# INVESTIGATION OF ATBF1 AS A MULTI-TUMOR SUPPRESSOR GENE

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BY

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AUGUST, 2005

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# ABSTRACT

# INVESTIGATION OF ATBF1 AS A MULTI-TUMOR SUPPRESSOR GENE

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ATBF1 is a transcription factor which inhibits Myb oncoprotein and increases p21 promoter activity. Recently, ATBF1 gene was shown to be expressionally downregulated and harbored frequent mutations in prostate cancers. ATBF1 resides on chromosome 16q22.3-23.1 which is deleted recurrently in numerous types of tumors including hepatocellular carcinoma, breast cancers, glial tumors and colorectal cancers. In this study we aimed to assess ATBF1 gene's involvement in HCC, breast cancer, glial cancers and colorectal cancers development. We analyzed the DNA samples of these tumors for ATBF1 gene mutations. We focused on the regions which harbored high frequency of mutations in prostate cancers. Our study revealed frequent 3 and 6 bp polymorphisms on glutamine rich, and therefore possible transactivation domains. We showed homozygous 3 and 6 bp alterations were heterozygous in the matching histologically normal tissues in 3 cases; supporting the tumor suppressor role of ATBF1 gene in tumors tested, according to Knudson's two-hit hypothesis. In addition, we were able to identify single base pair substitutions on ATBF1 gene, and one of them is highly likely to be somatic. Based on our results, we hypothesize that ATBF1 gene is a multi-tumor suppressor gene, although further investigation of this gene is necessary.

*Keywords:* hepatocellular carcinoma, tumor suppressor gene, chromosome16q, LOH.

# ÖZET

# ATBF1 GENİNİN BİR MULTİ-TÜMÖR BASKILAYICI GEN OLARAK İNCELENMESİ

Özkan Aydemir
Moleküler Biyoloji ve Genetik Bölümü Yüksek Lisansı
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ATBF1, Myb onkoproteinini baskılayan ve p21 promotor aktivitesini arttıran bir transkripsiyon faktörüdür. Değişik organ kanserlerinde sıklıkla delesyona uğrayan 16q22.3-23.1 bölgesinde yer alan ATBF1 geninin prostat kanserlerinde tümor baskılayıcı gen olduğu ve sıklıkla mutasyona uğradığı gösterilmiştir. 16q22.3-23.1 bölgesinin prostat kanserlerinin yanısıra hepatoselüler karsinom, meme kanserleri, glial tümörler ve kolorektal kanserler dahil olmak üzere bir çok kanser çeşidinde kaybolduğu gösterilmiştir. Bu çalışmada amacımız, ATBF1 geninin hepatoselüler karsinom, meme kanserleri, glial tümörler ve kolorektal kanserlerin gelişimindeki rolünü incelemektir. Bunun için sözkonusu kanser örneklerinde ATBF1 geninde mutasyonların olup olmadığı araştırılmıştır. Çalışmamız ATBF1 geninin prostat kanserlerinde sık mutastona uğrayan bölgelerine odaklanmıştır. Çalışmamızda sözkonusu geninin glutaminden zengin, olası transaktivasyon bölgelerinde sıklıkla 3 ve 6 baz polimofizmler taşıdığını gösterilmiştir. 3 örnekte, bu polimorfizmlerin tümör dokularında homozigot, normal dokularda ise heterozigot olduğu; dolayısıyla ATBF1 geninin Knudson Hipotezi'ne göre bu tümörlerde tumor baskılayıcı gen olarak görev yapıyor olabileceği gösterilmiştir. Ayrıca, ATBF1 geninde amino asit değişikliklerine sebep olan tek baz değişiklikleri de gösterilmiştir. Çalışmalarımızın sonuçlarına dayanarak, ATBF1 geni ile ilgili daha fazla çalışma yapılması gerektiğini ve ATBF1 geninin bir multi-tümör baskılayıcı gen olduğu hipotezini öne sürüyoruz.

*Anahtar sözcükler:* hepatoselüler karsinom, tümör baskılayıcı gen, kromozom 16q, LOH.

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# ABBREVIATIONS

| | |
|---|---|
| AFP | Alpha-fetoprotein |
| AFP-GC | Alpha-fetoprotein producing gastric cancer |
| APC | Apolipoprotein C |
| APN | aminopeptidase-N |
| ATBF1 | AT Motif-Binding Factor 1 (transcript variant A, unless otherwise stated) |
| ATBF1-A | AT Motif-Binding Factor 1 transcript variant A |
| ATBF1-B | AT Motif-Binding Factor 1 transcript variant B |
| bp | base pairs |
| BRAF | v-Raf Murine Sarcoma Viral Oncogene Homolog B1 |
| BRCA1 | Breast Cancer 1 |
| BRCA2 | Breast Cancer 2 |
| CCND1 | Cyclin D1 |
| CDKN2A | Cyclin-Dependent Kinase Inhibitor 2A |
| CGH | Comparative Genomic Hybridization |
| CNS | Central Nervous System |
| c-Myb | v-Myb Avian Myeloblastosis Viral Oncogene Homolog |
| DNA | Deoxyribonucleic Acid |
| EDTA | ethylenediaminetetraecetic acid |
| ERBB2 | v-ERB-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 |
| EtBr | Ethidium Bromide |
| HCC | Hepatocellular Carcinoma |
| HNF1 | Hepatocyte Nuclear Factor 1 |
| LOH | Loss of Heterozygosity |
| MADH4 | Mothers Against Decapentaplegic, Drosophila Homolog of 4; SMAD4 |
| MMC | Mitomycin-C |
| mRNA | messenger RNA |
| MSI | Microsatellite Instability |
| MYC | v-Myc Avian Myelocytomatosis Viral Oncogene Homolog |
| MyoD | Myogenic Differentiation Antigen 1 |

| | |
|---|---|
| PAGE | Polyacrylamide Gel Electrophrosesis |
| PCR | Polymerase Chain Reaction |
| PBS | Phosphate-buffered Saline |
| PIAS3 | Protein Inhibitor of Activated STAT3 |
| PIK3CA | Phosphatidylinositol 3-Kinase, Catalytic, Alpha |
| PTEN | Phosphatase and Tensin Homologue |
| RB1 | Retinoblastoma |
| RNA | Ribonucleic Acid |
| SEMA3B | Semaphorin 3B |
| siRNA | Small interfering RNA |
| ST7 | Suppressor of Tumorigenicity 7 |
| STAT3 | Tris-Boric Acid-Edta |
| TSG | Tumor Suppressor Gene |
| TP53 | Tumor Protein p53 |
| ZFH2 | Zinc Finger Homeodomain 2 |

# 1. INTRODUCTION

## 1.1. Tumorigenesis

Cancer is a chronic disease characterized by clonal expansion of cells that progressively develop from normal cells to invasive cancer cells via intermediate steps and this progressive process called tumorigenesis or oncogenesis. (Fearon and Vogelstein 1990)

There are more than 100 distinct type of cancer, and an organ may develop different subtypes of tumors giving rise to an almost infinite numbers of possible combinations of genetic and epigenetic modifications. Even though there are several kind of tumors with similar signaling and regulatory pathways were involved in tumorigenesis.

Tumorigenesis is a multistep event where cells have to attain many capabilities, such as self adequacy in growth signals, irresponsiveness to anti-growth signals, escaping apoptosis, unlimited reproductive capacity, angiogenesis, and finally invading surrounding tissues and metastasis, to avoid normal regulation of tissue homeostasis. Each of these capabilities is gained by a genetic alteration in cells' genome. These alterations include gain of function mutations in oncogenes and loss of function mutations in tumor suppressor genes; and might be as gross as chromosomal losses, amplifications or rearrangements, or as small as point mutations in growth regulatory genes (Hanahan and Weinberg 2000).

A better understanding of growth regulatory genes, their functions and alterations is essential to comprehend development of tumors and to find new therapeutic strategies against cancer. Analysis of chromosomal aberrations is a useful tool for identifying the chromosomal regions having the genes important for tumor formation. Frequent chromosomal deletions and amplifications generally involve regions containing known tumor suppressors and oncogenes, respectively, which causes a change in the expression level of these genes (gene dosage). A change in

the gene dosage usually gives cancer cells a growth advantage over non-cancerous cells. For instance, known oncogenes ERBB2, MYC and CCND1 reside on the chromosomal regions that are amplified in tumors, while tumor suppressors PTEN and CDKN2A are located in regions that are lost in some tumors. Aside from affecting the gene dosage, chromosomal aberrations also cause the loss of a functional copy of a tumor suppressor with a germ line mutation in the other allele, as in the cases of RB1, BRCA1 and BRCA2. Identifying cancer associated genes is also essential to develop gene-targeted therapies. To date many such therapies are approved and proved affective such as herceptin, a monoclonal antibody against ERBB2 receptor, in breast cancer with ERBB2 amplification; and gleevec, a tyrosine kinase inhibitor, in chronic myeloid leukemia (Albertson et al. 2003).

### 1.2. Genetic Aberrations in Tumors

According to Knudson's "two-hit" hypothesis, both copies of a tumor suppressor gene have to be eliminated to cause cancer formation. This elimination occurs in various ways, one of which is the elimination of one copy by an inherited or acquired loss of function mutation; and elimination of the other copy by the loss of chromosomal region in which the functional allele resides. Therefore, regions of LOH serve as a guide for identifying tumor suppressor genes. In positional cloning method, a minimal deletion region is derived from a number of LOH studies. Subsequently, the candidate genes located on this minimal deletion region are investigated by expression or mutation analysis. A TSG in a LOH region is expected to have mutations in the remaining allele or a decrease in its expression in tumors, possibly caused by an epigenetic silencing. A number of TSGs are identified by using this method including MADH4 in pancreatic carcinoma, SMARCB1 in rhabdoid tumors of kidney, PPP2R1B in lung and colon carcinomas and SEMA3B in small cell lung cancer. While some tumor suppressor genes are frequently mutated in a variety of tumors, some are mutated in a very high rate in some tumors and with a low rate in others; yet some are mutated only in a limited type of tumors. For instance, TP53 is mutated in almost all tumors with a rate of 10-74 %, APC is mutated in 60 % of colon cancers and 18 % of breast carcinomas but only in 1-7% of gastric cancer, prostates and melanomas; and VHL mutations are exclusive to renal cell carcinoma (60%) (Presneau et al. 2003).

Tumor suppressor types of genes are involved in a wide variety of human cancers and are frequently detected through an apparent loss of alleles in polymorphic markers. Large number of allelic loss studies point that there are several chromosomal regions frequently deleted but few tumor suppressor genes located at these site are discovered such as PTEN, MADH4, SEMA3B. Thus, regions of the human genome which demonstrate high frequency LOH in human cancers are likely to harbor as yet undiscovered tumor suppressor genes and are the subject of much investigation (Presneau et al. 2003).

Genetic alterations accumulating in a single cancer cell in addition to TSGs also involve oncogenes. The Ras oncogenes N-*RAS*, H-*RAS*, and K-*RAS* are mutated in 30% of human cancers. In the post genomic era new oncogenes are identified by dissecting signaling pathways such as RAS–RAF–MEK–ERK–MAP kinase pathway. Using this strategy, genetic alterations in the BRAF and PIK3CA genes in a variety of cancers have been reported. Mutational activation of proto-oncogenes is not the only way to activate these genes. Many cancer cells contain multiple copies of structurally normal proto-oncogenes for instance some breast and colorectal cancers often amplify MYC. Amplification of the NMYC gene is consistently associated with late stage neuroblastomas. In addition, chromosomal translocations may create novel fusion proteins that in turn may act as oncogenes such as BCR-ABL translocation in leukemias (Weir et al. 2004).

### 1.3. 16q LOH

A review by Knuutila et.al.(1999) summarizes reports of DNA copy number losses in human cancers. According to the review, 23% of the all 73 tumors lost chromosomal region 16q22. Chromosome arm 16q is lost in 60% of hepatocellular carcinomas, 30% of breast cancers, 25% of oligodendrogliomas, 25% of gliomas and 37% of medulloblastomas. There are more specific regions on chromosome 16q which are deleted in several cancers such as loss of 16q21-24 in 70% of HCC, 16q22-qter in 40% of breast carcinoma, 16q21-qter in 8% of Neuroblastoma, 16q21-qter in 16% of medulloblastoma (Knuutila et al. 1999).

A comperative genomic hybridization (CGH) study on 26 HCC samples demonstrated 14 out of 26 samples had LOH at chromosome arm 16q. A minimal deleted region was shown to be 16q21-23 (Zondervan et al. 2000).

A combined use of microsatellite instability (MSI) and CGH analysis performed on HCC samples revealed that 16q21-23.3 was lost in 52% of the samples and it was one of the regions with the highest number of genomic imbalances (Wang et al. 2001).

Thus, chromosome 16q shows LOH at a high frequency in a variety of tumor types, which suggests this region harbors at least one tumor suppressor gene whose loss may contribute to development of these tumors.

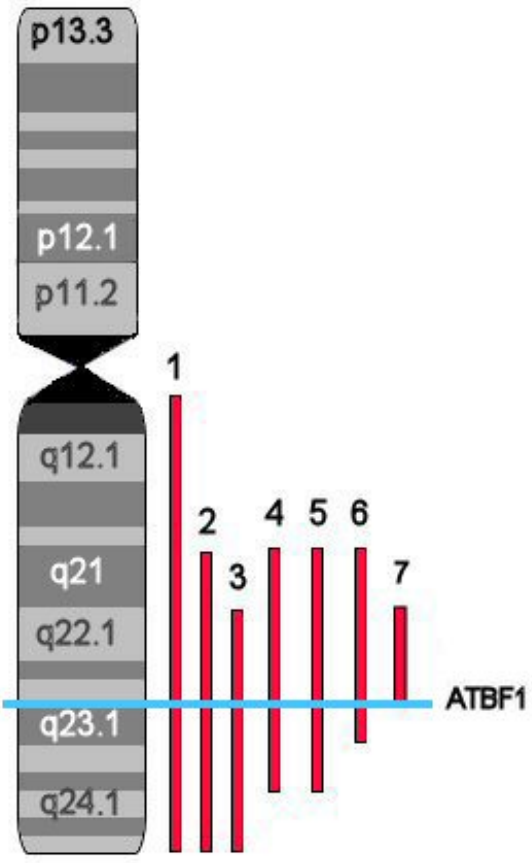


**Figure 1.** Schematic representation of LOH regions in different tumors.
*Line 1*: Lost in HCC, breast cancers, oligodendrogliomas, gliomas and medulloblastomas.
*Line 2*: Lost in neuroblastoma and medullablastoma.
*Line 3*: Lost in breast carcinoma.
*Line 4*: Lost in HCC.
*Line 5*: Lost in HCC.
*Line 6*: Lost in HCC
*Line 7*: Lost in all tumors
*Blue Line*: Indicates the location of ATBF1 gene on chromosome 16. (Knuutila et al. 1999; Wang et al. 2001; Zondervan et al. 2000)

### 1.4. ATBF1

ATBF1 was first identified as a transcription factor that binds to an AT-rich motif in the human α-fetoprotein gene enhancer. This protein contains four homeodomains and 17 zinc finger motifs which binds to DNA; and long stretches of acidic residues, serine/threonine rich segments, proline, glutamine and glycine rich domains suggest a transcription regulatory role for ATBF1 protein (Morinaga et al. 1991). ATBF1 has been shown to repress transcriptional activity of AT-rich enhancer of AFP gene, possibly by means of competing with HNF1 which binds to and activates similar AT-rich promoter and enhancer regions of AFP (Yasuda et al. 1994). Later, another ATBF1 transcript, 3.3 kb longer than the first one, was identified and named ATBF1-A; first transcript then named ATBF1-B. This transcript encoded a 404 kDa protein with 4 homeodomains and 23 zinc finger domains and has similar expression patterns to ATBF1-B (Miura et al. 1995). Human ATBF1 gene is mapped to chromosome 16q22.3-q23.1 (Yamada et al. 1995).

A drosophila protein, ZFH2, which has three homeodomains with high homology to those of ATBF1, was shown to regulate neuron-specific dopa decarboxylase gene (Lundell and Hirsh 1992). ATBF1 was shown to be expressed at high levels in pre- and post-natal mouse brain but was present in small amounts in adult brain and shown to be important in neuronal differentiation (Ido et al. 1994). These information suggest that ATBF1 may also play an important role in regulation of genes related to human CNS development and differentiation (Ido et al. 1994; Lundell and Hirsh 1992; Yasuda et al. 1994). In vitro and in vivo binding of ATBF1 to v-Myb oncoprotein C terminus shows that ATBF1 may repress biological activities of this oncogene in chicken fibroblasts (Kaspar et al. 1999). ATBF1 also negatively regulates the expression of aminopeptidase-N (APN) gene in the crypts and base of villi in small intestine and thereby affect enterocyte differentiation and maturation (Kataoka et al. 2000). Besides its activator role in neuronal and enterocyte differentiation, ATBF1 is also involved in regulation of myogenic differentiation; in a somewhat more complex way. ATBF1-A and ATBF1-B has been shown to inhibit and promote myogenic differentiation of myoblast cells,

respectively; possibly by regulating MyoD and myogenin expression and thereby regulating the expression of muscle specific genes (Berry et al. 2001).

Kataoka et. al. (2001) reported that AFP producing gastric cancer cells expresses trace amounts of ATBF1 whereas non-AFP producing gastric cancer cells expresses relatively high levels of ATBF1. Since the promoter region of AFP gene in gastric cell lines producing AFP was not methylated in most cases; ATBF1 seems to be mainly responsible for downregulation of AFP in these cell lines (Kataoka et al. 2001). Due to the inhibitory effects of ATBF1 on c-Myb oncoprotein (Kaspar et al. 1999), absence of ATBF1 expression in AFP producing gastric cancers may cause the highly malignant nature of this cancer (Kataoka et al. 2001).

Besides its transcription regulation and DNA binding features, ATBF1 also has ATPase activity in the presence of double or single stranded DNA or RNA; and its ATPase activity is dependent on intact homeodomain IV and zinc finger 21 domains since mutation of these domains caused a dramatic decrease of ATBF1's ATPase activity (Kawaguchi et al. 2001).

In a study by Van Roy et.al. (2001), a neuroblastoma cell line, SJNB-12, was shown to have amplified chromosomal parts containing ATBF1 gene in the form of double minutes, and it was suggested that the anomalous expression of ATBF1 caused by this amplification may account for development of neuroblastoma given the involvement of this gene in neurogenesis (Miura et al. 1995).

Existence of ATBF1 expression in one out of four cervical cancer cell lines but none of the eight normal cervical tissues supports this gene's involvement in cervical tumorigenesis (Li et al. 2002).

Elevated ATBF1 mRNA expression levels is associated with better prognosis in breast cancer due to no lymph node metastasis, although this relation was not shown at protein expression levels of ATBF1 (Zhang et al. 2005).

A study by Ninomiya et. al. (2002) showed that two ATBF1 isoforms regulate AFP expression in hepatoma cell lines HuH-7, huH-1 and HepG2. Whereas ATBF1-A isoform acted as a repressor of AFP expression in all three cell lines, relatively high level of ATBF1-B in HepG2 was shown to counteract its isoform's effect and activate AFP production; consistent with the view that N-terminal part of ATBF1-A, which is absent in ATBF1-B isoform, is mainly responsible for this proteins repressor activity (Ninomiya et al. 2002).

Mitomycin-C (MMC), a chemotherapeutical agent, increases ATBF1 expression in an AFP-GC cell line, Ist-1, and leads to up to 7 fold increase in p21 (Waf1/Cip1) promoter activity by binding to p53 protein. MMC treatment causes elevated levels of ATBF1 which also inhibits c-Myb activity. This, together with p21 activation might account for increased susceptibility of MMC treated gastric cancer cells to killer cells and entry to apoptosis (Miura et al. 2004).

In an attempt to identify ATBF1 interacting proteins by yeast two hybrid screening, PIAS3 protein is found to interact with ATBF1. Co-transfection experiments confirmed this interaction in HepG2 cells and its inhibitory effect on IL-6 induced STAT3 signaling (Nojiri et al. 2004).

Recently, Sun et.al. (2005) narrowed down the LOH region in chromosome 16q22 in prostate cancer cell lines and xenografts using microsatellite markers; and defined a minimal LOH region containing ATBF1 gene. In nearly half of the prostate cancers they tested, ATBF1 gene expression was downregulated compared with the normal prostates. Furthermore, they identified 22 somatic mutations, some of which are frameshifting deletions and non-sense mutations, of ATBF1 gene. The high rate of mutations in high grade tumors relative to low grade tumors may indicate that ATBF1 mutations are correlated with the aggressiveness of tumors. In addition, introduction of siRNA against ATBF1 in an ATBF1 positive cell line elevated cell proliferation, whereas expression of ATBF1 in an ATBF1 negative cell line reduced colony forming efficiency, indicating the functional importance of ATBF1 gene in prostate cancer (Sun et al. 2005).

# 2. AIM

AT-Motif Binding Factor 1 (ATBF1) is located on chromosome 16q22.3-q23.1 (Yamada et al. 1995). Although chromosomal region containing ATBF1 was shown to be amplified in neuroblastoma in one report (Van Roy et al. 2001), common deletions of chromosome 16q in various tumors including neuroblastoma (Knuutila et al. 1999) makes ATBF1 a very likely candidate tumor suppressor gene in a variety of tumors.

The inhibitory role of ATBF1 on transcriptional activity of c-MYB (Kaspar et al. 1999) and activation of p21 promoter by ATBF1 (Miura et al. 2004) further suggests that ATBF1 is a candidate tumor suppressor gene.

Recent data showing ATBF1 is somatically mutated in prostate cancer makes ATBF1 an eligible candidate for further analysis in other tumors.

Therefore, the aim of our research is to study involvement of ATBF1 gene as a multi-tumor suppressor gene in a variety of cancers including HCC, gliomas, colon cancers and breast cancers.

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# 3. MATERIALS AND METHODS

## 3.1. Tumor Samples

Tumor samples used in this study include a set of 50 HCC samples of varying racial origins from a previously used collection (Unsal et al. 1994), 95 glial tumors with 5 normal glial tissues from Turkish patients provided by Dr. Hasan C. Ugur (Ibni Sina Hospital, Ankara University)

## 3.2. Cell Lines

The cell lines used in this study are: 14 HCC cell lines; Snu182, Snu398, Snu387, Snu423, Snu475, Snu449, PLC, Focus, HepG2, Hep40, Mahlavu, Hep3B, SK Hep1 and Huh7; 8 breast cancer cell lines; MDA-MB-453, T-47-D, BT-474, HCC1937, MCF-7, MDA-468, HME-1 and MDA-MB-231; 13 colorectal cancer cell lines; SW48, CO115, WiDr, SW480, LS411, KM12, Lovo, HCT15, HT29, TC7, colo205, SW620 and HCT8. Breast cancer cell line DNAs and cDNAs were kindly supplied by Dr. Isik Yulug and Dr. Uygar H. Tazebay respectively.

### 3.2.1. Reagents for Tissue Culture

DMEM, trypsin/EDTA and penicillin / streptomyocin were obtained from Biochrom, RPMI 1640 was obtained from Kibbutz Beit Haenek Israel and fetal bovine serum was obtained from Sigma. Tissue culture flasks, petri dishes, 15 ml polycarbonate centrifuge tubes with lids and cryotubes were purchased from Costar Corp. (Cambridge, England).

### 3.3. Solutions and Reagents

Ethidium Bromide (EtBr)

10mg/ml in water (stock solution)

30ng/ml (working solution)

10X TBE Buffer Solution

108g Tris

55g Boric Acid

8.3g EDTA

dissolved in 1lt of deionized water.

6X Loading Buffer Solution

30% Glycerol

0.04% Bromphenolblue

0.04% Xylene Cyanol

$\Delta dH_2O$

Agarose Gel Solution for PCR

| | |
|---|---|
| 50 ml | 1X TBE |
| 0.75g | Agarose |

Acrylamide/ Bisacrylamide 30% stock

29g Acrylamide

1g Bisacrylamide

dissolved in 100 ml $\Delta dH_2O$

15% Non-Denaturing Polyacrylamide Gel Solution

| | |
|---|---|
| 10X TBE | 10 ml |
| 30% acryl./bisacryl. | 50 ml |
| $\Delta dH_2O$ | 40 ml |

mixed and filtered.

10X Phosphate-buffered saline (PBS)

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $Na_2HPO_4$ | 14.4 g |
| $KH_2PO_4$ | 2.4 g |

dissolved in 1 lt of water and pH is adjusted to 7.4.

### 3.4. DNA and RNA Isolation, cDNA Preparation

DNA and RNA are isolated from frozen tissues, cultured cells or blood by using Qiagen DNeasy Tissue Kit (#69506) and Qiagen RNAeasy Midi Kit (#75144) according to manufacturer's instructions. cDNAs from RNA are prepared using 2-3 µg RNA using Qiagen Omniscript RT Kit (#205111) according to manufacturer's instructions.

### 3.5. Mutation Analysis

#### 3.5.1. Sequencing

For sequencing analysis, we chose the region on exon 9, which harbored a high number of mutations in the study by Sun et. al. (2005). (Figure 2)

**Table 1. Primers used in this study**.

| Primer | Sequence from 5' to 3' |
|---|---|
| ATBF1 Del F | GAGTCTGCAGGAGGCAATTC |
| ATBF1 Del R | AGGGTCTTTGTCTGGGGAAG |
| ATBF1 9F | AGAGGCCAATCGGAAGAAAC |
| ATBF1 9R | AAAGCCAAAGTTCTCCAGCA |
| ATBF1 9R2 | TGCTGCAGGTGAGCTTGA |
| ATBF1 9F3 | CTGTTTAACCCCACCCTCCT |
| ATBF1 9R3 | GTCCCAGTCAGTGTCAGTGC |

#### 3.5.1.1. PCR

##### 3.5.1.1.1. Primers

Primers are designed using website program (primer3_www.cgiv0.2).

We designed primers for sequencing (ATBF1 9F/9R) covering bases 5796-6535 on exon 9, on which 5 of the total 35 mutations resided (Figure 2). PCR reactions were done with Fermantas Taq DNA Polymerase (#EP0402) enzyme

##### 3.5.1.1.2. PCR conditions for ATBF1 9F/9R primers

Reaction Conditions

10 min. denaturation at 94 $^{o}$C,
35 cycles of
30 sec. at 94 $^{o}$C,
30 sec. at 58 $^{o}$C
40 sec. at 72 $^{o}$C.
10 min. at 72 $^{o}$C.

Reaction Ingredients

1X Buffer
15mM dNTP mix
1.5mM $MgCl_2$
20 pmol Forward Primer
20 pmol Reverse Primer
1.25u Taq Polymerase
100ng DNA
$\Delta$ $H_2O$

#### 3.5.1.1.3. PCR Purification

PCR products are purified using Qiagen PCR Purification Kit (#28106) or Millipore $PCR_{96}$ Cleanup Kit (#LSKC09604) according to manufacturer's instructions. After purification, 3µl of products are run on a 1.5% agarose gel with 3µl Mass Ruler DNA ladder and quantified under UV light using Bio-Rad MultiAnalyst software.

### 3.5.1.2 Sequencing Reactions

Purified PCR products are sent to Iontek Corp. (Istanbul, Turkey) for sequencing analysis.

### 3.5.1.3 Sequencing Data Analysis

Sequencing data are analyzed using Mutation Explorer (DEMO) version 2.41 (Softgenetics Inc.) or Chromas version 2.24 (Technelysium Pty Ltd) softwares.

## 3.5.2. Agarose Gel Electrophoresis

We used agarose gel electrophoresis to identify the frequent 21-24 bp deletions on exon 10 which were shown in the study by Sun et. al. (2005).

#### 3.5.2.1. PCR

#### 3.5.2.1.1. Primers

We designed primers (ATBF1 Del F/R) covering bases 10780-10915 on exon 10, on which 10 of the total 35 mutations resided. 9 of these mutations are 21-24 bp deletions (Figure 2). PCR reactions were done with Fermantas Taq DNA Polymerase (#EP0402) enzyme.

#### 3.5.2.1.2. PCR conditions for ATBF1 Del F/R primers

| Reaction Conditions | Reaction Ingredients |
|---|---|
| 10 min. denaturation at 94 $^{o}$C, | 1X Buffer |
| 35 cycles of | 15mM dNTP mix |
| 30 sec. at 94 $^{o}$C, | 1.5mM $MgCl_2$ |
| 20 sec. at 59 $^{o}$C | 20 pmol Forward Primer |
| 20 sec. at 72 $^{o}$C. | 20 pmol Reverse Primer |
| 10 min. at 72 $^{o}$C. | 1.25u Taq Polymerase |
| | 100ng DNA |
| | $\Delta$ $H_2O$ |

### 3.5.2.2. Agasore Gel electrophoresis

PCR products of ATBF1 del F/R primer are mixed with 6X loading buffer and loaded in 3% agarose gel; run at 90 V for 2 hours. Other PCR products are run in 1.5% agarose gels and run at 120 V for 45 minutes. Gels are visualized under UV light using Bio-Rad GelDoc and MultiAnalyst software.

## 3.5.3. Polyacrylamide Gel Electrophrosesis (PAGE) and Heteroduplex Analysis

We performed PAGE analysis to confirm 3-6 bp alterations on ATBF1 gene.

### 3.5.3.1. PCR

#### 3.5.3.1.1. Primers

We designed a reverse primer (ATBF1 9R2) to amplify a 150 bp region containing 13 of the 3-6 bp alterations. ATBF1 9F primer is used as forward primer for this reaction. We also designed a primer pair (ATBF1 9F3/R3) to amplify a 170 bp. region containing one 3bp alteration. . PCR reactions were done with Fermantas Taq DNA Polymerase (#EP0402) enzyme.

### 3.5.3.1.2. PCR conditions for ATBF1 9F/9R2 and ATBF1 9F3/9R3 primers

| Reaction Conditions | Reaction Ingredients |
|---|---|
| 10 min. denaturation at 94 $^{o}$C, | 1X Buffer |
| 35 cycles of | 15mM dNTP mix |
| 20 sec. at 94 $^{o}$C, | 1.5mM $MgCl_2$ |
| 15 sec. at 56 $^{o}$C | 20 pmol Forward Primer |
| 15 sec. at 72 $^{o}$C. | 20 pmol Reverse Primer |
| 10 min. at 72 $^{o}$C. | 1.25u Taq Polymerase |
| | 100ng DNA |
| | Δ $H_2O$ |

### 3.5.3.1.3. PAGE Analysis

500 µl of 10 % Ammonium persulfate (APS) and 50 µl of Temed is added to 50 ml of 15 % PAGE solution. The solution is poured into gel apparatus and left for polymerization for 1 hour. 4-15 µl of PCR product is mixed with 3 µl of 6 x loading buffer and loaded into an electrophoresis apparatus and run at 20W for overnight. After electrophoresis, gel is stained with 0.5 µg / ml EtBr for 30 min and visualized under UV light.

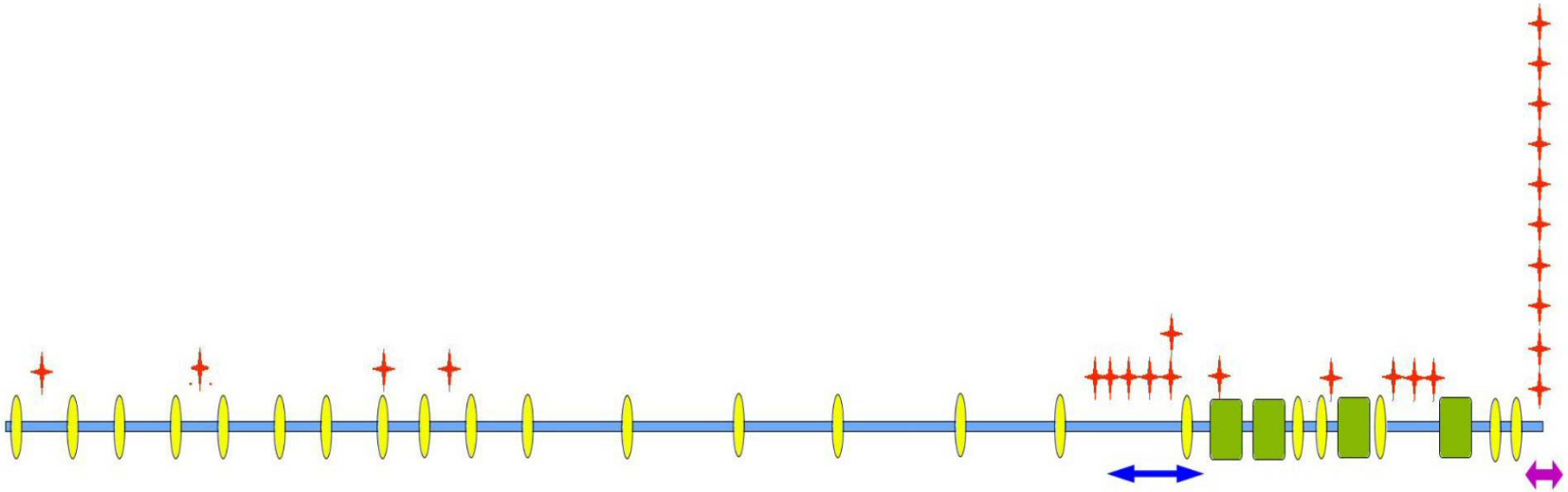

**Figure 2. ATBF1 Domains and Mutations:** This figure shows the domains of ATBF1 protein. Yellow ellipses show Zinc Finger domains; green squares show homeobox domains. Red stars indicate the mutations found in prostate cancers (8 intronic mutations and 1 gross deletion- exons 1 through 4- are not shown in the picture) by Sun et. al. (2005). Arrow headed lines at the bottom shows the regions screened for mutations in this study (blue on exon 9 and purple on exon 10).

# 4. RESULTS

## 4.1. Sequencing Analysis of ATBF1 Gene Exon 9

### 4.1.1. Mutations in Breast Cancer Cell Lines

8 breast cancer cell lines are screened for hot spot mutations on exon 9 (bases 5796-6535) by sequencing. MDA-468 cell line is shown to have a homozygous 3bp insertion (5897-5898 ins CAA). MCF7 cell line carried a heterozygous 6bp deletion (5891-5896 del. CAACAA).

### 4.1.2. Mutations in Colon Cancer Cell Lines

13 colon cancer cell lines are screened for hot spot mutations as breast cancer cell lines. LS 411 cell line is shown to have a heterozygous 3bp insertion (5897-5898 ins CAA). TC7 cell line carried a heterozygous 3bp insertion (6043-6044 ins. CAG) and a heterozygous substitution (6143 C-G, 1824 L/V). Co115 had a heterozygous 3bp deletion (5891-5893 del. CAA). (Figure 3)

### 4.1.3. Mutations in HCC Cell Lines

Analysis of 14 HCC cell lines showed no mutation in the mutation region on exon 9.

### 4.1.4. Mutations in HCC Tumors

30 HCC tumor tissues are screened for mutations by aforementioned methods. 3 of them showed heterozygous 6bp deletion (5891-5896 del. CAACAA); 2 tumors had a homozygous 6bp insertion (5897-5898 ins CAACAA); 2 of them carried a 3bp insertion (5897-5898 ins CAA), one homozygous and the other heterozygous. In addition 2 tumors had substitution mutations (6228 A-C, 1852 Q/P), one was homozygous and the other was heterozygous. We analyzed the matching histologically-normal tissue from 3 patients (T29 [hom. 5897-5898 ins CAACAA],

T39 [hom. 5897-5898 ins CAA] and T47 [hom. 5897-5898 ins CAACAA]) and they were heterozygous for the alterations. This shows these alterations caused the loss of a normal copy of ATBF1 in tumors. (Figures 4-7)

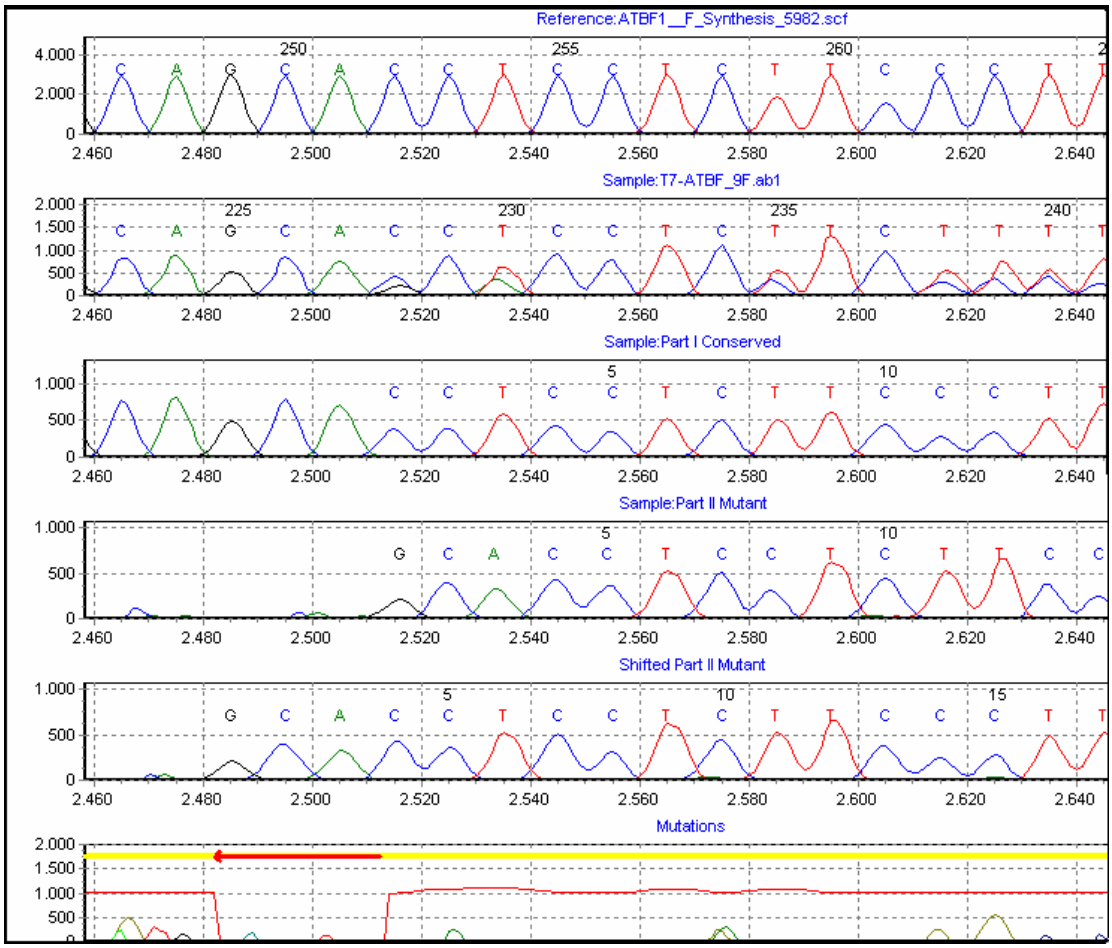


**Figure 3.** A heterozygous 3bp insertion (CAG) in TC7 cell line, compared with GeneBank sequence. First row shows wild type GeneBank sequence. Second row is the electrophorogram of TC7 cell line sequence. Third row is the sequence from second row that matches wild type sequence. Fourth row is the sequence from second row that does not match wild type sequence. Fifth row is 3 bases shifted form of fourth row so as to match wild type sequence (showing the allele has a 3bp insertion).

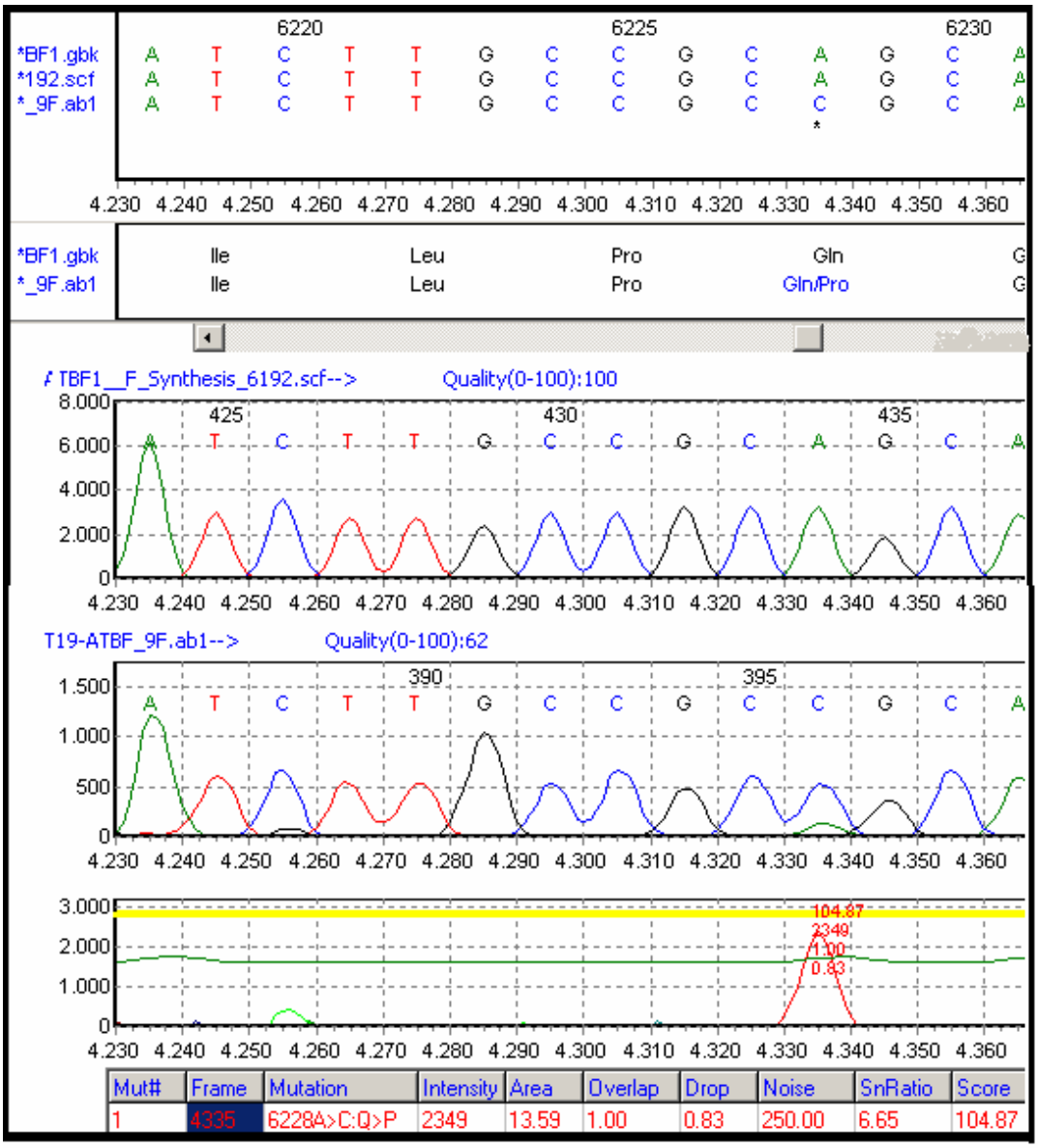


**Figure 4.** A homozygous 6228 A-C, 1852 Q-P substitution in HCC tumor T19, compared with GeneBank sequence.

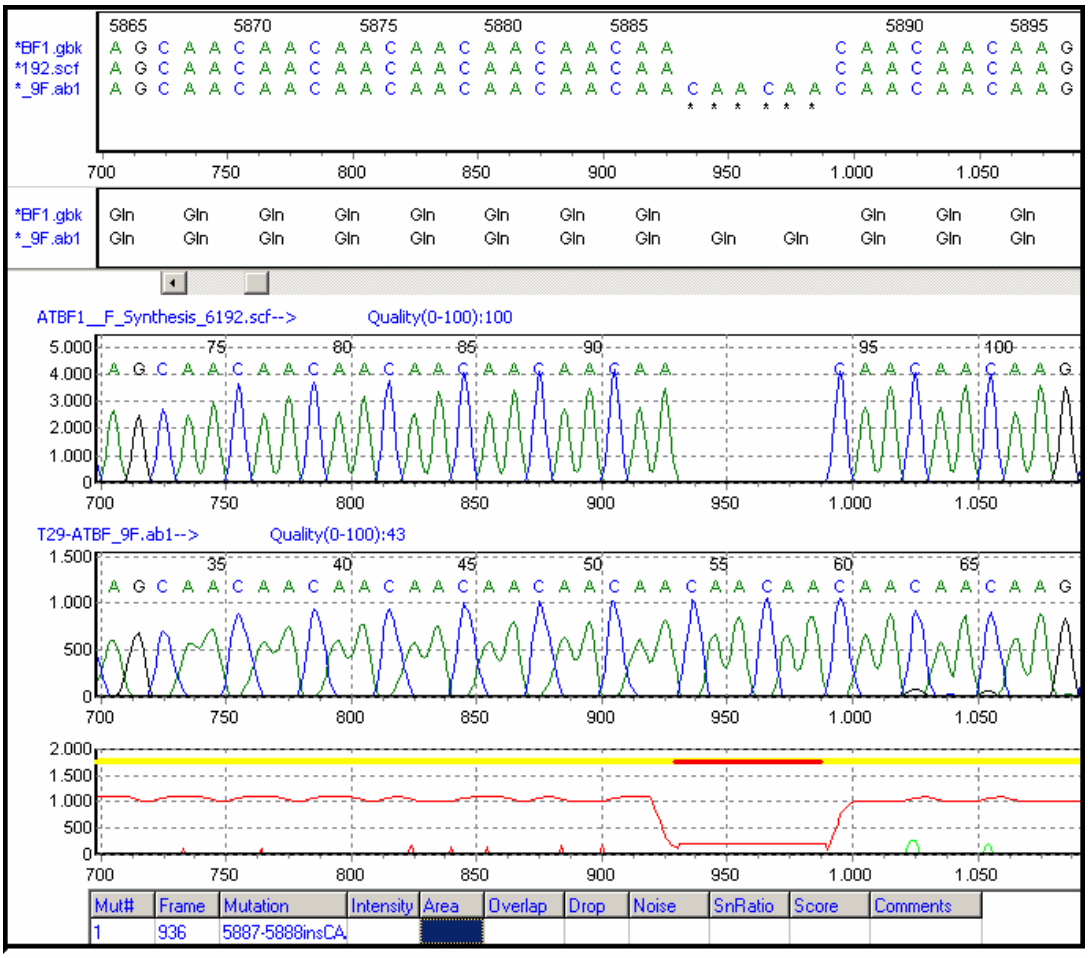


**Figure 5.** A homozygous 6bp insertion (CAACAA) in HCC tumor T29, compared with GeneBank sequence.

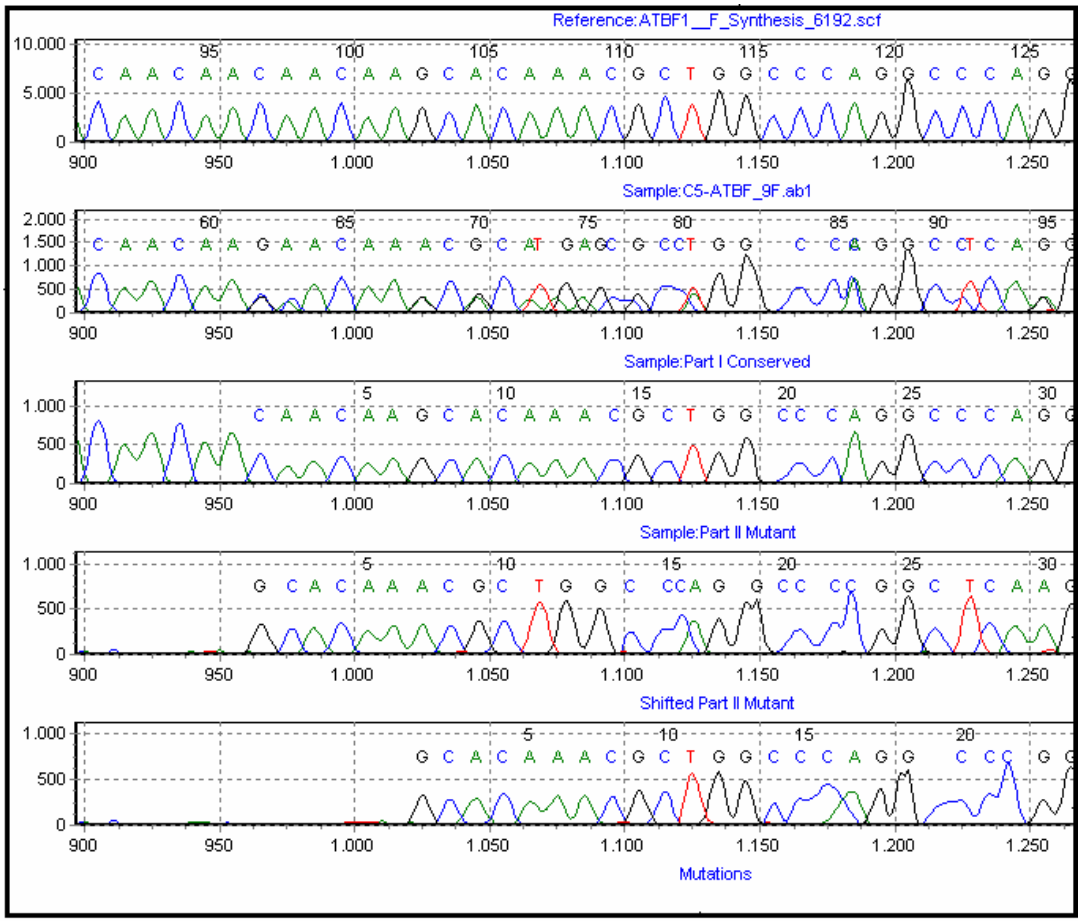


**Figure 6.** A heterozygous 6bp deletion (CAACAA) in HCC tumor C5, compared with GeneBank sequence.

### 4.1.5. Mutations in Glial Tumors

50 glial tumor samples are used for mutational analysis. One tumor showed a heterozygous substitution (6137 C/G, 1822 L/V). Another tumor had a heterozygous 6bp deletion (5864-5869 del. CAGCAA). Moreover, there was another tumor with a heterozygous 6bp deletion (5891-5896 del. CAACAA).

## 4.2. Agarose Gel Electrophoresis

To analyze the existence of frequent 21-24 bp deletions on exon 10, we amplified a 136 bp region covering the deletions in various tumor samples and cell lines. Then we run the amplified products on a 3% agarose gel. We screened a set of samples including 14 HCC cell lines, 50 HCC tumors, 8 breast cancer cell lines, 13 colon cancer cell lines and 50 glial tumors. Of the 135 samples screened, only Huh7 cell line was shown to carry a heterozygous 21 bp deletion. (Figure 7)

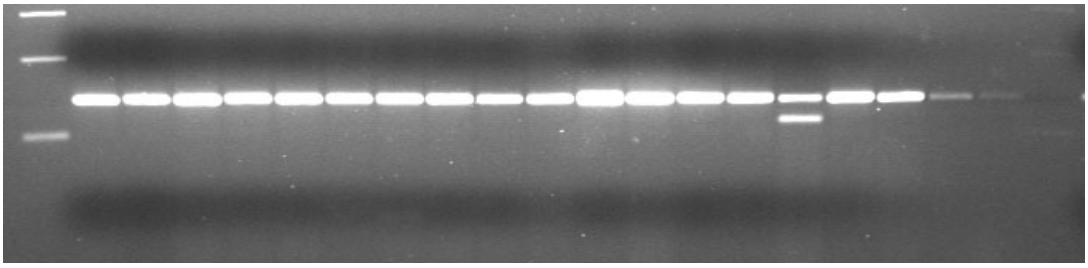

**Figure 7.** Gel picture showing heterozygous 24 bp deletion in Huh7 cell line.

## 4.3. PAGE and Heteroduplex Analysis

Page analysis was carried out to confirm 3-6bp alterations in ATBF1 gene which were determined by sequencing. In addition, we performed PAGE analysis on 11 HCC tumors from Turkish patients to determine the 3-6bp alterations.

Heteroduplex analysis was used to determine 6bp alterations on ATBF1 gene and one 3bp alteration that was only seen in TC7 colorectal cell line by sequencing (Figures 9-10).

PAGE and heteroduplex analysis confirmed the mutations found by sequencing. Furthermore, 11 tumors from Turkish patients did not show any 3-6bp alterations. (Figure 8-9)

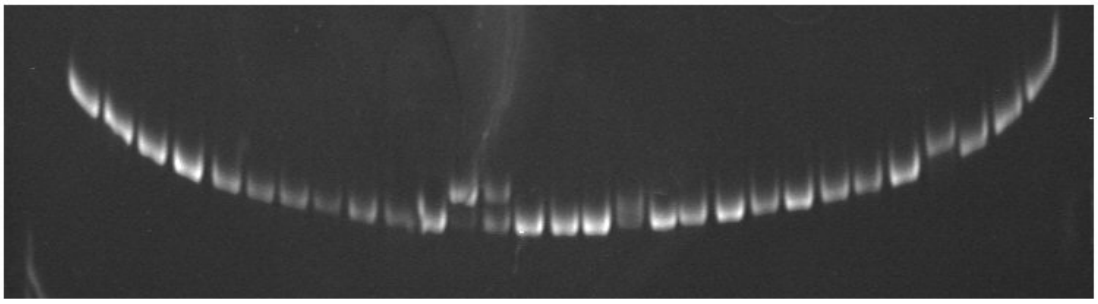

**Figure 8:** Gel picture from PAGE analysis. First 11 lanes are analysis of Turkish HCC patients which showed no 3-6 bp alteration. Lane 12: T47 HCC tumor with 6bp homozygous deletion. Lane 13: Normal tissue corresponding to T47. Lane 17: T31 tumor with 3bp heterozygous deletion. Lane 26: MDA-468 breast cancer cell line with 3bp homozygous deletion. Other lanes are HCC tumors or cell lines with no alterations (sequenced).

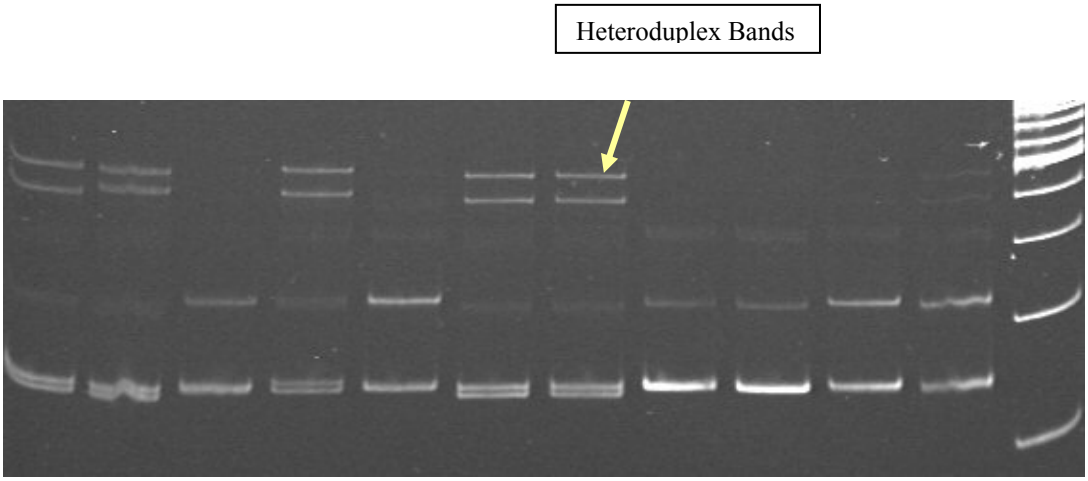


**Figure 9:** Gel picture from heteroduplex analysis for 6bp alterations. Lane1: G68 glial tumor with 6bp heterozygous deletion. Lane 2: G63 glial tumor with 6bp heterozygous deletion. Lane 4: T47 HCC tumor with 6bp homozygous 6bp insertion (a wild type product is added to make heteroduplex bands). Lane 6: J5 HCC tumor with 6bp heterozygous deletion. Lane 7: C5 HCC tumor with 6bp heterozygous deletion. Other lanes are the HCC tumor samples with no 6bp alteration (sequenced).

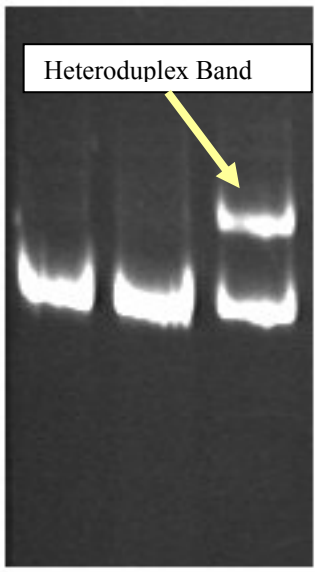


**Figure 10:** Gel picture from heteroduplex analysis for 3bp alteration in TC7 colorectal cancer cell line. Lane 1: Huh7 cell line (wt). Lane 2: SNU475 cell line (wt). Lane 3: TC7 cell line with 3bp heterozygous insertion.

**Table 2. Summary of alterations found in ATBF1 gene**

| SAMPLE | TISSUE TYPE | ORIGIN | MUTATION | STATUS | EFFECT ON PROTEIN |
|---|---|---|---|---|---|
| G63 | Glial | Turkish | del.5864-5869 | Het. | Del. of 2 Glutamine residues |
| G68 | Glial | Turkish | del. 5891-5896 | Het. | Del. of 2 Glutamine residues |
| G70 | Glial | Turkish | subs 6137 C-G | Het. | 1822 L/V |
| Huh7 | HCC | | del. 10814-10837 | Het. | Del. of 8 aminoacids |
| C5 | HCC | China | del. 5891-5896 | Het. | Del. of 2 Glutamine residues |
| J3 | HCC | Japan | del. 5891-5896 | Het. | Del. of 2 Glutamine residues |
| J5 | HCC | Japan | del. 5891-5896 | Het. | Del. of 2 Glutamine residues |
| T19 | HCC | African | substitution 6228 A-C | Hom. | 1852 Q/P |
| T29 | HCC | African | 5897 ins. CAACAA | Hom. | Ins. of 2 Glutamine residues |
| T31 | HCC | African | 5897 ins. CAA | Het. | Ins. of 1 Glutamine residue |
| T39 | HCC | African | 5897 ins. CAA | Hom. | Ins. of 1 Glutamine residue |
| T43 | HCC | African | substitution 6228 A-C | Het. | 1852 Q/P |
| T47 | HCC | African | 5897 ins. CAACAA | Hom. | Ins. of 2 Glutamine residues |
| MCF7 | Breast | | del. 5891-5896 | Het | Del. of 2 Glutamine residues |
| MDA-468 | Breast | | 5897 ins. CAA | Hom.. | Ins. of 1 Glutamine residue |
| TC7 | Colorectal | | 6055 ins. CAG | Het. | Ins. of 1 Glutamine residue |
| TC7 | Colorectal | | subs 6143 C-G | Het | 1824 L/V |
| LS 411 | Colorectal | | 5897 ins. CAA | Het. | Ins. of 1 Glutamine residue |
| Co115 | Colorectal | | del. 5891-5893 | Het. | Del. of 1 Glutamine residues |

# 5. DISCUSSION

Cancerous cells have to eliminate many steps of growth regulation in order to survive and continue their uncontrolled growth. One of the mechanisms to bypass this regulation is to eliminate the function of tumor suppressor genes (Hanahan and Weinberg 2000). There are many ways to alter the function of a tumor suppressor gene such as downregulation of its expression genetically or epigenetically, deletion of one or both alleles of the gene, inactivating mutations and so on (Albertson et al. 2003).

Identifying tumor related genes is crucial to have a better understanding of tumor development, mechanisms underlying tumor formation and to discover new treatments for cancer. Discovering tumor related genes are also vital to develop gene-targeted therapies such as herceptin and gleevec (Albertson et al. 2003).

One of the wildly used methods to identify tumor suppressor genes is positional cloning method. This method utilizes the fact that chromosomal regions that are lost during tumorigenesis generally contain tumor suppressor genes. Minimal deletion regions in tumors are detected by LOH analysis and the genes in these regions are screened for mutations or expressional silencing because according to Knudson's "two-hit" hypothesis, both copies of a tumor suppressor gene have to be eliminated to cause cancer formation (Presneau et al. 2003).

Chromosome arm 16q is lost frequently in many tumors including HCC, breast cancer and glial tumors. LOH studies showed that there are some minimally deleted regions on chromosome arm 16q showing these regions are expected to contain tumor suppressor genes. Different studies point different size of deleted regions such as 16q22, 16q21-24, 16q21-qter and 16q22-qter (Knuutila et al. 1999). Fine mapping studies on chromosome 16q show several distinct minimally deleted region and suggest that this chromosome arm hosts at least two different tumor suppressor genes (Callen et al. 2002; Cleton-Jansen et al. 2001; Driouch et al. 1997). One of these genes, WWOX is located on 16q24 has cloned recently and alterations of this gene has been shown in several cancers (Bednarek et al. 2000; Paige et al. 2001)

Despite chromosome 16q22 is also frequently lost in a variety of tumors; studies had failed to identify a tumor suppressor gene in this region until recently. Sun et.al. (2005) showed that chromosome arm 16q22 was lost in prostate tumors and identified a minimally deleted region containing the gene ATBF1. They demonstrated that in nearly half of the prostate cancers ATBF1 gene expression was downregulated and ATBF1 harbored somatic mutations causing loss of function of ATBF1 in prostate cancers. They also functionally showed that ATBF1 was a tumor suppressor gene in prostate cancer.

Contrary to accumulating data in favor of association of ATBF1 gene as a tumor suppressor in tumor development; one study by Li et. al. (2002) showed that ATBF1 was expressed in 1 out of 4 cervical cancer cell lines but in none of the eight normal cervical tissues. However, this seemingly contradictory data should be further analyzed to show the ATBF1 expressing cell line has an intact copy of this gene. If there is an alteration of this gene with a dominant negative effect, it would be an additional data in support of ATBF1 as a tumor suppressor gene.

Some tumor suppressor genes are frequently mutated in exclusively one type of tumor (such as VHL gene in renal carcinoma), whereas some are mutated in multiple types of tumors (such as p53) (Presneau et al. 2003). This fact, together with frequent LOH on chromosome 16q in other tumors (Knuutila et al. 1999) supports the idea that ATBF1 may be involved in development of other tumors as well.

Our aim in this study was to investigate role of ATBF1 gene as a multi-tumor suppressor gene in tumors from a variety of tissues including HCC, breast cancer, colorectal cancer and glial tumors.

We have concentrated only on the regions that were highly mutated in prostate cancers according to the recent study by Sun et. al.(2005). Since the mutations of ATBF1 are accumulated in well defined regions of the gene similar to BRAF, PIK3CA and p53 mutations we thought that mutation screening of these regions would give a preliminary idea about the involvement of this gene in several cancers (Davies et al. 2002; Samuels et al. 2004; Soussi and Lozano 2005).

In this study, we identified a total of 19 alterations in ATBF1 gene. 13 of the alterations were 1 or 2 glutamine insertions or deletions in the same glutamine-consecutive sequence ($Q_{19}$ 1723-1741). Another glutamine insertion (6055insCAG) was in another glutamine rich region. One alteration on exon 10 (del. 10814-10837) was only present in Huh7 cell line. Other than insertions or deletions, we also identified 4 single nucleotide substitutions causing amino acid changes.

Our analysis on ATBF1 exon 10 was limited to the frequent 7-8 aminoacid deletion (del. 10814-10837). We screened 14 HCC cell lines, 50 HCC tumors, 8 breast cell lines, 13 colon cancer cell lines and 50 glial tumors for this alteration and found that only Huh7 cell line had this deletion.

On the mutation region on exon 9; HCC cell lines had no mutations (0/14, 0%), HCC tumors carried 9 alterations (9/30, 30%), Colorectal cancer cell lines showed 4 alterations in 3 cell lines (3/13, 23%), breast cell lines showed 2 alterations (2/8, 25%) and glial tumors had 3 alterations (3/50, 6%).

Furthermore, our polyacrylamide gel electrophoresis analysis on 11 HCC samples from Turkish patients showed no 3 or 6bp alterations.

Two of these alterations (del. 5891-5896 and 5897 ins. CAA) were previously shown to be somatic polymorphisms (due to their occurrences in healthy individuals) in prostate cancer. 3 of the point mutations that we identified, Leu1824Val and Gln1852Pro (2), were also considered as polymorphisms due to the existence of the same alterations in matching normal tissue or in healthy individuals, respectively (Sun et al. 2005).

Our analysis on 3 histologically-normal matching tissues of HCC tumors with homozygous 3 and 6 bp insertions showed that these alterations were heterozygous in normal samples. This data shows that the wild type allele was lost during tumor development and these insertions may provide a growth advantage for the cancer cells. Although, Sun et.al. (2005) showed these alterations were also present in 5% (3/40) of the normal population, our data shows that these alterations are more frequent in HCC samples (12%, 7/55) and in all tumors we analyzed (10%, 13/126).

However, the predominance of these alterations in Chinese (14%, 1/7), Japanese (25%, 2/8) and African (57%, 4/7) patients rises the possibility that these alterations might be polymorphisms which are frequent in these populations. To determine whether these alterations are benign polymorphisms or have a causal effect on tumor development, a population screening for these alterations and functional studies showing the effect of these changes need to be done. However, the fact that it is difficult to obtain a large enough sample collection containing DNAs from Chinese, Japanese and African populations makes the population screening quite challenging for us.

Glutamine or Proline rich domains are important acidic transactivation domains for transcription factors in organisms ranging from budding yeast to mammalians (Remacle et al. 1997). Mutations disrupting these regions were shown to impair transactivation capabilities of transcription factors such as p73 (Takada et al. 1999). This suggests glutamine rich regions of ATBF1 are highly likely to be transactivation domains and frequent 3 and 6 bp alterations that we identified in glutamine rich regions of ATBF1 may disrupt its activity as a transcription activator.

Finally, we have found frequent ATBF1 genetic alterations in HCC, breast cancer, glial tumors and colorectal cancers. Even though most of these alterations seems to be polymorphisms, some of them are highly likely somatic such as 24 bp deletion in Huh7 cell line, 1822L/V mutation in a glial tumor. In addition, three tumor samples displaying homozygous insertions found to be heterozygous when tested in normal counterparts, suggesting somatic loss of a normal allele. Thus, ATBF1 gene fits well in to Knudson's two-hit hypothesis. Taken together, somatic alterations and highly frequent polymorphisms of ATBF1 gene found in different cancers and work published by Sun et al.(2005) allow us to speculate that ATBF1 gene is a multi-tumor suppressor gene and genetic alterations of unstudied regions as well as epigenetic alterations of this gene are worth studying.

Our future perspectives include determining if the alterations we found in ATBF1 have any functional effect on growth regulation and tumor development. Moreover, we are planning to study the outcomes of ATBF1 alterations on its inhibitory effect on c-Myb and activating effect on p21 promoter.

We will also investigate expressional regulation of ATBF1 in different tumors since it is possible that in some tumors ATBF1 gene loses its function by repression of expression. To do this, we are planning to carry out real-time PCR experiments to quantify and compare ATBF1 mRNA levels in tumors and normal tissues. If we find downregulation of this gene in tumors, we will analyze its promoter region for its methylation status.

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