PCR-RFLP Based Analysis of Functional Polymorphism at MicroRNA-629-Binding Site in the 3′-Untranslated Region of *NBS1* Gene in Prostate Cancer Patients in Local Population

By

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1. Cancer - Genetic aspects
Cancer - Environmental aspects
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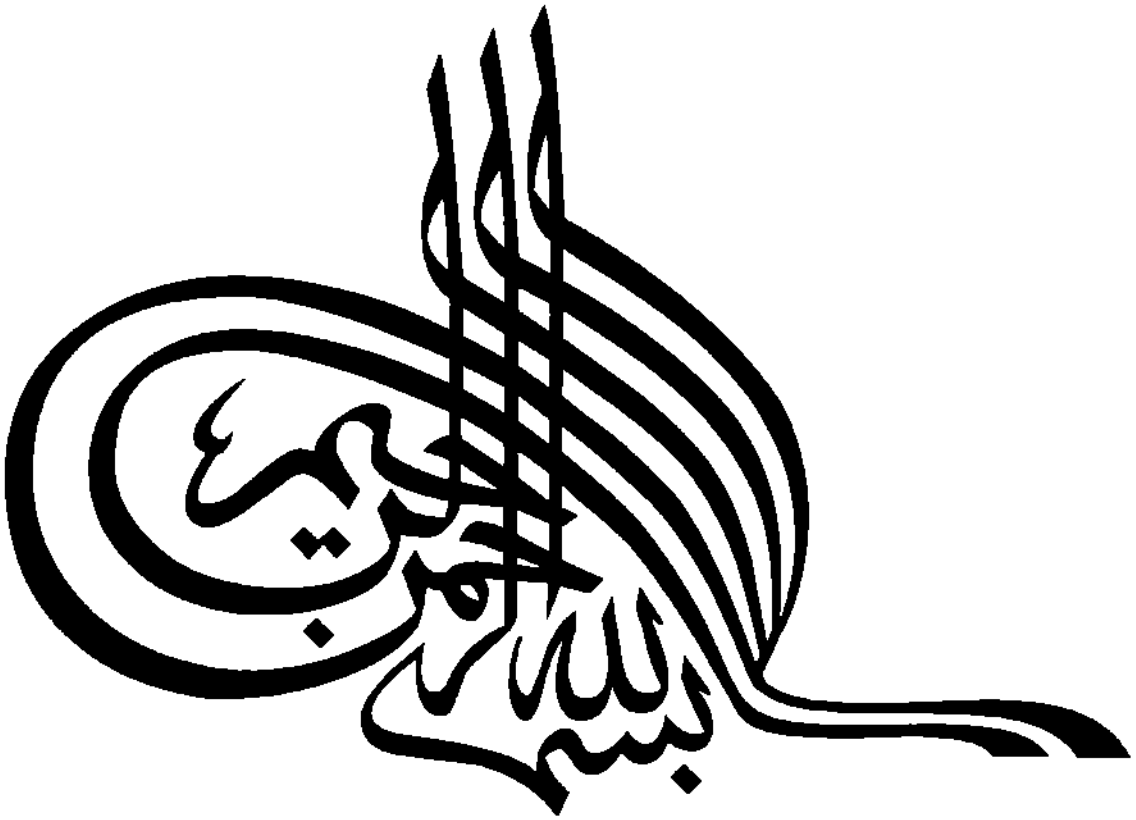

In the name of Allah,
the Most Beneficent,
the Most Merciful

Department of Bioinformatics and Biotechnology

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FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms Shahzadi kanwal and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University Islamabad for the M S Degree in Biotechnology

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A thesis submitted to Department of Bioinformatics and Biotechnology,

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fulfilment of requirement for the award of the

Degree of MS Biotechnology

DEDICATED

TO

MY

WORLD'S BEST

BELOVED

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GIT1	G-protein-coupled receptor kinase-interacting protein 1
HR	Homologous recombination
HRPC	Hormone refractory prostate cancer
IR	Ionizing irradiation
$KHCO_3$	Potassium carbonate
KLK3	Kallikrein-related peptidase 3
KRL	Kahuta Research Laboratories
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
NBS1	Nijmegen breakage syndrome 1
NH_4Cl	Ammonium chloride
NHEJ	Non-homologous end joining
P	p-value
Pca	Prostate cancer
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNAi	RNA interference
rpm	Revolution per minute
rs	Reference SNP ID Number
SDS	Sodium dodecyl sulphate
SNPs	Single nucleotide polymorphism
SPSS	Software package Used for Statistical Analysis
ssRNA	Single stranded RNA

STL	Sodium chloride, tris-HCl and EDTA
TBE	Tris borate-ethylene diamine tetra acetic acid
TL	Tris EDTA
Thr	Threonine
TRBP	Transactivation Responsive RNA binding Protein
TRIM33	Tripartite motif-containing 33
UV	Ultra violet
Xpo5	Exportin protein
µL	Microliter
χ^2	Chi-square vale

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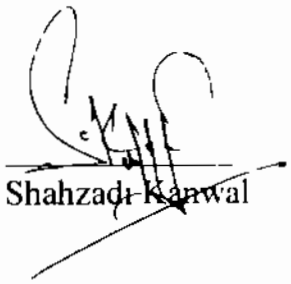
DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition No part of the thesis has been previously presented for any other degree

Date 28-3-16



Shahzadi Kanwal

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In the name of **Allah Almighty**, The most gracious and The most merciful The creator, The most supreme Whose mercy enabled me to accomplish this task and bestowed me with success He blessed me and my parents with resources, audacity and imperturbability I plead and implore to **Allah Almighty** that He continues to bless me, my teachers and all human beings Next to the entire messenger, **Hazart Muhammad (Peace Be Upon Him)** Who is the internal touch of guidance and knowledge For the humanity as a whole and is an ever inspiration for all the learned means

I take this opportunity to thank all those who helped me in this research study First of all I would like to express my deepest appreciation and most heartfelt gratitude to my supervisor Dr Sumbul Khalid (assistant professor, department of bioinformatics and biotechnology, IIUI Islamabad) for her keen interest and persistent encouragement Despite her busy and tiring schedule she was always there for me and ready to help me in the best probable way I'm very grateful for her timely help whenever I needed

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Shahzadi Kanwal

LIST OF ABBREVIATIONS

Acronym	Abbreviation
ACD	Acid Citrate Dextrose
AIPC	Androgen-independent prostate cancer
AR	Androgen Receptor
Arg	Arginine
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutation
bp	Base pair
°C	Degree Celsius
CDK	Cyclin-dependent kinase
DBD	DNA binding domain
DGCR8	DiGeorge syndrome critical region 8
dH_20	Distilled water
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotides Triphosphate
DSBs	Double-strand breaks
EDTA	Ethylene Diamine Tetra Acetic Acid
Fig	Figure
FISH	Fluorescence in situ hybridization

GIT1	G-protein-coupled receptor kinase-interacting protein 1
HR	Homologous recombination
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SPSS	Software package Used for Statistical Analysis
ssRNA	Single stranded RNA

STE	Sodium chloride, tris-HCl and EDTA
TBE	Tris borate-ethylene diamine tetra acetic acid
TE	Tris EDTA
Thr	Threonine
TRBP	Transactivation Responsive RNA binding Protein
TRIM33	Tripartite motif-containing 33
UV	Ultra violet
Xpo5	Exportin protein
µL	Microliter
χ^2	Chi-square vale

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ABSTRACT

Prostate cancer is a genomically complex disease and data obtained through laboratory methodologies has considerably improved our understanding of the dysregulation of intracellular signaling cascades Moreover, microRNAs have also revolutionized our knowledge of cancer biology and it is now known that miRNAs considerably modulate wide ranging target genes In the present laboratory research, we studied 3' UTR C/T and A/G polymorphism in NBS1 gene in 60 prostate cancer patients and 60 healthy individuals without any previous clinical history, using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis For rs13312986 A>G genotypes, AA was 75% in patients and 70% in controls AG was 25% in patients and 30% in controls GG was 0% in patients and none was detected in control Allelic frequencies for A was 0 875 in patients and 0 85% in controls Allelic frequencies for G were 0 125% in patients and 0 15% in controls P value was insignificant for rs13312986 A>G genotypes For rs14448 T>C genotypes, TC was 71 6% in patients and 66 6% in controls TT was 28 3% in patients and 33 33% in controls CC was not detected either in patients or controls T allele was 0 64% in patients and 0 66% in controls C allele was 0 35% in patients and 0 33% in controls P value was insignificant for rs14448 T>C genotypes Future studies surely will demystify detailed mechanisms, as miRNA regulation of NBS1 is incompletely studied and exploration of these modulations will be helpful in getting a step closer to personalized medicine

INTRODUCTION

Prostate cancer (Pca) is a multifaceted and genomically complex disease and research over the decades has gradually shown tremendous breakthroughs in our understanding of the prostate cancer biology Using high-throughput technologies, it has been shown that overexpression of oncogenes inactivation of tumor suppressor genes, loss of apoptosis, genetic epigenetic mutations dysregulation of Androgen Receptor (AR) mediated intracellular signaling are some of the most extensively studied molecular mechanisms In-vitro studies have shown that Androgen treatment induced DNA damage in prostate cancer cells

AR has emerged as one of the most deeply investigated target for prostate cancer therapy Substantial fraction of information has been added into the existing web of knowledge and increasingly it is being realized that AR has evolved various mechanisms to transduce the signals to downstream effectors in absence or at low levels of androgen There are some exciting pieces of evidence which shed light on the DNA repair, chromosomal translocations and genomic rearrangements in prostate cancer (Farooqi *et al* , 2012)

Prostate cancer is one of the most pervasive malignancies around the world constituting a genuine health issue Clinically localized disease can be successfully treated whereas the disease which is spread throughout an organ or the body remains mostly lethal Increasingly it is being realized that miRNA plays an important role in deregulation These are single-stranded, small non-coding, untranslated RNAs which post-transcriptionally modulate wide ranging genes by interacting directly with messenger RNA (mRNA)

Increasingly it is being realized that Pca is the most common diagnosed cancer in men and is the second leading cause of death (Gronberg *et al* , 2003) Androgen is essential for almost all prostate cancerous cell as well a normal prostate tissues for their survival and growth Radical prostatectomy or radiation is used to treat localized cancer however Androgen ablation therapy is suggested for more advance cancers (Javidan *et al* , 2005, Deitch *et al* , 2005)

Substantial fraction of information has been added into existing pool of knowledge that the most protruding participant in the progression of AIPC is the androgen receptor (AR), a protein that binds to androgen and act as a transcription factor to regulate a wide pattern of gene involved in heterogeneous processes including growth and proliferation represented in Fig 1 3 (Chodak *et al* 1992 Ruizeveld de Winter *et al* , 1991, Sadi *et al* , 1991)

The role of AR in prostate cancer development has been researched in many studies however the precise molecular mechanisms that occur in the development to androgen independence remain largely unexplored and as a result there is no compelling therapies against AIPC (Attar *et al*., 2009, Debes *et al*., 2004, Feldman *et al*., 2001)

DNA damage consistently over deluge cellular action that response to DNA damage DNA damage action can develop malignancy due to diminishing the fidelity and efficiency of the DNA repair NBS1 protein that is integral part of RAD50-MRE11-NBS1 complex (MRN) play crucial role in cellular action to DNA damage and maintain integrity of chromosomes Nijmegen breakage syndrome (NBS) occur due to mutation in NBS1 gene NBS is a hereditary recessive genetic disorder which may increase risk of developing malignancy

It has previously been reported that gene–environment interaction between rs2735383CC genotypic variant and exposure to ionizing radiation (IR) contributed to an increased cancer risk (Yang et al, 2012) rs2735383CC variant genotype carriers had been noted to show an elevated cancer risk when exposed to high or medium level of radiation in an earlier published report (Yang et al, 2012) In accordance with the findings that radiations induced double stranded breaks (DSBs) in human cells and promoted cancer development and progression, it has been assumed that rs2735383CC genotypic variant having lower expression of NBS1 and defective DNA damage repair ability, interaction between rs2735383CC genotypic variant and radiation may increase risk of cancer (Yang et al, 2012)

In Chinese population it has been noted that chromatid breaks were notably higher in X-ray radiation exposed lymphocyte cells from carriers of rs2735383CC homozygote (Yang et al, 2012) It was concluded that rs2735383G>C variation contributed to an increased risk of lung cancer possibly through miR-629 mediated targeting of NBS1 (Yang et al, 2012)

The aims and objectives of the present study are to

- ✓ To study genetic variation at microRNA-629-binding site in the 3'-untranslated region of *NBS1 gene* in prostate cancer patients
- ✓ There is no previously existing data with reference to germ line mutations and somatic mutations identified in *NBS1 gene* in prostate cancer patients of Pakistani population
- ✓ To probe the genotyping and allelic frequency of *NBS1 gene* associated with risk of prostate cancer
- ✓ To get further understanding about the genetic basis of prostate cancer

Literature Review

Prostate cancer is one of the most pervasive malignancies around the world, constituting a genuine health issue Clinically localized disease can be successfully treated whereas the disease which is spread throughout an organ or the body remains mostly lethal

In males, prostrate is an accessory gland and located on the floor of the pelvis and surrounds the neck of urethra and bladder Normal prostate does not block the flow of urine from the bladder however enlarged prostate exerts pressure on the bladder and urethra and blocks the flow of urine (see Figure 2 1) Prostate cancer progresses through different stages from stage 0 to stage 4 and is represented in Fig 2 2

2.1 Epidemiology

Prostate cancer (Pca) is the second prevailing cancer in males, peculiarly affecting older men over 80% of cases are investigated after age 65 Even though 10% of men with prostate malignancy die of this disease Therefore in Asian countries the most approbate access to the management of Pca is probably different and consequently individuate Pca screening and treatment strategies on the basis of epidemiological hallmark and socioeconomic status of each country are desired

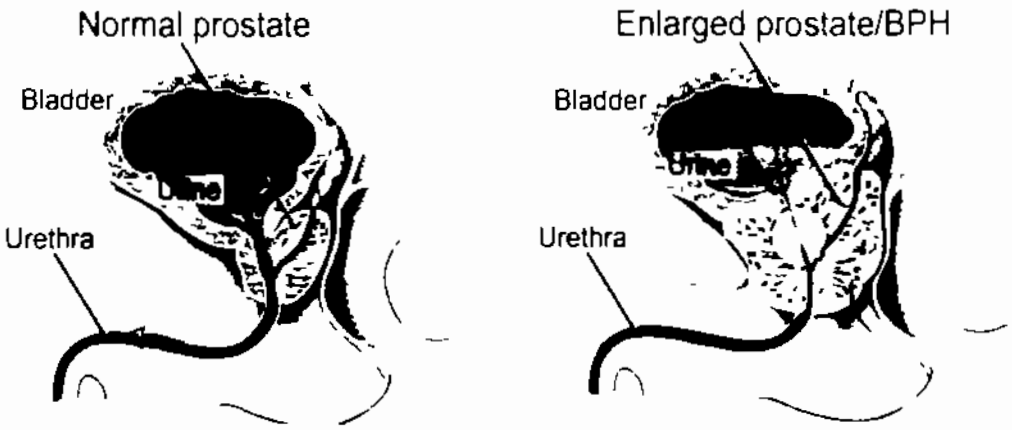

Fig 2 1 Anatomy of normal and enlarged prostate (Retrieved from http //www cancer gov/images cdr/live/CDR462221)

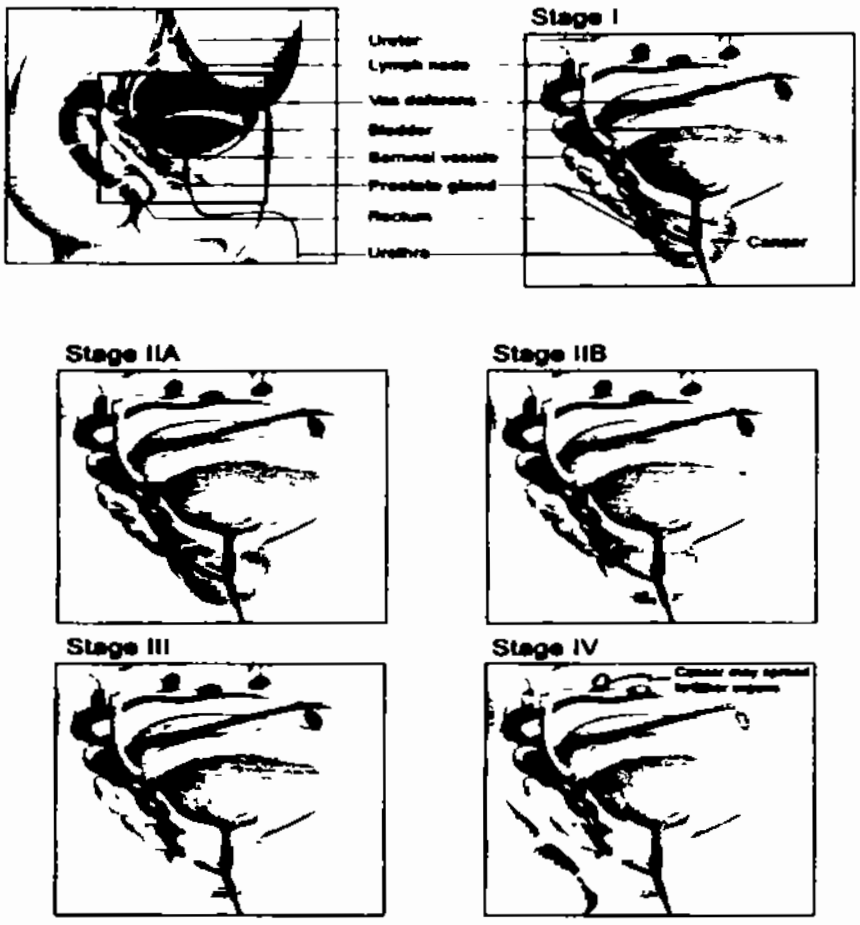

Fig 2 2 Different stages of prostate cancer (Retrieved from http //www cancer gov/images/cdr/live/CDR442273)

2.2 Androgen receptor: Progression model of Pca

It is intriguing that most of the tumor have become androgen independent but still depend upon the androgen receptor (AR) signaling Mechanism that upgrade AR signaling in androgen-depleted condition comprise AR gene amplification, change in the balance of AR cofactor AR mutation, increase in the steroidogenic precursors and activation through 'outlaw pathways Numerous other AR-independent "bypass" pathways has been demonstrated to achieve apparently during androgen independence

MicroRNA concentration and change in the epigenetic impression have also been suspected in the development of androgen independent prostate cancer Understanding of molecular mechanisms that facade to the development of androgen independent prostate cancer will concede for better and improved therapeutic approach that mark key pathways and molecules that are indispensable for these cell to survive In spite of very efficient at minimizing cancer growth this treatment hereafter selects for those cells that are no longer susceptible to such therapy resulting in a persist lethal cancer i e androgen-independent prostate cancer (AIPC) (Denmeade *et al* , 2002, Isaacs *et al* , 2002)

Cancerous cell has accomplish the ability to grow in the absence of androgen in advance stage of Pca so, hormone therapy is no longer effective and this is the stage at which most patients develop AIPC Despite androgen independence occurs but most cancers will become androgen independent because these cancerous cell mutate themselves in such a way that enhance their growth in the absence of vital survival factor such as androgens (See fig 2 3) Interestingly term AIPC is less widely acknowledged because threatening form of cancer still depend upon androgen signaling However hormone refractory prostate cancer (HRPC) is becoming more widely acknowledged (Crawford *et al* , 2010, Petrylak *et al* , 2010)

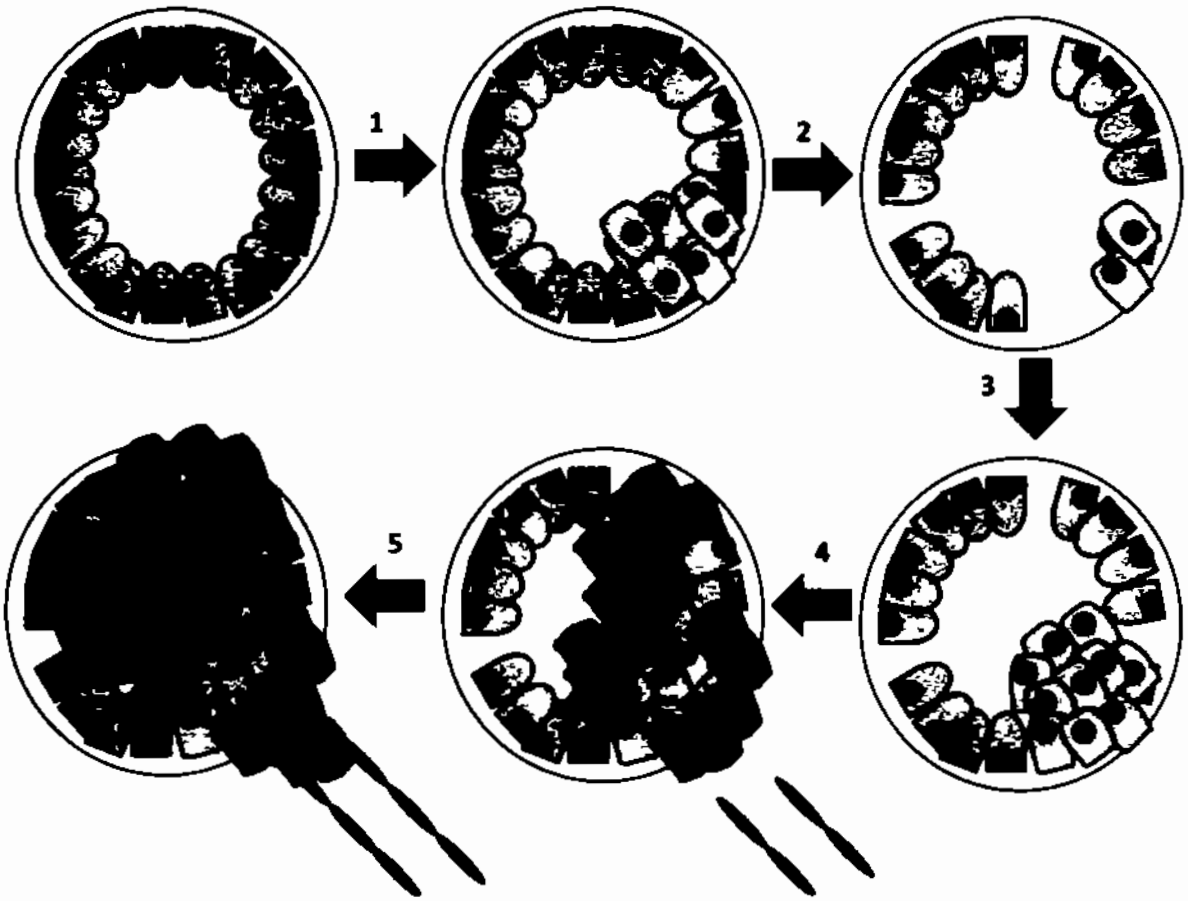

Fig. 2 3· Progression to androgen independence in prostate cancer cells Different cancer causing forms happen whereby few prostate cells multiply wild (2) Prostate growth cells are at first androgen subordinate along these lines androgen deprivation treatment is effective in pulverizing these disease cells (3) A few cells can survive under this treatment hence keep multiplying (4) Cells are presently androgen autonomous and increase ensuing changes bringing about expanded angiogenesis (5) Androgen-independent prostate cancer (AIPC) starts to metastasize to inaccessible locales (Punit Saraon *et al.*, 2011)

There is a list of exciting findings which has helped us in developing a better understanding of the underlying mechanisms of prostate cancer It has previously been convincingly revealed that that initial proliferation of prostate cancer cells is androgen dependent and accordingly pharmaceutical inhibition of androgen may be effective against these cancer cells However androgen exposure induces rewiring of intracellular signaling cascades which make prostate cancer cells resistant against different androgen deprivation therapies

2.3 Androgen Receptor signaling: Important Modulator of Prostate cancer progression

Testosterone is primary androgen produced by the body released by Leydig cells of the testes In small quantity are also produce in the adrenal glands Normally free circulating testosterone is not bound to steroid hormone binding protein It enters into prostate cells and binds with 5-alpha-reductase, which results in its conversion into a potent metabolite dihydrotestosterone (DHT) DHT binds to AR protein represented in Fig 2 4 Testosterone itself can bind to the AR but DHT has much more greater potency and have higher affinity (Radmayr *et al* , 2008)

AR nuclear accumulation triggers transcriptional regulation of different genes involved in proliferation AR protein is structurally composed of ligand-binding domain, DNA binding domain (DBD) and amino-terminal domain Targeting of amino-terminal domain has proved to be an efficient strategy to inhibit activity of the AR (Andersen *et al* , 2010) Cytosolically located conformational re-organized androgen bound AR gets sequestered from heat shock protein/proteins (HSPs) and enters into the nucleus AR tethered to DNA sequences through its DNA binding domain AR regulated different genes which modulate prostate cancer progression and metastasis Kallikrein-3 encoded by Kallikrein-related peptidase 3 (*KLK3*) is a cancer biomarker reportedly modulated by AR

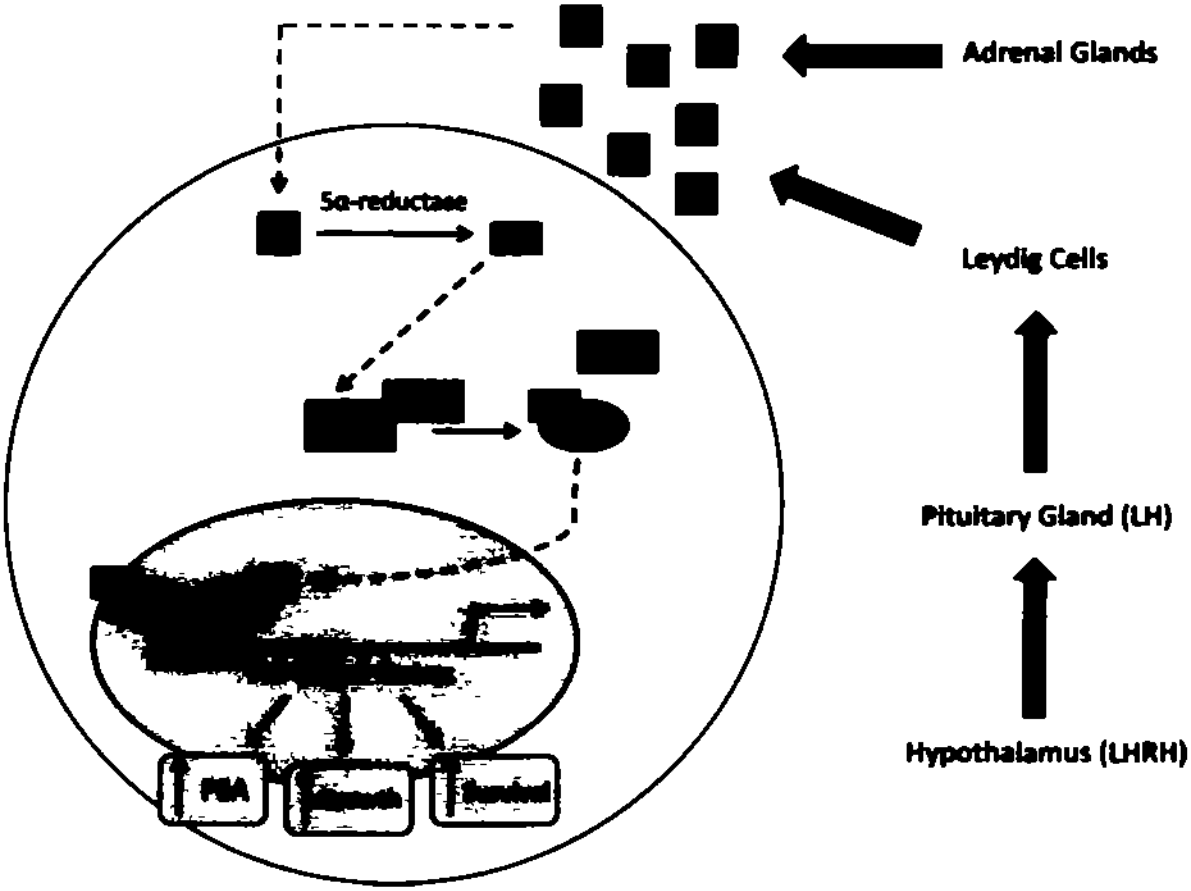

Fig. 2.4 Mechanisms of the androgen action and androgen receptor induced signals in prostate cells (Radmayr *et al.*, 2008)

2.4 Nijmegen breakage syndrome 1

Nijmegen breakage syndrome (NBS1) the autosomal recessive disorder was first observed in 1981 in the patients who lived in Nijmegen, Holland Confluence of information suggested that NBS patient s cell have increased level of damage to chromosomes and prone to ionizing irradiation (IR) Increasingly it is being realized that IR caused most dangerous DNA lesions NBS patients cell are sensitive to all DSB inducing mutagens NBS1 gene is found to be responsible for Nijmegen breakage syndrome in 1998 (Carney *et al* , 1998, Matsuura *et al* 1998, Varon *et al* 1998)

Nibrin is the gene product of NBS1 and contains a 26 kDa fragment (p26-nibrin) the BRC I and FHA domains complementary to the N-terminal portion of nibrin and a 70 kDa (p70-nibrin) originated by alternatively initiated translation from cryptic upstream start codon (Master *et al* 2001)

Nibrin additionally with Rad50 and Mre11 is a part of MRN complex that is trimeric and preserves between mammals and yeast Confluence of information show that nibrin is phosphorylated at certain sites in response to introduction of double-strand breaks (DSBs) The kinase dependable is the essential initiator of cellular reaction to double-strand break, ataxia telangiectasia mutation (ATM), bi allelic transformation of which prompts ataxia telangiectasia (A T) a huddle with numerous components additionally observe in NBS

Post-translational modifications of Nibrin are modulated by different enzymes Ionizing radiation induced phosphorylation of nibrin is further controlled by the deacetylase SIRT1 SIRT1 hypo acetylates nibrin for efficient kinase induced phosphorylation (Yuan *et al* , 2007)

It is becoming progressively more understandable that nibrin play important role in trigging auto phosphorylation of ataxia telangiectasia mutation (ATM) in response to DNA-damage Deoxyribonucleic acid (DNA) is, simply put, the molecule used to encode all the genetic information required by the living organism to maintain its life functions Therefore its stability and integrity is of absolute importance DNA is actually not a "full-protected" static substance – it is a chemical entity composed of nucleotides attached to a sugar-phosphate backbone Such a structure can be damaged by environmental factors such as irradiation, chemicals, and ultraviolet rays (UV)

The DNA can also undergo oxidative damage by free radicals, which are byproducts of metabolism One serious result of these chemical changes in DNA can be double-strand breaks (DSBs) in the chromosome Be it environmental or internal, DNA damage must be repaired as the accumulation of unrepaired DNA can lead to aging and disease Organisms have developed various processes to detect and repair DNA breaks There are two main double-strand break (DSB) repair pathways homologous recombination (HR) and non-homologous end joining (NHEJ) One protein complex the Mre11-Rad50-NBS1 (MRN), has diverse roles in both HR and NHEJ Each protein component of this versatile complex contributes to the overall structure and functions

2.5 Multiple roles of the MRN complex in DSB repair

MRN complex may involve at different steps in double-stranded break (DBS) along with DNA end processing, break recognition and cell signaling cascade And MRN complex that is primary damage sensor may play role in DBS repair in both yeast and human cells (D Amours & Jackson *et al* 2002) Importance of Rad50-MRE11-NBS1 gene can be observed when any one out of these three genes is disarray resulting in embryonic lethality or cellular non-viability (Xiao & Weaver *et al*., 1997 Luo *et al*., 1999, Zhu et al , 2001) Patient of NBS manifest deficient immune system radiation sensitivity and high risk of malignancy (Shiloh *et al*., 1997) NBS and AT (ataxia telangiectasia) both are phenotypically same, (A-T) a related radiation sensitivity disarray (Stewart *et al*., 1999)

2.6 Cellular features of NBS

Cells illustrate pleiotropic phenotype which are isolated from NBS patients viz the hyper-sensitivity to ionizing radiation, chromosome impairment, impaired cell cycle checkpoints (Shiloh *et al*., 1997) Cellular studies have shown that homologous recombination reduced notably in NBS defective cells

The drift of chromosomal mutation is 2 5± 4 fold elevated in NBS as compare to the normal cells when lymphocytes are exposed to radiations, resulting in chromosomal break That is similar to ataxia telangiectasia cells and these breaks are tethered to the chromatid type, implying defects in the repair of double-stranded breaks during the S-phase of cell cycle Furthermore, susceptibility of ionizing radiation of Nijmegen breakage syndrome heterozygotes has not been proved however many attempts are made

Recently Data obtained through Fluorescence in situ hybridization (FISH) analysis revealed significantly enhanced chromosomal impairment in lymphoblastic cells derived from the NBS heterozygotes. When NBS deficient cells are exposed to ionizing radiation it illustrate abnormal cell cycle regulation although this deformity is limited in nature And due to radiation unable to conceal their DNA synthesis also intending some deficiency in intra S Phase check points Furthermore along with deficiency in intra S phase checkpoint control interruption in G1 and G2 checkpoints are also recognized in the NBS cells

2.7 Vital role of NBS1 in homologous recombination

It is becoming progressively more understandable that NBS1 has a vital role in double-stranded break repair as it coordinate the MRN complex for nuclease action and repair to damage sites It is intriguing that there are two pathways to rejoin/repair double-stranded breaks viz homologous recombination (HR) and non-homologous end joining (NHEJ) NHEJ has proteins such as DNA-PKcs, Ku70/80 and DNA ligase IV and proteins for the HR are RAD51, RAD 52 and RAD 54 It has been observed that these proteins are immensely specific for particular double-stranded break repairing pathway (Haber *et al*, 1998)

Increasingly it is being realized that NBS1 functions in homologous recombination (HR) relatively in non-homologous end joining (NHEJ) On the contrary it is recognized that non-homologous end joining (NHEJ) are tantamount from wild type cells manifesting that NBS1 is not vital for non-homologous end joining (NHEJ) And is sustained by manifestation that V(D)J recombination, less commonly known as somatic recombination which is to be initiated by non-homologous end joining is prevalent in NBS patients (Harfst *et al*, 2000) MRN Complex is essential for Ig class switch recombination events (Pan *et al*, 2002 Petersen *et al* 2001, van Engelen *et al* 2001)

Data obtained through high throughput technologies has considerably improved our understanding that NBS1 played a vital role in regulation of the V (D) J recombination through suppression of inter chromosomal recombination NBS1 suppresses inter chromosomal homologous recombination (HR) and non-homologous end joining (NHEJ) through MRN complex formation Deregulation or functional inactivation may cause instability of genome mainly through translocation as recognized in the NBS cells

2.8 Role of NBS1 in cell-cycle checkpoint control

Confluence of information suggested that NBS defective cells are radiosensitive and upon treatment with radiomimetic drugs or exposure to ionizing radiation, it show defects in cell cycle check points

Substantial fraction of information has been added into existing pool of knowledge that NBS1 and ATM both are involved in the intra S phase checkpoint controls over the SMC1 phosphorylation

2.9 Role of NBS1 in telomere stability maintenance

Confluence of information show that telomeres are highly functional nucleoprotein structures which maintain the genome integrity by protecting and stabilizing the linear chromosomal ends Telomeric DNA comprised of tandem array of the 5-26 base pair repetitive sequence of G-rich that align 5′ to 3′ end and these also assist as the binding sites for various particular telomeric proteins

Ataxia telangiectasia (AT) is clinically classified as the premature aging syndrome (Metcalfe *et al*., 1996) Clinically premature aging is not examined in the NBS although NBS patient's blood cell have reduced length of telomeres (Ranganathan *et al* 2001) Due to this abnormal telomeric shortening in the NBS cells, NBS1 shows interaction with telomere binding proteins i e TRF1 and TRF2 in mammalian telomeres (Zhu *et al*., 2000, Wu *et al* 2000b) And this interaction can be recognized only at the early S phase of cell cycle where telomeric elongation take place, though MR complex binds to the TRF2 during the whole cell cycle implying that the NBS1 play role in telomere replication after the formation of MRN complex like for the DNA repair (Ranganathan *et al* 2001)

Increasingly it is being realized that NBS1 play role in telomere maintenance to accomplish G-strand (telomeric 3′ overhang) Hence throughout the cell cycle telomeric fusion take place and telomeric fusion transfer to the decedents due to breakage fusion bridge cycles which leads to the chromosome mutation (Lo *et al*., 2002)

2.10 Micro RNA

MicroRNAs are a class of small non-coding RNAs usually 10-25 nucleotides that after their processing bind to specific mRNA and degrade it before transcription They were first discovered in 1993 in non-mammalian species and were characterized to play a pivotal role in development and differentiation in the year 2001 The transcription of miRNAs begins under the supervision of RNA Polymerase II in nucleus that generates a primary miRNA molecule consisting of 5 cap and a poly A tail at 3' end Most of miRNAs genes are transcribed from the intronic region of DNA and are either transcribed as a single gene or cluster

This newly synthesized pri-miRNA then undergoes further processing by micro-processing complex consisting of RNAse III enzyme DROSHA and a RNA binding protein DGCR8 (DiGeorge syndrome critical region 8) that trims this molecule in to double stranded pre-miRNA characterized by its hairpin loop structure DROSHA contains two domains for RNAse III that deliberately excises the long structure of Pri-miRNA

This Pre-miRNA is then transported out of the nucleus by the aid of Exportin protein (Xpo5) these protein carry two NLS nuclear localizing sequences that transfer Pre-miRNA out of the nucleus The energy required for this transportation come Ran GTPase Upon entering the cytoplasm Pre-miRNA is further processed by another RNAse III enzyme named Dicer1 DICER activity transform Pre-miRNA in to mature miRNA consisting of a leading strand This mature miRNA is then loaded on to the RNA induced Silencing complex that already carries TRBP (Transactivation Responsive RNA binding Protein and AGO (Argonaut proteins) There are different views about the mechanism of action of TRBP and AGO, most appraisal view suggests that TRBPs aid in physiological binding of different types of AGO proteins with DICER This activity in turns promote guiding strand to bind to the target mRNAs in the presence of GW 18 proteins and gene silencing of targeted mRNAs

2.10.1 History of miRNA

Lee *et al* in 1993, recognized that the lin-4 gene is interpreted into the ~ 22 nucleotide long RNA which inhibits the lin-14 gene through RNA interference (RNAi) in *Caenorhabditis elegans* (nematode) It is also concluded that the lin-4 gene may associate with the regulatory, small non-translated RNA Let-7, second microRNA in *Caenorhabditis* elegans, *Drosophila* and human was recognized in the 2000 (Reinhart *et al*., 2000)

Moreover with discovery of ~22 nucleotide RNAs in 2001(Lagos-Quintana *et al* 2001 Lee and Ambros *et al*., 2001, Lau *et al*., 2001) named them as microRNAs (miRNAs)

It is interesting to note that in human genome ~1000 miRNAs are predicted to exist (Berezikov *et al*., 2005) Sanger miRBase (the microRNA database) was created to administer the database of microRNA sequence and regulate nomenclature in 2006 (Griffiths-jones *et al*., 2006) It is intriguing that in March 2011, about 1037 human microRNA were commentate at the miRBase

2.10.2 MicroRNA genomic location

It is intriguing that many microRNAs are positioned in noticeable regions that are far from the protein coding genes (Lee *et al*., 2001, Ambros *et al*., 2001) These microRNA genes are assert from their own promoter In addition ~40% of the microRNA are positioned in introns of the protein coding genes, generally with equivalent orientation same as host genes (Rodriguez *et al*., 2004) Moreover some microRNAs are positioned far from the other microRNA loci while other are positioned adjacent to one another and considered to be elucidate in the clusters (Altuvia *et al* 2005) MiRBase (the microRNA database) elucidate cluster as group of microRNAs that are positioned not beyond 10kb (kilo base) pair of each other

2.10.3 MicroRNA biogenesis

Confluence of information suggest that several investigators have been efficiently reviewed microRNA biogenesis (Bartel *et al*., 2004, Chen *et al*., 2005, Kim *et al*., 2005, Lomari and Zamore *et al* 2005) Briefly, miRNA is produced by RNA polymerase II and it is termed as primary-miRNA (pri miRNA) and the process is endogenous (inside the cell) Primary mi-RNA form distinct hairpin shaped, stem looped secondary structures containing an enzyme named Drosha (a RNAase III endonuclease) and fundamental cofactor DGCR8/Pasha

Drosha and DGCR8 combine together and act on the pri-miRNA and cut some region of pri-miRNA and make it shorter and is termed as pre-miRNA ~60-70 nucleotide long This pre-miRNA enters into cytoplasm with the help of protein i e Exportin 5 (Exp 5) member of Ran transport receptor family) So once this pre-miRNA enter into the cytoplasm this will undergo further stages of dicing i e cleavage after this dicer act on this pre-miRNA and activate it properly

Dicer is an RNAase III type of enzyme it is double-stranded RNA (dsRNA) specific endonuclease Dicer will bind with this pre-miRNA and cleave it and results in 3 overhang of 2 nucleotide and 5 monophosphate region and this complex is termed as miRNA miRNA* duplex ~15-20 nucleotide long

Finally miRNA miRNA* duplex is unwounded to produce mature miRNA and miRNA* with the help of helicase And then this mature miRNA is incorporated into a RISC (RNA induces silencing complex)

RISC is consisting of protein slicer and Argonaut (AGO 1-4) then this mature miRNA binds with RISC complex and facilitates gene silencing via binding to 3' untranslated region UTR sequences which are complementary to the miRNA sequence (Meister *et al*., 2004) Then RISC complex along with guide strand i e ~ 2-8 nucleotide long will migrate to the actual targeted sequence of single stranded RNA (ssRNA) leading to the translation inhibition or degradation of mRNA target represented in Fig 2 5

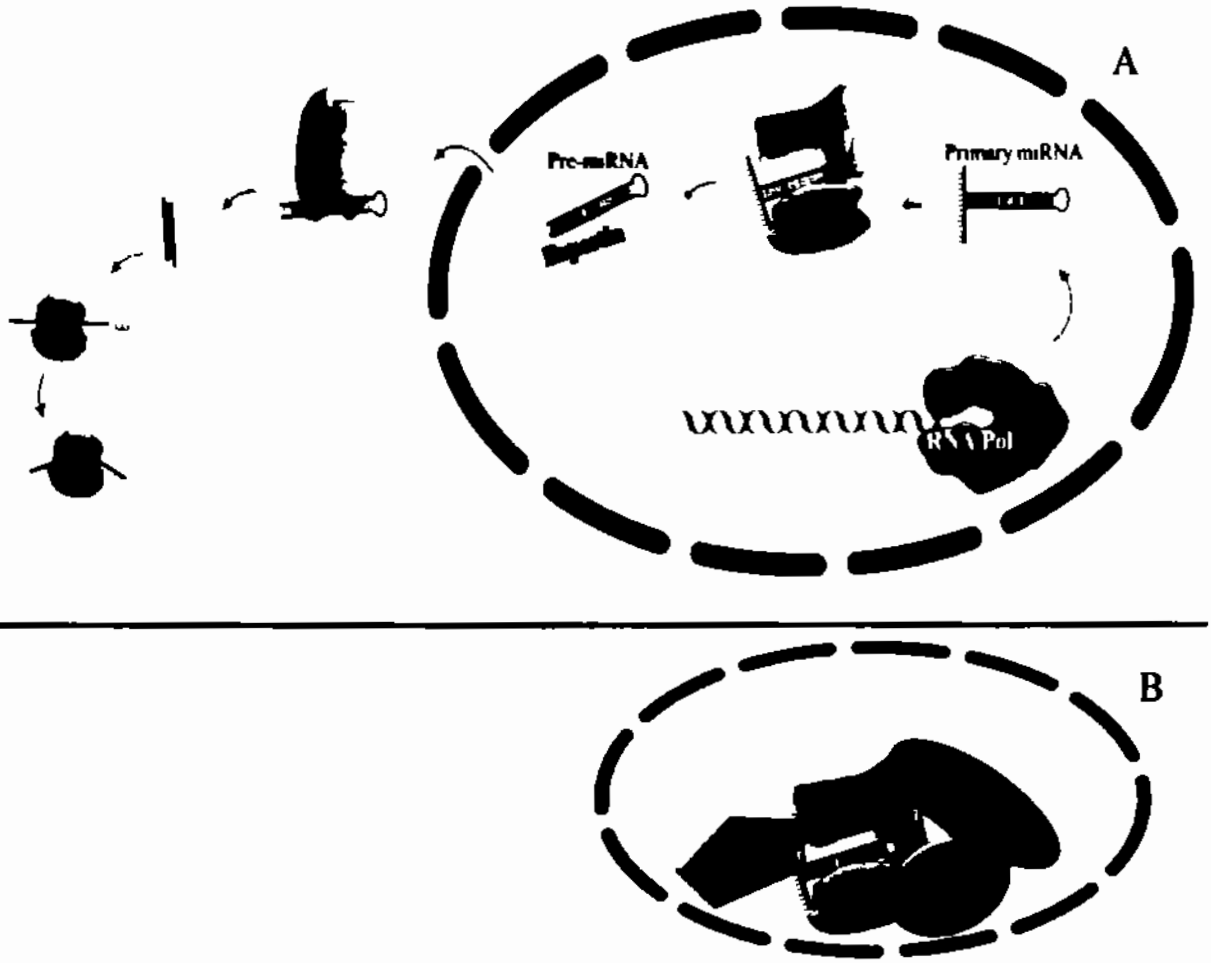

Fig 2 5 Schematic representation of miRNA biogenesis (Farooqi *et al*., 2012)

2.10.4 MicroRNAs regulation in cancer

It is intriguing that loss of apoptosis and abnormal growth lead to malignancy or cancer formation in the cell It is becoming progressively more understandable that microRNA involve in regulation of apoptosis and cell growth MicroRNA function in many pathological and biological processes lead to malignancy initiation, progression and metastasis formation MicroRNA regulate gene control in DNA damage repair DDR and its entanglement in malignancy progression will help in understanding the impact of these small molecules in the DNA damage or repair as well as chemo and radio-resistance mechanism

Furthermore studies acknowledge both upregulation and downregulation of microRNA in all substantial tumor and the leukemia Interestingly, while some microRNAs seem to have a tissue-specific function and are only expressed in specific cancers, others are universally over expressed or under expressed in cancer (Chan *et al* , 2005, Iorio *et al* , 2005, Volinia *et al* , 2006)

2.10.5 miRNA profiling in carcinogenesis

It is intriguing that microRNAs mediated all the hallmarks of malignancy including apoptosis, tissue invasion, uncontrolled replicative ability, metastasis and sustained angiogenesis (Hanhan and Weinber *et al* , 2011) MicroRNA can act both as oncogenes or tumor suppressor gene depending upon their target MicroRNA has highly complex regulatory machinery as one messenger RNA can be targeted by the multiple microRNAs Confluence of information suggest that microRNAs take on hundreds of targets synchronously as these are the tempting targets for therapy of cancer It is interestingly to noted that miRNA play role in tumorigenesis so it's become attractive target for novel therapies either in combination with the chemotherapeutical agents or may be alone

2.10.6 MicroRNAs as Oncogene

Confluence of information clearly suggest miRNA mediate targeting of tumor suppressor genes There is an overwhelmingly increasing list of the target reported to be quantitatively controlled by oncogenic miRNA In tumor when miRNA showed increased expression is considered as oncogenes/ oncomirs, involve in tumor progression by negatively inhibition of the tumor suppressor genes

Good example of an oncogenic microRNA is mir-17-92 and is located at the chromosome 13q31 the genomic locus that is exaggerate in different types of lymphoma and lung cancer also including diffuse large B cell lymphoma (Hayashita *et al* , 2005, He *et al* , 2005b)

It is intriguing that mir-17-92 involve in lung cancer and increase risk of lung cancer cell growth (Hayashita *et al* , 2005) Substantial fraction of information has been added into existing pool of knowledge that miR-17-92 is associated with the c-Myc (Avian myelocytomatosis virus oncogene cellular homolog) gene expression C-Myc is known to be as best characterized oncogene and both c-Myc and miR-17-92 are associated with the expression of the cell cycle transcription factor gene E2F1 (E2F Transcription Factor 1) is a Protein Coding gene (O'Donnell *et al* , 2005) Confluence of information show that if c-Myc gene is not properly expressed and show dysfunction it may lead to human cancer (Cole and McMahon *et al* , 1999)

Other oncogenic microRNAs are miR-373 and miR-372 reportedly involved in regulation of cancer as oncogenes in the human testicular germ cell tumor And these two oncogenic miRNAs are associated with the increase tumor development and cell proliferation by overcoming p53-mediated CDK (Cyclin-dependent kinase) inhibition or may possibly by direct inhibition of LATS2 expression (tumor suppressor gene) (Voorhoeve *et al* , 2006)

2.10.7 MicroRNA as tumor suppressor gene

Increasingly it is being realized that miR let-7 may highly maintain including worm to human being (Pasquinelli *et al* , 2000), and may also associated with the developmental stages and timings (Johnson *et al* , 2003, Johnston and Hobert *et al* , 2003, Miska *et al* , 2004 Thomson *et al* , 2004) Decreased levels of miR let-7 are investigated during early developmental stages of the animal and in differentiated adult tissues there are increased levels of miR-let-7 expression has been observed (Miska *et al* , 2004, Thomson *et al* , 2004) It is intriguing that if miR let-7 express inappropriately it leads to oncogenic loss of differentiation (Reinhart *et al* , 2000)

Recent studies reveals that miR let-7 directly targets RAS oncogene by its negative regulation result in repression of translation (Johnson *et al* , 2005) It is interestingly noted that there is decreased level of the miR let-7 and increased level of RAS protein in lung tumor tissues rather than the normal tissues implying that negative regulation of RAS by miR let-7 is a tool for miR let-7 to function as a tumor suppressor gene in case of lung oncogenesis represented in Fig 2 6 (Johnson *et al* , 2005)

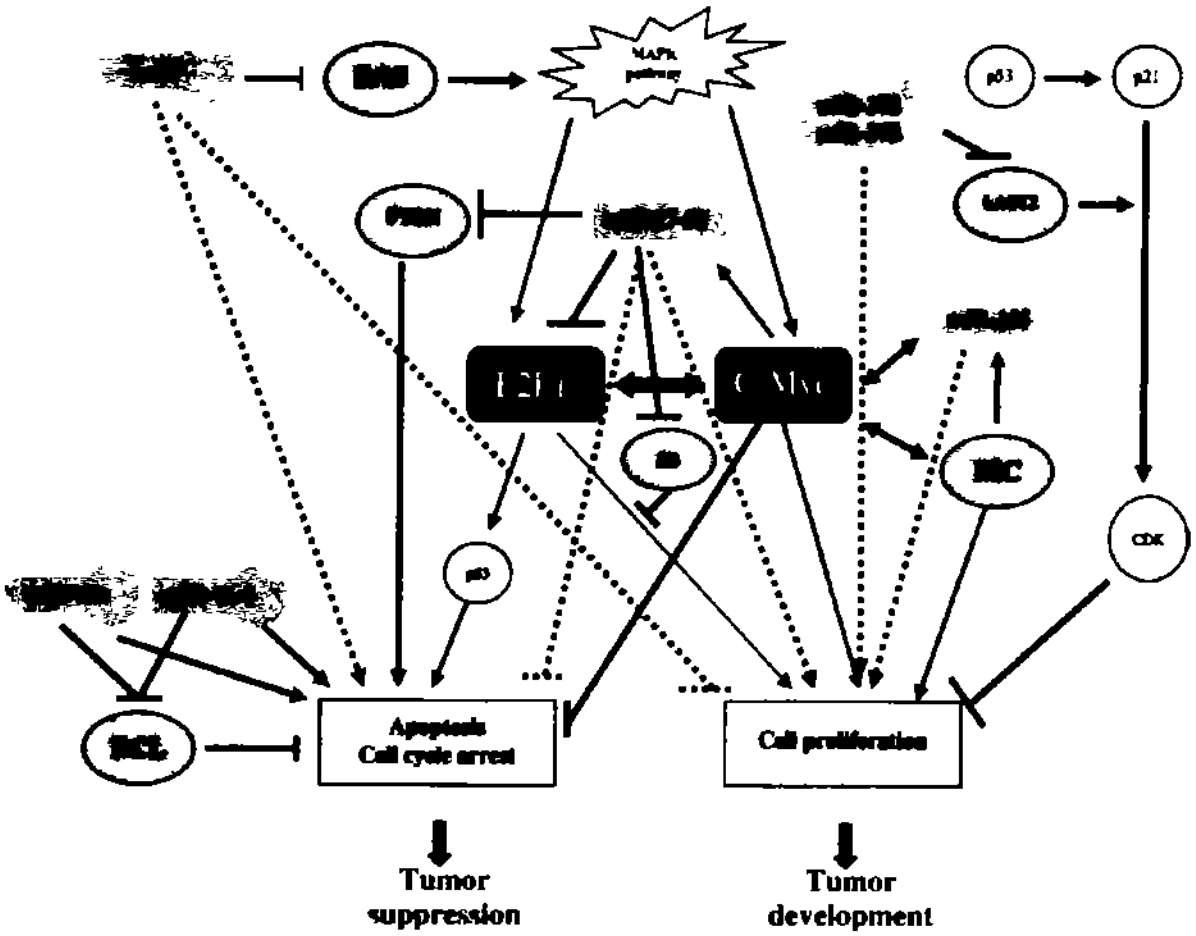

Fig 2 6 A schematic model showing the molecular mechanisms of microRNA-involved cancer pathogenesis (Baohong Zhang *et al* , 2006)

2.10.8 miRNA mediated suppression of cancer in xenografted mice

Increasingly it is being realized that activation of pro-survival signaling in cancer cells induces resistance against chemotherapeutic drugs Interestingly, there is tremendously accumulating experimental evidence of targeting of key proteins of pro-survival signaling using different strategies It has lately been shown that ectopic expression of miR-506 in lung cancer cells considerably repressed NFKB (Yin *et al* , 2014)

Growing body of evidence substantiates the fact that upregulated expression of hTERT is associated with the growth and metastasis of gastric tumor Intriguingly miR-1207-5p and miR-1266 remarkably inhibited tumor formation in mice (Chen *et al* , 2014)

It has recently been convincingly revealed that over-expression of miR181b in metastatic breast cancer cells notably repressed metastasis formation in immunodeficient mice miR-181b negatively regulated CXCL1 and -2 as evidenced by in-vitro analysis of curcumin treated cancer cells (Kronski *et al* , 2014)

miR-520c-3p mediated control of translation initiation factor, eIF4GII is also noted to play an essential role Inoculating, miR-520c-3p overexpressing cancer cells in mice notably inhibited tumor formation (Mazan-Mamczarz *et al* , 2014)

miR-656 is also a tumor suppressor reported to exert its inhibitory effects on glioma cell proliferation via negative regulation of Bone morphogenetic protein 2 There is a direct piece of evidence suggesting that non-canonical BMP/MAPK and canonical BMP/Smad pathways were inhibited in miR-656 overexpressing cancer cells Moreover, ectopic expression of miR-656 induced regression of tumor in mice (Guo *et al* , 2014)

Confluence of information suggested that downregulation of miR-193a-3p/5p induced lymph node metastasis and tumor node metastasis Overexpressing miR-193a-3p/5p in Non-Small Cell Lung Cancer Cells considerably repressed proliferation and migration Detailed mechanistic insights highligbted that miR-193a-3p/5p exerted its growth inhibitory effects via negative regulation of ERBB4 and S6K2 (Yu *et al* , 2015)

miRNA mediated inhibitory effects on angiogenesis are also being studied Concordant with this notion, a recent study provided compelling evidence that introducing miR-542-3p in tumor-bearing mice induced regression of tumor, angiogenesis, and metastasis (He *et al* , 2014)

G-protein-coupled receptor kinase-interacting protein 1 (GIT1) is involved in regulation of Oral Squamous Cell Carcinoma Invasion In-vivo study revealed that injecting GIT1-siRNA expressing C9-lung-IV2 cells into SCID mice significantly reduced lung metastasis It was experimentally verified that expression of miR-491-5p resulted in substantially reduced targeting of C9-lung-IV2 cells to lung 24 hours after injection (Huang *et al* , 2014)

2.10.9 Role of MicroRNA 629 in cancer

miR-629 was reported to be significantly higher in gastric cancer patients as compared to controls (Shin *et al*, 2015) Viral E6 and E7 oncoproteins are essential for induction of transformation and maintenance of the tumorigenic phenotype of HPV-infected cervical cancer cells It has been convincingly revealed that higher intracellular levels of miR-629–5p are noted in E6/E7 expressing cervical cancer cells (Honegger *et al* , 2015)

miR-629 has been shown to modulate different genes Tripartite motif-containing 33 (TRIM33) is reportedly targeted by miR-629 that consequently inhibits TGFβ/Smad signaling cascade In clinical ccRCC specimens, downregulated TRIM33 levels associated notably with pathologic stages and grades Detailed mechanistic insights revealed that TGFβ-induced/Smad activation was significantly suppressed in cancer cells treated with miR-629 inhibitor TRIM33 level was considerably enhanced in miR-629 inhibitor treated cancer cells and there was markedly reduced structural association of Smad4 with Smad2/3 TGFβ treatment induced an upregulation of expression levels of EMT-related factors in miR-629 mimic transfected renal cell carcinoma cells (Jingushi *et al* , 2015)

MATERIALS AND METHODS

3.1 Subjects

Prostate cancer patient's blood samples using informed consent were provided by KRL Hospital Islamabad along with the clinical evaluation and diagnosis The present study was conducted in the institute of Biomedical and Genetic Engineering (IBGE), Islamabad

3.2 Blood sample collection

The patients were assessed by personal information and clinical investigations all information was included in a clinical questionnaire The patients were informed about the objectives of the study and consent form was signed by every participant 5ml blood was drawn from each participant and collected in Acid Citrate Dextrose (ACD) vacutainer (BD Franklin Lakes NJ USA) which contained 2 5ml acid-citrate-dextrose solution, the final volume was 7 5ml This ACD vacutainer serve as preservative and anticoagulant for the fresh blood sample The vacutainer tubes were inverted few times immediately and stored at 4°C till further processing

3.3 Identity number allotment

All the samples were assigned specific identity numbers as PCA-001 PCA-002 and so on Moreover an individual taken as controls were also given i d as CPCA-001, CPCA-002 and so on

3.4 Extraction of genomic DNA from whole blood samples

The genomic DNA from all of the samples was isolated by following standard organic method which recruits two chief organic chemicals namely Phenol and Chloroform (Sambrook and Russell 2001) The protocol was slightly modified as per requirement

The DNA extraction was performed in strict accordance to the following steps

3.4.1 Methodology

DNA extraction procedure was completed in three days The detail of the procedure is as follow

Day 1

1 The samples were transferred to 50ml falcon tubes and 3X volume of cell lysis buffer (KHCO3, NH4Cl and 0 5M EDTA) was added Tubes were placed on ice for 30 min

2 Then the samples were centrifuged at 1200rpm for 10 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130)

3 The supernatant was discarded and the pellet at the base of the tube was re- suspended

4 If the pellet was reddish then again 10ml of cell lysis buffer was added The sample tubes were centrifuged at 1200rpm at 4°C for 10 minutes

5 The supernatant was discarded and the pellet was re-suspended

6 To the pellet, 4 75ml of STE (Sodium Chloride, Tris-HCl, and EDTA) and 250µl of 10% SDS (sodium dodecyl sulfate) were added

7 To the above mixture 10µL of proteinase K enzyme (20mg/ml) (Fermentas, Lithuania) was added and samples were incubated at 55°C for overnight in shaking water bath (Orbit Shaker Bath, Lab-Line, USA)

Day 2

8 The samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8) agitated for 10 minutes and kept on ice for 10 minutes Spun at 3200rpm for 30 min at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130), removed the supernatant with 1ml cut tip into the separate labeled 15ml centrifuge tubes

9 To the samples 5ml of chilled chloroform isoamyl alcohol (24 1) was added and agitated for 10 minutes and kept on ice for 10 minutes Samples were spun at 3200rpm

for 30 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130) The supernatant was separated with cut tip into the separate labeled 15ml centrifuge tubes

10 To the isolated supernatant added 500µL of 10M ammonium acetate and 5ml of chilled Isopropanol and agitated until DNA precipitates as visible white threads The samples were then placed overnight at -20°C (or for 15 minutes at -70°C)

Day 3:

11 The samples were then centrifuged at 3000rpm for 60 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130) The supernatant was discarded and the DNA pellet was loosened by tapping the 15ml centrifuge tube

12 Then the loosened DNA pellet was washed with 5ml of chilled 70% ethanol 3200rpm for 40 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130)

13 The supernatant was discarded and the DNA pellet was dried

14 The dried pellet was dissolved in TE (10mM Tris, 1mM EDTA) buffer (the volume buffer added according to the size of the pellet)

15 The complete DNA dissolving in TE buffer was achieved by incubating the sample tube in shaking water bath at 55°C (Orbit Shaker Bath, Lab-Line, USA)

16 The DNA samples were then transferred to 1 5 ml labelled Eppendorf tubes and stored at 4°C

3.5 Composition of the solutions used in DNA extraction

3.5.1 Cell-lysis buffer

This buffer is composed of three chemicals as

i KHCO3 (Potassium Carbonate) 1gm/L

ii NH4Cl (Ammonium Chloride) 8 29gm/L

iii 0 5 M EDTA (Ethylene Diamine Tetra Acetate) 0 34gm

Cell lysis buffer cleaves the cell membranes and exposes the chromatin material of the nucleated cells

3.5.2 STE buffer

This buffer provides a saline environment to the newly exposed chromatin material

i 3M NaCl (Sodium Chloride) 33 3ml

ii 1M Tris-HCl buffer (pH8 0) 4 0ml

iii 0 5M EDTA (pH8 0) 2 0ml

The above reagents were mixed and volume was made up to 1 liter with dH2O

3.5.3 DNA dissolving buffer

This buffer is used for dissolving the DNA and is composed of

i 10mM Tris-HCl (pH8 0)

ii 0 1mM EDTA

3.5.4 SDS solution

10 % solution of sodium dodecyl sulfate or sodium lauryl sulfate helps in protein degradation

3.5.5 Chloroform-isoamyl alcohol

This solution is made in 24 1 v/v for 500ml total volume as

i Chloroform 480ml (24)

ii Isoamyl alcohol 20ml (1)

3.6 Assessment of quality and quantity of DNA

The Optical density (OD) of DNA samples were measured at 260nm and 280nm by Spectrophotometer (Bio-Rad Laboratories, USA) Optical density (OD) ratio for each sample was calculated as

OD = Absorbance at 260nm / Absorbance at 280nm

The quality of genomic DNA was determined by taking ratio of ODs at these two wavelengths (260/280) The ratio ranges between 1 7-1 9 was considered best If the ratio comes out to be greater than 1 9 then it may have phenolic contamination and the ratio below 1 7 is considered to have proteinic contamination Those samples having ODs out of this range (1 7-1 9) were resuspended for the second time and their ODs were calculated again

Quantification was done using formula

Abs 260nm*50=DNA concentration (µg/ml)

3.7 Working solution of DNA

25ng/µl working solution of DNA was prepared from the stock DNA solution by using the following ratio

DNA stock dH2O

60µL 140 µl

3.8 Reconstitution of primers

Lyophilized primers were mixed with calculated amount of deionized water to make a stock solution of 100 µM of each primer From these 100µM stock primer solutions, 20µM working solutions for each primer were prepared by taking 20µl stock solution and 80µl of distilled water PCR amplifications were performed by using 20µM primer solutions

3.9 PCR analysis for NBS1 gene

Polymerase chain reaction was carried out to amplify the NBS1 gene in which polymorphism was present

3.9.1 Primers for NBS1 3'UTR

In order to determine 3'UTR polymorphism in the *NBS1* gene PCR was performed by the following primers designed for the analysis Details of primers are given in table 3 1

3.9.2 PCR methodology

After optimization with reproducible results, all samples were amplified at optimized conditions of PCR The master mix for all PCR reactions with specific primer pair was prepared in 1 5mL Eppendorf tube Total reaction volume for a single PCR reaction was 25µL All the PCR reagents with their concentrations are summarized in the table 3 2

3.9.3 Thermal profile of PCR reaction for *NBS1* 3' UTR

The above PCR was carried out in 96 well thermal cycler (Thermo Electron Corporation Mill ford, USA) at the following thermal stages, steps and cycles as shown in table 3 3

3.9.4 PCR-RFLP of rs14448T>C polymorphism of *NBS1* gene

For *NBS1* genotyping the amplified DNA fragments harboring the SNP were digested with *RsaI* restriction enzyme Digestion was performed on all samples by adding 1µL *RsaI* enzymes. 3µL of digestion buffer for *NBS1* adjusted with dH2O for final volume of 20µL of PCR reaction mixture It was mixed gently and spins down for few seconds The reaction mixture was incubated at 37°C for 16 hours The RFLP products were checked on 2 5% agarose gel containing ethidium bromide The agarose gel electrophoresis was done at 200 volts for 30 minutes using Bio-Rad Power PAC 3000

Table 3 1: Sequence of the primers used for the genotyping of NBS1 rs14448T>C

Regions	Primer	Sequence
NBS1 3'UTR	Forward primer	5' GCAGAGACCTGTGGGAAGCGA 3
	Reverse primer	5' ACCGTAGCTGCCCTGGTAGGT 3

Table 3.2: PCR reagents with their concentrations used for PCR

Sr. No.	Reagents with initial concentration.	Volume used (µl)	Total volume for 100 reactions
1	d H2O	10 8 µl	1080 µl
2	10x PCR buffer (Fermentas, Lithuania)	2 5 µl	250 µl
3	MgCl2 (Fermentas, Lithuania)	1 5 µl	150 µl
4	2 0 mM dNTPs	1 0 µl	100 µl
5	5U/µl Taq DNA polymerase (Fermentas, Lithuania)	0 2 µl	20 µl
6	10 µM Forward primer	1 µl	100 µl
7	10 µM Reverse primer	1 µl	100 µl
8	25 ng Sample Genomic DNA	5 µl	5 µl
9	Triton/Glycerol	2 0 µl	200 µl

Table 3 3: Thermal profile of PCR reaction for NBS1 rs14448T>C

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1	95 °C	4 Minutes	01 Cycle
Stage 2	Step 1	95 °C	45 Seconds	35 Cycles
	Step 2	60 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

3.9.5 PCR-RFLP of the rs13312986A>G, polymorphism of *NBS1* gene

For *NBS1* genotyping the amplified DNA fragments harboring the SNP were digested with AluI restriction enzyme Digestion was performed on all samples by adding 1µl AluI enzymes, 3µL of digestion buffer for *NBS1* adjusted with dH2O for final volume of 20µL of PCR reaction mixture It was mixed gently and spins down for few seconds The reaction mixture was incubated at 37°C for 16 hours The RFLP products were checked on 2 5% agarose gel containing ethidium bromide The agarose gel electrophoresis was done at 200 volts for 30 minutes using Bio-Rad Power PAC 3000

Table 3.4: Sequence of the primers used for the genotyping of *NBS1* rs13312986A>G

Regions	**Primer**	**Sequence**
NBS1 3'UTR	Forward primer	5' GCAGAGACCTGTGGGAAGCGA 3
	Reverse primer	5' ACCGTAGCTGCCCTGGTAGG1 3'

Table 3 5· Thermal profile of PCR reaction for *NBS1* rs13312986A>G

No. of Stage	**No. of Step**	**Temperature**	**Time**	**No. of Cycles**
Stage 1	Step 1	94 °C	5 Minutes	01 Cycle
Stage 2	Step 1	94 °C	45 Seconds	35 Cycles
	Step 2	55 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

3.10 Analysis of PCR products by using agarose gel electrophoresis

Analysis of PCR product was performed by using agarose gel electrophoresis

Following reagents were used in the process of agarose gel electrophoresis

a) 10x TBE buffer (Iris-Borate- Ethylene diamine tetra acetic acid)

b) 6X Gel loading dye (Fermentas, Lithuania)

c) 10mg/ml Ethidium Bromide

d) 2% Agarose (Promega)

e) 100bp DNA Ladder (Ferments Lithuania)

3.10.1 Preparation of 10X TBE buffer stock

i Tris-base (promega) = 107 8gm/liter

ii Boric acid (promega) = 55 02gm/liter

iii EDTA (Ethylene diamine tetra acetic acid) (Bio Rad) = 9 04gm/liter

All reagents were dissolved in 800ml deionized water using magnetic stirrer and finally 1000ml volume was made in measuring cylinder and stirred to mix well

3.10.2 Preparation of 1X TBE buffer stock

10X TBE buffer = 200mL

dH2O = 1800mL

3.10.3 Preparation of 2.5% agarose gel

For 20cm×20cm agarose gel pouring plate 300 ml of 2 5% agarose was made as

i Ion free fine agarose (Promega) = 6 5gm

ii 1X TBE = 300ml

iii Glycerin = 300µl

iv Ethidium bromide (10mg/ml) = 5µl

Mix 6.5gm agarose, 300ml 1X TBE and 300µl glycerin in Pyrex bottle and heat in oven with loose bottle cap until agarose boils to mix in liquids. Add 5µl Ethidium bromide in the agarose gel solution and shake to mix. Set the 20cm×20cm gel plate with combs at suitable distance and pour the gel solution. The gel was polymerized in 30-45 minutes.

3.11 Gel electrophoresis and gel documentation

Before loading the sample in the gel, 5µl of the PCR product was mixed with 6X gel loading buffer (Fermentas, Lituania). Then samples were loaded on agarose gel. In the first well of gel 100bp DNA marker was loaded as size reference for amplified target DNA. The gel was run for 35 minutes using the Maxicell EC360-M electrophoretic gel system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 200 constant volts by using BioRad Power Pack 3000 (BioRad, USA) in 1X TBE buffer. DNA bands were visualized under UV illumination and photographed using Syngene gel documentation system (Gene Genius, Syngene, UK).

3.12 Statistical analysis

The following statistical tests were performed for the data.

3.12.1 Hardy-Weinberg Equilibrium

The frequency of NBS1 SNP was calculated by Hardy- Weinberg Equilibrium equation.

$$p^2 + 2pq + q^2 = 1$$

The allele frequency of the study groups was calculated by using the following formula.

$$\text{Allele Frequency} = \frac{\text{No. of that allele in study group}}{\text{Total no. of allele}}$$

3.12.2 Chi square test and p-value

To find the association of the NBS1 gene with the prostate patients the p value and Chi square test was performed by using statistical program SPSS (Version 20.0) and as described by Preacher (Preacher, 2001). A p-value < 0.05 was considered as statistically significant whereas p value >0.05 was taken as non-significant

RESULTS

In the present laboratory research, we studied 3' UTR C/T and A/G polymorphism in NBS1 gene in 60 prostate cancer patients and 60 healthy individuals without any previous clinical history, using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis For rs13312986 A>G genotypes, AA was 75% in patients and 70% in controls AG was 25% in patients and 30% in controls GG was 0% in patients and none was detected in control Allelic frequencies for A was 0 875 in patients and 0 85% in controls Allelic frequencies for G were 0 125% in patients and 0 15% in controls P value was insignificant for rs13312986 A>G genotypes For rs14448 T>C genotypes, TC was 71 6% in patients and 66 6% in controls T1 was 28 3% in patients and 33 33% in controls CC was not detected either in patients or controls T allele was 0 64% in patients and 0 66% in controls C allele was 0 35% in patients and 0 33% in controls P value was insignificant for rs14448 T>C genotypes

4.1 *NBS1* 3' UTR (rs14448 T>C) genotyping

For rs14448 T>C genotypes, TC was 71 6% in patients and 66 6% in controls T1 was 28 3% in patients and 33 33% in controls CC was not detected either in patients or controls T allele was 0 64% in patients and 0 66% in controls C allele was 0 35% in patients and 0 33% in controls *P* value was insignificant for rs14448 T>C genotypes As shown in Fig 4 1 and Table 4 1

4.2 *NBS1* 3' UTR (rs13312986 A>G) genotyping

For rs13312986 A>G genotypes, AA was 75% in patients and 70% in controls AG was 25% in patients and 30% in controls GG was 0% in patients and none was detected in control Allelic frequencies for A was 0 875 in patients and 0 85% in controls Allelic frequencies for G were 0 125% in patients and 0 15% in controls *P* value was insignificant for rs13312986 A>G genotypes As shown in Fig 4 2 and Table 4 2

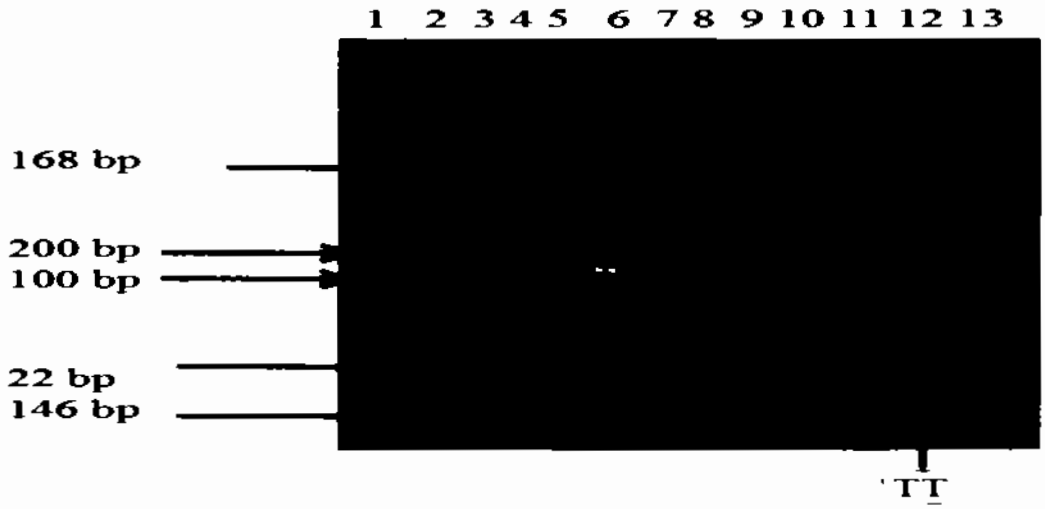

Fig 4.1: For rs14448 T>C, TT is 168 bp, TC is 168, 146, 22bp and CC is146, 22bp Lane 1 is ladder, Lane 2-12 is TC and Lane 13 is TT.

Table 4.1: Analysis of rs14448 T>C genotypes in patients and controls

Study Group	rs14448 T>C genotypes (Percentage)			Allele Frequencies	
	TC	TT	CC	T	C
Patients (n=60)	43 (71.6)	17 (28.33)	0	0 64	0.35
Control (n=60)	40 (66.66)	20 (33.33)	0	0.66	0.33
p-value	0.67	0.524	1	1	0.974

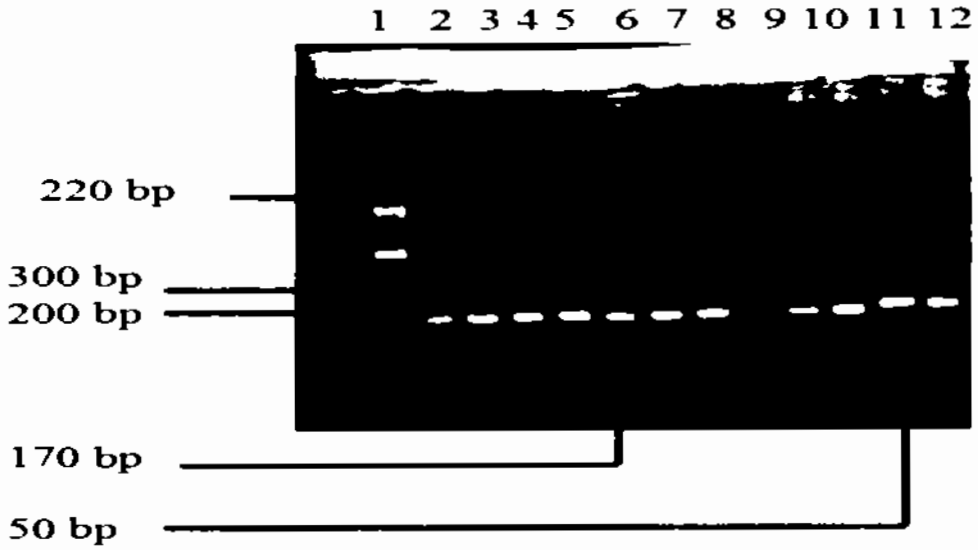

Fig 4.2: For rs13312986 A>G genotypes, AA is 170, 50 bp. AG is 220, 170, 50bp and GG is 220 bp Lane 1 is ladder. Lane 2-8 is AG, Lane 9 is AA.

Table 4.2: Analysis of rs13312986 A>G genotypes in patients and controls

Study Group	rs13312986 A>G genotypes			Allele Frequencies	
	AA	AG	GG	A	G
Patients (n=60)	45 (75%)	15 (25%)	0	0.875	0 125
Control (n=60)	42 (70%)	18 (30%)	0	0 85	0 15
***p*-value**	0.67	0.499	1	1	0 964

DISCUSSION

Prostate cancer (Pca) is a multifaceted and genomically complex disease and research over the decades has gradually shown tremendous breakthroughs in our understanding of the prostate cancer biology Prostate cancer is one of the most pervasive malignancies around the world constituting a genuine health issue Clinically localized disease can be successfully treated whereas the disease which is spread throughout an organ or the body remains mostly lethal Increasingly it is being realized that miRNA plays an important role in deregulation Increasingly it is being realized that Pca is the most common diagnosed cancer in men and is the second leading cause of death (Gronberg *et al* , 2003)

Androgen receptor (AR) has emerged as one of the most deeply investigated target for prostate cancer therapy It is intriguing that AR has emerged as one of the most deeply investigated target for prostate cancer therapy The role of AR in prostate cancer development has been researched in many studies, however the precise molecular mechanisms that occur in the development to androgen independence remain largely unexplored and as a result there is no compelling therapies against AIPC (Attar *et al* , 2009, Debes *et al* 2004, Feldman *et al* 2001)

It has been reported that *NBS1* gene polymorphism might affect function of gene and may lead to cancer susceptibility We hypothesized that *NBS1* gene polymorphism is associated with the risk of lung cancer Two independent case-control studies was conducted in Southern and Eastern Chinese population From these studies it is concluded that there was no significant association found for tag SNP's rs13312986 and rs14448 whereas rs2735383CC variant genotype carriers had been noted to show an elevated cancer risk when exposed to high or medium level of radiation in an earlier published report It was concluded that rs2735383G>C variation contributed to an increased risk of lung cancer possibly through miR-629 mediated targeting of NBS1 (Yang et al 2012)

miR-629 was reported to be significantly higher in gastric cancer patients as compared to controls (Shin *et al* 2015) It has been convincingly revealed that higher intracellular levels of miR-629–5p are noted in E6/E7 expressing cervical cancer cells (Honegger *et al* , 2015) Tripartite motif-containing 33 (TRIM33) is reportedly targeted by miR-629 that consequently inhibits TGFβ/Smad signaling cascade (Jingushi *et al* , 2015)

The novelty of this study is that there is no work done on prostate cancer before In this study we examined the *NBS1 gene* polymorphism in prostate cancer patients in Pakistani population The results didn't show any significant association in prostate cancer patients For rs13312986 A>G genotypes, AA was 75% in patients and 70% in controls AG was 25% in patients and 30% in controls GG was 0% in patients and none was detected in control Allelic frequencies for A was 0 875 in patients and 0 85% in controls Allelic frequencies for G were 0 125% in patients and 0 15% in controls P value was insignificant for rs13312986 A>G genotypes For rs14448 T>C genotypes, TC was 71 6% in patients and 66 6% in controls TT was 28 3% in patients and 33 33% in controls CC was not detected either in patients or controls T allele was 0 64% in patients and 0 66% in controls C allele was 0 35% in patients and 0 33% in controls P value was insignificant for rs14448 T>C genotypes

To summarize, our investigation on *NBS1* gene polymorphisms, this mutation did not show statistically significant association between prostate cancer patients and controls Further studies are required to explore the detail mechanism, as miRNA regulation of NBS1 is not completely studied

Conclusion

Future studies surely will demystify detailed mechanisms, as miRNA regulation of NBS1 is incompletely studied and exploration of these modulations will be helpful in getting a step closer to personalized medicine

Future perspectives

Future studies must converge on investigation of mRNA levels of NBS1 from breast cancer patients and additional miRNAs, which possibly can modulate expression of NBS1 NBS1 is reported involved in regulation of DNA damage repair and future studies must converge on cell culture based studies using various phytochemicals to analyze changes in expression profiles of NBS1 and miR-629 Moreover, it needs to be studied in detail, if NBS1 is quantitatively controlled by other miRNAs This information will be helpful in a broader and deeper analysis on miRNA regulation of DNA damage signaling network

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