EXPRESSION PROFILES OF DIFFERENTIALLY EXPRESSED GENES OF RAT MAMMARY ADENOCARCINOMA IN VARIOUS TUMOR CELL LINES AND EFFECTS OF SOME ANTIOXIDANTS

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ABSTRACT

Cancer is the most frequent reason of death in humans after heart disease. Most of the cancers are caused by mutations in tumor suppressor genes that play distinct roles in tumor formations. If a tumor suppressor gene is mutated, it loses its function to control cell division which may lead to overgrowth of cells that leads to tumor formation. It is important to identify the genes involved in tumor formation and metastasis to develop new cancer prevention and treatment methods.

In a previous study, differentially expressed genes were identified between poorly metastatic and highly metastatic cell lines of rat mammary adenocarcinoma R3230AC. Eight cDNA clones from poorly metastatic CAb.D5 cell line and six cDNA clones from highly metastatic LN4.D6 cell line were identified (Gunes and Carlsen, 2003). The aim of this study was to investigate expression profiles of these differentially expressed genes in a set of different adenocarcinoma cell lines to find if they have any relation with metastasis. Cell culture and stocks of sixteen different cell lines were prepared and RNA from these cells were isolated. After synthesizing cDNA, RT-PCR analysis was carried out using primers specific for each cDNA clone. It was found that the gene clones FF-10 and SG-1 were expressed in non-metastatic cells but not in metastatic cells suggesting that they may have a tumor suppressive potential.

Antioxidants are chemicals that prevents oxidation and free radical damages on cells. Because there is some evidence that antioxidants may prevent tumor formation, effects of antioxidants such as green tea catechins, beta carotene, lycopene as well as zeolite were examined on cancer cell growth and expression profiles of the differentially expressed genes. The cells were treated with different amounts of antioxidants and the cell growth was determined by MTT assay. The effects of antioxidants in gene expression were identified by RT-PCR analysis. At 100µM and higher concentrations, epigallocatechingallate (EGCG), epigallocatechin (EGC) and beta carotene significantly inhibited cell growth. Lycopene affected the cell growth at 3µM concentration. FH-2 expression decreased by 1.8-fold with lycopene treatment. In addition, EGCG increased the SG-1 expression by 1.14-fold. No effects of antioxidants as well as zeolite on the expression of other differentially expressed genes were observed. For further studies, investigation of expression profiles of differentially

expressed genes in primary and secondary tumors of human will give more definitive results for the metastatic relevance of these genes.

ÖZ

Kanser insanlar arasında, kalp hastalığından sonra en sık görülen ölüm nedenidir. Kanserlerin çoğu mutasyonlar nedeniyle oluşur. Tümör baskılayıcı genler tümör oluşumlarınnda belirgin rol oynarlar. Eğer bir tümör baskılayıcı gen mutasyona uğrarsa, tümörü oluşturan fazla hücre bölünmesine öncülük edebilecek olan hücre bölünmesini kontrol etme fonksiyonunu yitirir.Yeni tedavi edici ve önleyici metodlara sahip olmak için tumör oluşumu ve metastazda etki gösteren genleri saptamak önemlidir.

Bir önceki çalışmada, sıçan meme adenokarsinomasında (R3230AC) az metastatik ve çok metastatik olan hücre hatları arasında farklı ifade edilen genler saptanmıştır. Az metastatik hücre hattı CAb.D5'dan sekiz, çok metastatik hücre hattı olan LN4.D6'dan altı klon elde edilmiştir (Güneş ve Carlsen, 2003). Bu verilere göre, bu çalışmanın amacı, farklı ifade edilen bu genlerin metastatik ilgileri olup olmadığını bulmak için bir seri farklı adenokarsinoma hücre hatlarında ifade edilme profillerini araştırmaktır. On altı farklı hücrenin hücre kültürü ve stokları hazırlanmış ve bu hücrelerden RNA izole edilmiştir. cDNA sentezinden sonra, her cDNA klonu için kullanılan özel primerlerle RT-PCR analizi gerçekleştirilmiştir. Buna göre FF-10 ve SG-1 genlerinin tümör baskılayıcı potansiyele sahip olma durumunu işaret eder biçimde metastatik olmayan hücrelerde sentezlendiği ama metastatik olan hücrelerde sentezlenmediği görülmüştür.

Antioksidanlar hücrelerde oksidasyonu ve serbest radikal zararını önleyici kimyasal maddelerdir. Antioksidanların tümör oluşumunu engelleyebileceğini belirten bazı kanıtlar nedeniyle, yeşil çay, beta karoten, likopen ve bunların yanısıra zeolitin kanser hücre büyümesi ve farklı olarak ifade edilmiş genlerin ifade edilme profilleri üzerine etkileri incelenmiştir. Hücreler farklı miktarlarda antioksidanlara maruz bırakılmış ve hücre büyümeleri MTT metoduyla izlenmiştir. Antioksidanların gen ekspresyonu üzerindeki etkileri RT-PCR analizi ile belirlenmiştir. Yüz mikromolar ve üzeri konsantrasyonlarda epigallokateşingalleyt (EGCG), epigallokateşin (EGC) ve beta karoten belirgin biçimde hücre büyümesini engellemiştir. Likopen üç mikromolar konsantrasyonda hücre büyümesini etkilemiştir. FH-2 geninin ekspresyonu likopen etkisiyle 1.8-kat azalmıştır. Ek olarak, EGCG, SG-1 geninin ekspresyonunu 1.14-kat arttırmıştır. Ayrıca antioksidanlardan zeolitin farklı ifade edilen genlerin ekspresyonu

üzerine etkisi görülmemiştir. Daha ilerki çalışmalar için farklı ifade edilmiş bu genlerin primer ve sekonder insan tümörlerinde eskpresyon profillerinin incelenmesi bu genlerin metastatik ilgisi için daha kesin sonuçlar elde etmemizi sağlayacaktır.

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ABBREVIATIONS

β-ME	:2-Mercaptoethanol
DMSO	:Dimethyl Sulfoxide
EGC	:Epigallocatechin
EGCG	:Epigallocatechin gallate
EDTA	:Disodium Salt Dihydrate
FBS	:Fetal Bovine Serum
FCS	:Fetal Calf Serum
GAPDH	:Glyseraldehyde-3-phosphate dehydrogenase
GTC	:Green Tea Catechin
MTT	:3-{4,5-dimethylthiazol-2-yl}-2,5 diphenyl tetrazolium bromide
OD	:Optical density
PBS	:Phosphate-Buffered Saline
PCR	:Polymerase Chain Reaction
RPMI-1640	:Roswell Park Memorial Institude-1640
RT-PCR	:Reverse transcriptase polymerase chain reaction
TAE	:Tris Acetate
THF	:Tetrahydrofurane

CHAPTER 1

INTRODUCTION

Cancer is one of the major health problems in the world. It is the second most frequent cause of death in industrialized countries. Men have 44% chance of having malignancy, while women have 38% chance of having malignancy at some point during their lifetime.

Cancer is a disease characterized by uncontrolled cell division and invasion of these cells to the other tissues where they can be localized. Cancer is caused by genetic aberrations activating oncogenes and inactivating tumor suppressor genes. Most of the mutations leading to cancer are acquired during the evolution of cancer which is termed as tumorigenesis. Some mutations may also be inherited. Furthermore, environmental factors play a role in cancer formation. Studying the molecular mechanisms of malignancies provide development of new tools for diagnosis, prognosis and treatment of cancer (Porkka and Visakorpi 2004).

Cell proliferation is a normal, physiologic process in which mutations may lead to cancer formation changing the order of this process. Tumor suppressor genes have role in regulating cell proliferation and cell death. When a mutation occurs in a tumor suppressor gene, this change may result in unregulation of cell growth causing tumor formation. If the tumor does not spread any other part of the body, it is called benign tumor. If it does, then it is called malignant tumor (Rieger 2004). This is why tumor suppressor genes have a great importance for exploring new ways in cancer research.

1.1. Tumor and Tumor Formation

When cells can not be controlled in growth and division, they form a mass of tissue which is called *tumor.* The term *tumor* originally means “swelling” but this term is now primarily used to denote abnormal growth of tissue. Tumors are divided into two groups. Benign tumors are tumors that do not spread into other tissues. They grow slowly in the originated area and have a good prognosis. This does not mean that they can not cause a problem. Benign tumors can cause damage depending on the tissue they grow such as brain. The other group of tumors is malignant tumors. Malignant tumors are tumors that invade the surrounding tissues and different areas of body.

Malignant tumors grow fastly (Artner, 2001). The differences between benign and malignant tumors can be summarized as;

Benign vs Malignant

- Benign cells have limited growth potential while malignant cells proliferate rapidly
- Benign cells are localized, but malignant cells metastasize
- Benign cells have fibrous capsule, malignant cells have no enclosing capsule
- Benign cells rarely recur after removal, malignant cells form irregular shape with poorly defined borders
- Benign cells are much different than parent cells (degrees of anaplasia), malignant cells are much different than parent cells (degrees of anaplasia) (Figure 1.1).

  For malignant cells morphological changes include;

  1. pleomorphism of nuclei and cells

  2. coarsely distributed DNA

  3. increased number of nucleoli

  4. disorderly cell growth (abnormal mitosis) (Porth, 1998)

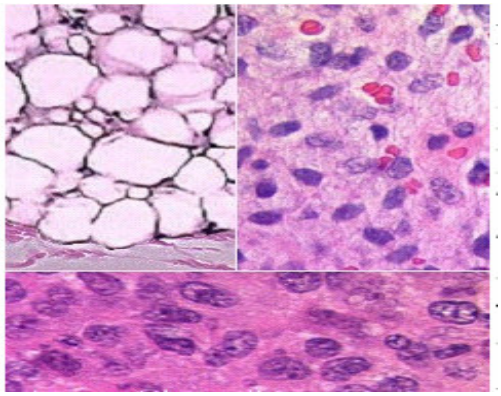


Figure 1.1. The upper part of the figure on the left shows the fat-tissue including on the left benign *Lipoma* and on the right malignant *Liposarcoma*. In Lipoma cells are large and it is well-differentiated. The cellular structures are typical for the origin of tissue. The capsule at the bottom of the Lipoma can be seen as a sign of non-invading benign neoplasm. Liposarcoma shows lack of differentiation. There is no capsule and the cells are different from the original tissue in structure. The cells are smaller than the normal with varying shapes. The lower part shows the anaplastic carcinoma including variable

cellular, nuclear size and shape. The tissue of origin can not be determined (Source: Artner 2001).

Cell proliferation normally takes place in the regulation of cell cycle. It is controlled by growth factors binding to receptors on the cell surface. These receptors are connected to signaling molecules that convey message from receptor to the nucleus. All these regulate the protein formation which cause continous cell division. If any mutation occurs in any of the genes encoding these proteins, cell proliferation can not be controlled resulting in tumor occurance (King 1996).

A cancer is formed from a tumor, but a tumor is not always cancerous. Cancer is first described for breast carcinoma (Hippocrates) and malignant tumors have been found in Egyptian mummies nearly 5000 years old. Ancient medical writings such as Edwin Smith and Ebers papyrusses mention about tumors about 3500 years ago. The Greek term “karkinos” means cancer and it is described for the abnormal cells. This latin description for cancer is first used by Galen (199 a.Chr.). Cancer is now being used as malignant growth of cells. (Artner 2001)

1.2. Carcinogenesis and Stages of Tumor Initiation

All cells have a standard growth cycle and physical specifications. When cells are effected by carcinogens, changes in their growth cycle and physical specifications are seen, which is termed as carcinogenesis. Carcinogenesis have several steps. Damage by carcinogenic factors occurs at the beginning of malignant transformation and is called initiation. These damage cause changes in proteins, DNA, or signaling pathway. The initiation is followed by promotion. Promotion step includes further damage on cells in which genome damage can not be repaired. Increase in clonal growth is seen in promotion step. Initiation and promotion can be endogenous (genetic mutations) or exogenous (chemicals or viral). During carcinogenesis, the normal program including differentiation and proliferation of the cell changes. Henceforth, growth, angiogenesis, invasion, and metastasis become possible in carcinogenesis step. Progression is the final step in carcinogenesis in which a number of cells grow forming a mass called tumor. Factors such as immune system response, localization, blood support and the rate of growth play an important role in this process. Metastasis is the last stage in which a

tumor becomes a cancer. Tumor progression and occurance of metastatic capacity need additional changes in gene expression (Artner 2001, Yoshida et al. 2002).

1.3. Cancer and Metastasis

Cancer is a genetic disease mostly arising from accumulation of several mutations. Cancer cells over proliferate by their failure of cell cycle control (Griffiths 2002). These cells continously divide and lead to formation and growth of tumors. Another important case for cancer formation is the acquired ability of cancerous cells to leave the primary tumor and forming colonies at secondary sites (Lodish 2002). This process is known as metastasis and it is caused by the changes of cell to cell adhesion of tumor cells (Tepass 2001). These cells find a way to escape from primary tumor and travel within the circulatory or lymph system until they attach themselves to a new site which is known as secondary site (Lodish 2002). In this secondary site these cells invade the tissue by activating various molecules. For example, if breast cancer spreads to the lung, the secondary tumor is made up of abnormal breast cancer cells, not abnormal lung cancer cells. This means the disease in the lung is the metastatic breast cancer, not lung cancer. Usually, carcinomas spread by hematogenous route, and the sarcomas spread by lypmhatic route. Adrenal glands, brain, liver and bones are the most common parts of the body for metastasis. There are also some types of tumors that only seed in particular organs. For example, prostate cancer usually metastases to the bones while the stomach cancer mostly metastases to the ovary in women. Cancer cells may also spread to regional lymph nodes around the primary tumor site. This is called nodal involvement or regional disease (King 1996, Nicolson 1991).

In metastasis, to be able to leave the original tumor site and migrate to other parts of the body, malignant cells attach to extracellular matrix (ECM) by degrading the proteins surrounding and separating the tumor from the adjoining tissue. To complete the metastatic journey of the tumor cell, this is followed by two events, cell migration and angiogenesis (Ahmad and Hart 1997).

Cell migration is the movement of cells from one part of the body to another (Figure 1.2). Cell migration is properly required for normal organ and structural formations. When
cells fail to migrate or migrate to inappropriate parts, abnormal development is observed. Metastasis occurs by the migration of cancer cells from primary tumor site to secondary sites where they invade normal tissue causing proliferation and formation of new tumors (Tepass 2001).

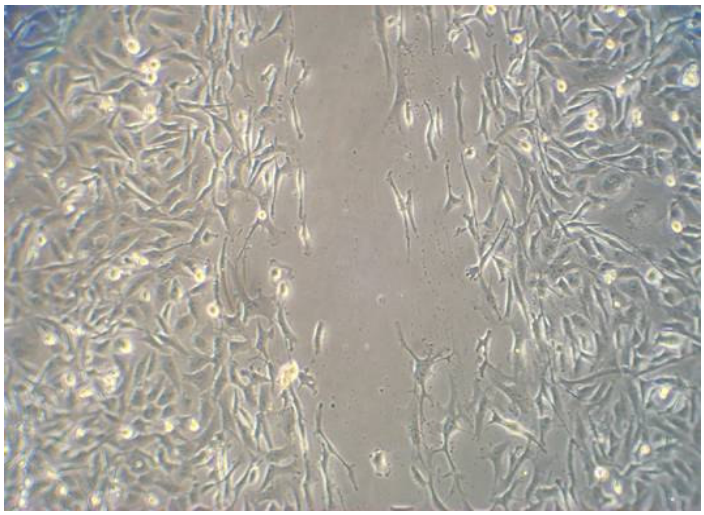


Figure 1.2. The cells are moving and migrating. (Source: http://cf.unc.edu/our/slides/Weisner04_files/slide0001_image002.jpg)

During cancer research, it is discovered that for tumors to grow beyond 2mm in three dimensions, one of the required events is the formation of the new blood vessels which is called angiogenesis . Tumor angiogenesis is the proliferation of a new blood vessel system to supply oxygen and nutrient intake and removal of waste materials.

Tumor cells send signals to normal host tissue and these signals activate the genes related to growth of blood vessels. Such new blood vessels for tumor cells are thin-walled so that tumor cells can easily exit the primary site to metastasize. The angiogenesis consists of stages which are 1) dissolution of the basement membrane, 2) endothelial cell migration, 3) endothelial cell proliferation, 4) vascular loop formation and 5) development of new basal membrane (Ahmad and Hart 1997, Hanahan and Weinberg 2000).

Shortly, the metastatic cascade can be figured as;

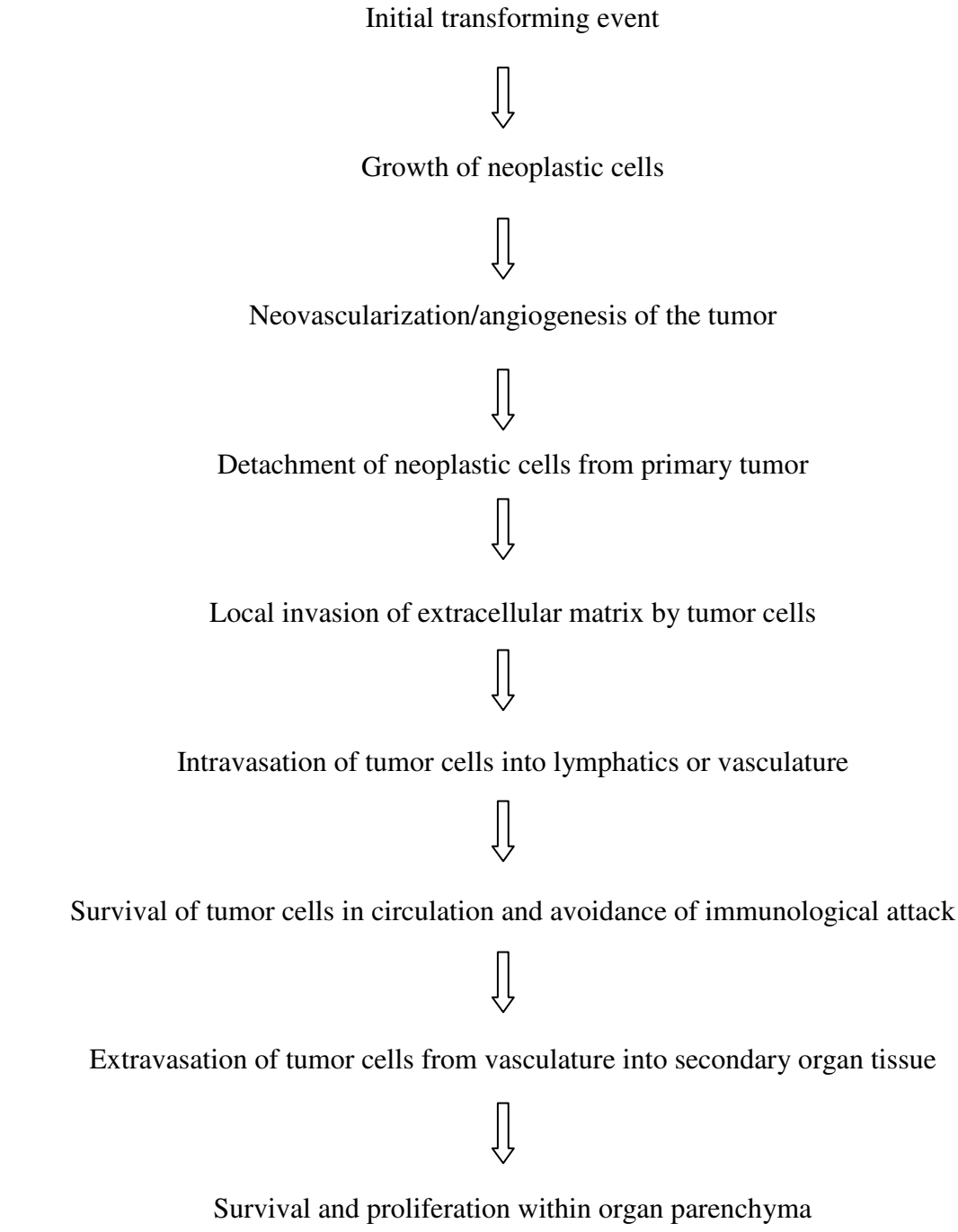


Figure 1.3. The metastatic cascade (Ahmad and Hart 1997).

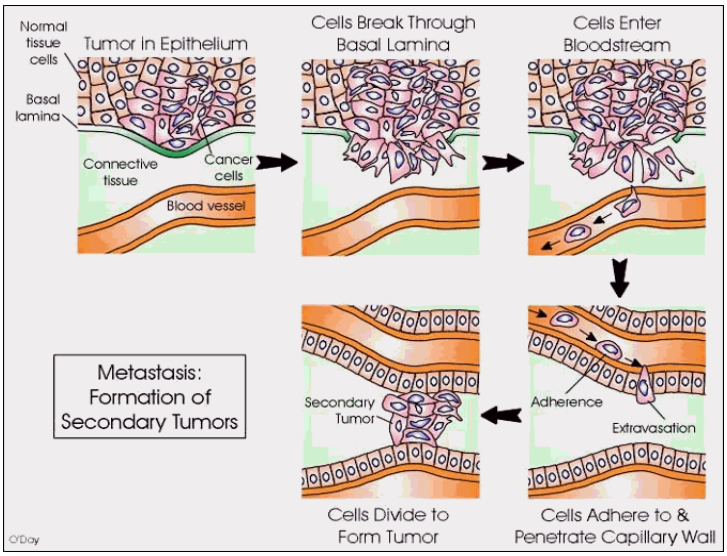


Figure 1.4. Stages of metastasis beginning from primary tumor, spreading via blood and lymph system and formation of new tumors in the secondary site (Source: http://207.68.36.7/ths/ science/apbio/ch5-8.gif).

Finally, for a successful metastasis each step required for production of metastases must be completed (Figure 1.4). If one step fails, the metastasis can not be completed.

1.4. Factors Causing Cancer

Cancer is not the result of a single effect, multiple changes can lead to cancer. There are many different factors causing cancer such as hereditary factors, viral factors, radiation, chemical factors, immune system factors, environmental factors, life style and dietary factors (Artner 2001).

Several mutations are needed for a cell to become cancerous. These mutations involve oncogenes and tumor suppressor genes. Mutations may have different causes. Some of them belongs to the specific cancer cases. Smoking is related with lung cancer, exposure to radiation or ultraviolet radiation from sun is associated with skin cancer (melanoma). Chemicals and free radicals also cause mutations and form cancer. Mutations can also be inherited such as BRCA1 gene which is a factor in developing breast and ovarian cancer. Some types of viruses also cause mutations. Herpesviruses,

Papillomaviruses, and retroviruses are called as oncoviruses carrying oncogenes and tumor suppressor inactivating genes in their genomes (Rieger 2004, Artner 2001).

It is not possible to define the initial cause for any specific cancer. By the developing molecular biology techniques, it is possible to charactarize the mutations in a tumor. For example, tumor suppressor gene p53 is known as "the guardian of the genome" because of its potential, when mutated, to cause formation of most tumors.

1.5. Tumor Suppressor Genes

Tumor suppressor genes are genes whose loss of function may lead to malignancy (Osborne et al. 2004). Tumor suppressor genes are normally found in our cells. They function in regulating diverse cellular activities such as cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell differentiation and migration and tumor angiogenesis. Over the last 15 years many tumor suppressor genes have been identified (Sherr 2004). When a mutation occurs in a tumor suppressor gene, this may result in tumor formation or growth. A mutation in a tumor suppressor gene is like an automobile with a dysfunctional brake, the car does not stop even the driver attempts to stop it (Vogelstein and Kinzler 2004).

The first experiments about the activity of tumor suppressor genes were done including somatic cell hybridization experiments by Henry Harris and his colleagues in 1969 (Cooper 2000). Isolation of tumor suppressor genes in the late 1980s and 1990s has improved the studies on genetic alterations in human cancer cells (Yokota 2000). Retinablastoma (Rb) was the first identified tumor suppressor gene in 1986 (Friend et al. 1986). Later on, more than 20 tumor suppressor genes have been identified to date (Haber and Harlow 1997, Fearon et al. 1997). The second identified tumor suppressor gene was p53 which is now very popular in cancer research. Inactivated p53 is very common among most of the mutations taking role in tumor initiation. This makes p53 the most common target for genetic alterations in human cancers (Cooper 2000).

Tumor suppressor genes are mostly recessive at the cellular level because they need to be mutated for both copies of the gene pairs to be able to cause malignancy. Mutations in tumor suppressor genes mostly occur as the result of aging and/or environmental factors. A mutation of a tumor suppressor gene can be inherited from a parent. When a mutation in the second copy occurs the process of tumor formation

begins. This is also called "two-hit theory" (Knudson 1971). "Two-hit" hypothesis states that tumor suppressor genes are inactivated following a recessive mutation in one allele and the loss of other wild-type allele (Weir et al. 2004). The genes related with hereditary cancer are mostly tumor suppressor genes.

Identification of tumor suppressor genes are more difficult than oncogenes. They are important for marker development as their loss may be related with the metastatic potential. They also provide new aspects for therapeutic targets. The studies done about the mechanisms of loss of metastatic suppressor gene expression suggest that metastatic suppressor genes are not mutated but are differentially expressed (Steeg et al. 2003).

Table 1.1 (cont**.).** Selected Tumor Suppressor Genes and Associated Protein Function (Table modified from Table 1 of Fearon ER. Tumor suppressor genes. In: Vogelstein B, Kinzler KW, eds. The Genetic Basis of Human Cancer, 2nd Edition. McGraw-Hill: New York, NY. In press.)

Gene	Associated inherited cancer syndrome	Cancers with somatic mutations	Presumed function of protein
RB1	Familial retinoblastoma	Retinoblastoma, osteosarcoma, SCLC, breast, prostate, bladder, pancreas, esophageal, others	Transcriptional regulator; E2F binding
TP53	Li-Fraumeni syndrome	Approx. 50% of all cancers (rare in some types, such as prostate carcinoma and neuroblastoma)	Transcription factor; regulates cell cycle and apoptosis
INK4a			
p16	Familial melanoma, Familial pancreatic carcinoma	Approx. 25-30% of many different cancer types (e.g., breast, lung, pancreatic, bladder)	Cyclin-dependent kinase inhibitor (i.e., Cdk4 and Cdk6)
p19^{ARF}	?Familial melanoma?	Approx. 15% of many different cancer types	Regulates Mdm-2 protein stability and hence p53 stability; alternative reading frame of *p16/INK4a* gene

(cont.on next page)

APC	Familial adenomatous polyposis coli (FAP), Gardner syndrome, Turcot's syndrome	Colorectal, desmoid tumors	Regulates levels of β-catenin protein in the cytosol; binding to microtubules
BRCA1	Inherited breast and ovarian cancer	Ovarian (~10%), rare in breast cancer	DNA repair; complexes with Rad 51 and BRCA2; transcriptional regulation
BRCA2	Inherited breast (both female and male), pancreatic cancer, ?others?	Rare mutations in pancreatic, ?others/	DNA repair; complexes with Rad 51 and BRCA1
WT-1	WAGR, Denys-Drash Syndrome	Wilms' tumor	Transcription factor
NF-1	Neurofibromatosis type 1	Melanoma, neuroblastoma	p21ras-GTPase
NF-2	Neurofibromatosis type 2	Schwannoma, meningioma, ependymoma	Juxtamembrane link to cytoskeleton
VHL	von-Hippel Lindau syndrome	Renal (clear cell type), hemangioblastoma	Regulator of protein stability
MEN-1	Multiple endocrine neoplasia type 1	Parathyroid adenoma, pituitary adenoma, Endocrine tumors of the pancreas	Not known
PTCH	Gorlin syndrome, hereditary basal cell carcinoma syndrome	Basal cell skin carcinoma, medulloblastoma	Transmembrane receptor for sonic hedgehog factor; negative regulator of smoothened protein
PTEN/MMAC1	Cowden's syndrome; sporadic cases of juvenile polyposis syndrome	Glioma, breast, prostate, follicular thyroid carcinoma, head and neck squamous carcinoma	Phosphoinositide 3-phosphatase; protein tyrosine phosphatase
DPC4	Familial juvenile polyposis syndrome	Pancreatic(~50%), approx. 10–15% of colorectal cancers, rare in others	Transcriptional factor in TGF-β signaling pathway
E-CAD	Familial diffuse-type gastric cancer; Lobular breast cancer	Gastric (diffuse type), lobular breast carcinoma, rare in other types (e.g., ovarian)	Cell-cell adhesion molecule

(cont.on next page)

LKB1/STK1	Peutz-Jeghers syndrome	Rare in colorectal, not known in others	Serine/threonine protein kinase
SNF5/INI1	Rhabdoid predisposition syndrome (renal; or extra-renal malignant rhabdoid tumors), choroid plexus carcinoma medulloblastoma; central primitive neuroectodermal tumors)	Rare in rhabdoid tumors, choroid plexus carcinoma, medulloblastoma	Member of the SWI/SNF chromatin ATP-dependent remodeling complex
EXT1	Hereditary multiple exostoses	Not known	Glycosyltransferase; heparan sulfate chain elongation
EXT2	Hereditary multiple exostoses	Not known	Glycosyltransferase; heparan sulfate chain elongation
TSC1	Tuberous sclerosis	Not known	Not known; cytoplasmic vesicle localization
TSC2	Tuberous sclerosis	Not known	Putative GTPase activating protein for Rap1 and rab5; golgi localization
MSH2, *MLH1* *PMS1, PMS2*, *MSH6*	Hereditary non-polyposis colorectal cancer	Colorectal, gastric, endometrial	DNA mismatch repair

1.6. Cancer Research and Model Organisms

Metastasis is the most lethal stage of a cancer. The gene expression analysis of known tumor suppressors and oncogenes provide an understanding approach of context of early tumorigenesis and role of these genes in cancer formation (Hahn and Weinberg. 2002). Developments in complete sequencing of human genome have provided an acceleration in discovery of cancer-related genes and pathways (Baak et al. 2003). Some organisms have metabolic events similar to the events in humans. These organisms led scientists create models helping to understand processes that take place in and cause cancer. Model organisms including Drosophila melanogaster and the mouse have been used to perform experiments to study genetic alterations leading to cancer (Griffith 2002). The experiments done by Chakraborty and Yamaga (2003) provided

information about regulation of metastasis by exploring the effects of in vitro fusion of poorly metastatic melanoma cells with macrophages from mice which produced hybrids with metastatic potentials. Tumor formation and development in Drosophila constitutes another model for understanding cancer mechanisms. Especially, it is used in genetic techniques allowing rapid identification and characterization of related genes (Potter 2000).

The search for metastasis-suppressor genes was first initiated in the late 1980s (Yoshida et al. 2000). Identifying these genes are important as they have a functional role in the acquisition of metastatic ability. This research needs well-characterized in vivo (animal) models completed with experimental studies. However, in vitro models do not directly reflect in vivo metastasis (Welch D.R. 1997). As the metastasis is a complex and multigenic phenotype, several markers are needed to distinguish metastatic ability of tumor cells. Observing tumor cell growth at metastatic level is an important clinical target since inhibition of such growth requires identification of genes or proteins regulating metastatic colonization (Yoshida et al. 2000). Thus, gene expression profiling especially detected by sensitive methods such as RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) has become an issue of interest (Gomella et al. 1997).

Finally, the identification of genes and pathways involving tumor growth and metastasis will provide us both understanding of the cancer biology and will give new targets for early detection and treatment of cancer. Gene expression profiling has also provided information for tumor subclassification (Garnis et al. 2004). Additionally, working with model organisms will hopefully provide answers to many questions in terms of the molecular mechanisms of metastasis.

1.7. Antioxidants

Several anticarcinogens and antimutagens have the ability to inhibit propagation of peroxidation processes by peroxy radicals which is called as antioxidant property (Pziezak 1986). Peroxy products (H_2O_2, ROOH) and different oxygen radicals (-OH, -O_2, ROO-, ARO-) have an important role in carcinogenesis (Chiu et al. 1982). According to many studies, it is suggested that dietary antioxidants are critical in cancer prevention and research on this area is gaining interest (Cutler 1984).

An antioxidant is a chemical which prevents oxidation of other chemicals. In biological systems, during oxidation, highly reactive free radicals are produced. These free radicals may damage the other molecules in the body by reacting with them. The research on antioxidant function in cancer treatment is a rapidly evolving area. They have been comprehensively studied for their ability to prevent cancer (Singh and Lippman 1998).

In many studies, it was suggested that antioxidants may interfere with alkylating agents (Labriola and Livingston 1999). Antioxidants have been put forward to increase cell death (apoptosis) by this mechanism (Chinery et al. 1997, Mediavilla et al. 1999). Several animal studies have been published showing decreased tumor size and/or increased longevity with the combination of chemotherapy and antioxidants (Seifter et al. 1984, Berry et al. 1984).

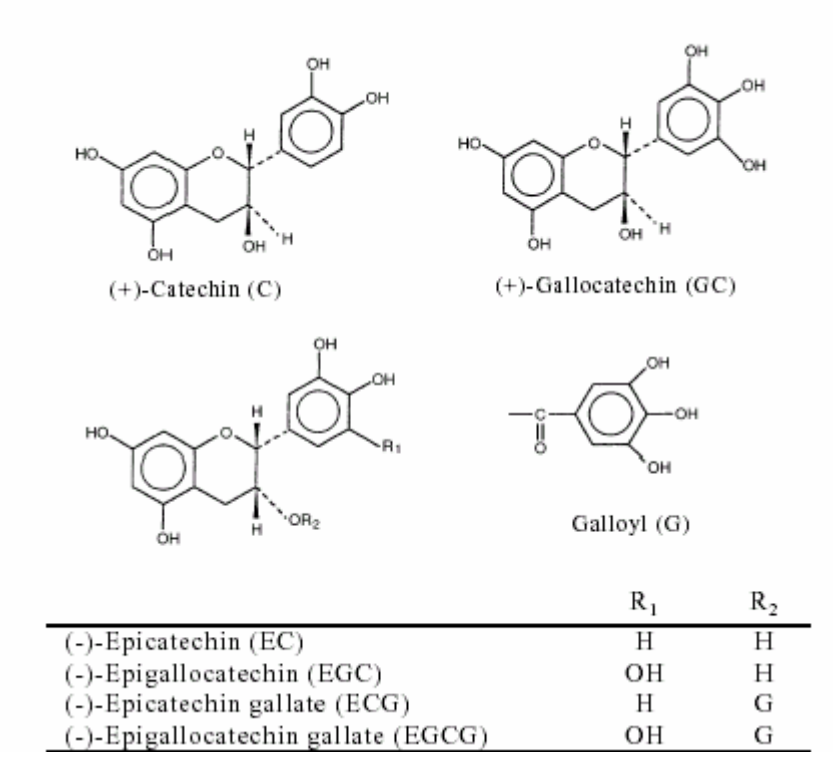
Many chemicals, such as retinol (vitamin A or beta-carotene), ascorbic acid (vitamin C), vitamin E (tocopherol), selenium, melatonin, coenzyme Q10, n-acetylcysteine, glutathione, and flavanoids have been used as antioxidants for cancer treatment and prevention studies (Halliwell 1999).

1.8. Green Tea Catechins

There is increasing evidence that specific substances found in certain foods such as phenolic compounds found in plants. Flavanoids are powerful antioxidants in vitro and they are found in tea. They have been identified as cancer preventing components (Middleton et al. 1994, Smith et al. 2001, Lynn-Cook et al. 1999). Tea has been shown to inhibit tumorigenesis at the initiation, promotion and progression stages of cancer. Their mechanisms have been suggested as including; 1) antioxidant activity, 2) ability to inhibit nitrosamine reactions, 3) modulation of carcinogen-metabolising enzymes, 4) ability to inhibit cell proliferation (Dreosti et al. 1997). Some studies have shown that there is an inverse association between green tea drinking and stomach cancer (Bushman 1998, Setiawan et al. 2001).

There are mainly three types of tea that are green tea, black tea and oolong tea-differing in chemistry and how they are produced. A typical green tea beverage contains 30-42% catechins by dry weight. These catechins are (-)-epigallocatechin (EC), (-)-

epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG).



	R_1	R_2
(-)-Epicatechin (EC)	H	H
(-)-Epigallocatechin (EGC)	OH	H
(-)-Epicatechin gallate (ECG)	H	G
(-)-Epigallocatechin gallate (EGCG)	OH	G

Figure 2.1. Structure of green tea polyphenols (Lin and Liang, 2000).

The effects of EGCG and the other polyphenols on cancer prevention may be the result of decreased cell transformation and proliferation or increased apoptosis. In many studies, in vitro, tea catechins, especially EGCG have been shown to cause inhibition in growth of various human tumor cell lines including melanoma, lung cancer, breast cancer, leukemia, and colon cancer (Chung et al. 2001, Yang et al. 1998).

1.9. Beta Carotene

Carotenoids are naturally occuring pigments synthesized by plants and microorganisms, but not by animals. Beta carotene is a group of carotenoids which has two cyclo hexene type end groups (Goodwin 1980).

By the early 1980s large number of epidemiological studies have shown that beta carotene can prevent cancer (Willett et. al 1984). Several studies have shown that beta carotene and other carotenoids have lipid-soluble antioxidant activity. Beta carotene is studied mostly in homogenous lipid solutions, in membrane models and in

intact cells resulting as a less effective antioxidant than α-tocopherol (Woodall et al. 1997). In recent studies, increase in lung cancer cells after treatment with beta carotene suggested that beta carotene might possess co-carcinogenic potential. Epidemiological and experimental observations have indicated that fruits and vegetables rich in carotenoids may have a role in cancer treatment and prevention (Paolini et al. 2003).

Beta carotene is scavenger of peroxyl radicals at low oxygen tension (Burton and Ingold 1984). The antioxidant actions of beta carotene are based on its ability to bind peroxyl radicals.

1.10. Lycopene

Lycopene is a natural pigment and it is only synthesized by plants and microorganisms. It is a carotenoid and an acyclic isomer of beta carotene. Recent studies on lycopene has focused on its antioxidant properties (Rao and Agarwal 2000). Lycopene has been shown as an inhibitor on the growth of human endometrial, mammary and lung cancer cells and it was reported as more effective than α or β carotene (Levy et al. 1995). Epidemiological studies on the role of lycopene in relation to chronic diseases have focused primarily on cancers (Rao and Agarwal 2000). Some scientists have reported inverse association of lycopene with breast cancer risk (Potischman et al. 1990, Zhang et al. 1997).

1.11. Zeolites

Zeolite is a popular and important group of minerals for industrial and other purposes. Zeolite is crystalline aluminosilicate with fully cross-linked open framework structure made up of corner sharing SiO_4 and AlO_4 tetrahedra. The name “zeolite” comes from the Greek words zeo (to boil) and lithos (stone). It was named by Swede minerologist Fredrich Cronstdet who explored the mineral in 1756.

The main structure of a zeolite is constituted by SiO_4 and/or AlO_4. Most significant features of this structure are pores and cavities providing ability to release water in these pores and cavities at high temperatures without losing its original form; and possessing loosely bounded, exchangeable cations. Therefore it can successfully be used in adsorption, ion exchange and dehydration processes. More than 40 natural and

more than 150 artifical types of zeolites are being used today in fields such as agriculture and stockbreeding, pollution control, energy applications, and mining and metallurgy (Breck 1974, Tsitsishvili et al. 1992).

Clinoptilolite is a type of natural zeolite which is most abundant in nature. In clinoptilolite, changes can be seen in the composition of framework and exchangeable cations such as $K^{+,}$, Na^{+}, Ca^{2+}. Clinoptilolite can also be used for adsorption processes and ion exchange separations like other types of natural zeolites (Ackley et al. 1992). Clinoptilolite has been also suggested that it may show diverse biological activities. It has been reported as a potential supportive agent for anticancer therapy (Pavelić et al. 2001).

1.12. Thesis Objectives

Identification of differentially expressed tumor genes have an important place in cancer research to get a better understanding the mechanisms of malignancy and to develop markers. In a previous study, there were differentially expressed genes between poorly metastatic CAb.D5 and highly metastatic LN4.D6 cell lines of rat mammary adenocarcinoma R3230AC. In CAb.D5 8 cDNA clones (FA-8: rat alpha 2 collagen IV; FC-1: rat aldehyde dehydrogenase; FC-6: rat proalpha 1 collagen III; FC-7: ribosomal protein L4; FF-10: osteoblast/osteocyte Factor 45; FH-2: rat cytokeratin 8; SG-1) and in LN4.D6 6 cDNA clones (RB-8: neutrophil chemoattractant 2; RB-9: human LGN protein; RD-4: mouse aspartylaminopeptidase; RE-1: rat keratin (C K5) mRNA; RF-5: mouse keratin (epidermal) type 1) were identified (Gunes and Carlsen 2003).Based on the previous findings, the objectives of this study were;

- investigating the expression profiles of differentially expressed genes in various (16) adenocarcinoma and tumor cell lines by reverse transcriptase polymerase chain reaction (RT-PCR) in order to see the metastatic relevance of these genes
- investigating the effects of antioxidant compounds like green tea catechins, beta carotene, lycopene as well as a silica mineral zeolite on the growth of tumor cell lines and expression profiles of differentially expressed genes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Cell lines were kindly supplied from some colleagues as listed below. For cell culture experiments, Roswell Park Memorial Institute (RPMI)-1640 medium and Trypsin were purchased both from Sigma Chemical Company and Biological Industries Chemical Co. Fetal Bovine Serum (FBS), trypan blue, gel loading solution, MTT, sodium bicarbonate, Green tea catechin (GTC), Epigallocatechin gallate (EGCG), β carotene, Epigallocatechin (EGC), Lycopene, phenol were purchased from Sigma Chemical Company. DMSO, sodium citrate, sodium chloride were purchased from Merck Chemical Co. Fetal Calf Serum (FCS) was purchased from Biological Industries Ltd. Penicilin Streptomycine was purchased from Biochrom Chemical Co. Ethidium Bromide, 2-Mercaptoethanol, EDTA (Disodium Salt Dihydrate), sodium phosphate (monobasic and dibasic) were purchased from Amresco Chemical Co. DEPC was obtained from Serva Chemical Co. Tetrahydrofurane (THF) was purchased from Lachema Co. Zeolites were kindly supplied by Prof. Dr. Semra Ülkü, Chemical Engineering Deparment of Izmir Institute of Technology. Tissue culture plates were from Corning Star, and polypropylene tubes and disposable pipettes were obtained from Greiner.

For RNA isolation, Nucleospin® RNA II Kit and for cDNA synthesis by RT-PCR, Advantage™ RT-for-PCR Kit were purchased from BD Biosciences, Clontech.

2.2. Methods

2.2.1. Preparation of Cell Lines

There were sixteen different cell lines used for this project. Rat mammary adenocarcinoma cell lines CAb.D5, LN4.D6, 13762 CT, 13762 WT and rat prostate adenocarcinoma cell lines. AT-1 and MATLyLu were supplied by Dr. S.Carlsen,

Saskatchewan Cancer Agency, Canada. Rat prostate adenocarcinoma cell lines AT-2, AT-3 and MATLu were supplied by Dr. J.A.Schalken, University Medical Center, Experimental Urology, Nijmegen, Netherlands. Human mammary adenocarcinoma cell lines MCF-7, HBL-100, MDA-MB-453, T-47-D were supplied by Dr. M. Öztürk, Bilkent University. Human prostate adenocarcinoma cell lines LNCAP, PC-3 were supplied by Dr. Ahmet Şanlıoğlu, Akdeniz University and DU145 was supplied by GATA. Human cervix adenocarcinoma cell line HeLa and human colon adenocarcinoma cell line CAC0-2 were supplied by Dr. I. Gürhan, from Ebiltem, Ege University.

When the cell lines arrived at the laboratory, they were incubated for 2-4 hours in a CO_2 incubator at 37 ˚C in a humidified atmosphere. At the end of incubation period plaque of the flask was opened carefully and entrance of the flask was swapped with ethanol. Then culture medium was poured slowly in another vessel until 10-15 ml remained. The entrance of the flask was swapped again with ethanol and incubated overnight in a CO_2 incubator at 37 ˚C in a humidified atmosphere. Approximately 20 hours later cells were prepared for trypsinization.

For trypsinization the medium was removed from the flask and phosphate buffered saline (PBS) at pH: 7.4 was added to wash and get rid of unwanted proteins in the medium. After washing, trypsin was added to cover the surface of the cells and waited for 15-30 seconds. Trypsin was decanted until a few drops remained and incubated for 2-5 minutes depending on the cell line. At the end of the incubation, 10 ml medium (RPMI 1640 supplemented with 8% FCS, 100 µg/ml streptomycine, and 100 u/ml peniciline, pH: 7.1) was added by pipetting to disperse the adhesive tumor cells. The cell suspension was transferred into 15 ml falcon tube and centrifuged at 800 rpm twice (for 6 minutes). After removing the supernatant, cells were washed twice and the cells resuspended in the medium. Then cells were dissolved with 3 ml RPMI 1640 by pipetting up and down and 1 ml cell suspension was put into eppendorf tube to count the cells . After that, cell count was performed by using a hemacytometer. The viability of the cells was determined by the trypan blue exclusion method. The total number of cells was calculated based on the formula below.

$$\textit{Number of cells/ml} = \textit{Number of cells counted in 25 square} \times \textit{Dilution factor} \times 10^4$$

2.2.2. RNA Isolation

RNA isolation was carried out using Nucleospin® RNA II Kit (BD Biosciences, Clontech).

The cells ($1x10^6$) were put into 1.5 ml eppendorf tube and centrifuged at 8000 rpm (for 30 sec). In the tube, 350 µl RA1 solution and 3.5 µl β-ME were added and mixed by vortexing. In order to clean the cell lysate, the mixture was passed through the Nucleospin filter column by spinning at 11000 rpm for 1 min. 350 µl of 70% ethanol was added into the lysate and mixed by vortexing. A Nucleospin column was put into a 2 ml collection tube and the sample was loaded into the column. The tube was centrifuged at 8000 rpm for 30 sec. The flowthrough was discarded. Then 350 µl of Buffer MDB was added and centrifuged at 11000 rpm for 1 min. The flowthrough was discarded at the end of the centrfiugation. DNAse I mixture reconstituted before and 95 µl of DNAse I was added to the Nucleospin column and incubated at room temperature for 15 min. After incubation, 200 µl of Buffer RA2 was added and centrifuged at 8000 rpm for 30 sec. The 2 ml Nucleospin column was changed by a new one after the flowthrough was discarded. Then 600 µl of Buffer RA3 was added and the tube was centrifuged at 8000 rpm for 30 sec. The tube was placed into the column after the flowthrough was discarded. Again, 250 µl of Buffer RA3 was added and this time tube was centrifuged at 11000 rpm for 2 min. When the flowthrough was discarded the column was placed into 1.5 ml microcentrifuge tube. After that, RNA was eluted by adding 60 µl of DNAse free water and centrifuged at 11000 rpm for 1 min. The RNA isolated was quantified by UV spectroscopy. The RNA samples were stored at -80˚C until used.

2.2.3. Quantification of RNA

The quantity and quality of the RNA samples were determined by analysis of absorption profiles at 260 nm and 280 nm. If the ratio of absorption at 260 nm/280 nm was 1.8+0.01, the samples considered as pure RNA (Maniatis et al. 1982).

2.2.4. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) Analysis

Reverse transcriptase polymerase chain reaction is the technique that provides the transcription of an RNA strand into a DNA complement using reverse transcriptase enzyme. The complementary DNA is then amplified by Polymerase Chain Reaction (PCR). In this study, cDNA synthesis was achieved by the protocol in Advantage™ RT-for-PCR Kit (BD Biosciences, Clontech). All dilutions and additions were carried out on ice and all the reagents were spinned before usage. In a sterile 0.5 ml microcentrifuge tube, 12.5 µl of RNA sample including DEPC- treated water was added. According to the amount of RNA sample, 1 µl of random hexamer or the oligo (dt) primer was added. The RNA was heated at 70 ˚C for 2 min and then put on ice rapidly. A master mix including 4 µl of 5x Reaction Buffer, 1 µl dNTP mix (10 mM each), 0.5 µl Recombinant RNAse Inhibitor, and 1 MMLV Reverse Transcriptase for each RNA sample was prepared. Then 6.5 µl of master mix was added to each tube. The contents of the tube was mixed by pipetting up and down and incubated at 42 ˚C for 1 hr. After incubation, the tube was heated at 94 ˚C for 5 min to stop cDNA synthesis and to destroy DNAse activity. After the tube was spinned down, the reaction was diluted to a final volume of 100 µl by adding 80 µl of DEPC-treated water. Finally that mixture was vortexed and spinned again. The cDNA sample was stored at -80 ˚C until used. In each PCR reaction 2 µl of cDNA sample was used.

2.2.5. Polymerase Chain Reaction (PCR)

PCR is used to amplify DNA with primer pairs specific for each cDNA clone. All PCR reactions were carried out in 50 µl reaction volumes. In the PCR mixture 2 µl cDNA was mixed with 0.2 mM dNTP, 3 Mm $MgCl_2$, 1x PCR buffer, 2U Taq Polymerase and 1 µM of each primer. Amplifications were carried out in a DNA thermal cycler (Techne Progen). The initial denaturation was at 94 ˚C for 2 minutes and the final extension was at 72 ˚C for 10 minutes for all the primer pairs. Thirty-five cycles were applied, and the annealing temperatures was 54 ˚C.

The PCR products were electrophorased on 1.5% agarose-ethidium bromide gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA; pH:8.0) at 90 V for 40 minutes. Gels were visualized in a gel documentation system (Vilber Lourmat, France).

2.2.6. Determination of Cell Growth by MTT Assay

Green Tea Catechins including Epigallocatechin gallate (EGCG), Epigallocatechin (EGC), Green tea catechin (GTC); Lycopene and β carotene were used as antioxidants to see the effect of them on cell growth and gene expression profiles.

The in vitro tetrazolium-based colorimetric assay was used to measure cell growth after treatment with antioxidants. This method includes the formation of purple water insoluble formazan crystals from yellow water-soluble substrate 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by the effect of mitochondrial enzymes of metabolically active cells.

The cell lines were grown until they reach 90% confluency. After trypsinization, the cells were plated in 96 wellplate at a concentration of $2x10^4$ cell/well as duplicate. Then they were incubated at 37 ˚C for 1 day in humidified 5% CO_2 incubator.

The green tea catechins were prepared in DMSO getting the final concentrations as 1µM, 10µM, 50µM, 100µM and 200µM. After incubating the cells for 1 day, 4µl of each dilution was added into the wells. They were incubated at 37˚C for 3 days in 5% CO_2 incubator. After incubation, the medium was removed and 200µl fresh medium was added. Then 20µl MTT (5mg/ml in PBS) was added and left for incubation at 37˚C for 4 hrs in 5% CO_2 incubator. Into each well 200µl DMSO was added and they were incubated for 5 min at room temperature on orbital shaker at 150rpm. Finally the absorbance of each well was read by 540nm microplate reader.

Lycopene was dissolved in 5% tetrahydrofurane (THF). Effects of lycopene at 3, 6, 9,and 18 micromole on cell growth was determined with MTT assay.

Zeolites at 0.5mg/ml, 5mg/ml, 10mg/ml, and 20mg/ml were dissolved in RPMI 1640 medium by shaking on orbital shaker at 220rpm overnight at room temperature. Next day, each sample was centrifuged to remove the zeolite particles and supernatant was used to treat the cells. Cells were plated at $2.5x10^4$ cells/well in 96 wellplates. Cells were allowed to grow at 37˚C in humidified 5% CO_2 incubator overnight. Medium was removed and 200µl supernatant obtained from zeolite solution at different concentration

was added to each well. Cells were allowed to grow 48 hrs. After removing the medium, fresh medium was added into wells. Then, 20µl MTT solution (5mg/ml in PBS) was added and incubation was carried out for 4 hrs at 37˚C in humidified 5% CO_2 incubator. The formazan crystals formed were dissolved by addition of 200µl DMSO into each well. Optical density of color formation in each well was measured at 570nm on a microplate reader after 10 min incubation.

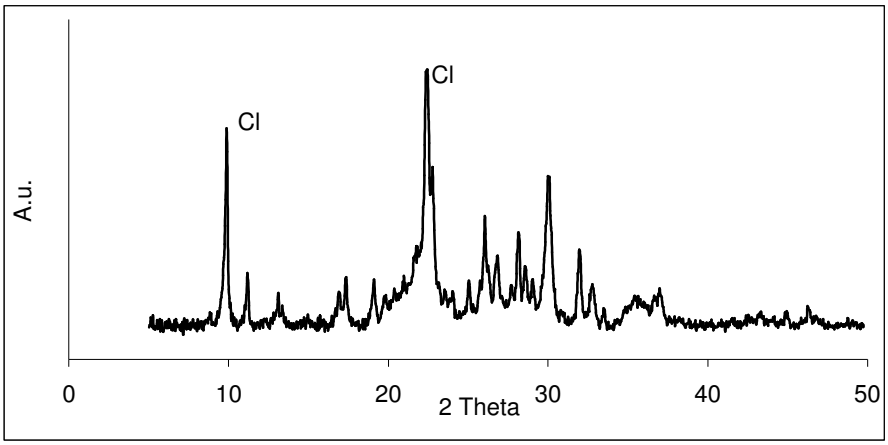


Figure 2.1. X-Ray diffraction pattern of natural zeolite

According to the X-ray diffraction pattern the zeolite used in this study was found as clinoptilolite rich natural zeolite.

The clinoptilolite content of natural zeolitic mineral was calculated by using Relative Intensity Ratio (RIR) methods. This method has been measured for one or more reflection and can be defined as the intensity of o peak of interest for a given phase divided by the intensity of a peak from a standard (50:50 mixture) (Bish and Chipera, 1995). The content of clinoptilolite was found aroun 80 %. Further study (chemical and thermal) is required for the identification of the mineral used.

2.2.7. Quantification of Gene Expression Level with RT-PCR

Cells were treated with antioxidants and zeolite as it was explained in cell growth (3.3). These reagents were EGCG, EGC, GTC, and beta carotene at 100µM, lycopene at 9µM and zeolite at 0.5mg/ml, 5mg/ml, 10mg/ml, and 20mg/ml concentrations. RNA was isolated from cells treated with each of these antioxidants. After synthesis of cDNA, each differentially expressed gene was amplified with a specific primer pair by PCR. PCR products were resolved in 1.5% agarose-ethidium

bromide gel. Intensity of DNA bands in the gel was quantified by gel documentation system (Vilber Lourmat, France) and the level of mRNA for each gene was normalized to the level of the GAPDH, internal standard.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Expression Profiles of Differentially Expressed Genes in Different Cell Lines

The expression profiles of previously described genes of rat mammary adenocarcinoma cells were investigated in various tumor cell lines by using RT-PCR. cDNA at different concentrations for each reaction were used for PCR. There was a linear relationship between log of the signal (amplified cDNA product) and log of the cDNA input (data not shown). Therefore, cDNA concentrations was chosen at a concentration in which there was a linear increase in the PCR product for each gene. The results of expression patterns of each differentially expressed gene are given below:

FA-8: (Rat alpha 2 collagen IV): When the expression level of the FA-8 clone was examined in a set of metastatic and non-metastatic rat tumor cell lines, it was expressed in non-metastatic CAb.D5 and AT-1 cells. However, no expression was observed in other non-metastatic cell lines (Figure 3.1.A).

There was no singificant expression of FA-8 in metastatic LN4.D6 cell line. As it was highly expressed in CAb.D5 cells and not expressed in LN4.D6 cells, FA-8 has been suggested to have a relationship with low metastatic potential of CAb.D5 cells (Gunes and Carlsen 2003). Even though there was almost no expression of FA-8 in LN4.D6 cells, it was expressed in other metastatic cells (Figure 3.1.A). This result suggests that FA-8 may not be related with non-metastatic potential of the cell.

FC-1: (Rat aldehyde dehydrogenase): Expression pattern of FC-1 clone did not correlate with the metastatic status of the cell because it was expressed in both metastatic and non-metastatic cell lines (Figure 3.1.B). In addition, no expression was observed in 13762 CT and AT-2 non-metastatic rat cell lines. Even though FC-1 expression was less than that of non-metastatic CAb.D5 cell. The expression level of FC-1 was 4-fold, 1.3-fold, and 2-fold less in metastatic cells LN4.D6, MATLu, MATLyLu respectively compared to non-metastatic cell line CAb.D5 (Table 3.2).

Because FC-1 was not expressed in other non-metastatic cell lines 13762 CT and AT-2, it may be suggested that FC-1 does not have metastatic suppression potential.

FC-6: (Rat pro-alpha 1 collagen III): According to the expression level of FC-6 clone in the cell lines, it was significantly expressed in non-metastatic CAb.D5 cells (Figure 3.1.C). In non-metastatic cell lines 13762 CT and AT-2 and metastatic cell line AT-3, there was no expression of FC-6. There were 3.2-fold, 4.2-fold, 5-fold and 2.4-fold expression levels of FC-6 clone in metastatic LN4.D6, MATLu, MATLyLu and non-metastatic AT-1 cells orderly (Figure 3.2).

An important step in metastatic cascade is the invasion of the extracellular matrix by tumor cells (Ahmad and Hart 1997). Extracellular matrix is made up of basement membrane and interstitial connective tissue. Collagen fibers are main components of interstitial connective tissue which functions as a barrier for tumor cell invasion. Therefore, a decrease in collagen fibers in LN4.D6 cells may be responsible for the cells to become metastatic.

However, comparison of the expression level of FC-6 clone in metastatic and non-metastatic cell lines (Figure 3.1.C) indicates that rat pro-alpha 1 collagen III is not a reason for the cells to become non-metastatic because no expression was observed in non-metastatic AT-2 and 13762 CT cells.

FF-10: (Osteoblast/osteocyte factor 45): FF-10 clone was highly expressed in non-metastatic CAb.D5 and AT-1 cells (Figure 3.1.D). Besides, there was no expression of FF-10 in other metastatic and non-metastatic cell lines.

Previously, FF-10 was described as osteoblast/osteocyte factor 45 (Petersen et al. 2000). These researchers found the expression of this gene only in bone but not in other tissues.

In this current study, FF-10 was expressed in non-metastatic CAb.D5 and AT-1; however, no expression was observed in other non-metastatic cell lines. Because there was no significant expression of FF-10 in any metastatic cell line but strong expression in non-metastatic CAb.D5 and AT-1 cell lines, it may be suggested that FF-10 prevents these cells from being metastatic.

FH-2: (Rat cytokeratin 8): When FH-2 expression was examined, the most abundant expression was seen in non-metastatic AT-1 cells when compared to other cells (Figure 3.1.E). However, FH-2 was also expressed in metastatic AT-3 and

MATLu cells having a closer level of expression for each other. In non-metastatic CAb.D5 cells, FH-2 expression was seen less than others while it was the least in metastatic MATLyLu cells.

Cytokeratins are the members of the intemediate multigene family and their expression change with the cell state (Simonishi et al. 2000). In addition, cytokeratins may be used as biomarkers to distinguish primary from metastatic carcinoma (Tot 2002). Furthermore, cytokeratin expression patterns help to discriminate types of breast cancer (Bocker et al. 2002). In a research, it was demonstrated that FH-2 was poorly expressed or not expressed in the cells of metastatic human breast carcinoma (Zoli et al. 1997). In another study, FH-2 was shown to be expressed significantly and highly in NSCLC (non-small-cell-lung cancer) than SCLC (small-cell-lung cancer) cells suggesting FH-2 may become a novel tumor marker especially in NSCLC patients. However, FH-2 expression was detected in both metastatic and non-metastatic rat cell lines.

Finally, FH-2 expression was correlated with increased invasiveness in vitro and in vivo in many cancers (Schaafsma et al. 1993). According to the results of the present study, further investigations should be done to see its metastatic relevance.

Rat SG-1: When the rat SG-1 clone was investigated in different cell lines, it was expressed in non-metastatic cell CAb.D5, AT-1, and 13762 CT significantly (Figure 3.1.F). In comparison with the quantity of each expression level of rat SG-1 in cell lines, the most abundant expression was in CAb.D5 cells, and then in 13762 CT and AT-1 cells orderly. In other words, expression of SG-1 clone was consistant in three non-metastatic cell lines However, no expression was observed in metastatic cell lines LN4.D6 and MATLu. On the other hand, slight expression was detected in MATLyLu and AT-3 metastatic cell lines.

Taken together, these results suggest that SG-1 may have tumor metastasis suppressor function. Transfection of metastatic cells with this gene and decrease in metastatic potential of the cells will confirm this result.

Human SG-1: When human SG-1 was examined to see the expression levels in various cell lines, the housekeeping gene GAPDH and human SG-1 were interfered with each other (Figure 3.1.G). Therefore, they were examined in separate PCR reactions. Human SG-1 was mostly expressed in non-metastatic cell lines including CAb.D5, AT-1, MDA-MB-453, and HBL-100. Moreover, it was expressed in

metastatic MATLu cell line appearantly. No expression was observed in LN4.D6 metastatic cell line.

RB-8: (Neutrophil chemoattractant 2): According to the expression pattern of RB-8 clone in each cell line, it was seen that there was no correlation with metastatic characteristic (Figure 3.1.H). RB-8 was expressed in both metastatic and non-metastatic cell lines. The quantifications for expression levels of RB-8 in these cells were similar to each other or the same with each other. It was only not expressed in non-metastatic cells AT-1 and AT-2.

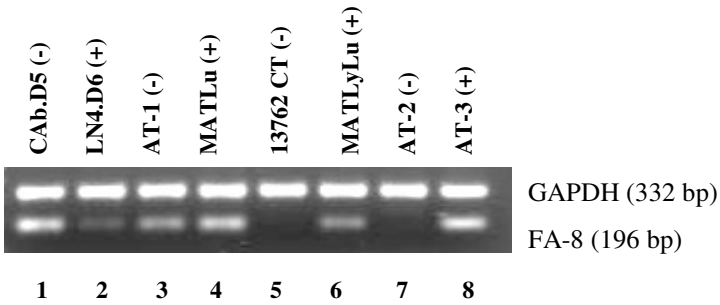
RB-8 was demonstrated as expressed only in LN4.D6 cells and it was identified as a target to investigate its cell specific expression and relation with metastatic potential of cells (Gunes and Carlsen 2003). RB-8 was expressed in both non-metastatic and metastatic cell lines in this study. The reason for the expression of RB-8 in CAb.D5 cells unlike the results of previous study might be due to the sensitivity of the techniques, Northern blot and RT-PCR, used in two different study. That is why it is not possible to indicate any metastatic relation of RB-8 clone before any other investigations have been done.

RE-1: (Rat keratin (CK 5) mRNA): Among the cell lines that RE-1 was examined, it was only expressed in metastatic cell line LN4.D6 (Figure 3.1.I). There was no expression in other metastatic and non-metastatic cell lines.

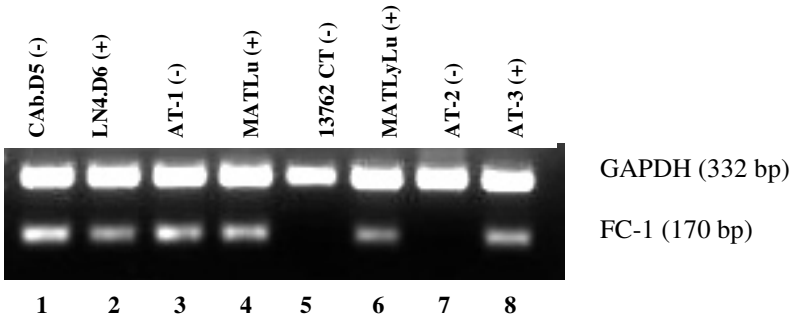
Based on the results of this study, cell specific expression of RE-1 most likely is not involved in metastatic potential of LN4.D6 cells. However, it is possible that high level expression of this gene may have synergistic effect on the induction of metastatic formation in the cell. Similarly, Hansson et al. (2001) indicated that RE-1 was highly expressed in malignant cells.

RF-5: (Mouse keratin (epidermal) type 1): When RF-5 was examined in various cell lines, abundant expression was seen in LN4.D6 compared to CAb.D5 cell line which has 1.4-fold less expression than that of LN4.D6. However, no expression was observed in other cell lines (Figure 4.1.J). In a previous study, RF-5 expression was found 2-fold higher in metastatic cells than non-metastatic cells (Pencil et al. 1993). Therefore, upregulation and down regulation of cell specific expression level of RF-5 may be involved in metastatic potential of the cells.

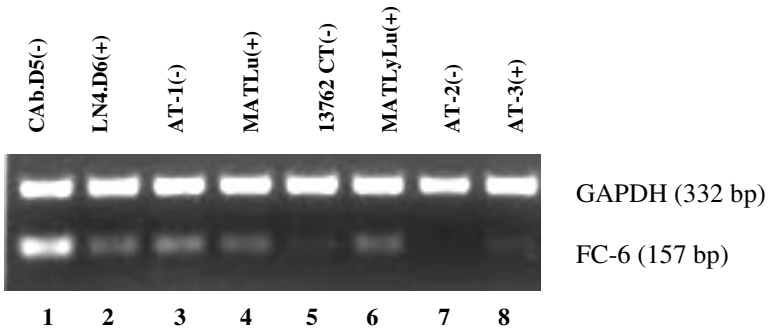
A) FA-8



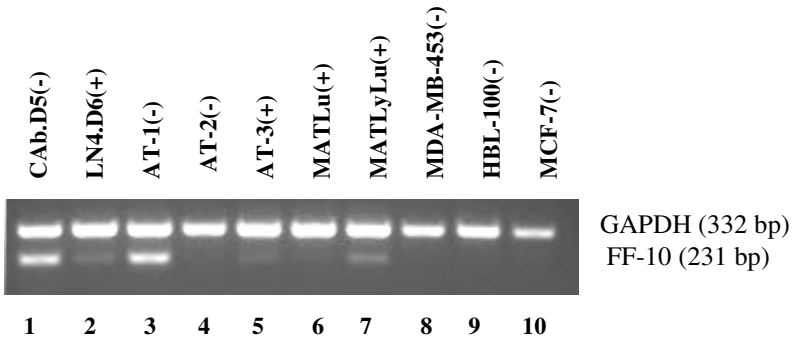
B) FC-1



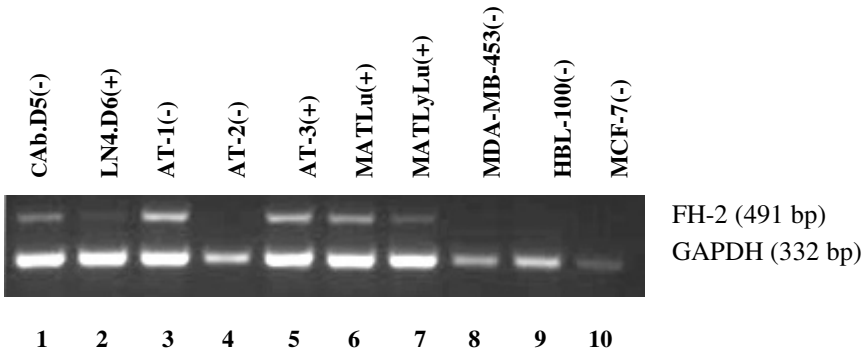
C) FC-6



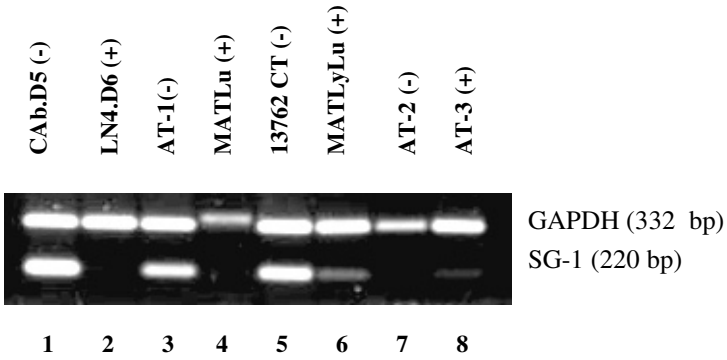
D) FF-10



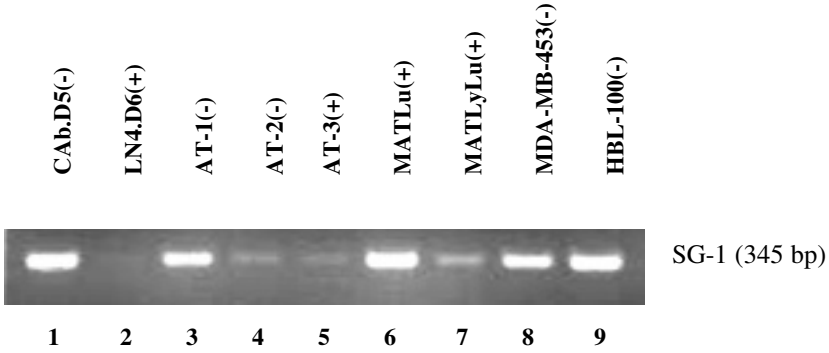
E) FH-2



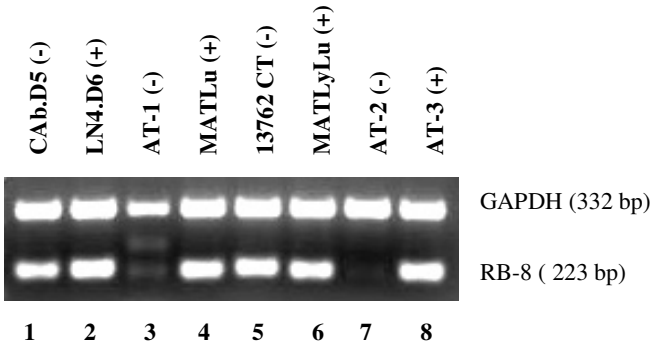
F) Rat SG-1



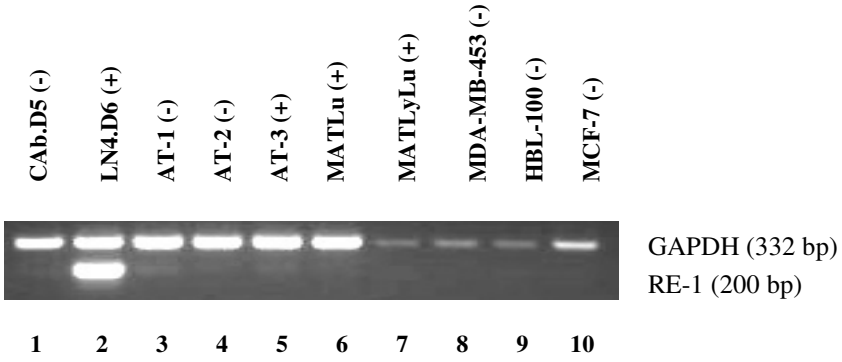
G) Human SG-1



H) RB-8



I) RE-1



J) RF-5

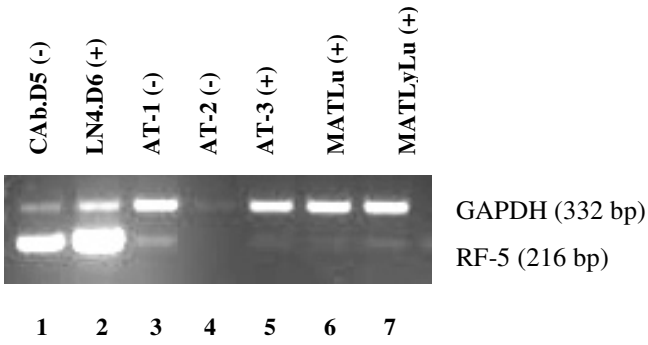


Figure 3.1. Expression profiles of differentially expressed genes in different cell lines. GAPDH was used as internal control. Metastatic cell lines were designated as (+); non-metastatic cell lines were designated as (-).

Even though expression levels of other differentially expressed genes were planned to analyze in a variety of cell lines, they could not be determined because primers which were designed for FC-7, FG-6, RB-9, RD-4 and RE-12 did not work. In addition, primers for human homolog of FF-10 were designed and tested in human tumor cell lines; however, it was expressed in every tumor cell line, examined and there was no significant difference in the expression level of FF-10 among the cell line (data not shown).

Table 3.1. Relative expression levels of mRNA of each differentially expressed gene.

Clones	**Cell lines**										
	CAb.D5	**LN4.D6**	**AT-1**	**AT-2**	**AT-3**	**MATLu**	**MATLyLu**	**MDA-MB-453**	**HBL-100**	**MCF-7**	**13762 CT**
FA-8	116	29	64	NE	116	89	54	ND	ND	ND	NE
FC-1	58	39	54	NE	43	41	32	ND	ND	ND	NE
FC-6	102	31	41	NE	NE	24	20	ND	ND	ND	NE
FF-10	77	29	64	NE	24	NE	29	NE	NE	NE	ND
FH-2	37	NE	61	NE	52	48	32	NE	NE	NE	ND
Rat SG-1	99	NE	67	NE	NE	NE	27	ND	ND	ND	78
RB-8	98	110	NE	NE	117	106	100	ND	ND	ND	110
RE-1	NE	121	31	33	28	NE	NE	NE	NE	NE	ND
RF-5	160	234	64	NE	13	20	31	ND	ND	ND	ND

Expression levels of differentially expressed genes were determined with RT-PCR analysis. The level of mRNA was quantified with gel documentation system (Vilber Lourmat, France) and normalized to the level of GAPDH, internal control.

3.2. Effects of Antioxidants and Zeolite on Cell Growth

Because there is some evidence in the literature that antioxidants have effect on the growth of tumor cells, effects of green tea catechins, beta carotene, lycopene on the growth of CAb.D5 and LN4.D6 tumor cells as well as some other tumor cell lines were investigated. In addition, a silica mineral zeolite was used to treat cells to see the effect on the cell growth.

3.2.1. Effects of Green Tea Catechins and Beta Carotene on Cell Growth

The effects of green tea catechins and beta carotene on the cell growth of HeLa and DU145 cell lines were examined in variable concentrations. When we compared the effect of green tea catechins and beta carotene on HeLa cells, general inhibition of all those antioxidants on cell growth was seen at 50 µM concentration (Figure 3.3.1.A). Especially, at 100 µM and higher concentrations EGCG, EGC, GTC and beta carotene showed similar and most significant effect on cell growth. EGCG was the primarily inhibiting green tea catechin at lower concentrations in comparison with the others. Both EGCG and EGC showed a decrease on HeLa cell growth beginning from 10 µM concentration. GTC was the least effecting catechin on cell growth among other antioxidants.

Another investigation for green tea catechins and beta carotene was done on the cell growth of prostate adenocarcinoma DU145 cells. Similar to the effects of those catechins and beta carotene on HeLa cells, EGCG, EGC, GTC and beta carotene effected growth of DU145 cells significantly at 50 µM concentration. Those four antioxidants effected DU145 cell growth most at 100µmole and higher concentrations with a range of similar value interval of each antioxidant. For DU145 cells, primarily inhibiting agents were EGCG effecting 2-fold and EGC effecting 1.5-fold among other antioxidants.

Among green tea catechins, EGCG and EGC were mostly indicated as remarkable inhibitors against the growth of tumor cells like HTC116 human colorectal tumor cells (Uesato et al. 2001). In another study, EGCG was also reported as the most

effective apoptosis inducing polyphenol present in green tea (Azam et al. 2004). EGCG was used for human pancreatic carcinoma cells at 0, 25, 50, 100 and 200 µM/L. Growth of human pancreatic carcinoma cells were suppressed by EGCG in a doze-dependent manner (Moriatsu et al. 2002). EGCG was also significant in our results for cell growth inhibition.

Many studies have shown an inverse relationship between beta carotene intake and various cancers. Levin et al. (1997) showed that beta carotene is an efficient antioxidant in inhibition of tumor cell growth in rats. In our experiments, beta carotene displayed average inhibition on tumor cell growth.

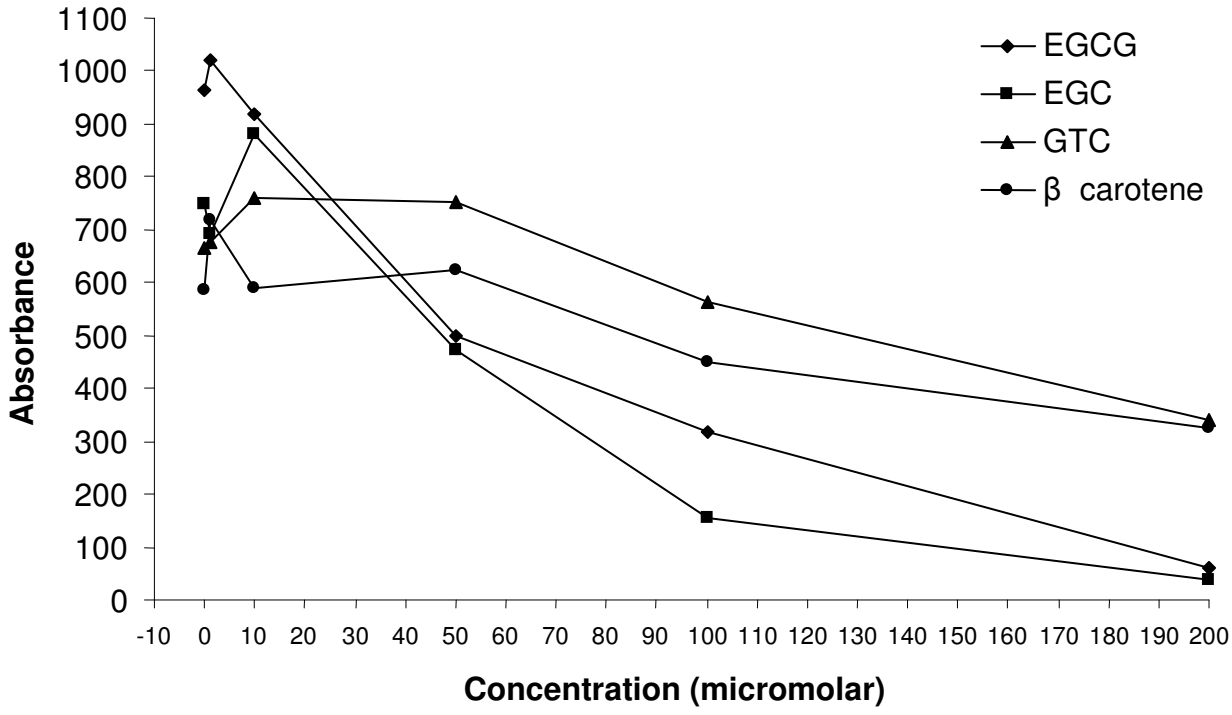


Figure 3.2.A. Effects of green tea catechins and beta carotene on the growth of HeLa cells. Data are the average of duplicate samples. Difference among absorbance value of the duplicate samples were less than 2%. The experiment was repeated twice with the similar results.

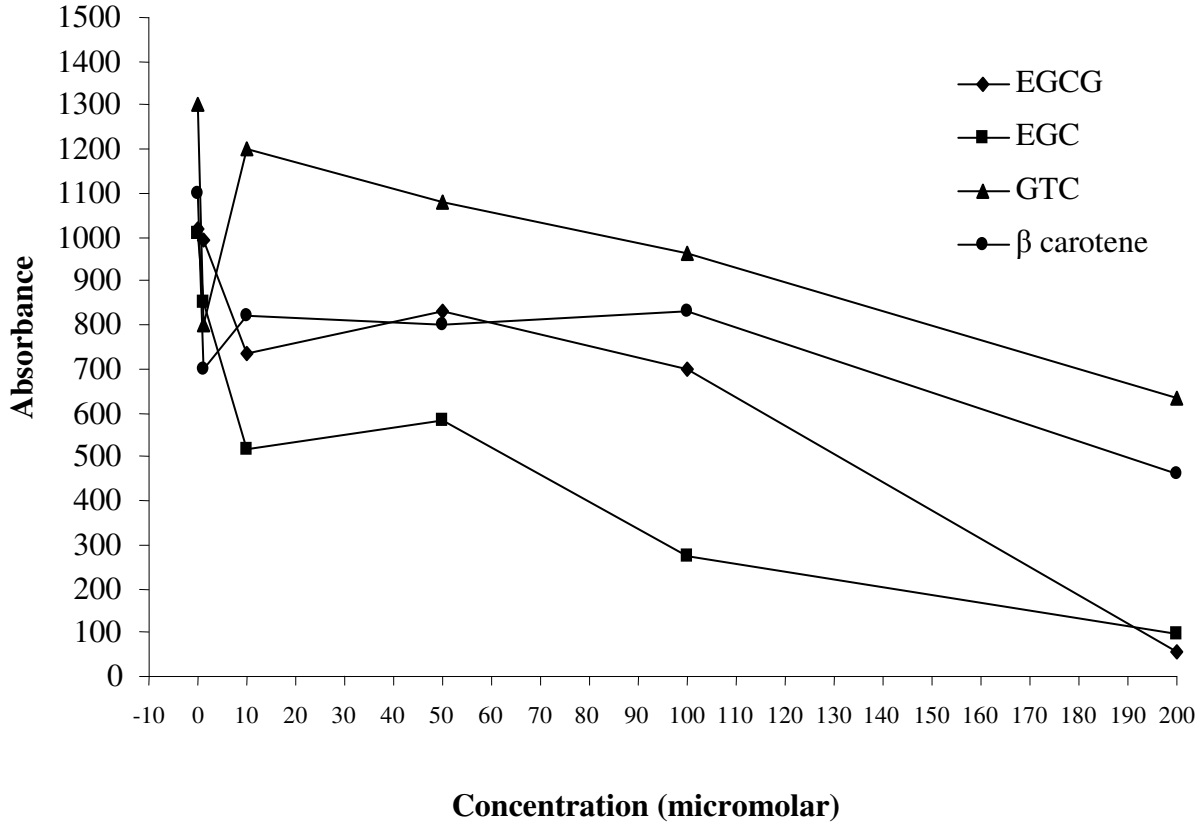


Figure 3.2.B. Effects of green tea catechins and beta carotene on the growth of DU145 cells. Data are the average of duplicate samples. Difference among the absorbance value of duplicate samples were less than 2%. The experiments were repeated twice with the similar results.

3.2.2. Effects of Lycopene on Cell Growth

Lycopene was the most effective on the growth of CAb.D5 cells compared to other cell lines. For example, at 3 µM concentration lycopene decreased the CAb.D5 cell growth 1.7-fold compared to untreated cells and it remained almost at the same level at 6 µmole and 9 µM lycopene concentrations. Until 6µM concentration, lycopene decreased LN4.D6 cell growth regularly. When the effect of lycopene was compared with each of cell lines including CAb.D5, LN4.D6, DU145 and HBL-100; lycopene showed highest decrease on cell growth at 9 µM concentration.

Lycopene was shown to be a powerful inhibitor of endometrial, mammary (MCF-7), and lung (NCI-H 226) human cancer cells proliferation (Giovannucci et al. 1995). Several groups have shown inhibition by lycopene of cancer cell growth on tissue culture experiments (Dorgan et al. 1998). It was demonstrated that lycopene inhibits mammary, endometrial, lung and leukemic cancer cell growth in a dose-dependent manner (IC_{50}~ 2 μM) (Amir et al. 1999, Levy et al. 1995). It was most significant at 9 μM concentration of lycopene in our study for inhibiting the cell growth of treated cell lines.

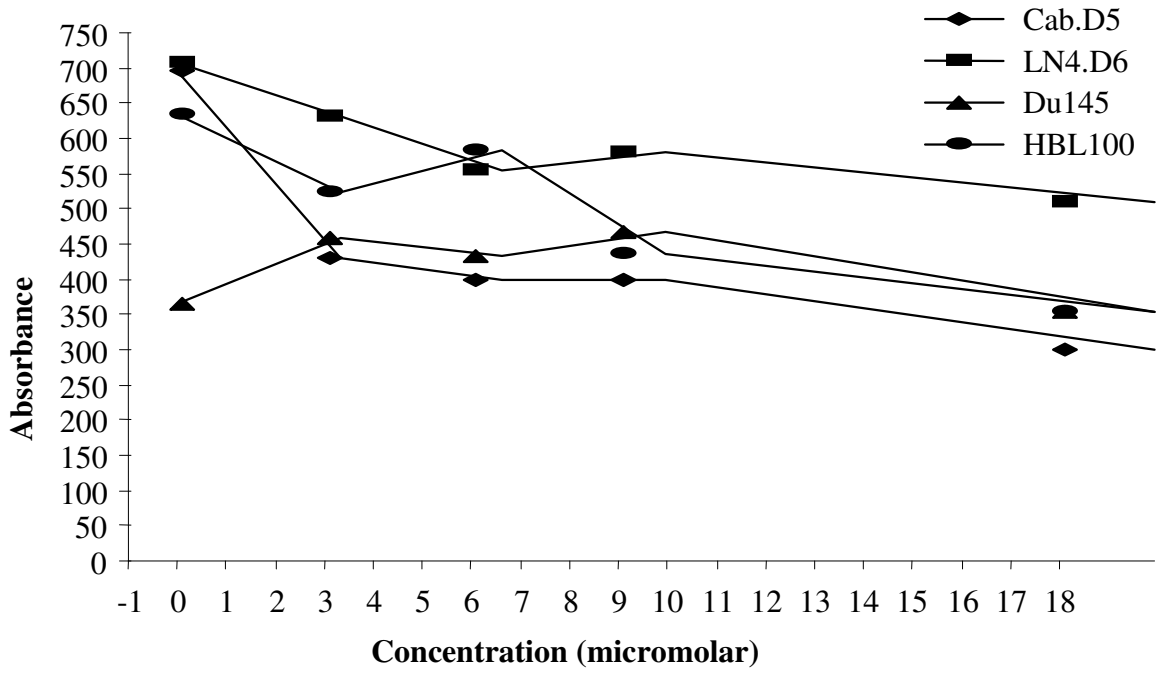


Figure 3.3. Effect of Lycopene on the growth of CAb.D5, DU145, LN4.D6, HBL100 cell lines. Data are the average of duplicate samples. Difference among the absorbance value of the duplicates were less than 2%. Experiment was repeated twice with the similar results.

3.2.3. Effects of Zeolite on Cell Growth

Pavelić et al. (2001) has extensively examined the effect of zeolite clinoptilolite on tumor development and prevention in both in vitro and in vivo models. Therefore, in this present study we wanted to see how zeolite clinoptilolite will affect growth of

metastatic and non-metastatic tumor cells LN4.D6 and CAb.D5 as well as some other cancer cell lines.

The inhibitory effect of zeolite on CAb.D5, LN4.D6, AT-1, and MATLyLu cells showed different levels at different concentrations. In CAb.D5 cells, zeolite decreased cell growth at 5mg/ml concentration and it was highly effective at 50mg/ml concentration when compared to untreated cells. For LN4.D6, decrease on cell growth was gradually seen at 0.5mg/ml, 5mg/ml, and 50mg/ml reaching 2.5-fold less than untreated LN4.D6 cells. The effect of zeolite on AT-1 cell growth was similar to LN4.D6. There was a gradual decrease but it was less than the decrease seen in LN4.D6 cells. In MATLyLu and CAb.D5 cells, 0.5mg/ml concentration of zeolite showed no inhibitory effect. At 5mg/ml and 50mg/ml, zeolite decreased the cell growth of MATLyLu. Zeolite at 100mg/ml concentration showed opposite effect, increasing the cell growth in comparison with the untreated cells.

Zeolite clinoptilolite has been demonstrated to induce expression of $P21^{WAF1/CIP1}$ and $P27^{KIP1}$ tumor suppressor proteins. Thus, it inhibited cell growth of several cancer cell lines (Pavelić et al. 2001). The results of this current study is consistent with their results in which zeolite clinoptilolite inhibited the tumor cell growth at almost every concentration tested. However, zeolite at 100mg/ml concentration, it reconstituted the tumor cell growth and in some cases it increased the cell growth 1.6-fold and 1.8-fold in LN4.D6 and AT-1 cells compared to untreated cells (Figure 3.3.3). More detailed analysis of the cell culture content in the future studies will explain the reason for an increase of cell growth at 100mg/ml zeolite cocentration. It was also suggested as a potential regulator of immune system (Ueki et al. 1994, Aikoh et al. 1998). Finally, when we consider our results, there should be additional investigations of zeolite effect on cell growth. Further studies are necessary to reveal the effects of zeolites on cell growth.

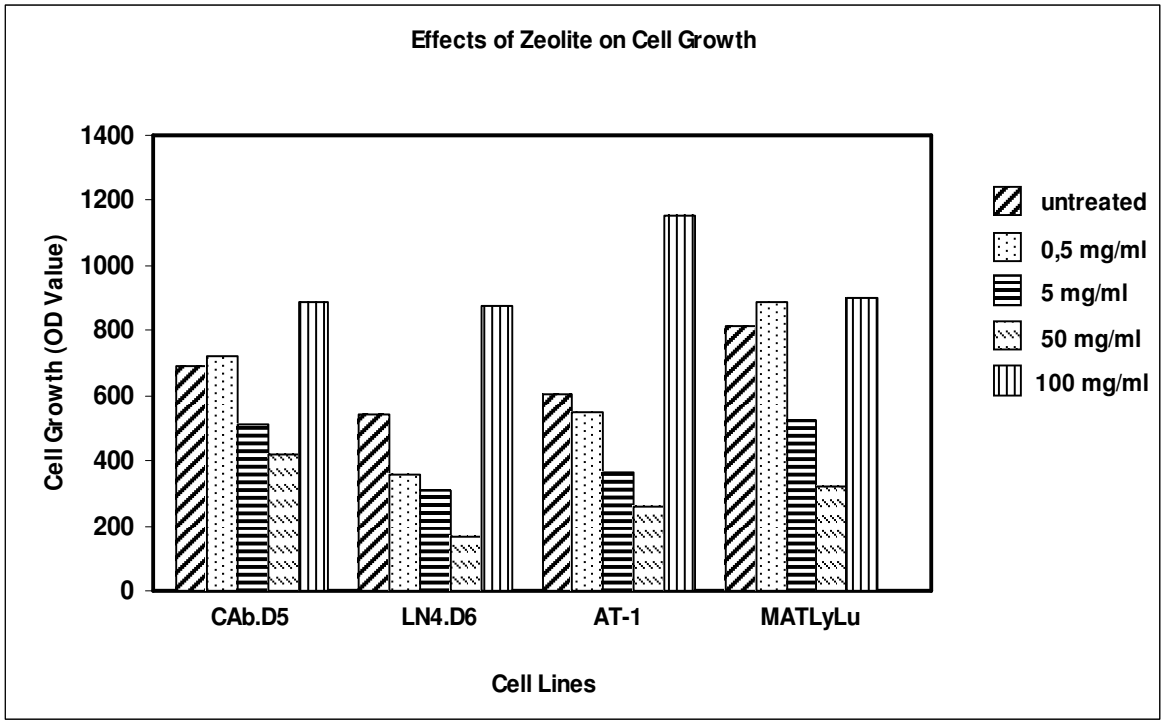


Figure 3.4. Effects of zeolite on the growth of different tumor cell lines at different concentrations. Data are the average of duplicate samples. Difference among the absorbance value of the duplicates were less than 2%. Experiment was repeated twice with the similar results.

3.3. Effects of Antioxidants and Zeolite on the Expression of Differentially Expressed Genes

In order to see how antioxidants will effect the expression of differentially expressed genes, cells were treated with green tea catechins, beta carotene, lycopene and zeolite. After that RNA isolation was carried out and gene expression level was determined with RT-PCR analysis.

3.3.1. Effects of Green Tea Catechins and Beta Carotene on Gene Expression

SG-1: When SG-1 expression was examined after EGCG, EGC, GTC and beta carotene treatment, there were similar results for EGC, GTC, and beta carotene. In

comparison with untreated CAb.D5 cells, SG-1 expression was the highest in EGCG treated CAb.D5 cells. Relative levels of SG-1 mRNA were lower than the that of untreated CAb.D5 in EGC, GTC, and beta carotene treated cells (Figure 3.4.1.A).

FF-10: According to the expression levels of FF-10 after catechin and beta carotene treatment, FF-10 was expressed in each EGCG, EGC, GTC and beta carotene treated cells at similar levels. However, the FF-10 expression was 1.3-fold less in antioxidant treated cells (Figure.3.5.B2).

FH-2: In FH-2 expression after catechin and beta carotene treatment, there were similar results among EGCG, ECG, and beta carotene treated CAb.D5 cells. However, the amount of expression of treated cells were lower than the untreated CAb.D5 cells. FH-2 expression was inhibited 1.8-fold compared to untreated cells in GTC treated cells if we compare it with the other treated and untreated cells (Figure 4.4.1.C).

FA-8: When FA-8 expression was investigated in EGCG, ECG, GTC and beta carotene treated and untreated cells, the expression level of FA-8 was almost the same in all conditions (Figure 3.4.1.D).

p53: When p53 expression was investigated in EGCG, EGC, GTC and beta carotene and untreated CAb.D5 cells, gradual inhibition in p53 expression was observed (Figure 3.4.1.E). The expression levels increased in each antioxidant orderly. p53 was the lowest in EGCG treated CAb.D5 cells. The expression level in EGC treated cells were 1.25-fold higher than that of EGCG treated cells while p53 expression was also 1.25-fold higher in GTC treated cells than the expression level of p53 in EGC treated CAb.D5 cells. Among treated cells, the highest expression potential was seen in beta carotene treated cells. p53 was highestly expressed in untreated cells when compared to treated cells. This result may suggest that EGCG, EGC, and GTC have inhibitory effect on the expression of p53 in CAb.D5 cells.

EGCG was indicated to have anti-cancer activity through expression of several genes involved in cell proliferation and cell-matrix interactions (McLoughlin et al. 2004). RT-PCR analysis of 20μM EGCG treatment on mouse calvarial primary osteoblastic cells resulted in reducing in expression of matrix metalloproteinases (MMPs) significantly. Also, EGCG significantly inhibited osteoblast formation at 20μM concentration (Yun et al. 2004). Similar to previous studies, EGCG inhibited p53 expression by 1.9-fold compared to untreated cells.

A)

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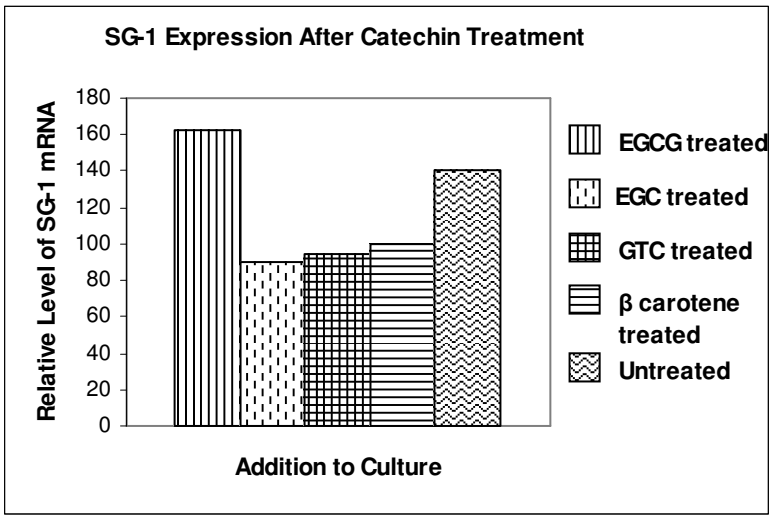
1 2 3 4 5

2)

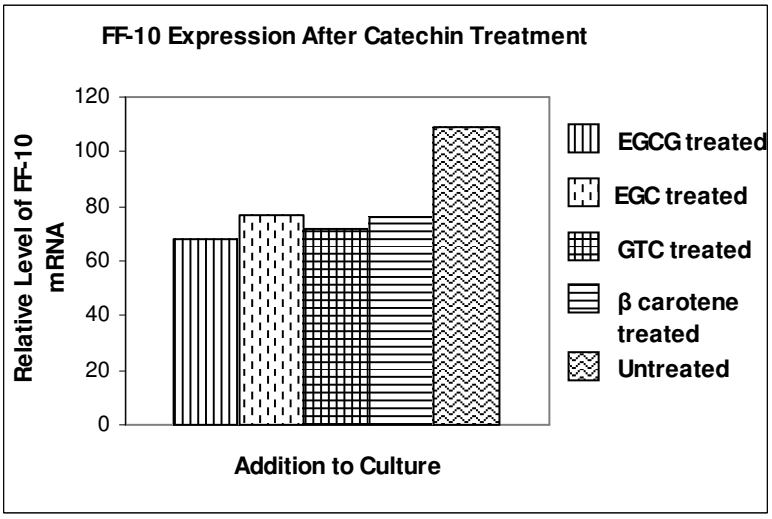


B)

1)

1 2 3 4 5

2)



C)

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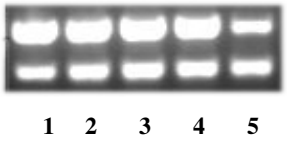
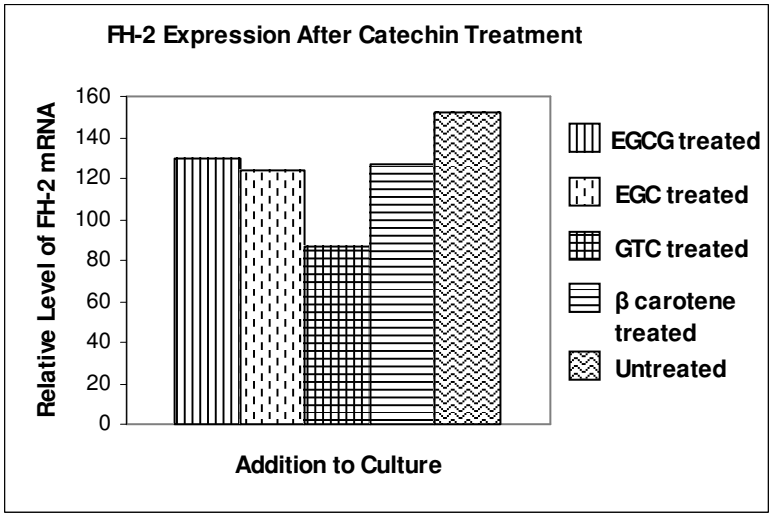
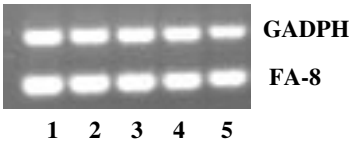

FH-2
GAPDH
1 2 3 4 5

2)

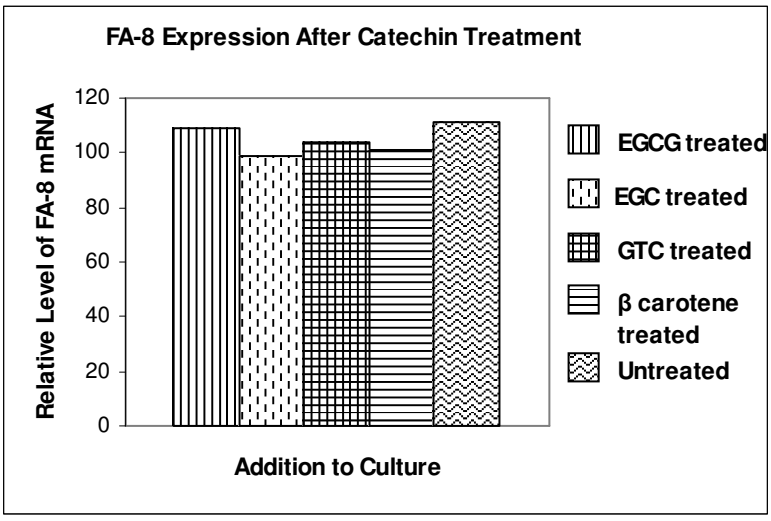

FH-2 Expression After Catechin Treatment
Relative Level of FH-2 mRNA
160
140
120
100
80
60
40
20
0
Addition to Culture
EGCG treated
EGC treated
GTC treated
β carotene treated
Untreated

D)

1)

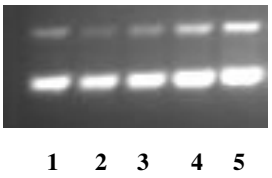

GADPH
FA-8
1 2 3 4 5

2)


FA-8 Expression After Catechin Treatment
Relative Level of FA-8 mRNA
120
100
80
60
40
20
0
Addition to Culture
EGCG treated
EGC treated
GTC treated
β carotene treated
Untreated

E)

1)



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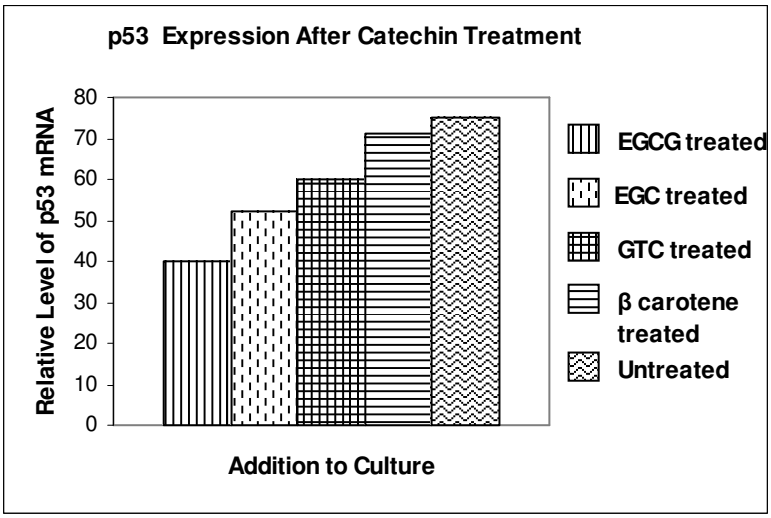


Figure 3.5. Expression profiles of differentially expressed genes in CAb.D5 cell line after treatment with green tea catechins. Lane 1: EGCG treated , Lane 2: EGC treated, Lane 3: GTC treated, Lane 4: beta carotene treated, Lane 5: untreated CAb.D5 cells.

3.3.2. Effects of Lycopene on Gene Expression

Effect of lycopene on gene expression was examined among lycopene treated and untreated CAb.D5 cells for SG-1, FF-10 and FH-2 clones. According to the results, lycopene treated cells did not show any change in the expression of SG-1 clone. On the other hand, FF-10 expression was decreased almost 2-fold in lycopene treated CAb.D5 cells in comparison with the untreated CAb.D5 cells. FH-2 expression was not inhibited after lycopene treatment considering the untreated CAb.D5 cells, either.

In epidemiological studies, an inverse relation between lycopene and breast cancer risk was detected by some investigators (Zhang et al 1997, Jarvinen et al. 1997). Lycopene intake reduced 50% of mortality from cancers. The results of this study support the previous studies because SG-1 seems to have potential metastasis suppressor gene and its expression was not inhibited with lycopene. In contrast, FF-10 expression was decreased by 2-fold with lycopene treatment compared to untreated cells.

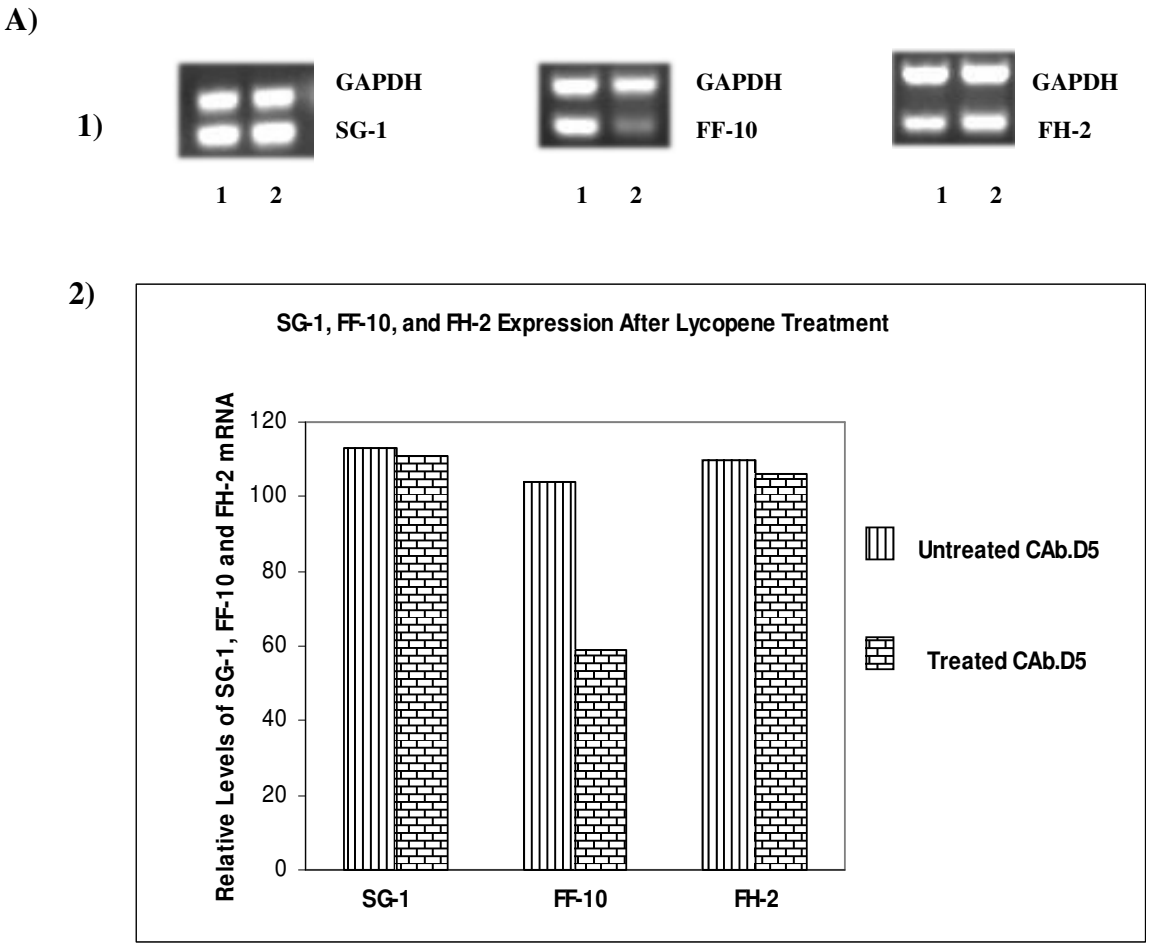


Figure 3.6. Effect of lycopene on SG-1, FF-10 and FH-2 expression. Lane 1: untreated, Lane 2: lycopene treated CAb.D5 cells.

3.3.3. Effects of Zeolite on Gene Expression

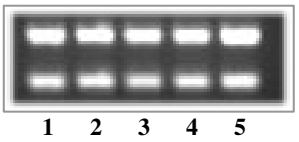
The effect of zeolite on the expression of SG-1, FF-10, FH-2, FA-8 and p53 was determined after treatment of non-metastatic CAb.D5, AT-1 cells and metastatic LN4.D6, MATLyLu cells at different concentrations of zeolite. The identification of gene expression effect was achieved by RT-PCR analysis.

When we compare the results of gene expression levels of zeolite treated cells with untreated cells, it was easily seen that there was no significant difference among them. Zeolite was reported to induce expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ tumor suppressor proteins (Pavelić et al. 2001). Therefore, our results are not consistent with the results of Pavelić et al. (2001). In the literature, zeolite effect on gene expression is

not widely investigated. There should be detailed studies done for gene expression effect of zeolite trying different concentrations.

A)

1)



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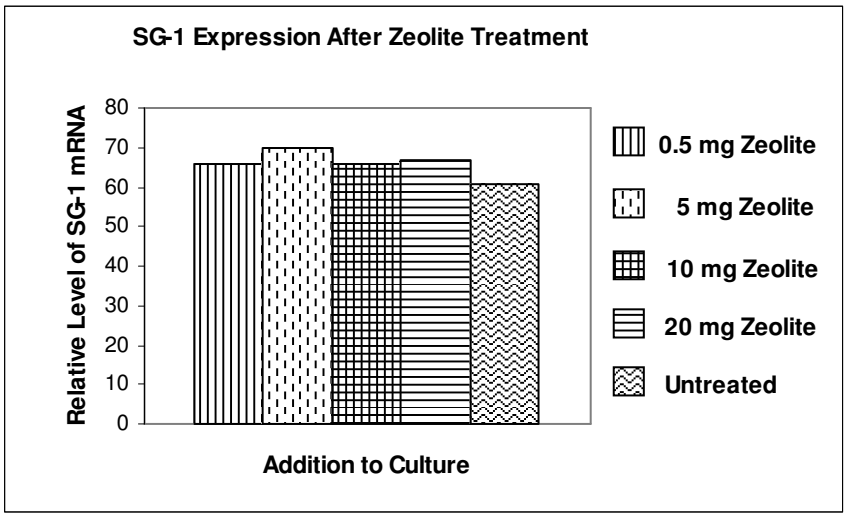
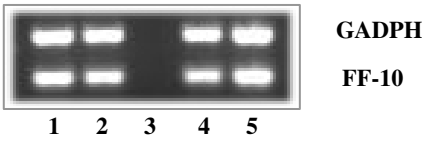


B) **1)**



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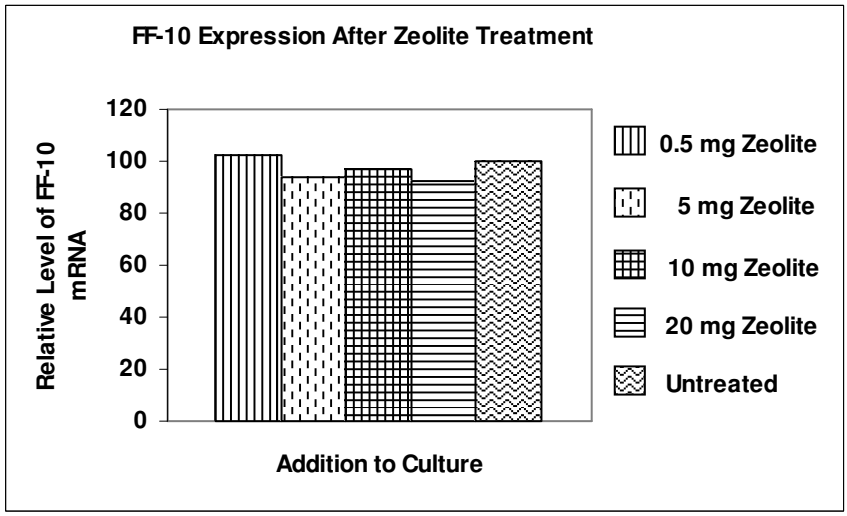
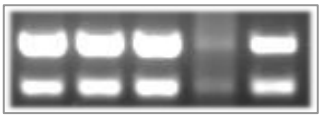


C) 1)

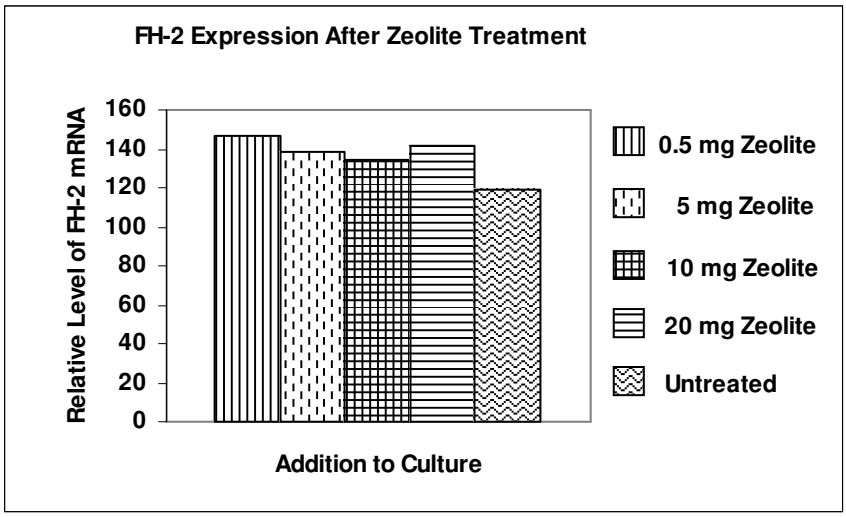


FH-2

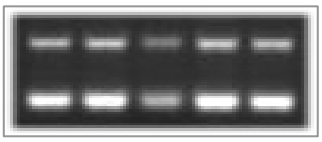
GADPH

1 2 3 4 5

2)



D) 1)

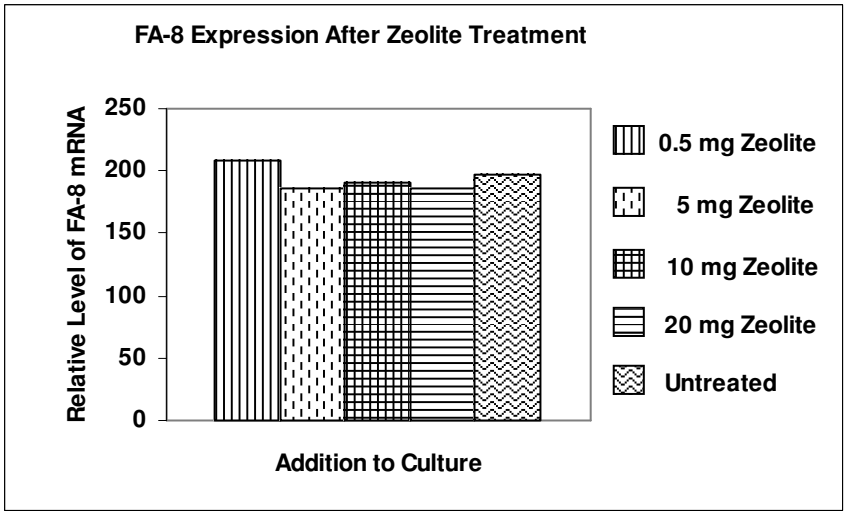


GADPH

FA-8

1 2 3 4 5

2)



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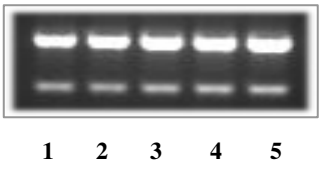

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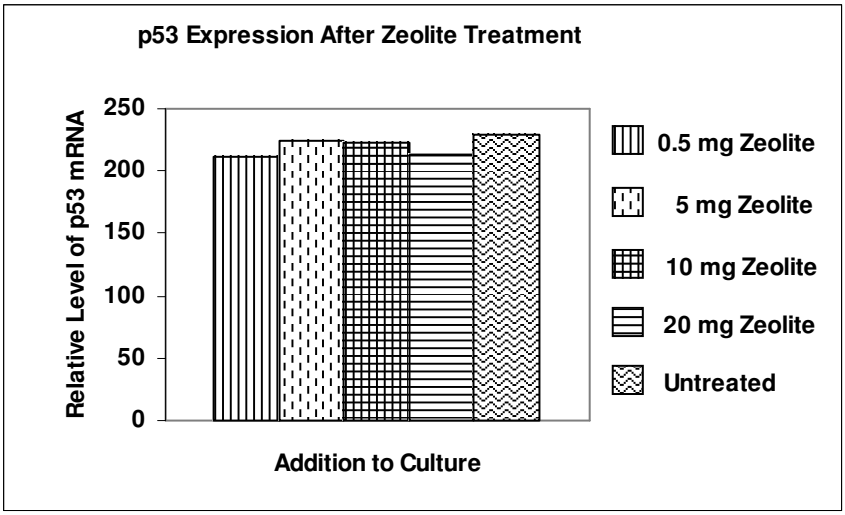

Figure 3.7. Expression profiles of differentially expressed genes in CAb.D5 cell line after treatment with zeolite. Lane 1: 0.5mg/ml zeolite treated, Lane 2: 5mg/ml zeolite treated, Lane 3: 10mg/ml zeolite treated, Lane 4: 20 mg/ml zeolite treated, Lane 5: untreated CAb.D5 cell lines.

CHAPTER 4

CONCLUSION AND FUTURE EXPERIMENTS

Cancer progress through different and related histopathological stages. Progression occurs by the accumulation of genetic alterations and during this, changes in gene expression patterns occur. Mutational and expression analysis of tumor suppressor genes in the early tumorigenesis are important to indicate the role of these genes in metastatis and cancer progression. According to the heterogenity of tumor cells, in primary tumors, it is not possible to separate the cells with respect to their metastatic potentials. However, rapid advances in cancer research provided scientists to investigate the gene expression changes at early and late stages of cancer progression.

In the previous study there were eight cDNA clones identified in poorly metastatic CAb.D5 cell lines and six cDNA clones identified in highly metastatic LN4.D6 cell lines. In this study, expression profiles of these differentially expressed genes were investigated in a set of adenocarcinoma cell lines to indicate their metastatic association. Among the primers designed for differentially expressed genes, primers for FC-7, FG-6, RB-9, RD-4 and RE-12 did not work. Therefore, expression profiles of the other differentially expressed genes (FA-8, FC-1, FC-6, FF-10, FH-2, RB-8, RE-1, RF-5 and SG-1) were examined in different adenocarcinoma cell lines.

cDNA clones designated with FF-10 and SG-1 were initially detected to be expressed in non-metastatic cell line CAb.D5. Their expression was generally observed in non-metastatic tumor cell lines in this study as well. In addition, the clones RB-8, RE-1 and RF-5 were expressed in metastatic LN4.D6 cell line in a previous study. However, RB-8 expression was observed in both metastatic and non-metastatic cell lines in this current study. RE-1 was exclusively expressed in LN4.D6 cell line compared to other metastatic and non-metastatic cell lines. RF-5 expression was detected in both CAb.D5 and LN4.D6 cell lines; however, RF-5 expression was almost 1.5-fold higher in LN4.D6 cells. In contrast to other clones explained above, the clones FF-10 expression was observed only in non-metastatic CAb.D5 and AT-1 cell lines. Moreover, when SG-1 expression was examined in three pairs of metastatic and non-metastatic rat adenocarcinoma cell lines, it was only expressed in non-metastatic cell lines. This result suggest that the clone SG-1 may have metastatic suppressor function.

In further studies, transfection of cDNA constructs of SG-1, FF-10, RE-1 and RF-5 will help to elucidate the function of these genes in the metastatic process.

Many studies have been done on the effect of antioxidants on tumor and tumor metastatasis. In this present study, various tumor cells were treated with green tea catechins including EGCG, ECG, GTC, beta carotene, lycopene and additionally with zeolite. Different concentrations of antioxidants were used to treat the cells and MTT assay was performed to see their effects on the tumor cell growth. The effects of green tea catechin and beta carotene at different concentrations on tumor cell growth exhibited the similar pattern in which a significant decrease in the cell growth was observed at 100 μM of EGCG and EGC. Cell growth gradually decreased after treatment with GTC and beta carotene. Among the green tea catechins EGC was the most effective on the inhibition of tumor cell growth. In addition, effect of lycopene on different tumor cell growth was examined and found that lycopene at 3 μM showed the significant effect on the inhibition of CAb.D5 cell growth. Moreover, effect of zeolite on cell growth was investigated and shown that at 5mg/ml and 50mg/ml concentrations, zeolite exhibited an inhibitory effect on the growth of cell lines examined. However, at 100mg/ml, zeolite induced the tumor cell growth. Further study is required to have more results for the effect of zeolite on cell growth.

Finally, the effect of green tea catechins, beta carotene, lycopene, and zeolite was investigated on the expression of differentially expressed genes. Compared to other green tea catechins, EGCG did not inhibit SG-1 expression in fact it increased the expression 1.2-fold compared to untreated cells.

It has been suggested that in the next decade, there will be serious developments in cancer research by the help of new data on cancer genetics, epigenetics, genomics and gene expression. Continued work with model organisms will hopefully provide answers to many questions especially in the molecular mechanisms of cancer. The identification of genes involved in cancer progression will enhance understanding the biology of this process as well as provide new targets for early diagnosis and treatment of cancer. As gene expression profiling gave rise to the subclassification of tumors, our results will be a step for indicating the metastatic relevance of these different tumor types. Investigations on antioxidant effect is a developing area that may provide new drug discoveries, exploring chemopreventive agents and treatment approaches on cancer patients and in malignant situations.

In conclusion, except SG-1, metastatic relevance of other differentially expressed genes were not observed in different adenocarcinoma cell lines. However, it will be important to look at expression profiles of especially SG-1, FF-10, RE-1 and RF-5 in primary and secondary human tumors in order to confirm their metastatic relevance.

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APPENDIX A

Cell Lines and Their Metastatic Characteristics

- **Rat Mammary Adenocarcinoma Cell Lines**

  CAb.D5: Poorly metastatic

  LN4.D6: Metastatic

  13762 CT: Non-metastatic

  13762 WT: Metastatic

- **Rat Prostate Adenocarcinoma Cell Lines**

  AT-1: Non-metastatic

  AT-2: Non-metastatic

  AT-3: Metastatic

  MATLu: Metastatic

  MATLyLu: Metastatic

- **Human Mammary Adenocarcinoma Cell Lines**

  MDA-MB-453: Poorly metastatic

  MCF-7: Non-metastatic

  HBL-100: Non-metastatic

  T47D: Non-metastatic

- **Human Prostate Adenocarcinoma Cell Lines**

  LNCap: Non-metastatic

  PC-3: Poorly metastatic (buna metastatic deniyor bazı makalelerde)

  DU145: Metastatic

- **Human Cervix Adenocarcinoma Cell Line and Human Colon Adenocarcinoma**

  HeLa: Metastatic