Pt 6): p. 1806-9.

36. Liu, B., et al., *Identification of a proviral structure in human breast cancer.* Cancer Res, 2001. **61**(4): p. 1754-9.
37. Zammarchi, F., et al., *MMTV-like sequences in human breast cancer: a fluorescent PCR/laser microdissection approach.* J Pathol, 2006. **209**(4): p. 436-44.
38. Melana, S.M., et al., *Characterization of viral particles isolated from primary cultures of human breast cancer cells.* Cancer Res, 2007. **67**(18): p. 8960-5.
39. Melana, S.M., et al., *Detection of human mammary tumor virus proteins in human breast cancer cells.* J Virol Methods, 2010. **163**(1): p. 157-61.
40. Jackson, R.B. and C.C. Little, *The Existence of Non-Chromosomal Influence in the Incidence of Mammary Tumors in Mice.* Science, 1933. **78**(2029): p. 465-6.
41. Bittner, J.J., *The Milk-Influence of Breast Tumors in Mice.* Science, 1942. **95**(2470): p. 462-3.
42. Duesberg, P.H. and P.B. Blair, *Isolation of the nucleic acid of mouse mammary tumor virus (MTV).* Proc Natl Acad Sci U S A, 1966. **55**(6): p. 1490-7.
43. Coffin, J.M., *Structure and Classification of Retroviruses*. 1st ed ed. 1992.
44. Ryan, F.P., *Human endogenous retroviruses in health and disease: a symbiotic perspective.* J R Soc Med, 2004. **97**(12): p. 560-5.
45. Morinaga, N., Y. Kaihou, and M. Noda, *Purification, cloning and characterization of variant LukE-LukD with*

strong leukocidal activity of staphylococcal bi-component leukotoxin family. Microbiol Immunol, 2003. **47**(1): p. 81-90.

46. Teramoto, Y.A., R.D. Cardiff, and J.K. Lund, *The structure of the mouse mammary tumor virus: isolation and characterization of the core.* Virology, 1977. **77**(1): p. 135-48.
47. Luther, S.A. and H. Acha-Orbea, *Mouse mammary tumor virus: immunological interplays between virus and host.* Adv Immunol, 1997. **65**: p. 139-243.
48. Acha-Orbea, H., et al., *Interplays between mouse mammary tumor virus and the cellular and humoral immune response.* Immunol Rev, 1999. **168**: p. 287-303.
49. Herman, A., et al., *Superantigens: mechanism of T-cell stimulation and role in immune responses.* Annu Rev Immunol, 1991. **9**: p. 745-72.
50. Golovkina, T.V., J.P. Dudley, and S.R. Ross, *B and T cells are required for mouse mammary tumor virus spread within the mammary gland.* J Immunol, 1998. **161**(5): p. 2375-82.
51. Tsubura, A., et al., *Intervention of T-cells in transportation of mouse mammary tumor virus (milk factor) to mammary gland cells in vivo.* Cancer Res, 1988. **48**(22): p. 6555-9.
52. Cohen, J.C. and H.E. Varmus, *Endogenous mammary tumour virus DNA varies among wild mice and segregates during inbreeding.* Nature, 1979. **278**(5703): p. 418-23.

53. Karapetian, O., et al., *Retroviral infection of neonatal Peyer's patch lymphocytes: the mouse mammary tumor virus model.* J Exp Med, 1994. **180**(4): p. 1511-6.
54. Held, W., et al., *Superantigen-reactive CD4+ T cells are required to stimulate B cells after infection with mouse mammary tumor virus.* J Exp Med, 1993. **177**(2): p. 359-66.
55. Held, W., et al., *Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission.* Cell, 1993. **74**(3): p. 529-40.
56. Kim, M.H. and D.O. Peterson, *Stimulation of basal transcription from the mouse mammary tumor virus promoter by Oct proteins.* J Virol, 1995. **69**(8): p. 4717-26.
57. Yanagawa, S., et al., *Mouse mammary tumor virus with rearranged long terminal repeats causes murine lymphomas.* J Virol, 1993. **67**(1): p. 112-8.
58. Goodrich, D.W. and P.H. Duesberg, *Retroviral recombination during reverse transcription.* Proc Natl Acad Sci U S A, 1990. **87**(6): p. 2052-6.
59. Golovkina, T.V., et al., *Generation of a tumorigenic milk-borne mouse mammary tumor virus by recombination between endogenous and exogenous viruses.* J Virol, 1997. **71**(5): p. 3895-903.
60. Golovkina, T.V., *A novel mechanism of resistance to mouse mammary tumor virus infection.* J Virol, 2000. **74**(6): p. 2752-9.

61. Moore, D.H. and J.A. Holben, *Observations on the question of horizontal transmission of mouse mammary tumor virus.* Cancer Res, 1978. **38**(8): p. 2455-7.
62. Yamamoto, K.R., *Steroid receptor regulated transcription of specific genes and gene networks.* Annu Rev Genet, 1985. **19**: p. 209-52.
63. M., N.S.a.M.C., *Mammari neoplasia in mice.* . Advance Cancer Research, 1973. **17**: p. 353-414.
64. van Leeuwen, F. and R. Nusse, *Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer.* Semin Cancer Biol, 1995. **6**(3): p. 127-33.
65. Callahan, R., *MMTV-induced mutations in mouse mammary tumors: their potential relevance to human breast cancer.* Breast Cancer Res Treat, 1996. **39**(1): p. 33-44.
66. Nusse, R. and H.E. Varmus, *Wnt genes.* Cell, 1992. **69**(7): p. 1073-87.
67. Basilico, C. and D. Moscatelli, *The FGF family of growth factors and oncogenes.* Adv Cancer Res, 1992. **59**: p. 115-65.
68. Jakobovits, A., et al., *Two proto-oncogenes implicated in mammary carcinogenesis, int-1 and int-2, are independently regulated during mouse development.* Proc Natl Acad Sci U S A, 1986. **83**(20): p. 7806-10.
69. Wilkinson, D.G., et al., *Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse.* EMBO J, 1988. **7**(3): p. 691-5.

70. Grimm, S.L. and S.K. Nordeen, *Mouse mammary tumor virus sequences responsible for activating cellular oncogenes.* J Virol, 1998. **72**(12): p. 9428-35.
71. Peters, G. and C. Glover, *tRNA's and priming of RNA-directed DNA synthesis in mouse mammary tumor virus.* J Virol, 1980. **35**(1): p. 31-40.
72. Varmus, H.E., et al., *The origin and structure of endogenous retroviral DNA.* Ann N Y Acad Sci, 1980. **354**: p. 379-83.
73. Majors, J.E. and H.E. Varmus, *Nucleotide sequences at host-proviral junctions for mouse mammary tumour virus.* Nature, 1981. **289**(5795): p. 253-8.
74. Redmond, S.M. and C. Dickson, *Sequence and expression of the mouse mammary tumour virus env gene.* EMBO J, 1983. **2**(1): p. 125-31.
75. Xu, L., T.J. Wrona, and J.P. Dudley, *Strain-specific expression of spliced MMTV RNAs containing the superantigen gene.* Virology, 1997. **236**(1): p. 54-65.
76. Mertz, J.A., et al., *Mouse mammary tumor virus encodes a self-regulatory RNA export protein and is a complex retrovirus.* J Virol, 2005. **79**(23): p. 14737-47.
77. Massey, R.J. and G. Schochetman, *Gene order of mouse mammary tumor virus precusor polyproteins and their interaction leading to the formation of a virus.* Virology, 1979. **99**(2): p. 358-71.
78. Dickson, C. and M. Atterwill, *Composition, arrangement and cleavage of the mouse mammary tumor virus*

polyprotein precursor Pr77gag and p110gag. Cell, 1979. **17**(4): p. 1003-12.

79. Hizi, A., et al., *Analysis of gag proteins from mouse mammary tumor virus.* J Virol, 1989. **63**(6): p. 2543-9.
80. Hansen, M., et al., *Transport and assembly of gag proteins into Moloney murine leukemia virus.* J Virol, 1990. **64**(11): p. 5306-16.
81. Fleissner, E. and E. Tress, *Chromatographic and electrophoretic analysis of viral proteins from hamster and chicken cells transformed by Rous sarcoma virus.* J Virol, 1973. **11**(2): p. 250-62.
82. Elder, J.H., et al., *Distinct subsets of retroviruses encode dUTPase.* J Virol, 1992. **66**(3): p. 1791-4.
83. Menendez-Arias, L., et al., *Purification of immature cores of mouse mammary tumor virus and immunolocalization of protein domains.* J Virol, 1992. **66**(9): p. 5615-20.
84. Moore, R., et al., *Complete nucleotide sequence of a milk-transmitted mouse mammary tumor virus: two frameshift suppression events are required for translation of gag and pol.* J Virol, 1987. **61**(2): p. 480-90.
85. O'Hare, P. and R.W. Honess, *Identification of a subset of herpesvirus saimiri polypeptides synthesized in the absence of virus DNA replication.* J Virol, 1983. **46**(1): p. 279-83.
86. Sen, G.C., W. Zablocki, and N.H. Sarkar, *Gene order of murine mammary tumor virus gag proteins and env proteins.* Virology, 1980. **106**(1): p. 152-4.

87. Knight, A.M., et al., *Biochemical analysis of the mouse mammary tumor virus long terminal repeat product. Evidence for the molecular structure of an endogenous superantigen.* Eur J Immunol, 1992. **22**(3): p. 879-82.
88. Choi, Y., P. Marrack, and J.W. Kappler, *Structural analysis of a mouse mammary tumor virus superantigen.* J Exp Med, 1992. **175**(3): p. 847-52.
89. Damsky, C.H., et al., *Is there a role for actin in virus budding?* J Cell Biol, 1977. **75**(2 Pt 1): p. 593-605.
90. Sarkar, N.H. and J. Racevskis, *Expression and disposition of the murine mammary tumor virus (MuMTV) envelope gene products by murine mammary tumor cells.* Virology, 1983. **126**(1): p. 279-300.
91. Maldarelli, F., N.W. King, Jr., and M.J. Yagi, *Effects of cytoskeletal disrupting agents on mouse mammary tumor virus replication.* Virus Res, 1987. **7**(4): p. 281-95.
92. Indik, S., et al., *Rapid spread of mouse mammary tumor virus in cultured human breast cells.* Retrovirology, 2007. **4**: p. 73.
93. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
94. Friedlander, A. and R. Patarca, *Endogenous proviruses.* Crit Rev Oncog, 1999. **10**(1-2): p. 129-59.
95. Lee, Y.N. and P.D. Bieniasz, *Reconstitution of an infectious human endogenous retrovirus.* PLoS Pathog, 2007. **3**(1): p. e10.

96. Flockerzi, A., et al., *Human endogenous retrovirus HERV-K14 families: status, variants, evolution, and mobilization of other cellular sequences.* J Virol, 2005. **79**(5): p. 2941-9.

97. Knossl, M., R. Lower, and J. Lower, *Expression of the human endogenous retrovirus HTDV/HERV-K is enhanced by cellular transcription factor YY1.* J Virol, 1999. **73**(2): p. 1254-61.

98. Cordonnier, A., J.F. Casella, and T. Heidmann, *Isolation of novel human endogenous retrovirus-like elements with foamy virus-related pol sequence.* J Virol, 1995. **69**(9): p. 5890-7.

99. Gifford, R. and M. Tristem, *The evolution, distribution and diversity of endogenous retroviruses.* Virus Genes, 2003. **26**(3): p. 291-315.

100. Andersson, M.L., et al., *Diversity of human endogenous retrovirus class II-like sequences.* J Gen Virol, 1999. **80 (Pt 1)**: p. 255-60.

101. Wang-Johanning, F., et al., *Expression of human endogenous retrovirus k envelope transcripts in human breast cancer.* Clin Cancer Res, 2001. **7**(6): p. 1553-60.

102. Lower, R., *The pathogenic potential of endogenous retroviruses: facts and fantasies.* Trends Microbiol, 1999. **7**(9): p. 350-6.

103. Armbruester, V., et al., *A novel gene from the human endogenous retrovirus K expressed in transformed cells.* Clin Cancer Res, 2002. **8**(6): p. 1800-7.

104. Reus, K., et al., *HERV-K(OLD): ancestor sequences of the human endogenous retrovirus family HERV-K(HML-2).* J Virol, 2001. **75**(19): p. 8917-26.

105. Medstrand, P. and J. Blomberg, *Characterization of novel reverse transcriptase encoding human endogenous retroviral sequences similar to type A and type B retroviruses: differential transcription in normal human tissues.* J Virol, 1993. **67**(11): p. 6778-87.

106. Yin, H., et al., *Transcription of human endogenous retroviral sequences related to mouse mammary tumor virus in human breast and placenta: similar pattern in most malignant and nonmalignant breast tissues.* AIDS Res Hum Retroviruses, 1997. **13**(6): p. 507-16.

107. Willer, A., et al., *Two groups of endogenous MMTV related retroviral env transcripts expressed in human tissues.* Virus Genes, 1997. **15**(2): p. 123-33.

108. Allred, D.C., P. Brown, and D. Medina, *The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer.* Breast Cancer Res, 2004. **6**(6): p. 240-5.

109. Eccles, S.A. and D.R. Welch, *Metastasis: recent discoveries and novel treatment strategies.* Lancet, 2007. **369**(9574): p. 1742-57.

110. Ronckers, C.M., C.A. Erdmann, and C.E. Land, *Radiation and breast cancer: a review of current evidence.* Breast Cancer Res, 2005. **7**(1): p. 21-32.

111. Russo, J. and I.H. Russo, *The role of estrogen in the initiation of breast cancer.* J Steroid Biochem Mol Biol, 2006. **102**(1-5): p. 89-96.
112. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
113. Cianfrocca, M. and W. Gradishar, *New molecular classifications of breast cancer.* CA Cancer J Clin, 2009. **59**(5): p. 303-13.
114. Riester, M., et al., *A differentiation-based phylogeny of cancer subtypes.* PLoS Comput Biol, 2010. **6**(5): p. e1000777.
115. zur Hausen, H., *Papillomaviruses in the causation of human cancers - a brief historical account.* Virology, 2009. **384**(2): p. 260-5.
116. Schlom, J., S. Spiegelman, and D. Moore, *RNA-dependent DNA polymerase activity in virus-like particles isolated from human milk.* Nature, 1971. **231**(5298): p. 97-100.
117. Schlom, J. and S. Spiegelman, *Simultaneous detection of reverse transcriptase and high molecular weight RNA unique to oncogenic RNA viruses.* Science, 1971. **174**(11): p. 840-3.
118. Goedert, J.J., C.S. Rabkin, and S.R. Ross, *Prevalence of serologic reactivity against four strains of mouse mammary tumour virus among US women with breast cancer.* Br J Cancer, 2006. **94**(4): p. 548-51.

119. Stewart, T.H., et al., *Breast cancer incidence highest in the range of one species of house mouse, Mus domesticus.* Br J Cancer, 2000. **82**(2): p. 446-51.
120. Cotterchio, M., V. Nadalin, and M. Sauer, *Human breast cancer and lymphomas may share a common aetiology involving Mouse Mammary Tumour Virus (MMTV).* Med Hypotheses, 2002. **59**(4): p. 492-4.
121. Russ, J.E. and E.F. Scanlon, *Identical cancers in husband and wife.* Surg Gynecol Obstet, 1980. **150**(5): p. 664-6.
122. Wetchler, B.B. and B. Simon, *Carcinoma of the breast occurring in a husband and wife: a brief communication.* Mt Sinai J Med, 1975. **42**(3): p. 205-6.
123. Lawson, J.S., et al., *Mouse mammary tumor virus-like sequences in human breast cancer.* Cancer Res, 2010. **70**(9): p. 3576-85.
124. Reis-Filho, J.S. and S.R. Lakhani, *The diagnosis and management of pre-invasive breast disease: genetic alterations in pre-invasive lesions.* Breast Cancer Res, 2003. **5**(6): p. 313-9.
125. Abdel-Fatah, T.M., et al., *Morphologic and molecular evolutionary pathways of low nuclear grade invasive breast cancers and their putative precursor lesions: further evidence to support the concept of low nuclear grade breast neoplasia family.* Am J Surg Pathol, 2008. **32**(4): p. 513-23.

126. Simpson, J.F., *Update on atypical epithelial hyperplasia and ductal carcinoma in situ.* Pathology, 2009. **41**(1): p. 36-9.
127. Heaphy, C.M., et al., *Genomic instability demonstrates similarity between DCIS and invasive carcinomas.* Breast Cancer Res Treat, 2009. **117**(1): p. 17-24.
128. Allred, D.C., et al., *Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution.* Clin Cancer Res, 2008. **14**(2): p. 370-8.
129. Xu, S., et al., *Evidence of chromosomal alterations in pure usual ductal hyperplasia as a breast carcinoma precursor.* Oncol Rep, 2008. **19**(6): p. 1469-75.