DESIGNING A GOLD NANOPARTICLE BASED NANOCARRIER FOR MICRORNA TRANSFECTION INTO PROSTATE CANCER CELLS

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Submitted to the Institute of Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science
in
Biotechnology

Yeditepe University
2012

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DATE OF APPROVAL: ..../..../2012

ACKNOWLEDGMENTS

To begin with, I would like to thank to my supervisor Assoc. Prof. Mustafa Özen for giving me the chance to work with him throughout my master period and for his continuous support and encouragement since the very early stages of this work.

I am deeply grateful to my co-supervisor, Prof. Dr. Mustafa Çulha for providing the nanoparticles and Prof. Dr. Fikrettin Şahin for providing the cancer cell lines. I want to thank to Yrd. Doç. Dr. Ömer Faruk Bayrak for his help in technical support.

I am so thankful to my lab partners Ali Yasin Sonay, Şükrü Güllüoğlu, Serhat Sevli, Ömer Faruk Karataş, Esen Efeoğlu for helping and supporting me whenever I needed. I would also like to thank my friends especially Melis Uslu, Ayca Zeynep İlter, N. Başak Şentürk, Nihan Kılınç, Mine Altunbek, Ayşegül Doğan, Zeynel Demir and Kemal Keseroğlu for their continuous support and understanding whenever I needed.

I would like to thank members of İstanbul University, Cerrahpaşa Medical Faculty, Medical Genetic Group, Ayşe Emek, Seda Salman, Betül Yücetürk, Abdullah Çelebi, Z. Özlem Yüksel, Esra Güzel and İlknur Süer for their help during the lab work.

Lastly, but not the least, I thank to my lovely parents, Hacer Ekin and Mehmet Ekin, for their unconditional support and encouragement throughout my life. I am deeply grateful to my lovely brother Ahmet Ekin. In addition, I warmly thank Lalecan İşcanlı Ekin for her understanding and listening. I am also thankful to my aunts Aygül Yanık, Hadiye Yanık and other members of my family. They have given all the needed support during my research.

ABSTRACT

DESIGNING A GOLD NANOPARTICLE BASED NANOCARRIER FOR MICRORNA TRANSFECTION INTO PROSTATE CANCER CELLS

Cancer is the leading cause of death worldwide. Prostate cancer is one of the most frequent cancer types; therefore, research in this area can have large impact by saving lives of many. Nanotechnology has potential to facilitate advances in detection, diagnosis, and treatment of cancers. Especially, the efficient delivery of nucleic acids into cancer cells can be a significant improvement for medical and cell biology including the clinical treatment of genetic disorders. For instance, gold nanoparticles (AuNPs) have been used as a platform for novel experimental cancer therapy and have emerged as an alternative gene delivery system. Another important solution in cancer therapy is the use of microRNAs (miRNAs), which are 20-22 base nucleotides and induce post-transcriptional gene silencing, because they are inappropriately expressed by cancer cells. Particularly, the expression level of miRNAs that act as anti-oncogenes is frequently reduced in cancers because of chromosome aberrations. In the case of prostate cancer, hsa-miR-145, a specific type of miRNA, is down-regulated in prostate tumors in comparison to normal prostatic tissues. As a result, an efficient delivery of extra hsa-miR-145 to prostate cancer cells can assist the treatment of prostate cancer. In this thesis, we used an engineered AuNP as a nanocarier to deliver miRNAs to prostate cancer cells. 13 nm AuNPs were modified with thiolated RNAs and then, the hsa-miR-145 was hybridized to the RNAs chemically attached to the AuNPs. We show that the proposed AuNPs help the delivery of large amount of hsa-miR-145 into cancer cells. We also showed that the delivery was more efficient when the AuNP-RNA-miRNA carrier complex was formed at an elevated temperature of 72 °C.

ÖZET

MİKRORNA'NIN PROSTAT KANSER HÜCRELERİNE TRANSFEKSİYONU İÇİN ALTIN NANOPARTİKÜL TEMELLİ NANO TAŞIYICI DİZAYNI

Kanser dünya genelinde ölümlerin başlıca sebebidir. En sık görülen kanser tiplerinden birisi prostat kanseridir. Bu nedenle bu alanla ilgili araştırmalar birçok hayatın kurtulmasında büyük etkiye sahip olabilir. Nanoteknoloji, kanserin ortaya çıkması, tanısı ve tedavisine olanak sağlayan bir potensiyele sahiptir. Özellikle, kanser hücreleri içerisindeki nükleik asitlerin etkili dağıtımı, tıp için önemli bir gelişme ve genetik bozukluklukların klinik tedavisini kapsayan hücre biyolojisi olabilir. Örneğin, altın nanopartikıllar yeni deneysel kanser terapisi için bir platform ve alternatif bir gen dağıtım sistemi olarak ortaya çıkmıştır. Kanser tedavisinde bir diğer önemli çözüm ise 20-24 baz nükleotid uzunluğunda ve yazılma sonrası gen ifadesinin başlandığı mikroRNA'ların kullanılmasıdır. Çünkü mikroRNA'lar kanser hücreleri ile uygun olmayan bir şekilde ifade edilebilir. Özellikle anti-onkogen olarak rol alan mikroRNA'nın ifade seviyesi kromozom değişimlerinden dolayı kanser içerisinde sıklıkla azalmıştır. Prostat kanseri durumunda mikroRNA'nın özel bir tipi olan hsa-miR-145, normal prostat dokularına nazaran prostat tümörleri içerisinde azalma gösterir. Prostat kanser hücrelerinde hsa-miR-145'in etkili dağıtımı prostat kanserinin tedavisine yardımcı olabilir. Bu tezde biz mikroRNA'ları prostat kanser hücrelerine dağıtmak için altın nanopartiküllü tiyol bağlı RNA'ları nanotaşıyıcı olarak tasarladık. 13nm- altın nanopartiküller ile tiyol bağlı RNA lar modifiye edildi. Sonra, altın nanopartiküllerle kimyasal olarak bağlanan RNA'lar hsa-miR-145 ile hibritlendi. Deneylerimizde gold nanopartıkılların büyük miktarda hsa-miR-145'in prostat kanser hücrelerine dağılmasına yardımcı olduğunu gösteriyoruz. Birde altın nanopartikül-RNA-mikroRNA taşıyan 72°C sıcaklıkta şekillendirildiği zaman çok etkili dahağılım gözlendi.

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LIST OF SYMBOLS / ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ml	Milliliters
µl	Microliters
ng	Nanogram
miRNAs	microRNAs
nts	nucleotides
oncomirs	oncogenic miRNAs
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNAP II	RNA polymerase II
PC3	Human Prostate Cancer Cell Line
PTGS	Post-Transcriptional Gene Silencing
SNPs	Single nucleotide polymorphisms
AuNPs	Gold nanoparticles
TNF	Tumor necrosis factor
PIN	Prostatic intraepithelial neoplasia
PZ	Peripheral zone
TZ	Transition zone
PGA	Poly(glycolic acid)
SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate
PBAE	Poly(β-amino ester)s
PEG	Polyethylene glycol
TEM	Transmisyon Elektron Mikroskobu
SiRNA	Small interfering RNA
PEI	Polyethyleneimine
PAH-Cit	Cis-aconitic anhydride-functionalized poly(allylamine)
-SH	Thiol

NH_2	Amino grubu

1. INTRODUCTION

1.1. CANCER AND NANOTECHNOLOGY

Cancer is a leading cause of death worldwide. Most of studies are trying to focus on the role of nanoscience in cancer diagnosis and therapy. Therefore, nanomaterials have led to a new discipline of nano-oncology [1, 2]. In the past years, the field of nanotechnology was first proclaimed by Professor Richard P. Feynman in 1959 (Nobel laureate in physics, 1965) with his famous Cal Tech Lecture entitled, "There's plenty of Room at theBottom" [3]. Afterwards, nanomaterials, can facilitate important advances in detection, diagnosis, and treatment of human cancers. In addition, nanomaterials, which measure 1–1000 nm, allow unique interaction with biological systems at the molecular level [2, 4]. In addition, there are biomarkers of cancer. These biomarkers or biomolecule markers comprise altered or mutant genes, RNAs proteins, carbohydrates, lipids, and small metabolite molecules, and their altered expressions that are correlated with a biological behavior or a clinical outcome. Cancer biomarkers are discovered by molecular profiling studies [5]. Especially, the efficient delivery of nucleic acids into cancer cells is a significant improvement for cell biology and medical applications, including the clinical treatment of genetic disorders. Thus, the use of nanomaterials, such as carbon nanotubes, iron oxide, silica, and AuNPs, have emerged as alternative gene delivery systems [6].

1.1.1. Gold Nanoparticles and Their Use in The Cancer Therapy

AuNPs have been used as a platform for novel experimental cancer therapy and used for treatment of human disease. AuNPs have been used as contrast agents in vitro based on their ability to scatter visible light. For example, in a model of colon cancer, it was shown that systemically delivered gold nanoparticles (size, approximately 33 nm) conjugated to tumor necrosis factor (TNF) collected in tumors [7]. Similarly, Au NPs have a high affinity for biomolecules. AuNPs chemically functionalized with alkylthiol-terminated oligonucleotides are highly stable in saline solutions and bind complementary nucleic acids in a very selective way. These distinctive properties have made oligonucleotide-functionalized Au NPs the centerpiece of several highly sensitive and selective assays for

biomolecule detection. Rosi, Nathaniel L.et al reported in studies that introduced the use of oligonucleotide functionalized 13 nm Au NPs as new antisense agents that take advantage of the nanoparticle-oligonucleotide conjugate. Several gene delivery systems have been developed that are based on using AuNPs [8].

1.1.2. Gold Nanoparticles

AuNPs have a number of desirable properties, including low cytotoxicity, easy size control, and well-developed surface chemistry for dense loading of functionalities. There are many kinds of modifications on gold nanoparticles. Elbakry et al. first developed the PEI/siRNA/PEIAuNP system to deliver siRNA into cells [9]. In the past, a lot of work had been done on improving the gene transfection efficiency based on PEI. PEI is the gold standard for gene delivery, which has strong escape capacity from endosome due to "proton sponge" effect. PAH-Cit, one kind of charge-reversal polymer, successfully used to fabricate polyelectrolyte multilayers [10]. Guo et al. reported in studies that charge-reversal PEI/PAH-Cit/PEI/MUAAuNPs were prepared with cationic PEI, charge-reversal PAH-Cit, and MUA-AuNPs by layer-by-layer assembly technique as nucleic acid carriers. Likewise, gene transfection and siRNA knockdown studies in vitro show high efficiency of the payload release owing to the charge reversion of PAH-Cit. Compared to the commercial transfection agents Lipofectamine and PEI, charge-reversal PEI/PAHCit/PEI/MUA-AuNP present higher efficiency at the same amount of siRNA. As a result, PEI and PAH Cit were used as opposite polyelectrolytes to modify gold nanoparticles in the study to improve gene transfection and gene silencing efficiency [11].

Lee et al. reported that the synthesis of the siRNA–AuNP (13nm) conjugates begins with modifying AuNPs with HS–PEG–NH2 (Mw 1000 Da). PEG was used to isolate the gold surface from disulfide bonds, since AuNPs could react with the disulfide bonds and induce release of siRNA. These NH2–PEG-modified AuNPs (NH2–PEG–AuNPs) exhibit excellent salt stability and can be salted up to 2.5 M NaCl. This high salt stability was important to ease siRNAconjugation procedures. They conjugated thiolated siRNA (antifirefly luciferase, HS-siRNA) to SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate) by the addition of excess HS–siRNA at pH 8.5 to displace the 2-pyridyldithio group of SPDP, still maintaining the disulfide linkage. Importantly, the loading of siRNA

per AuNP without SPDP was low. For one thing, the maximum loading of siRNA per particle was found to be at 2.5 M NaCl. To verify the cellular transfection of the PBAE–siRNA–AuNPs, gene knockdown was evaluated in a modifed HeLa cell line. The best two PBAEs (C32-228 and 221) exhibit significantly beter efficiency than the commercially available liposome-based delivery agent (Lipofectam) used as a positive control in accordance with manufacturer instructions. To summarize, the role of PBAEs for the cellular uptake of siRNA–AuNPs was further confirmed by TEM analysis. The PBAE poly(β-amino ester)s–siRNA–AuNPs were prepared with C32-221, the PBAE showing the best delivery efficiency, and exposed to the cell culture media under the same conditions studied for the gene knockdown experiments. In addition, the effective delivery of siRNA in to the cells were showed in the Figure 1.1 [12].

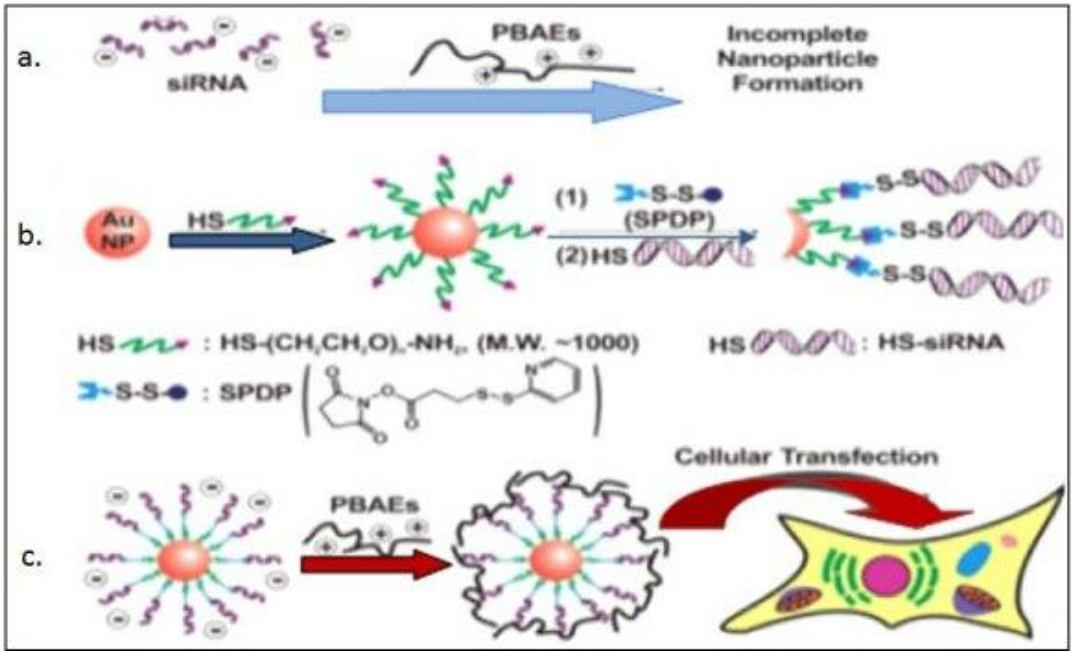

Figure 1.1. The effective delivery of siRNA in to the cells a. The scheme illustrating incomplete nanoparticle formation of siRNA combined with PBAEs. b. The synthesis of siRNA-modified AuNPs (siRNA–AuNPs) with a disulfide linkage. c. The complexation of siRNA–AuNPs with PBAEs, followed by cellular transfection (modified from [12])

1.2. PROSTATE CANCER

Cancer is the leading cause of death worldwide. Prostate cancer is one of the most frequent cancer types. Prostate cancer is the leading malignancy among men in the United States [13]. In addition, Prostate tumors show a range of clinical phenotypes, from sluggish to aggressively metastatic. Villers et al. [14] showed that prostate samples possess tumors on separated sites of tissue at the rates of 80% and 50%, separately. Some tumors are undeniably aggressive, progress quickly, and can result in significant morbidity and premature death of the patient [13]. Research into the genetic origins of prostate cancer has accelerated dramatically in recent years, [15] therefore, most of the studies are very important. For example, numerous gene expression studies have been conducted to characterize prostate cancer initiation and progression, and many of them have shown correlation with clinical outcome [16]. Especially, these studies expected to significantly improve the diagnosis and treatment of the prostate cancer.

1.2.1. Biology of Prostate Cancer

The biological function for the multifocal and histologic heterogeneity attributes of prostate cancer has not been fully crystallised. Two main theories attempt to explain this process: Firstly, multiclonality of the initial disease; and secondly, clone evolution from the initial disease. A second theory postulates a multiclonal evolution from the initial lesion. In studying the types of allelic loss at chromosome 8p12-21, the location of a tumor suppressor gene involved in early prostatic carcinogenesis, and at the BRCA1 locus on chromosome 17q21, the investigators found that the pattern was consistent with an independent tumor origin in 15 of 18 cases [17, 18].

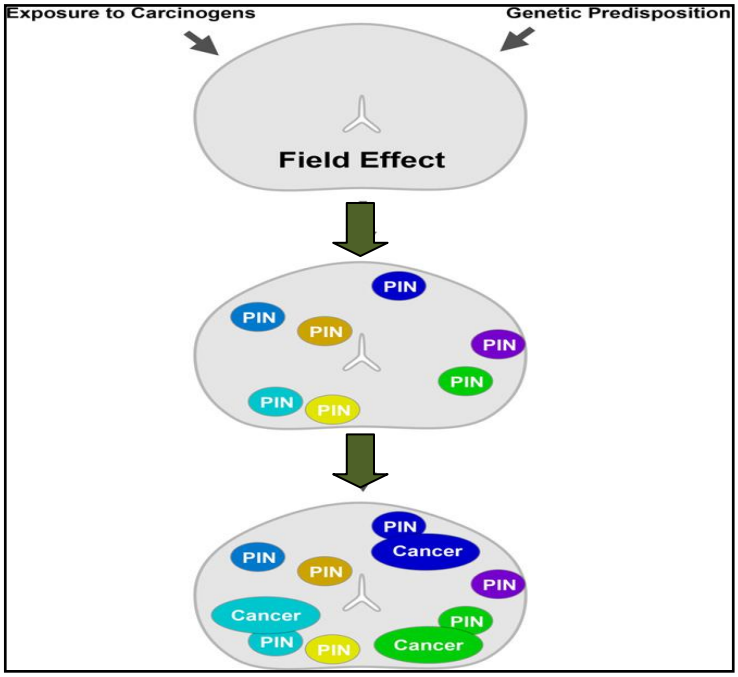

Figure 1.2. Carcinogenesis of prostate cancer (modified from [19])

The mechanism behind prostate carcinogenesis likely includes a combination of both obtained factors and inherited genetic predisposition. An underlying genetic predisposition could create a "field effect" upon which further environmental exposure to chemical and biologic carcinogens could lead to the formation of multiple precursor lesions. Through independent clonal expansion, these lesions would evolve into spatially separated and distinct cancers. High-grade PIN (prostatic intraepithelial neoplasia) is the precursor lesion of prostate cancer in most cases, particularly cancer arising in PZ (peripheral zone), although that relationship appears less certain for cancer originating in the transition and anterior zones. Carcinogenesis of prostate cancer were shown in the Figure 1.2 [19].

Anatomical zonal consideration in the prostate cancer are very important. Besides, the two most common zones of prostate cancer origin are the PZ and the transition zone (TZ) [20-22]. Initial studies showed that approximately 70% of tumors arose in the PZ, whereas 25% started in the TZ. In a study by Greene et al [23], PZ tumors were more likely to extend through the capsule and at a smaller volume. Although 20% of PZ tumors involved the seminal vesicles, hardly any TZ tumors did so. Furthermore, clinical distinction between tumors arising from these two zones remains of significance as TZ tumors have

been consistently shown to have more favorable pathologic features despite higher PSA levels and tumor volume at diagnosis [24, 25].

High-grade prostatic intraepithelial neoplasia (PIN) is the precursor lesion of prostate cancer in most cases, specifically cancer arising in PZ, although this relationship appears less certain for cancer originating in the transition and anterior zones. Like prostatic carcinoma, it has also been commonly found to be multifocal [26, 27]. The highest frequency of allelic imbalance has been found on chromosome arm 8p in 63% of foci of high-grade PIN and 91% of foci of prostate carcinoma [28]. In addition, there was a lower expression of tumor markers related to tumor growth rate (p53 and bcl-2) present in tumors arising in the TZ. Similarly, Shannon et al [29] found that TZ tumors had significantly lower rates of capsular penetration and positive surgical margins. They also found that a subset of TZ tumors tended to have features of high malignancy. Positive anterior and bladder neck margins were also more common in TZ than PZ carcinomas [30].

As a result, the most commonly deregulated tumor suppressor gene in prostate cancer is p53 as well as in other tumors. Role of P53 is inhibition proliferation in the case of DNA damage and driving cell into apoptosis [31].

1.3. MICRO-RNAs

MicroRNAs (miRNAs), have been discovered in animals and plants [32-34]. MicroRNAs (miRNAs) constitute a large class of philogenetically conserved single stranded RNA molecules of approximately 20 to 22 nucleotides (nt) (ranging 19–25 nt), long that induce post- transcriptional gene silencing [35-38]. The miRNAs lin-4 and let-7 were the first to be discovered and shown to function in Caenorhabditis elegans as triggers for a cascade of gene expression that regulates developmental events by Post-Transcriptional Gene Silencing (PTGS). Post-transcriptional gene silencing (PTGS) is accomplished by a multicomponent nuclease that targets mRNAs for degradation [34, 35, 39, 40].

About 60% of mammalian miRNA genes in human are located in introns of protein-coding genes, the remaining in intergenic non-coding transcriptional units; less often in exons and anti-sense orientation with the host gene [41-43].

Separately, bioinformatics studies reported that the human genome may contain up to 1000 miRNAs, over and above, 706 human miRNAs are in miRBase. MiRNA genes are spread throughout the genome and it is estimated that they account for 2–5% of human genes [44-46].

1.3.1. Roles of MicroRNAs (miRNAs)

MicroRNAs have functions in diverse human diseases. Most of studies have shown that miRNAs are important because miRNAs are regulators of a variety of essential biological processes, such as development, cellular differentiation, cell proliferation, apoptosis, haematopoiesis, stress resistance, fat metabolism, neural development, death and, importantly, tumourigenesis [47,48]. Studies of individual miRNAs have shown that they can act as oncogenes or tumor suppressor genes [49-52]. In addition, they are therefore referred to as ‘oncomirs’. Factors that are required for the biogenesis of miRNAs have also been associated with various cancers and might themselves function as tumour suppressors and oncogenes [53] as shown in Figure 1.3.

Some have been shown to negatively regulate well-known oncogenes: miR-15 and miR-16 have been reported to put down the antiapoptotic factor BCL2 and the let-7 miRNA family to target the RAS oncogene. In addition, there are some miRNAs, such as miR-143 and miR-145, that have been reported to be aberrantly expressed in several different cancer types, the miRNA signatures of cancers of different cellular origin seem to be unequalled [54-57].

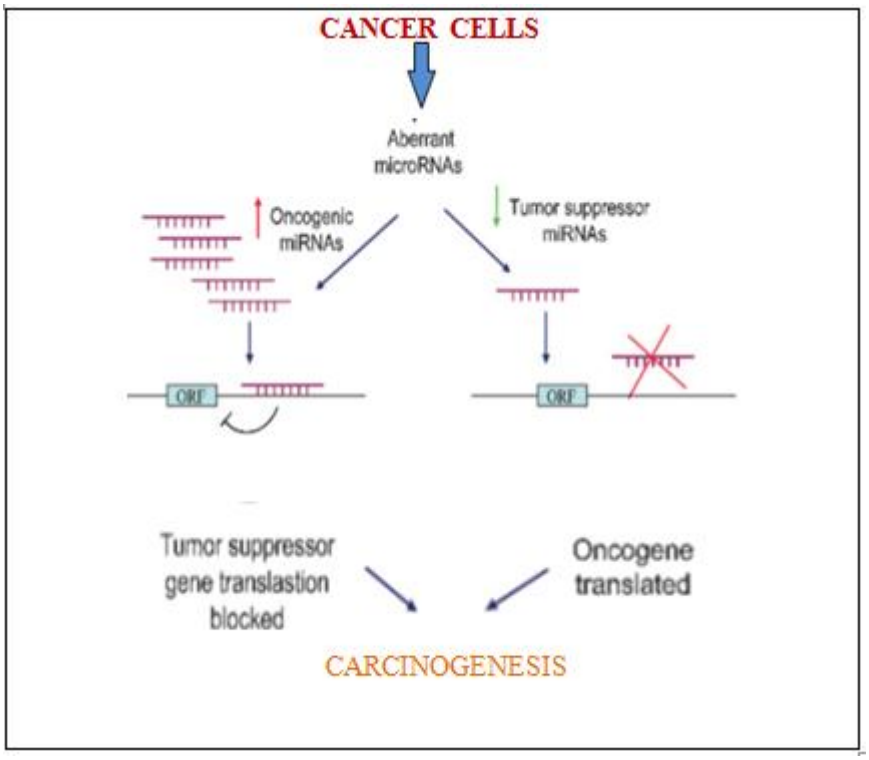

Figure 1. 3. MicroRNAs (miRNAs) as tumour suppressors and oncogenes (modified from [58])

Paranjape at al. [58] reported that down-regulation or loss of miRNAs with tumour suppressor functions might increase translation of oncogenes and hence formation of excess oncogenic proteins, leading to tumour formation. On the contrary, upregulation of oncogenic miRNAs might block tumour suppressor genes and also lead to tumour formation.

1.3.2. Role of MicroRNAs (miRNAs) in Cancer

Specifically, studies have appeared a strong correlation between abrogated expression of miRNAs and oncogenesis. For instance, mature miRNA levels of miR-143 and miR-145 are significantly decreased in colorectal tumours [59]. The tumour-suppressor roles of miR-143 and miR-145 might not be specific to colorectal tissue as their expression is also downregulated in breast, prostate, cervical and lymphoid cancer cell lines [56, 59]. MicroRNAs that are associated with human cancers were shown in the table 1.3.

Table 1.3. MicroRNAs that are associated with human cancers
(modified from [56, 59, 60])

MicroRNA (miRNA)	**Gene loci**	**Cancer association**	**Function**
miR-143, miR-145	Chromosome 5q32–33	Decreased abundance in colorectal cancer; downregulated in breast, prostate, cervical and lymphoid cancer cell lines; miR-145 is decreased in breast cancer.and miR-145 is associated with Breast,ovary, prostate,colon,liver,lung ca	TS (Tumour supressor)

.

1.3.3. Biogenesis of MicroRNAs (miRNAs)

Molecular mechanisms of miRNAs have only recently been clarified (Figure 1.4). MicroRNAs (MiRNA) production begins in the cell's nucleus and contains a series of RNA processing steps. MiRNA genes are transcribed by RNA polymerase II as long RNA precursors (pri-miRNAs), (500-3,000 bases) [52, 61-63]. In the nucleus, the pri-miRNAs are processed by the RNase III enzyme, in the human nucleus by Pasha (also known as DGCR8), Drosha and the double-stranded-RNA-binding protein [32, 64-70].

Separately, Pri-miRNAs are generally several kilobases in length, and comprise a 7-metyl guanosine cap structure and a poly (A) tail similar to protein-coding mRNAs. Drosha and DGCR8, endonucleases of the RNAase III family, give pre-microRNA ~70 bases [70,71]. The pre-miRNAs are then carried to to the cytoplasm by Exportin-5, in a Ran guanosine triphosphate-dependent manner (Ran-GTP) [83-85]. Dicer, another RNAase III, processes the pre-microRNA into double-stranded 21–25 nt long miRNA⁄miRNA* duplexes, each

strand of which bears 5’ monophosphate, 3’ hydroxyl group and a 3’ 2-nt overhang [70, 74].

Afterwards, the miRNA:miRNA* duplex is incorporated into the miRISC(RNA-induced silencing complex) complex. The mature miRNA strand is preferentially retained in the functional miRISC complex and negatively regulates its target genes. In addition, the miRNA biogenesis were showed in the Figure 1.4 [71].

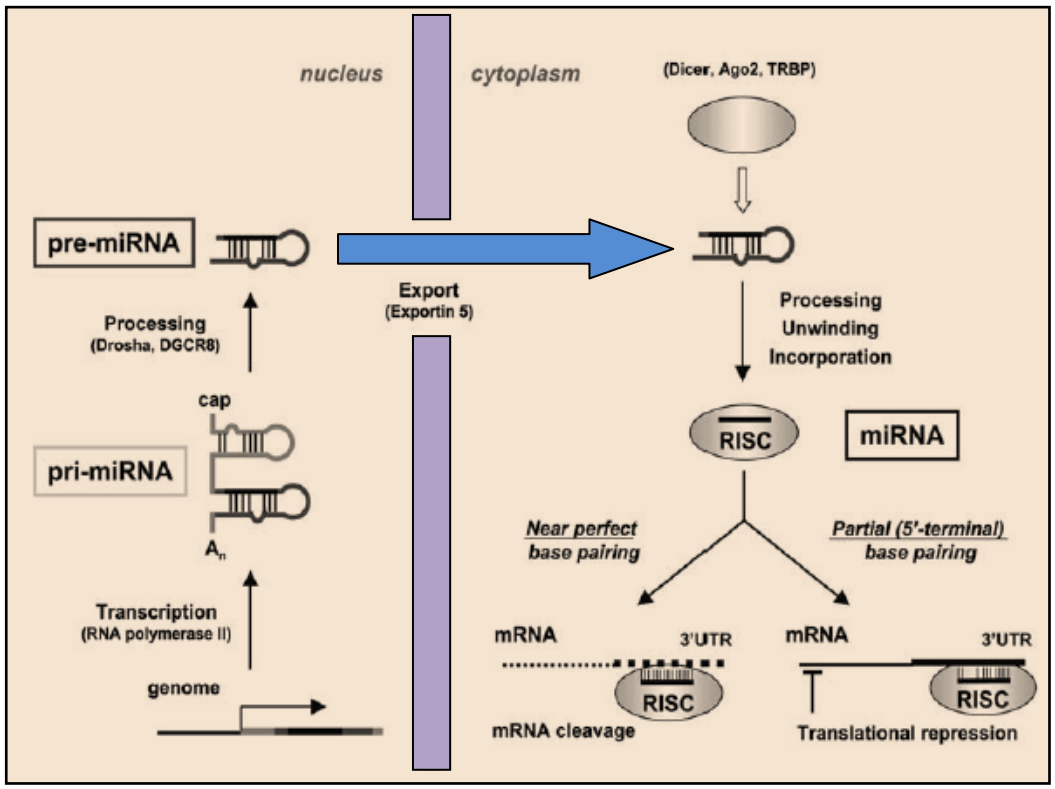

Figure 1.4. The miRNA biogenesis (modified from [71]) miRNAs are transcribed by RNA polymerase II and sequentially processed by drosha/DGCR8 and dicer. miRNA-loaded RISC causes the cleavage or translational silencing of target mRNAs

1.3.4. MicroRNAs (miRNAs) as Potential Diagnostic Biomarkers

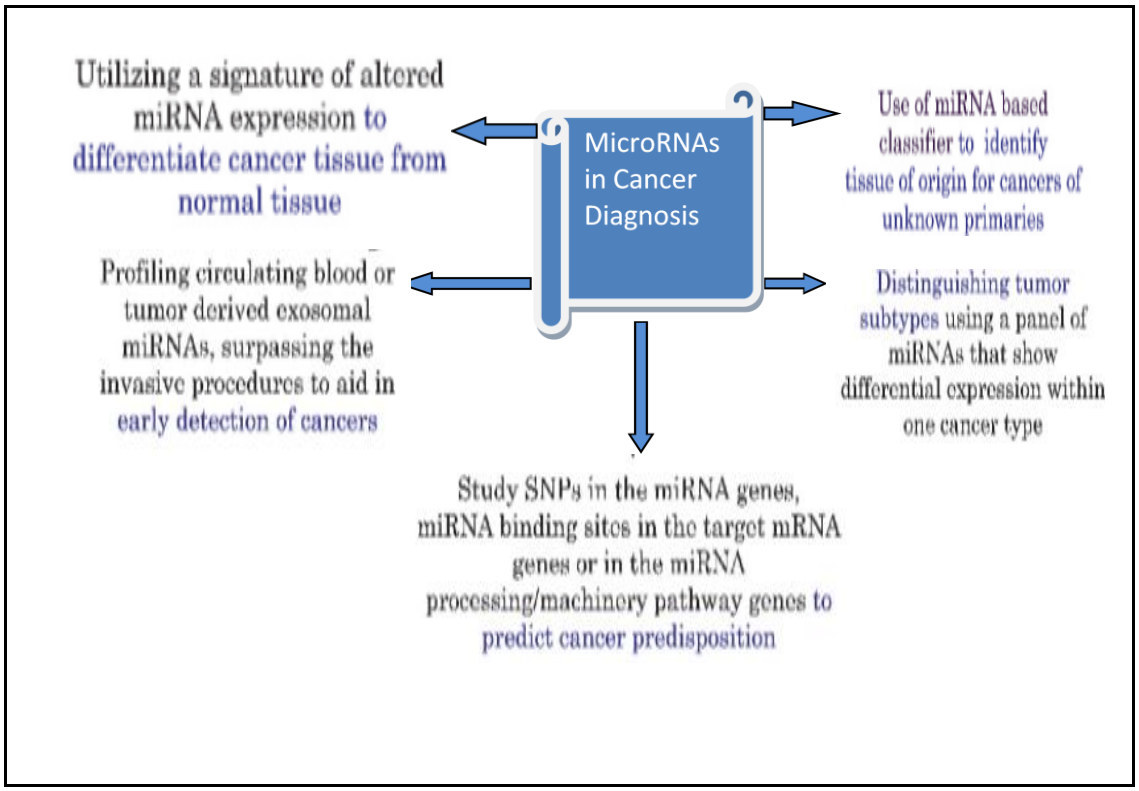

Figure 1.5. MicroRNAs (miRNAs) as potential diagnostic biomarkers (modified from [58])

Diverse aspects of miRNAs provide novel ways of utilising these in disease diagnosis. The unequaled miRNA signatures of different tumours distinguish the cancer from normal tissue. MiRNA classifiers can accurately identify the tissue of origin in the case of cancers of unknown primaries. Especially, blood-based miRNA profiling as a diagnostic test provides a non-invasive and fast alternative to traditional methods. Coupled with, miRNA patterns can also differentiate various tumour subtypes. Testing for miRNA-associated SNPs is another novel approach towards predicting cancer predisposition.

Single nucleotide polymorphisms (SNPs) in protein-coding genes can affect the functions of proteins and in turn influence the individual susceptibility to cancers has been well documented. SNPs that disrupt miRNA gene sequences have been associated with cancer risk. Similarly, profiling of blood miRNAs as a diagnostic test for colon cancer would be an advance in the field, and a non-invasive as well as an easy alternative to colonoscopy. Generally, diagnosis of metastatic cancers with an unknown primary site of origin is very

frustrating both for the patient and for the physician, and also poses huge treatment challenges [58].

As a result, expression of miRNAs is deregulated in human cancer including breast [75-77], glioblastoma [78], colon [79, 80], prostate [81], ovarian [82], and liver cancers. A real-time RT-PCR can be used to study miRNA. Takamizawa et al. [83] reported that certain let-7 family numbers are downregulated in lung cancer, and the reduced expression is significantly associated with the poor outcome of those patients. Besides, follow-up studies of large-scale global miRNA profiling further proved that expression signatures of miRNA genes have diagnostic and prognostic significance in leukemia [84] and lung cancer [85, 86].

1.4. AIM OF THE STUDY

The AuNPs have appeared as an attractive applicant for delivery of several agents, such as drugs, DNA fragments, and siRNA, into cells. We aimed to prepare a specific using gold nanoparticles that is able to carry hsa-miR-145 and to transfer this nanoparticle inside a prostate cancer cell lines. The hsa-miR-145 is down regulated in prostate tumors as compared to normal prostatic tissues. Therefore, we would like to up-regulate it in prostate tumors. To this purpose, the 13 nm- AuNPs- have been modified with thiolated RNAs for specific nanoparticle. Therefore, the MyCycler (Biorad, USA) instrument has been used- for hybridization of AuNPs- modified with thiolated RNAs and the sequence of the precursor microRNA miR-145 molecule. In addition, AuNPs have also been used as controls. Afterwards, the hybridized AuNPs and transfection reagent mixture have been transfected into the prostate cancer cell lines.

2. MATERIALS

2.1. INSTRUMENTS

The instruments used in this study are as follows:

- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore)
- CO_2 incubator (Nuaire NU5510/E/G, USA)
- Centrifuge (Hettich mikro 22R and SİGMA 2-5 centrifuge, Germany)
- Vortex (Stuart SA8, UK)
- LightCycler 480 Real-Time PCR System (Roche, Germany)
- PCR Thermal Cycler (Biorad MyCycler, USA)
- Nanophotometer (Implen)
- UV–Vis Spectrophotometer (PerkinElmer, USA)
- A Zetasizer Nano ZS instrument (Malvern, UK)
- -80 °C freezer (Thermo Forma -86 C ULT Freezer, USA)
- pH meter (Hanna instruments PH211, Germany)

2.2. EQUIPMENTS

The laboratory equipments used in this study are as follows:

- Serological pipettes 25, 10, 5, 2 ml (Grenier-Bio or Axygen, USA)
- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Cell culture flasks, T-25, T-75 and cell culture plates, 6-well, (TPP Switzerland or Grenier-Bio, Germany)

2.3. CHEMICALS

- 2-propanol (AppliChem A3928, Germany)
- Fetal Bovine Serum (FBS) Cell culture tested (Sigma F9665, Germany)
- Penicillin-streptomycin (Thermo Scientific SV30010 or Biochrom A2213, Germany)
- Trypsin-EDTA (Biochrom L2153, Germany)
- Absolute Ethanol (AppliChem A3678, Germany)
- Chloroform (Aldrich 528730, USA)
- Dulbecco's Phosphate Buffered Saline (DPBS) (PAN Biotech P04-53500, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Merck 108418, Germany)
- Trizol (Invitrogen 15596-018, USA)
- Sodium citrate (Merck, USA)
- $HAuCl_4 \cdot 3H_2O$ (Sigma, Germany)
- RPMI Medium (GIBCO, USA)

2.4. KITS AND SOLUTIONS

- TaqMan MicroRNA Reverse Transcription Kit (Applied Biosciences, Foster City, CA)
- Gene Expression Hybridization Kit 2XGE Hybridization Buffer(Agilent Technologies, USA)
- The AuNPs were modified with thiolated oligonucleotides (SynGen,Inc,USA)
- X- tremeGENE Transfection Reagent (Roche, Applied Science, Germany)
- Hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA)
- TaqMan Universal PCR Master Mix II (Applied Biosciences, Foster City, CA)
- MicroRNA specific primers (Applied Biosciences, Foster City, CA)

2.5. SAMPLES AND CELL CULTURE MATERIALS

PC3 (Human prostate cancer cell line) cell lines were collected from Yeditepe University Genetics and Bioengineering department. The cell culture media and other reagents including Trypsin-EDTA together with antibacterial and antifungal supplements are purchased from GIBCO (USA). The environmental conditions for culturing is in a 5% CO2 incubator with temperature set in 37 °C. The cell lines were passaged after reaching 70% confluency.

2.6. PRECURSOR MICRORNAs AND TRANSFECTION MATERIALS

The precursor microRNA miR-145 molecule, was purchased from Ambion (Foster City, CA, USA) Control transfections were performed by X-tremeGENE Transfection Kit of Roche (Applied Science, Germany) after optimizing the protocol for our cell lines.

2.7. RNA ISOLATION AND POLYMERASE CHAİN REACTİON (PCR) MATERIALS

For RNA isolation, total RNA was extracted by using Trizol reagent of Invitrogen Inc. (San Diego, CA). Equal amounts of RNA from isolates were used in cDNA synthesis by using MicroRNA Reverse Transcription kit Applied Biosciences (Foster City, CA). Quantitative PCR analysis was performed by Small RNA Assays with the Universal PCR Master Mix II Applied Biosciences (Foster City, CA). Quantitative PCR analysis assay for microRNAs and microRNA specific primers were purchased Applied Biosciences (Foster City, CA).

3. METHODS

3.1. PREPARATION OF GOLD NANOPARTICLES

3.1.1. Synthesis of Gold Nanoparticles (AuNPs)

AuNPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ with sodium citrate (Merck, USA), [87]. This procedure generates an average size of 13 nm AuNPs that are well characterized in the literature. Briefly, a 100mL (1 mM) of $HAuCl_4 \cdot 3H_2O$ (Sigma, Germany) solution was heated until to boil, and then 10 ml of 38.8 mM citrate solution was added quickly into boiling solution. The final solution was kept boiling for 15 min. The solution colour changes from yellow to deep red. As a result Figure 3.1. Shows TEM image of 13 nm – AuNPs

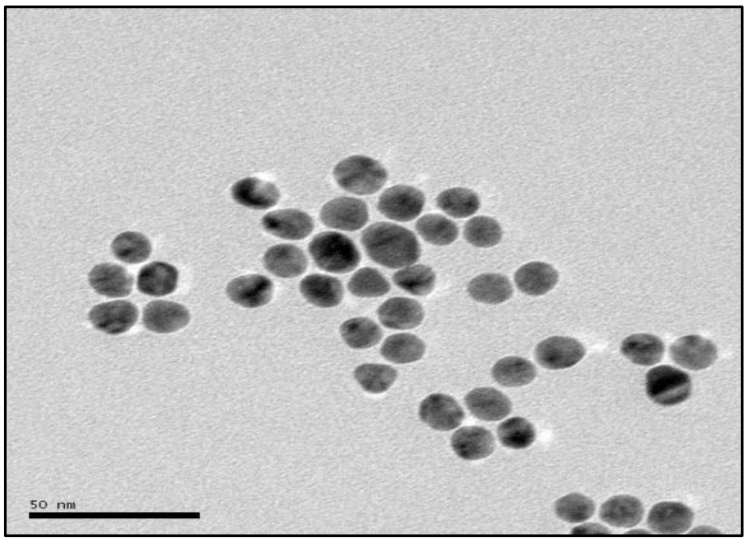

Figure 3.1. TEM image of 13 nm - AuNPs

3.1.2. Modification of Gold Nanoparticles (AuNPs)

The AuNPs were modified with thiolated oligonucleotide (-3'AAAAAAAAAAAUCUACGAUUCUACC5'), (SynGen,Inc, USA) as shown in Figure 3.2. For the attachment of oligonucleotide, an 2 µl of 200 µM was added into 1 mL AuNPs suspension and then the modified AuNPs were shaked for 24 h at 20 °C

3.1.3. Characterization of AuNPs

The synthesized AuNPs and AuNPs-oligonucleotide were characterized with UV–Vis Spectrophotometer (PerkinElmer, USA). Figure 3.2. Shows UV–Vis spectrum of 13 nm-AuNPs. The size distribution and zeta potential of AuNPs in suspensions were measured using a Zetasizer Nano ZS instrument (Malvern, UK) at 25 °C at 173° scattering angle with a 4 mW He–Ne laser. In addition, Figure 3.3. Hydrodynamic radii image of 13 nm - AuNPs. The samples of NPs were placed in standard Malvern zeta potential disposable capillary cells and polystyrene cuvettes for zeta potential and size measurements, respectively. All measurements were repeated three times.

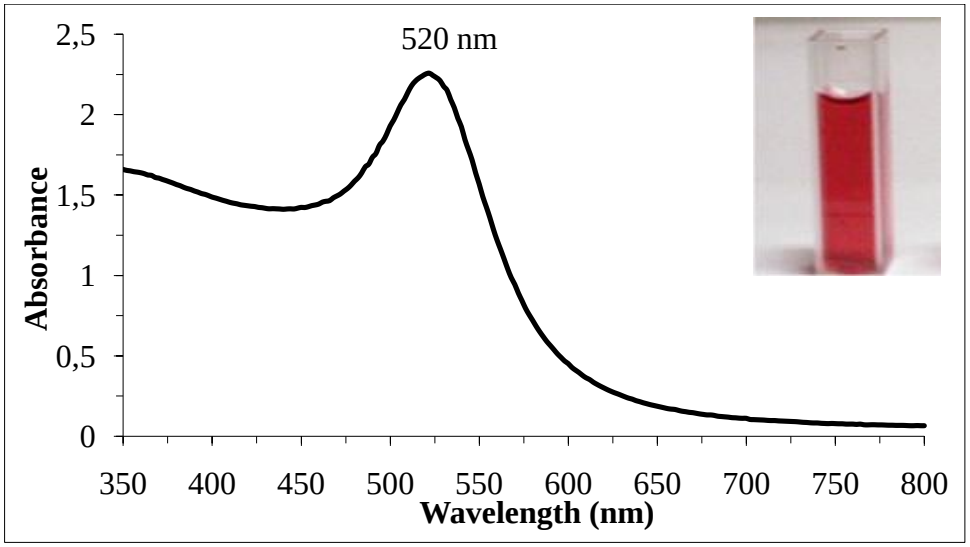

Figure 3.2. UV/ Vis Spectrum image of 13 nm – AuNPs

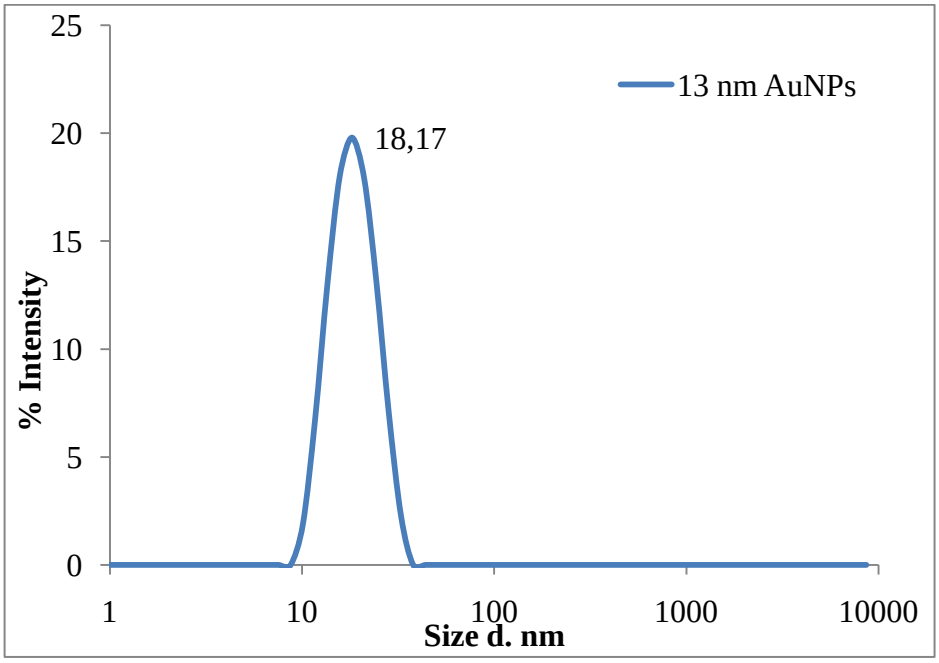

Figure 3.3. Hydrodynamic radii image of 13 nm - AuNPs

3.1.4. Influence of Temperature on Stability of AuNPs in Suspensions

A heating and cooling step of AgNP-RNA-miRNA complex was necessary to increase the transfection efficiency into the cells. The complex was heated to 72 and 90 ^{o}C to ıncrease the hybridization efficiency of the miRNA to the complamentary RNA chemically bound to the AuNPs. In order to make sure that the AuNPs were still stable after the heating step, a temperature gradient was emposed on the colloidal suspension containing AgNPs. The A Zetasizer Nano ZS instrument (Malvern, UK) at temperatures from 30 °C to 90 °C. Briefly, 10 µl of AuNPs, and 990 µl distilled water were added. In addition, we kept the suspension for 1 minute at each 10 °C intervals. As a result, this sample was placed in Glass cuvettes for size measurements. This separate measurement was repeated three times. Figure 3.4. shows the DLS spectrum.

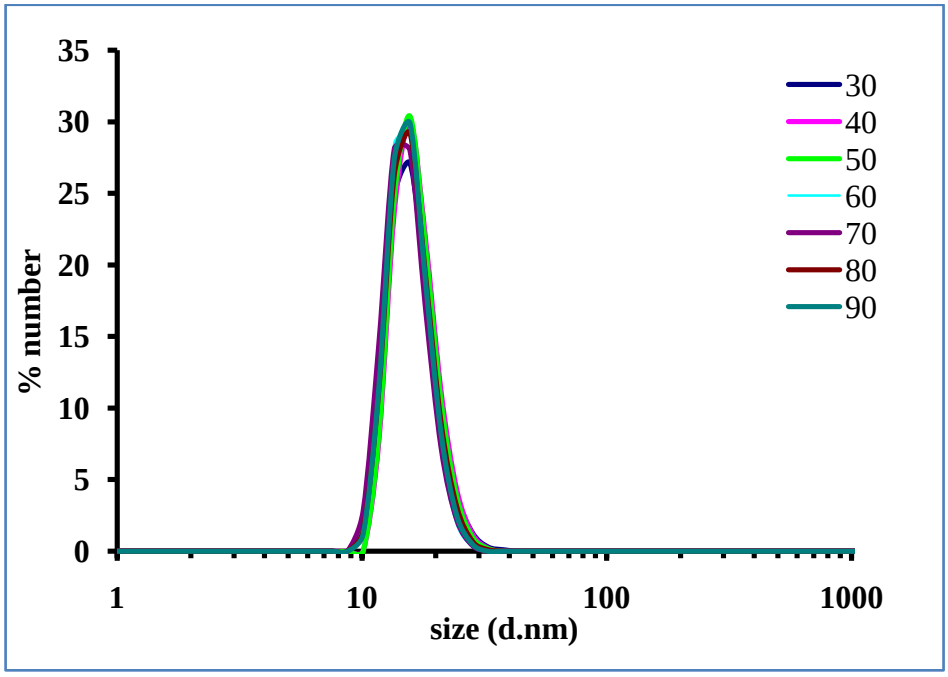

Figure 3.4. DLS spectrum of 13 nm - AuNPs

3.2. METHODS IN CELL CULTURE AND CELL PASSAGING

Prostate cancer cell lines were cultured with RPMI (GIBCO, USA) containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin/Antifungal (100 µg/ml streptomycin and 10,000 units/ml penicillin) solutions. The environmental conditions for culturing is in a 5% CO2 incubator with temperature set in 37 °C. The medium was then changed in the next 12-24 hours to remove the rest of the DMSO which is a toxic substance. The cell lines were passaged after reaching 70% confluency.

3.2.1. Cell Counting

Cell counting was performed using the hemocytometer. Following cell detachment, 10 µl of cell solution was added to hemocytometer. The cells were counted using inverted phase contrast microscope (Nikon, USA). The circled area on the hemocytometer represents 1/10000 of 1 ml.

The cells on this square were counted for 4 different times and then average of these numbers was used to calculate the number of cells per ml using the formula [88]:

$$\text{Cells/ml} = \text{the average number} \times 10^4 \times \text{dilution factor} \quad (3.1)$$

3.3. IN MICRORNA TARGET PREDICTION

The potential targets of hsa-miR-145 were predicted by bioinformatics algorithms. Computational algorithms have been developed for prediction of putative microRNA targets. The miRNA target prediction algorithms used for determining putative targets of hsa-miR-145can be found in [89]. These algorithms calculate the miRNA binding sites on 3'-UTR of genes algorithmically. This is the easy way of discovering potential targets.

3.4. PRECURSOR MICRORNAs

The precursor microRNA miR-145, inhibiting the miR-145 functions, molecules were purchased from Ambion (Foster City, CA, USA).

3.5. HYBRIDIZATION

Two groups were prepared for hybridization. Firstly, 10 μL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 μLof AuNPs - modified with thiolated RNAs and 40 μL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (AgilentTechnologies, USA) were added to the tubes. Afterwards, 50 μL of this mixture was transferred to 4 new tubes. The tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization in a 50 μL volume at 72°C for 2.5 min, and at 37 °C for 16 hours in Biorad MyCycler, USA instrument.

Secondly, 10 μL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 μLof AuNPs - modified with RNAs and 40 μL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies, USA) were added to the tubes. Afterwards, 50 μL of this mixture was transferred to 4 new tubes. The tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization in a 50 μL volume at 94°C for 1min, 72°C for 2.5 min, and 37 °C 16 hours in MyCycler (Biorad, USA) instrument.

The MyCycler (Biorad, USA) instrument were used for hybridization of the precursor microRNA miR-145 molecule with the thiolated RNAs that is modified with AuNPs.

3.6. TRANSFECTION OF PROSTATE CANCER CELL LINES

The miR-145 precursor molecule was transfected into the prostate cancer cell lines. The efficient lipofection method was chosen for the transfection. The number of cells was calculated for each group of incubation time. The approximate number of cells is 450000, 350000, 300000 for incubation times of 8, 16, 24, hours, respectively. The cells were grown in 6-well plate for 1 day and were washed with PBS just before transfection. Besides, the transfection of prostate cell lines and the amount of the reagent and initial cell amounts used were given below. For one well of 6-well plate; 2,5 μL X-tremeGENE transfection reagent was mixed with 250 μL of Opti-MEM, and 10 μL 10 pg of precursor microRNA was mixed with 250 μL of Opti-MEM. Afterwards, for the second well of 6-well plate; 2,5 μL X-tremeGENE transfection reagent was mixed with 250 μL of Opti-MEM. These mixes were incubated for 5 min at room temperature. Then, the transfection X-tremeGENE transfection reagent mix was added into precursor microRNA mix and the transfection mix was incubated for 20 minutes at room temperature.

The total 500 μL of these mixes were applied on the cells. After 1 hour, another 500 μL of Opti-MEM medium was added on plates. There were 7 groups in the transfected prostate cancer cell lines; 1: (+) Control of hsa-miR-145; There is the precursor microRNA miR-145 molecule and X-tremeGENE transfection reagent. 2: (-) Control 1; There is X-tremeGENE transfection reagent, 3: (-) Control 3; There is only Prostate cancer cell lines, 4: (-) Control 4: There is gold nanoparticles (Au- NPs), 5: (-) Control 2; There is AuNPs - modified with thiolated oligonucleotide. 6: Group 1: 10 μL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 μLof AuNPs - modified with thiolated RNAs. 40 μL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies, USA) was added to the tube. Afterwards, 50 μL of this mixture was transferred to 4 new tubes. The tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization in a 50 μL volume at 72°C for 2.5 min, and 37 °C 16 hours in a MyCycler (Biorad, USA) instrument. We hybridized the sequence of the precursor microRNA miR-145 molecule with the AuNPs- modified, thiolated RNAs. 7: For Group 2, 10 μL of hsa-

Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA) were added to the tube. Afterwards, 50 µLof this mixture was transferred to 4 new tubes. The tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization in a 50 µL volume 94°C for 1 min, at 72°C for 2.5 min, and at 37 °C 16 hours in a Biorad MyCycler, USA instrument. Similar to Group 1, we hybridized the sequence of the precursor microRNA miR-145 molecule with the AuNPs-modified, thiolated RNAs.

Afterwards, the total 150 µL of gold nanoparticles ((-) control 4) and AuNPs - modified with thiolated RNAs ((-) control 2) were applied on the cells and 350 µL of Opti-MEM medium was added on plates. After 1 hour, another 500 µL of Opti-MEM medium was added on plates. The total 200 µL of group 1 and group 2 was applied on the cells and 350 µL of Opti-MEM medium was added on plates. After 1 hour, another 500 µL of Opti-MEM medium was added on plates.

Total volume was 1 ml in per well. Cells were incubated for 24 hours. Afterwards, 600 µL of trypsin was added into the well. The samples were incubated for 5 minutes. Then, the transfected cell lines were transferred to 6 new tubes. The tubes centrifuged at 2500 g for 5 minutes. Following centrifugation, supernatant was discarded in the 6 tubes. At the end of the study, samples were stored at -80°C.

Likewise, we also conducted sodium acetate precipitation step in our experiment. In summary, 10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µL of AuNPs - modified with thiolated RNAs. and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA) were added to the tube. Afterwards, the tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization at 72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument. The volume of this mixture is 200 µL. This mixture was transferred to a new tube and 20 µL of 1 ml 3M NaOAc pH 5,2 was added; then, 440 µL of 20 ml 95% ethanol was added to the tube. The tube was centrifuged at 10,000 x g for 10 minutes at 4°C. After the centrifuge step, the sample was washed with 500 µL, 75% ethanol. The supernatant was discarded. Then, the pellet was dried and dissolved in 20 µL nuclease free water. As a

result, this mixture transfected into the prostate cancer cell lines and the cells were incubated for 24 hours.

3.7. RNA ISOLATION FROM CELLS

After transfection of PC3 cells, cells were transferred to a new tube. 500 µl TRIzol reagent was directly added to cell monolayer and scraped with pipette tip. After lysis of cells in TRIzol reagent by scrapping, additional 100 µl chloroform was added. The tubes were shaken vigorously by hand for 15 seconds, and were incubated for 10 minutes at 15 to 30°C for 2 to 3 minutes. The tubes centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the aqueous phase was transferred to a new tube and mixed with 250 µl isopropyl alcohol. The samples were incubated for 10 minutes at 4°C, and were centrifuged at 12,000 x g for 10 minutes at 4°C. Then, the supernatant was discarded; RNA pellet was washed with 500 µl 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. After the removal of supernatant, RNA pellet was dried via vacuum-dry and dissolved in 30 µl Rnase-free water, and was incubated for 10 minutes at 55°C to 60°C. RNA samples were stored at -80°C.

3.7.1. Quantification of RNA

3 µL of the RNA sample was used for measurement of the concentration. The concentrations of isolated RNA samples were determined by using spectrophotometer (Implen Nanophotometer). Rnase-free water was used as blank and RNA samples were measured at 260 nm. RNA concentration was calculated from the absorbance at 260 nm using equation below:

$$\text{Concentration of RNA sample (ng/µl)} = 40 \times A_{260} \times \text{dilution factor} \qquad (3.2)$$

As proteins give absorbance at 280 nm, the protein contamination in RNA samples was measured by calculating the A_{260}/A_{280} ratio.

3.8. POLYMERASE CHAIN REACTION

3.8.1. Reverse Transcriptase Polymerase Chain Reaction

The complementary DNA (cDNA) synthesis was prepared as shown in Table 3.2; Reverse transcriptase polymerase chain reaction (RT-PCR) for synthesis of complementary DNA (cDNA) MicroRNA Reverse Transcription kit from Applied Biosciences (Foster City, CA) was used. Aliquots of 15 ng/μL concentration from the total RNA of samples were used for specific cDNA synthesis with TaqMan Small RNA Assays primers. The control housekeeping gene for quantifying the relative expression was 5S-rRNA. Besides, small nuclear RNA, RNU43, were used as internal controls. Primer pairs for these controls and for miR-145 were obtained from Ambion. Each assay included a negative control. Therefore, the complementary DNA (cDNA) synthesis was prepared in two groups. Firstly, reaction mixture in of the hsa- pre mir 145 group; 7 μl reaction mixture that contains 1.5 μl 10 X RT Buffer, 0.15 μl dNTP Mix (100 mM total), 1.50 μL 10 X Reverse Transcription Buffer 1.00 μl MultiScribe Reverse Transcriptase, 0.19 μL RNase Inhibitor (20 U/μL), 4.16 μL Nuclease-free water was added to the tube. Afterwards, each 15-μL RT reaction consists of 7 μL master mix, 3 μL of 5 X RT primer, and 5 μL RNA sample. 15 μl total volume was obtained. Then, the reaction mixture was prepared separately for six samples. Secondly, reaction mix of the internal control (RNU43) housekeeping gene (RNU 43) group was prepared in the same way.

Table 3.1. The content of Reverse Transcriptase Polymerase Chain Reaction (RT – PCR) mixture for cDNA synthesis

Component	Master mix volume per 15-µL reaction
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10× Reverse Transcription Buffer	1.50 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	4.16 µL
Total volume	7.00 µL

Following parameter values to program the thermal cycler were used.

Table 3.2. The program for Reverse Transcriptase Polymerase Chain Reaction (RT –PCR)

Step	Time	Temperature
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	+4 °C

3.8.2. Quantitative Real-Time Polymerase Chain Reaction

The mRNA expression levels of PC3 genes in the cell lines were determined using TaqMan, Small RNA Assays PCR Kit in Roche, Life Cycle, 480 Real-Time PCR system. Quantitative Real-Time PCR analysis was performed by Small RNA Assays with the Universal PCR Master Mix II from TaqMan, Applied Biosciences (Foster City, CA). Quantitative Real-Time PCR analysis assay for microRNAs and microRNA specific primers were purchased from TaqMan, Applied Biosciences (Foster City, CA). Each reaction containing 1.00 µL TaqMan Small RNA Assay (20X), 10.00 µL TaqMan Universal PCR Master Mix II (2X), 7.67 µL Nuclease-free water and 10 ng 1.33 µL Product from RT reaction cDNA in a total volume of 20.00 µl was performed in conditions given in Table 3.4 that represents the PCR amplification/cycle graph or real-time PCR analysis. The specificity of PCR products were checked by fit-point analysis. Each sample was studied in triplicate. The quantification of results was done by using a Roche, Life Cycle, 480 Software Real-Time PCR. Small nuclear RNAs, RNU43, were used as internal controls. Primer pairs for these controls and for miR-145 were obtained from Ambion. Each assay included a blank negative control.

Table 3.3. Prepare the Quantitative Real-Time Polymerase Chain Reaction (qPCR) mix

Component	Volume per 20-µL Reaction	
	Single reaction	Three replicates
TaqMan Small RNA Assay (20X)	1.00 µL	3.60 µL
Product from RT reaction	1.33 µL	4.80 µL
TaqMan Universal PCR Master Mix II (2X)	10.00 µL	36.00 µL
Nuclease-free water	7.67 µL	27.61 µL
Total volume	20.00 µL	72.01 µL

Table 3.4. Quantitative Real-Time Polymerase Chain Reaction (qPCR) conditions

Step	Optional AmpErase UNG activity	Enzyme Activation	PCR	
			CYCLE (40 cycles)	
Temperature	HOLD	HOLD	Denature	Anneal/extend
Time	50 °C	95 °C	95 °C	60 °C
	2 minutes	10 minutes	15 seconds	60 seconds

4. RESULTS

4.1. MICROSCOPY IMAGE BEFORE TRANSFECTION OF PROSTATE CANCER CELLS

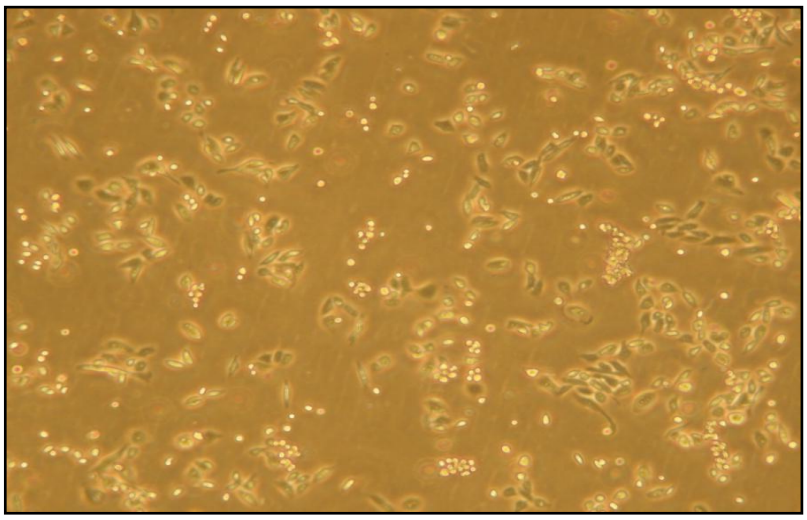

Figure 4.1. Microscopy image of prostate cancer cells. Prostate cancer cells are observed in the microscope (Nikon, USA), (10X magnification with full zoom of camera)

Before transfection of prostate cancer (PC3) cells, prostate cancer cell lines were cultured with RPMI (GIBCO, USA). The environmental conditions for culturing is in a 5% CO2 incubator with temperature set in 37 °C. In addition, the cell lines were passaged after reaching 70% confluency. Afterwards, Figure 4.1. Prostate cancer cells are observed in the microscope (Nikon, USA), (10X magnification with full zoom of camera).

4.2. TRANSFECTION OF PROSTATE CANCER CELLS WITH AuNP CARRIER HSA-MIR-145

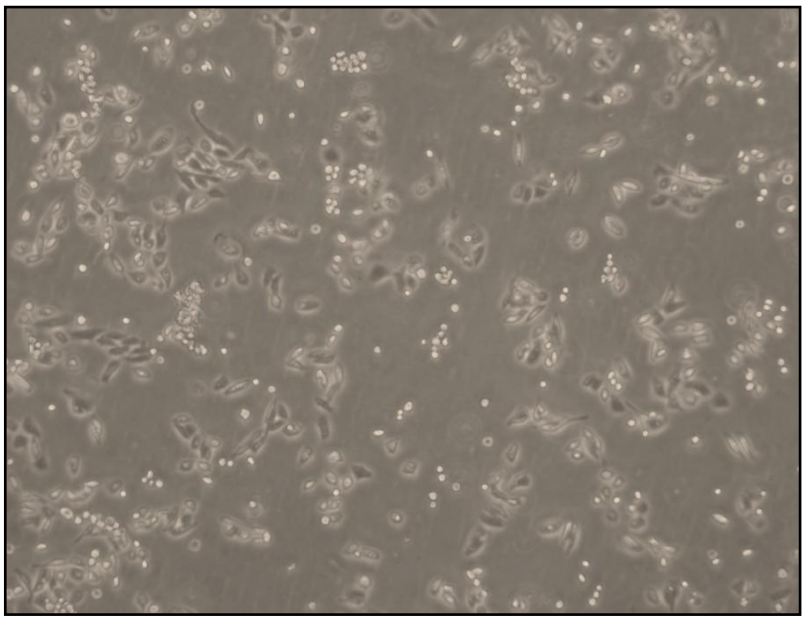

Figure 4.2. Microscopy image of transfection of prostate cancer cells with new nanoparticle carrier hsa-miR-145. (Nikon, USA), (10X magnification with full zoom of camera)

Prostate cancer cells with new nanoparticle carrier hsa-miR-145 transfected into the prostate cancer cells. These cells were incubated for 24 hours, After transfection, we could observe most of the prostate cancer cells in the microscope (Nikon, USA). In addition, Figure 4.2. Shows the transfection of prostate cancer cells with new nanoparticle carrier hsa-miR-145.

4.3. CONFIRMATION OF HSA-MIR-145 IN AuNP IN TRANSFECTED PROSTATE CANCER CELLS

AuNPs, with - 13 nm size were modified with RNAs, (SynGen, Inc, USA) to produce Particle A. as shown in Figure 4.3. For the attachment of oligonucleotide, an 2 µl of 200 µM was added into 1 mL AuNPs suspension and then the modified AuNPs were shaked for 24 h at 20 °C. Afterwards, the synthesized AuNPs and AuNPs-oligonucleotide were

characterized with UV–Vis Spectrophotometer (PerkinElmer, USA). Size distribution and zeta potential of NPs in suspensions were measured using a Zetasizer Nano ZS instrument (Malvern, UK) at 25 °C at 173° scattering angle with a 4 mW He–Ne laser.

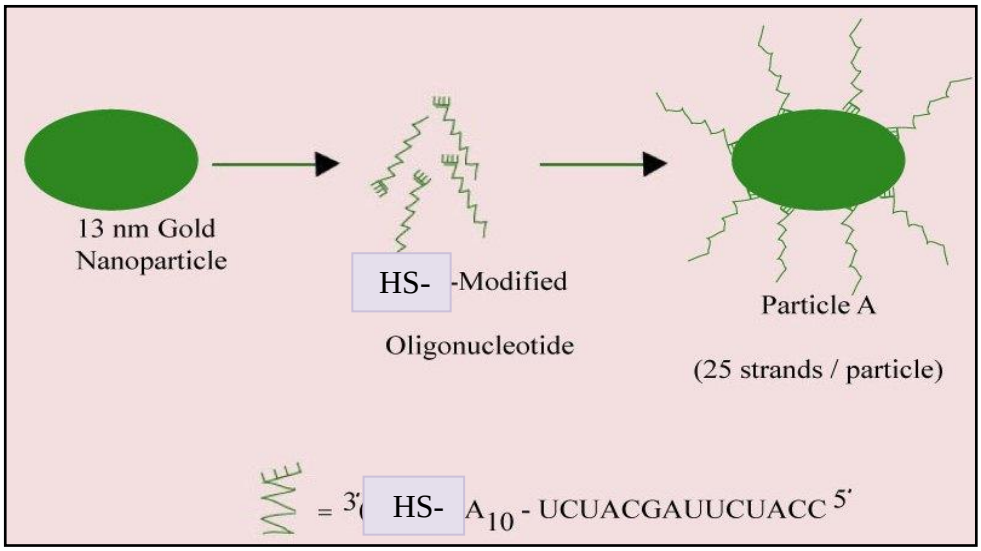

Figure 4.3. Schematic structure of 13 nm – AuNPs, - modified with thiolated oligonucleotide

4.4. THE RELATIVE EXPRESSION OF HAS-MIR-145 IN TRANSFECTED PROSTATE CANCER CELLS.

The up-regulation of miR-145 in prostate cancer cell lines was validated. Figures 4.4, 4.5, 4.6, and 4.7. Shows the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in different groups. Especially, hsa-miR-145 is down-regulated in prostate tumors compared to normal prostatic tissues. In addition, hsa-miR-145 is down-regulated in prostate cancer cell line. At the end of the study, we found favorable alteration in the relative expression of hsa-miR-145 in prostat cancer cell line. Specifically, hsa-miR-145 has been up- regulated in prostate tumors.

In our experiments, different groups were transfected into the prostate cancer cell lines. These cell lines were incubated for 24 hours. We used real-time RT–PCR assays conditions in prostate cancer cell lines (PC3), and TaqMan microRNA assays for individual miR-145 Applied Biosciences (Foster City, CA). 10 nanograms of total RNA

from each sample was subjected to reverse transcription reaction in 15 µl volume of RT-PCR assay at 16 °C for 30 min, at 42 °C for 30 min, at 85°C for 30 min, at 4°C for ∞. For quantitative PCR, 1.33 µl of the above-mentioned RT-PCR assay was used in duplicate as follows: 50 °C for 2 min, 95 °C for 10 min, and then 95 °C for 15 s and 60 °C for 60 s for 40 cycles in Roche, Life Cycle, 480 Software Real-Time PCR. Small nuclear RNAs, RNU43, were used as internal controls. Primer pairs for these controls and for hsa-miR-145 was obtained from Ambion. Each assay included a negative control, and the experiment was done in duplicate. The threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The signals were normalized against the relative quantity of control miRNA and expressed as $\Delta Ct=(Ct_{sample}-Ct_{control})$. Relative changes in expression were then calculated as $2^{(\Delta Ct)}$.

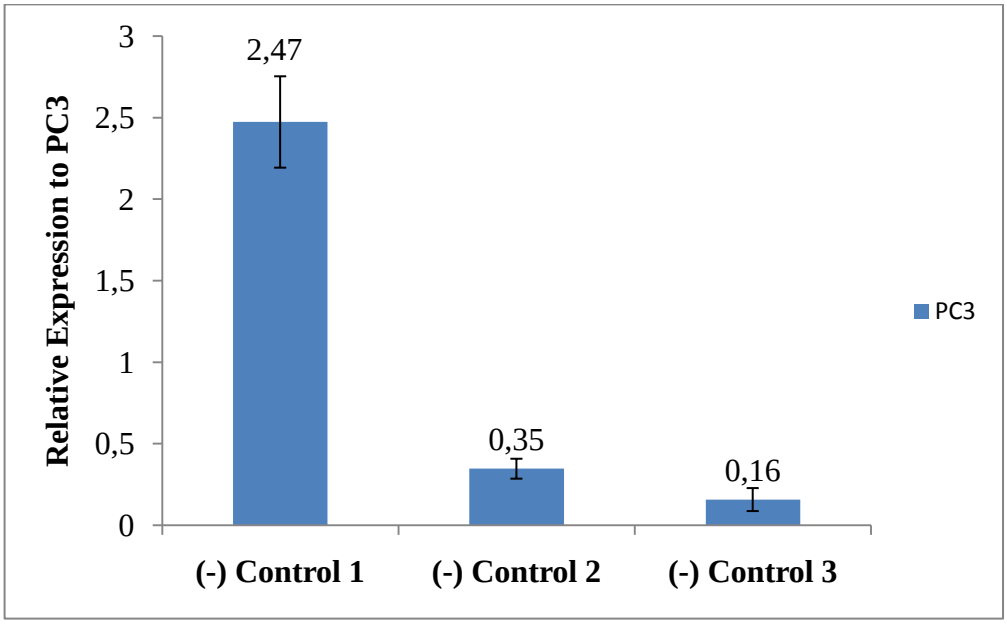

Figure 4.4. The relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 1, (-) Control 2 and (-) Control 3

Table 3.5. Identification of (-) Control 1, (-) Control 2 and (-) Control 3 in our study

GROUPS IN OUR STUDY	MIXTURES	PCR conditions
(-) Control 1	There is X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 2	There is AuNPs - modified with thiolated RNAs.	No PCR, transfected directly to the cells
(-) Control 3	(-) Control:There is only Prostate cancer cells.	No PCR, transfected directly to the cells

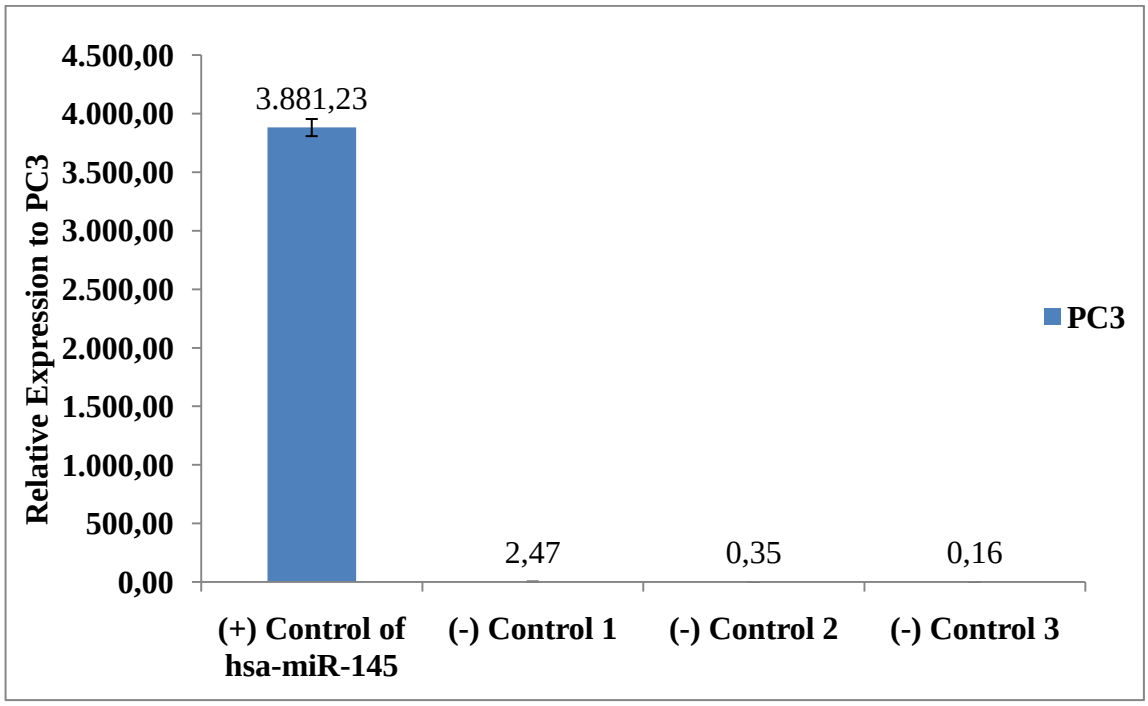

Figure 4.5. The relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145, (-) Control 1, (-) Control 2 and (-) Control 3

There are four different groups in Figure 4.5. These groups are: (+) Control of hsa-miR-145, (-) Control 1, (-) Control 2 and (-) Control 3 In the figure, the relative expression levels of hsa-miR-145 in transfected prostate cancer cell lines were plotted. Furthermore, because the relative expression levels of hsa-miR-145 in transfected prostate cancer cell lines for (-) Control 4 were found within normal values, we didn't include them in Figure

4.5. In our experiment, the table 3.5. Above is the list that we have used for Identification of (-) Control 1, (-) Control 2 and (-) Control 3.

Table 3.6. Identification of (+) control of hsa-miR-145, (-) Control 1, (-) Control 2 and (-) Control 3 in our study

GROUPS IN OUR STUDY	**MIXTURES**	**PCR conditions**
(+) Control of hsa-miR-145	The precursor microRNA hsa-miR-145 molecule and X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 1	There is X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 2	There is AuNPs - modified with thiolated RNAs.	No PCR, transfected directly to the cells
(-) Control 3	(-) Control: There is only Prostate cancer cells.	No PCR, transfected directly to the cells

In our experiment, the table 3.6. Above is the list that we have used for Identification of (+) control of hsa-miR-145, (-) Control 1, (-) Control 2 and (-) Control 3.

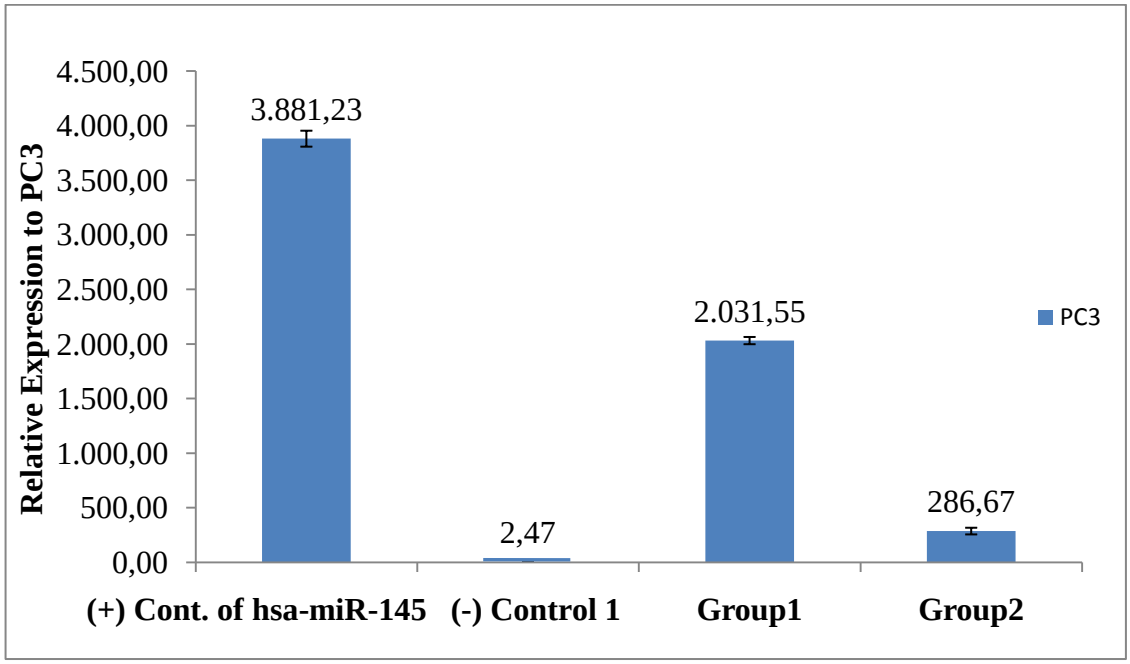

Figure 4.6. The relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145, (-) Control 1, group 1 and group 2

Figure 4.6. Shows the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145, (-) Control 1, group 1 and group 2. The relative expression for hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145is higher than the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 1. Furthermore, the relative expression data for hsa-miR-145 in transfected prostate cancer cell lines in (+) Control of hsa-miR-145 (mean = 3881.23) are higher than the amount in group 2 (mean = 286.67).

Table 3.7. Identification of (+) control of hsa-miR-145, (-) Control 1, group 1 and group 2 in our study

GROUPS IN OUR STUDY	MIXTURES	PCR conditions
(+) Control of hsa-miR-145	The precursor microRNA hsa-miR-145 molecule and X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 1	There is X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
Group 1	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies, USA).	72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument.
Group 2	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA)	94°C for 1min, 72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument.

In our experiment, the table 3.7. Above is the list that we have used for Identification of (+) control of hsa-miR-145, (-) Control 1, group 1 and group 2.

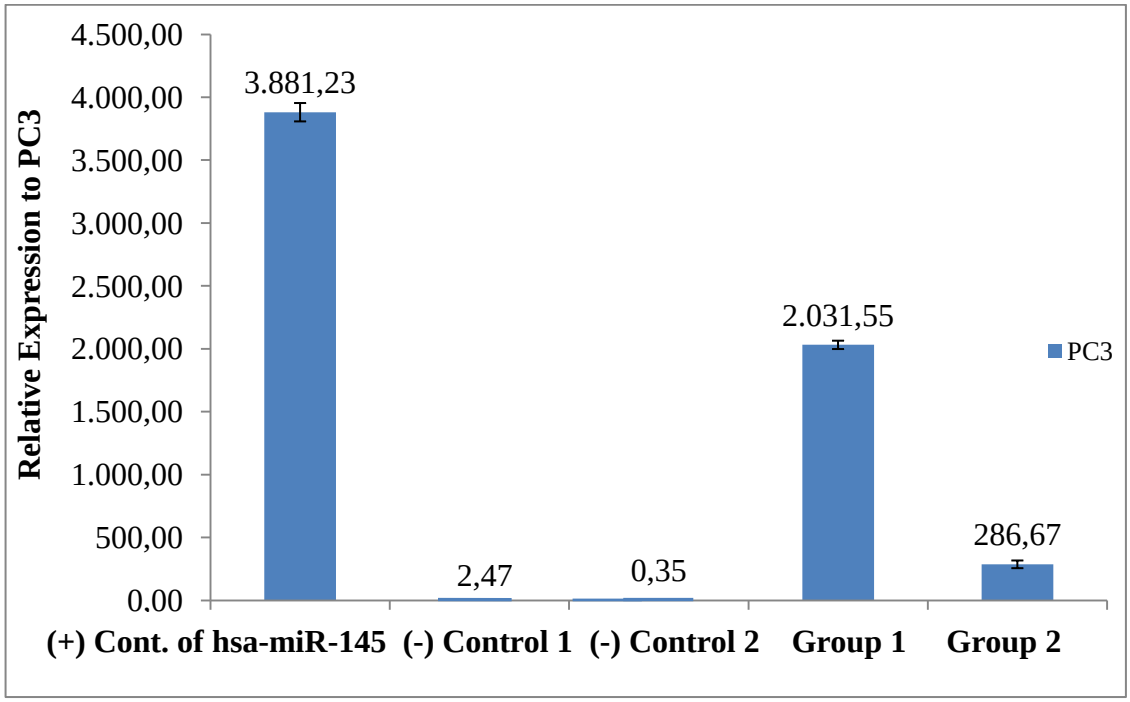

Figure 4.7. The relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145, (-) Control 1, (-) Control 2, group 1 and group 2

Figure 4.7. Shows the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (+) control of hsa-miR-145, (-) Control 1, (-) Control 2, group 1 and group 2. The relative expression for hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 1 is higher than the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 2.

Table 3.8. Identification of (+) control of hsa-miR-145, (-) Control 1, (-) Control 2, group 1 and group 2 in our study

GROUPS IN OUR STUDY	MIXTURES	PCR conditions
(+) Control of hsa-miR-145	The precursor microRNA hsa-miR-145 molecule and X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 1	There is X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 2	There is AuNPs - modified with RNAs.	No PCR, transfected directly to the cells
Group 1	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies, USA).	72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument.
Group 2	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA)	94°C for 1min, 72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument.

We hybridized the sequence of the precursor microRNA miR-145 molecule with the AuNPs- modified, thiolated RNAs. That is, we made it possible to complementing bases to connect to each other. In this hybridization process, we used a Biorad MyCycler. In addition, in our experiment, the table 3.8. Above is the list that we have used for identification of (+) control of hsa-miR-145, (-) Control 1, (-) Control 2, group 1 and group 2.

Table 3.9. Identification of groups in our study

GROUPS IN OUR STUDY	MIXTURES	RT-PCR conditions
(+) Control of hsa-miR-145	The precursor microRNA hsa-miR-145 molecule and X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 1	There is X-tremeGENE transfection reagent.	No PCR, transfected directly to the cells
(-) Control 3	(-) Control:There is only Prostate cancer cells.	No PCR, transfected directly to the cells
(-) Control 4	Crude; There is gold nanoparticles (Au- NPs)	No PCR, transfected directly to the cells
(-) Control 2	There is AuNPs - modified with thiolated RNAs.	No PCR, transfected directly to the cells
Group 1	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA).	72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument. in a Biorad MyCycler, USA instrument.
Group 2	10 µL of hsa- Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 µLof AuNPs - modified with thiolated RNAs and 40 µL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies,USA)	94°C for 1min, 72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument.

The results have been given above in Figures 4.4, 4.5, 4.6, and 4.7. The amount of relative expression for hsa-miR-145 in transfected prostate cancer cell lines in group 1 is very high (mean = 2132.48). This value is higher than the relative expression of hsa-miR-145 in

transfected prostate cancer cell lines in (-) Control 2 (mean=0.34, one-sided t-test at p=0.09) and group 2 (mean = 286.66, t-test significant at p=0.023). In adddition, the relative expression for hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 1 is higher than the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 2. Furthermore, the relative expression data for hsa-miR-145 in transfected prostate cancer cell lines in (+) Control of hsa-miR-145 (mean = 3881.23) are higher than the amount in group 2 (mean = 286.67). As an additional experiment, we also processed group 1 by first precipitating sodium acetate before transfection. This sample generated lower relative expressiom than the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in group 2. In addition, in our experiment, the table 3.9. Above is the list that we have used for identification of groups.

5. DISSCUSSION

Hsa-miR-145 is down- regulated in prostate tumors. Therefore, we wanted to up-regulate it in prostate tumors. In our study, we proposed to generate a specific nanoparticle from a set of gold nanoparticles that carries a hsa-miR-145 and to transfer this nanoparticle inside a prostate cancer cell lines. To that aim, we modified the AuNPs(13nm) with thiolated RNAs for specific nanoparticle. Then, there is a advantage alteration in the the relative expression of prostat cancer cell line. Then, these nanoparticles and different groups transfected into the Prostate cancer cell lines. These cells were incubated for 24 hours. At the end of the study, hsa-miR-145 is up- regulated in prostate tumors. Afterwards, we demonstrated that the release of hsa-miR-145 delivered by AuNPs - modified with thiolated RNAs is more efficient in the prostat cancer cell line. In addition, these novel gold nanoparticles can effectively enhance gene delivery efficiency and regulate gene expression in the context of miRNA.

There are novel studies utilizing gold nanoparticles (AuNPs) for the delivery applications that support our research findings. For instance, Nagasaki et al. prepared siRNA gold nanopartic complex that contained siRNA with thiol groupbound to pegylated gold nanoparticles (AuNPs) [90]. In addition, Lee at al. prepared PBAE–siRNA–AuNPs with C32-221, the best two PBAEs (C32-228 and 221). The PBAE showing the best delivery efficiency was studied for the gene knockdown experiments. The HeLa cell transfected by the PBAE–siRNA–AuNPs was shown in the TEM image. Numerous particles were observed as aggregates, in certain cases confined within endosomes in the cytosol. In conclusion, they described the development, optimization, and characterization of a new nanoparticulate siRNA delivery vehicle using PBAEs and AuNPs [12].

There are studies based on modification of gold nanoparticles. Giljohann et al. reported in their studies the synthesis and characterization of polyvalent RNA-goldnanoparticle conjugates (RNA-AuNPs) nanoparticles. Following synthesis, RNA-Au NPs were added to the cultures of HeLa cells. Afterwards, imaging studies revealed the throughout in the cytoplasm of HeLa cells after 6 hours. Flow cytometry confirmed the uptake of RNA-Au NPs in >99% of the cell population. After RNA-Au NPs were utaken by cells, they

examined the intracellular activity of RNA AuNP conjugates. They also conducted protein knockdown studies in HeLa cells using a transfected luciferase plasmid as a target for this model system. In addition, they hypothesized that the persistent knockdown of luciferase may be the result of the stabilization of the RNA on the nanoparticle. The enhanced stability they observed here for the RNA-Au NPs may be due to the same situation. Given their straightforward synthesis, these RNA-Au NPs, in addition to useful intracellular gene regulation materials, may be promising candidates for therapeutic and diagnostic applications [91].

In another important study, Guo, et al. demonstrated that the release of siRNA delivered by PEI/PAH-Cit/PEI/MUA-AuNPs is more efficient after being delivered inside the cells. In addition, the successful escape from endosomes is crucial for siRNA carriers to improve siRNA silencing efficiency. The penetration ability of cy5-siRNA/PEI/PAH-Cit/PEI/MUA-AuNP complexes into cells was investigated by confocal laser scanning microscopy. Images were taken to measure fluorescence in HeLa cells after transfection for 6 hrs. These novel gold nanoparticles can both effectively enhance gene delivery efficiency and regulate gene expression in the context of RNA interference, the surface of PEI/PAH-Cit/PEI/AuNPs was coated by a layer of polyelectrolyte bearing PEG and ligand moieties, the blood circulation time of PEI/PAH-Cit/PEI/AuNPs and the bioavailability of drugs would be improved [11]. As a result, gold nanoparticles conjugates can provide a significant advantage for the protection and delivery of miRNA.

On the other hand, Sevli et al. reported in their studies, the lipofection method was chosen for the transfection. Therefore, they added the transfection of miR-145 precursor in prostate cancer cell lines. Afterwards, the presence of mature miR-145 molecules and the duration of their stability were analyzed with microRNA. It was observed that after transfection of precursor-miR-145, mature miR-145 molecules were produced and degraded gradually in 3 days. In addition, the relative expression of miR-145 in transfected prostate cancer cell lines in terms of different growth time were observed in their study. Namely, the transfected cell lines were prepared to grow in 5 groups for different time periods as 8, 16, 24, 48, and 72 hours period. After 72 hours of incubation, the visible mature miR-145 molecules were very few. As a result, in their sudies, the lipofection method wasn’t found as an efficient method [92]. In our experiments, different groups

were transfected into the prostate cancer cell lines. For example, Group 1: 10 μL of hsa-Pre-miRNA 145 Ambion (Foster City, CA, USA), 150 μLof AuNPs - modified with thiolated oligonucleotide. 40 μL of Gene Expression Hybridization Kit 2X GE Hybridization buffer (Agilent Technologies, USA) was added to the tube. Afterwards, 50 μL of this mixture was transferred to 4 new tubes. The tubes were vortexed vigorously for 10 seconds. Each sample was subjected to hybridization in a 50 μL volume at 72°C for 2.5 min, and 37 °C 16 hours in a Biorad MyCycler, USA instrument. We hybridized the sequence of the precursor microRNA miR-145 molecule with the AuNPs- modified, thiolated oligonucleotide sequence. This mixture transfected into the prostate cancer cell lines. Afterwards, these cell lines were incubated for 24 hours. As a result, we performed a comparative analysis of the relative expression of hsa-miR-145 between different groups transfected into the prostate cancer cell lines. In addition, there is favorable alteration in the relative expression of prostat cancer cell line. The relative expression value of group 1 is the best among all groups. We reported experimental results that explained the efficiency of the investigated treatment process.

Mead; et al, reported in their studies, Mature miRNAs associate with an Argonaute protein and bind their mRNA targets, which are often in the 3' untranslated region (UTR), resulting in inhibition of translation or perhaps target mRNA degradation in animals. The "seed region" (bases 2–8 from the 5' end) contributes importantlly to miRNA-target recognition. miRNAs have been found to play important roles in apoptosis, cancer, development, differentiation, after general information, their used *An. stephensi* therefore,this species is an important malaria vector in Asia and it is becoming a model *Anopheline* species for physiological and genetics studies.after Ribonuclease Protection Assay (RPA) was used to examine ast-miR-76. Double-stranded oligos with a T7 promoter 5' of an antisense sequence of the miRNA were produced by annealing two single-stranded oligos. ast-miR-76

5'TTCGTTGTCGACGAAACCTGTTTTCTCCCTATAGTGAGTCGTATTA 3'

3'AAGCAACAGCTGCTTTGGACAAAAGAGGGATATCACTCAGCATAAT 5'

In their experiment, their used "mirVana miRNA Probe Construction Kit" (Ambion) . In addition, they utilized a "MEGAscript RNAi kit" (Ambion) to synthesize radiolabeled (^{32}P-UTP, Perkin-Elmer) antisense transcripts. Templates were synthesized for 4 hours, probe was added to 5 ug total RNA and denatured at 96°C for 3 minutes followed by 42°C hybridization overnight. Next, the hybridized samples were digested with RNase A/T1 for 45 minutes at 37°C to remove single-stranded RNA, and to trim unhybridized regions of the probes. Afterwards, Resuspended samples were denatured at 96°C for 5 minutes followed by a quick chill on ice before loading. RNA were separated using a 15% denaturing polyacrylamide gel at 150 V. Comparisons to miRBase also revealed sequences that match miRNAs from other organisms but were not reported in mosquitoes. The remaining *An. stephensi* small RNA sequences that matched the *An. gambiae* genome assembly were further analyzed to uncover new miRNAs from mosquitoes . These small RNAs were represented by 27 distinct sequences. Thirteen additional small RNA sequences had 1 mismatch to the *An. gambiae* genome assembly. To be conservative, we only considered these 13 sequences as possible miRNA candidates [93].

In another important study, Lodes et al. One issue that seems in these studies is the fact that the miRNA expression patterns seen in serum are not identical to those seen from miRNAs taken directly from cancer cell lines. In theirstudy, they found that although both the prostate cancer serum sample and the prostate cancer cell line. DNA array synthesis and microarray configuration Arrays for serum miRNA analysis were manufacture with 547 human miRNA sequences obtained from the Sanger Database version. Three probes were written for each miRNA: an anti-sensed wild-type version, a double-mutant control probe, in addition, a sense control version .Antisense controls were not included if the corresponding antisense miRNA existed in the databases. The double mutant control mutations were screened in order to maintain the same notional melting temperature (Tm) as the wild-type Tm. They were also designed to avoid perturbing or creating any secondary structure that might appear in the wild-type probe. They hybridized in their experiment. The content of hybridization. Mixture: Each filled with 30 μl of Pre-Hybridization Solution (CombiMatrix Corp) and incubated for 10 min at 45°C. MicroRNA was mixed with 9 μl 20× SSPE (Ambion), 4.8 μl BSA at 50 mg/ml (Ambion), 3.6 μl deionized formamide (Sigma) and 7.5 μl of 10% SDS (Ambion) and heated to 95°C for 3 min. 30 μl of each sample were added to sectored hybridization chambers, sealed with

aluminum tape, and incubated at 45°C for 16 hr with rotation. As a result, their, can speculate that the most obvious source of miRNAs that appear in the serum is a product of tumor cell lysis; however, it may also be possible that their appearance in the serum is the product of a form of active transport involving the formation of exosomes this study has shown that serum miRNAs are up-regulated in cancer patients as compared to normal donors. In a comparison of stages 3 and 4 prostate cancer sera and normal donor serum miRNA levels, we found that 15 miRNAs (miR-16, -92a, -103, -107, -197, -34b, -328, -485-3p, -486-5p, -92b, -574-3p, -636, -640, -766, -885-5p) were up-regulated in serum from prostate cancer patients compared to normal donor sera. Heat Map and cluster analyses show that serum miRNA signatures can also be used to separate cancer patients and normal donors in most cases [94].

The miRNA expression is crucial in oncogenesis.The pre-miRNA, in turn, is processed by Dicer to create the mature miRNA. Increased miRNA expression in cancers is associated with the down-regulation of tumor suppressors.The in situ detection of miRNAs has been assisted by the use of locked nucleic acid (LNA)–modified nucleotides. In addition, the LNA nucleotides are much more rigid in three-dimensional space, which results in a considerable increase in the T_m of the small LNA-modified oligonucleotide probe hybridized to its target miRNA. They have developed a novel method for the in situ detection of the mature miRNA based on the extension of the molecule after its hybridization to an ultramer template.In their experiment, the ultramer is at least 100 nucleotides in size. At its 5′ end, it consists of a series of four 20-nucleotide repeats (GACCCCTTAATGCGTCTAAA), ending at the 3′ end with the complementary sequence of the miRNA of interest. Separately, DNA or RNA polymerase will extend the miRNA using the ultramer as the template they repetitive sequence allows the use of a complementary oligonucleotide (TTTAGACGCATTAAGGGGTC) that can be added at the onset of the reaction that will inhibit the extension of the miRNA. Furthermore, the sequences of the different miRNAs studied by in situ hybridization with the LNA modified probes are provided. The probes (250 pmol) were made by Exiqon (Woburn, MA, USA), and include multiple modified LNA nucleotides dispersed throughout the sequence. he labeled probe was diluted in the in situ hybridization buffer (Enzo Life Sciences), added to the slide and denatured at 60°C for 5 min, and then hybridized at 37°C for 15 h. The ultramer extension method and RT in situ PCR, the negative control with the LNA in situ

method was done on the same slide as the test section. As a result, they have described a new method for the in situ detection of mature microRNAs. The major advantages of the ultramer extension method over the LNA probe method include that it is specific for the mature, active form of the miRNA, it is easier to optimize with a broader window between signal and it is much less expensive [95].

6. CONCLUSION

In this study, we demonstrated the transfection of miRNA into prostate cancer cell lines using an AuNP based carrier. The different groups transfected into the prostate cancer cells. These cells were incubated for 24 hours, after transfection, we could observe most of the prostate cancer cells under the microscope. As a result, we did comparative analysis of the relative expression of hsa-miR-145 between different groups transfected into the prostate cancer cell lines. In addition, there is favorable alteration in the relative expression of prostat cancer cell line. The relative expression value of group 1 is the best among all groups. The relative expression data for hsa-miR-145 in transfected prostate cancer cell lines in group 1 are higher than in the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 2. This sample is lower than the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in group 2. In addition, the relative expression data for hsa-miR-145 in transfected prostate cancer cell lines in (+) Control of hsa-miR-145 are higher than in the the relative expression of hsa-miR-145 in transfected prostate cancer cell lines in (-) Control 1. At the end of the study, hsa-miR-145 was observed as up- regulated in prostate tumors. In our study, we demonstrated the experimental results that explained the efficiency of the investigated treatment process. We think that dicer enzyme has cut off the AuNPs-modified thiolated RNAs in cancer cells to make hsa-miR-145 usable. Furthermore, our proposed mechanism of transfecting has-miR-145 to prostate cancer cell lines is a novel alternative to the existing way of using X-tremeGENE transfection reagent at comparable efficiency. Note that heating the AuNP-RNA- hsa-miR-145 complex breiefly to 70 ^{o}C and cooling helped to hybridization of the hsa-miR-145 to the complamentary sequence of RNA chemically attached to the AuNPs. However, increasing the temperature to 90 ^{o}C did not provide meaningful results. This suggests that the formed complex is transfected into the cell easily and the hsa-miR-145 is related into cytosol from endosomes.

7. FUTURE DIRECTIONS

In the future, cell transfection methods for delivery of small RNAs into the cancer cells. Furthermore, it may help us better understand the cancer process.We will use different buffers in cancer cells. Dicer enzyme may be cutting off the hsa- miRNA-145 from the gold nanoparticle (AuNPs) -modified thiolated oligonucleotide.We can use the dicer enzyme for understanding this mechanism. Further study is ncesseary to understand the mechanism of effective transfaction after a brief heating procedure.

Our study should be verified in other cell types and condition to find out better move universal goldnanoparticle to delivery small RNA in to cancer cells.

REFERENCES

1. Pomerantz, M. M., and M. L. Feedman, "The Genetics of Cancer Risk", *Cancer Journal,* Vol. 17, pp. 416-422, 2011.

2. Ferrari, M., "Cancer nanotechnology: opportunities and challenges", *Nat Rev Cancer*, Vol. 5, pp. 161–171, 2005.

3. Baker, R. J., A. Jr. Quintana, L. Pehlerel, M. Banazak- Holl al, D. Tomalia and E. Raczka, "The synthesis and testing of anti- acancer therapeutic nanodevices: Biomedical microdevices", *Oncogene,* Vol. 3, pp. 61-69, 2001.

4. Jain, K., "Nanotechnology in clinical laboratory diagnostics", *Clin Chim Act*, Vol. 358, pp**.** 37–54, 2005.

5. Nie, S., Y. Xing, J. G. Kim and W. J.Simons, "Nanotechnology Applications in Cancer", *Annu. Rev. Biomed*, Vol. 9, pp. 257-288, 2007.

6. Ryou, M. S., S. Kim, H. H. Jang, J. H. Kim, j. H. Yeom, M. S. Eom, Bae. J, M. S. Han and Lee. K, "Delivery of shRNA using gold nanoparticle–DNA oligonucleotide conjugates as a universal carrier", *ScienceDirect,* Vol. 398, pp. 542-546, 2010.

7. Kumar, B. K., P. R. Yadav, H. C. Goel, M. Moshahid and A. Rizvi, "Recent Developments In Cancer Therapy By The Use Of Nanotechnology", *Digest Journal Nanomaterials and Biostructures*, Vol. 4, No.1, pp. 1-12, 2009.

8. Rosi, N. L., D. A. Giljohann, C. S. Thaxton, A. K. R. Lytton-Jean, M. S. Han and C. A. Mirkin, "Oligonucleotide-Modified Gold Nanoparticles for Intracellular Gene Regulation", *Science,* Vol. 312, pp. 1027- 1030, 2006.

9. Elbakry, A., A. Zaky, R. Liebkl, R. Rachel, A. Goepferich and M. Breunig,"Layer-by-Layer Assembled Gold Nanoparticles for siRNA Delivery", *Nano Lett*, Vol. 9, pp. 2059–2064, 2009.

10. Liu, X. H., J. T. Zhang and D. M. Lynn, "Polyelectrolyte Multilayers Fabricated from 'Charge-Shifting' Anionic Polymers: A New Approach to Controlled Film Disruption and the Release of Cationic Agents from Surfaces", *Soft Matter*, Vol. 4, pp. 1688–1695, 2008.

11. Guo, Shutao., Y. Huang, Q. Jiang, Y. Sun, L. Deng, Z. Liang, Q. Du, J. Xing, Y. Zhao, P. C. Wang, A. Dong, and X-J. Liang, "Enhanced Gene Delivery and siRNA Silencing by Gold Nanoparticles Coated with Charge-Reversal Polyelectrolyte", *American Chemical Society*, Vol. 4, No.9, pp. 5505-5511, 2010.

12. Lee, J-S., J. J. Green, K. T. Love, J. Sunshine, R. Langer and D. G. Anderson," Gold, Poly(β-amino ester) Nanoparticles for Small Interfering RNA Delivery", *Nano Lett*, Vol .6, No.9, pp. 2402–2406, 2009.

13. Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu and M. J. Thun, "Cancer statistics, 2009", *CA Cancer J Clin*, Vol. 59, pp. 225-249, 2009.

14. Villers, A., J. E. McNeal, F. S. Freiha and T. A. Stamey, "Multiple cancers in the prostate. Morphologic features of clinically recognized versus incidental tumors", *Cancer*, Vol. 9, No.70, pp. 2313-2318, 1992.

15. Maroni, P. D. and E. D. Crawford, "Screening for prostate cancer in 2006: PSA in the 21st century", *N. C. Med. J*, Vol. 67, pp. 136–139, 2006.

16. Gelmann, E. P. and O. J. Semmes, "Expression of genes and proteins specific for prostate cancer", *J. Urol*, Vol. 172, pp. 23-27, 2004.

17. Bastacky, S. I., K. J. Wojno, P. C. Walsh, M. J. Carmichael and J. I. Epstein, "Pathological features of hereditary prostate cancer", *J Urol,* Vol. 153, pp. 987-992, 1995.

18. Cheng, L., S-Y. Song, T. G. Pretlow, F. W. Abdul-Karim, H-J. Kung, D. V. Dawson, W-S. Park, Y-W. Moon, M-L. Tsai, W. M. Linehan, M. R. Emmert-Buck, L. A. Liotta and Z. Zhuang, " Evidence of independent originof multiple tumors from patients with prostate cancer", *J Natl Can Inst,* Vol. 90, pp. 233-237, 1998.

19. Andreoiu, M. and L. Cheng, "Multifocal prostate cancer: biologic, prognostic, and therapeutic implications", *Human Pathology,* Vol. 41, pp. 781–793, 2010.

20. McNeal, J. E., D. G. Bostwick, R. A. Kindrachuk, E. A. Redwine, F. S. Freiha and T. A. Stamey, " Patterns of progression in prostate cancer", *Lancet,* Vol. 1, pp. 60-63, 1986.

21. Mc Neal, J. E., "Cancer volume and site of origin of adenocarcinoma in the prostate: relationship to local and distant spread", *Human Pathology,* Vol. 23, pp.258-266, 1992.

22. McNeal, J. E., E. A. Redwine, F. S. Freiha and T. A. Stamey, "Zonal distribution of prostatic adenocarcinoma: correlation with histologic pattern and direction of spread", *Am J Surg Pathol,* Vol. 12, pp. 897-906, 1988.

23. Greene, D. R., T. M. Wheeler, S. Egawa, J. K. Dunn and P. T. Scardino,"Acomparison of the morphological features of cancer arising in the transition zone and in the peripheral zone of the prostate", *J Urol,* Vol. 146, pp. 1069-1076, 1991.

24. Sakai, I., K. Harada, T. Kurahashi, K. Yamanaka, I. Hara and H. Miyake, "Analysis of differences in clinicopathological features between prostate cancers located in the transition and peripheral zones", *Int J Urol,* Vol. 13, pp. 368-372, 2006.

25. Sakai, I., K. Harada, I. Hara, H. Eto and H. Miyake, "A comparison of the biological features between prostate cancers arising in the transition and peripheral zones", *BJU Int*, Vol. 96, pp. 528-532, 2005.

26. Bostwick, D. G. and I. Meiers, "Neoplasms of the prostate", In: Bostwick DG, Cheng L, editors. *Urologic Surgical Pathology*, pp. 443-580, Philadelphia: Elsevier/Mosby; 2008.

27. Qian, J., R. B. Jenkins and D. G. Bostwick, "Detection of chromosomal anomalies and c-myc gene amplification in the cribriform pattern of prostatic intraepithelial neoplasia and carcinoma by fluorescence in situ hybridization", *Mod Pathol*, Vol. 10, pp. 1113-1114, 1997.

28. Emmert-Buck, M. R., C. D. Vocke and R. O. Pozzatti, "Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia", *Cancer Res*, Vol. 55, pp. 2959-2962, 1995.

29. Shannon, B. A., J. E. McNeal and R. J. Cohen, "Transition zone carcinoma of the prostate gland: a common indolent tumour type that occasionally manifests aggressive behaviour", *Pathology*, Vol .35, pp. 467-471, 2003.

30. Al-Ahmadie, H. A., S. K. Tickoo and S. Olgac, "Anterior-predominant prostatic tumors: zone of origin and pathologic outcomes at radical prostatectomy", *Am J Surg Pathol*, Vol. 32, pp. 229-235, 2008.

31. Ozen, M. and S. Pathak, "Genetic alterations in human prostate cancer: a review of current literature", *Anticancer Res*, Vol. 20, pp. 1905-1912, 2000.

32. Lee, R. C., R. L. Feinbaum and V. Ambros, " The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14", *Cell*, Vol. 75, pp. 843–854, 1993.

33. J. Li, R. Song, J. Messing and X. Chen, "CARPEL FACTORY, a Dicer homolog, and HEN1,a novel protein, act in microRNA metabolism in Arabidopsis thaliana", *Curr. Biol*, Vol. 12, pp. 1484–1495, 2002.

34. Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O . Radmark, S. Kim and V. N. Kim, "The nuclear RNase III Drosha initiates microRNA processing", *Nature,* Vol. 425, pp. 415–419, 2003.

35. Bernstein, Emily., A. A. Caudy, S. M. Hammond and G. J. Hannon, "Role for a bidentate ribonuclease inthe initiation step of RNA interference", *Nature,* Vol. 409, pp. 363–366, 2001.

36. Ozen, M., C. J. Creighton, M. Özdemir and M. Ittmann, "Widespread deregulation of microRNA expression in human prostate cancer", *Oncogene,* Vol.12, No.27, pp. 1788-1793, 2008.

37. Negrini, M. and G. A. Calin, "Involvement of MicroRNAs in Human Cancer: Discovery and Expression Profiling", *Springer Science+Business Media B.V,* Vol. 5, 2010.

38. Kwak, P. B., S. Iwasaki and Y. Tomari, "The microRNA pathway and cancer", *Cancer Science,* Vol. 101, pp. 2309–2315, 2010.

39. Wightman. B., I. Ha and G. Ruvkun, "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans", *Cell,* Vol. 75, pp. 855–862, 1993.

40. Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun, "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans", *Nature,* Vol. 403, pp. 901–906, 2000.

41. Baskerville, S. and D. P. Bartel, "Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes RNA", *ScienceDirect,* Vol. 11, pp. 241–247, 2005.

42. Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst and A. Bradley, "Identification of mammalian microRNA host genes and transcription units", *Genome Res,* Vol. 14, pp. 1902–1910, 2004.

43. Kim, V. N. and J. W. Nam, "Genomics of microRNA", *Trends Genet,* Vol. 22, pp. 165–173, 2006.

44. Bentwich, I., A. Avniel, Y. Karov, R. Aharonov, S. Gilad, O. Barad, A. Barzilai, P. Einat, U. Einav, E. Meiri, E. Sharon, Y. Spector and Z. Bentwich, "Identification of hundreds of onserved and nonconserved human microRNAs", *Nat Genet,* Vol. 37, pp. 766–770, 2005.

45. Berezikov, E., V. Guryev, J. van de Belt, E. Wienholds, R. H. A. Plasterk and E. Cuppen, "Phylogenetic shadowing and computational identification of human microRNA genes", *Cell,* Vol. 120, pp. 21–24, 2005.

46. Mirnezami, A. H., K. Pickard, L. Zhang, J. N. Primrose and G. Packham, "MicroRNAs: key players in carcinogenesis and novel therapeutic targets", *Eur J Surg Oncol*, Vol. 35, pp. 339–347, 2009.

47. Osaki ,M., F. Takeshita and T. Ochiya, "MicroRNAs as biomarkers and therapeutic drugs in human cancer ", *Biomarkers,* Vol. 13, pp. 658–670, 2008.

48. K, Sylvia., K. Shenouda and Alahari. Suresh, "MicroRNA function in cancer: oncogene or a tumor suppressor? ", *Cancer Metastasis Rev,* Vol. 28 pp. 369–378, 2009.

49. Bartel, D. P., "MicroRNAs: genomics,biogenesis, mechanism and function", *Cell*, Vol. 116, pp. 281-292, 2004.

50. Hayashita, Y., H. Osada and Y. Tatematsu, "Apolycistronic microRNA cluster, miR-17–92, is overexpressed in human lung cancers and enhances cell proliferation", *Cancer Res,* Vol. 65, pp. 9628–9632, 2005.

51. Si, M. L., S. Zhu, H. Wu, Z. Lu, F. Wu and Y. Y. Mo, "miR-21- mediated tumor growth. " *Oncogene,* Vol. 26, pp. 2799–2803, 2007.

52. Zhang, B., X. Pan, G. P. Cobb and T. A. Anderson " MicroRNAs as oncogenes and tumor suppressors", *Dev Biol,* Vol. 302, pp. 1–12, 2007.

53. Aurora –Kerscher, E. and F. J. Slack, "Oncomirs — microRNAs with a role in cancer ", *Nature Reviews,* Vol. 6, pp. 259-269, 2009.

54. Michael, M. Z., S. M. O' Connor, N. G. van Holst Pellekaan, G. P. Young and R. J. James, "Reduced accumulation of specific microRNAs in colorectal neoplasia", *Mol Cancer Res,* Vol. 1, pp. 882–891, 2003.

55. Akao, Y., Y. Nakagawa and T. Naoe, "MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers", *Oncol Rep,* Vol. 4, pp. 845–850, 2006.

56. Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo and S. Sabbioni "MicroRNA gene expression deregulation in human breast cancer", *Cancer Res,* Vol. 65, pp. 7065–7070, 2005.

57. Johnson, S. M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K. L. Reinert, D. Brown and F. J. Slack, "RAS is regulated by the let-7 microRNA family", *Cell,* Vol. 120, pp. 635–647, 2005.

58. Paranjape, T., F. J. Slack and J. B. Weidhaas, "MicroRNAs: tools for cancer diagnostics", *Gut,* Vol. 58, pp. 1546-1554, 2009.

59. Michael, M. Z., S. M. O'Connor, N. G. van Holst Pellekaan, G. P. Young and R. J. James, "Reduced accumulation of specific microRNAs in colorectal neoplasia: Reveals that mir-143 and mir-145 RNA levels are often reduced in colorectal cancer", *Mol. Cancer Res*, Vol. 1, pp. 882–891, 2003.

60. Negrini, M. and G. A. Calin, "Involvement of MicroRNAs in Human Cancer: Discovery and Expression Profiling", *Springer Science+Business Media B.V*, Vol. 5, pp. 69-104, 2010.

61. Lee, Y., K. Jeon, J. T. Lee, S. Kim, and V. N. Kim, "MicroRNA maturation: stepwise processing and subcellular localization", *EMBO J*, Vol. 21, pp. 4663–4670, 2002.

62. Cai, X., C. H. Hagedorn and B. R. Cullen, "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs", *RNA*, Vol. 10, pp. 1957–1966, 2004.

63. Lee. Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek, and V. N. Kim, "MicroRNA genes are transcribed by RNA polymerase II", *EMBO J,* Vol. 23, pp. 4051–4060, 2004.

64. Basyuk, E., F. Suavet, A. Doglio, R. Bordonne, and E. Bertrand, "Human let-7 stem-loop precursors harbor features of RNase III cleavage products", *Nucleic Acids Res*, Vol. 31, pp. 6593–6597, 2003.

65. Zeng, Y., and B. R. Cullen, "Sequence requirements for microRNA processing and function in human cells", *RNA*, Vol. 9, pp. 112–123, 2003.

66. Gregory, R. I., K-P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch and R. Shiekhattar,"The Microprocessor complex mediates the genesis of microRNAs ", Nature, Vol. 432, pp. 235–240, 2004.

67. Lee, Y. S., K Nakahara, J W Pham K. Kim, Z. He, E. J. Sontheimer and R. W. Carthew, "Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways", *Cell,* Vol. 117, pp. 69–81, 2004.

68. Cai, X., C. H. Hagedorn and B. R. Cullen, " Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs", *RNA*, Vol. 10, pp. 1957–1966, 2004.

69. Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting and G. J. Hannon, "Processing of primary microRNAs by the Microprocessor complex", *Nature,* Vol. 432, pp.231–235, 2004.

70. Shenouda, K. S. and A. K. Suresh, "MicroRNA function in cancer: oncogene or a tumor suppressor? ", *Cancer Metastasis Rev*, Vol. 28, pp. 369–378, 2009.

71. Tsuchiya, S., Y. Okuno and G. Tsujimoto, "MicroRNAs and Discovery of New Targets", in F. Innocenti (ed.), *Cancer Drug Discovery and Development: Genomics and Pharmacogenomics in Anticancer Drug Development and Clinical Response*, pp. 47-56, Humana Press, Totowa NJ, 2007.

72. Yi, R., Y. Qin, I. G. Macara, and B. R. Cullen, "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs", *Genes Dev*, Vol. 17, pp. 3011–3016, 2003.

73. Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg, and U. Kutay. "Nuclear export of microRNA precursors", *Science,* Vol. 303, pp. 95–98, 2004.

74. Kwak, P. B., S. Iwasaki and Y. Tomari, "The microRNA pathway and cancer", *Cancer Sci,* Vol. 101, pp. 2309–2315, 2010.

75. Hatfield, S. D., H. R. Shcherbata, K. A. Fischer, K. Nakahara, R. W. Carthew and H. Ruohola-Baker, "Stem cell division is regulated by the microRNA pathway", *Nature,* Vol. 435, pp. 974–978, 2005.

76. Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Ménard, J. P. Palazzo, A. Rosenberg, P. Musiani , S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini and C. M. Croce, "MicroRNA gene expression deregulation in human breast cancer", *Cancer Res,* Vol. 65, pp. 7065–7070, 2005.

77. Mattie, M. D., C. C. Benz, J. Bowers, K. Sensinger, L.Wong, G. K. Scott, V. Fedele, D. Ginzinger, R. Getts, and C. Haqq, "Optimized highthroughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies", *Mol Cancer,* Vol. 5, pp. 24, 2006.

78. Chan, J. A., A. M. Krichevsky and K. S. Kosik, "MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells", *Cancer Res,* Vol. 65, pp. 6029–6033, 2005.

79. Cummins, J. M., Y. He, R. J. Leary, R. Pagliarini, L. A. Diaz, T. Sjoblom, O. Barad, Z. Bentwich, A. E. Szafranska and E. Labourier "The colorectal microRNAome", *Proc Natl Acad Sci USA,Vol. 6,* pp. 28, 2006.

80. Bandres, E., E. Cubedo, X. Agirre, R. Malumbres, R. Zarate, N. Ramirez, A. Abajo, A. Navarro, I. Moreno, M. Monzo and J. Garcia-Foncillas, "Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues", *Mol Cancer,* Vol. 5, pp. 29, 2006.

81. Mattie, M. D., C. C. Benz, J. Bowers, K. Sensinger, L. Wong and G. K. Scott, "Optimized highthroughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies", *Mol Cancer*, Vol. 5, pp. 24, 2006.

82. Murakami, Y., T. Yasuda, K. Saigo, T. Urashima, H. Toyoda, T. Okanoue, and K. Shimotohno, "Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues", *Oncogene,* Vol. 25, pp. 2537–2545, 2006.

83. Takamizawa, J., H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, T. Harano, Y. Yatabe, M. Nagino, Y. Nimura, T. Mitsudomi and T. Takahashi, "Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival", *Cancer Res*, Vol. 64, pp. 3753–3756, 2004.

84. Calin, G. A., M. Ferracin, A. Cimmino, G. Di Leva, M. Shimizu, S. E. Wojcik, M. V. Iorio, R. Visone, N. I. Sever, M. Fabbri, R. Iuliano, T. Palumbo, F. Pichiorri, C. Roldo, R. Garzon, C. Sevignani, L. Rassenti, H. Alder, S. Volinia, C-G. Liu, T. J. Kipps, M. Negrini and C. M. Croce, "MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia", *N Engl J Med*, Vol. 353, pp. 1793–801, 2005.

85. Yanaihara, N., N. Caplen, E. Bowman, M. Seike, K .Kumamoto, M. Yi, R. M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G.A. Calin, C-G Liu, C. M. Croce and C. C. Harris, "Unique microRNA molecular profiles in lung cancer diagnosis and prognosis", *Cancer Cell*, Vol. 9, pp. 189–198, 2006.

86. Zhang, L., J. Huang, N. Yang, J. Greshock, M. S. Megraw, A. Giannakakis, S. Liang, T. L. Naylor, A. Barchetti, M. R. Ward, G. Yao, A. Medina, A. O'Brien-Jenkins, D. Katsaros, A. Hatzigeorgiou, P. A. Gimotty, B. L. Weber and G. Coukos, "microRNAs exhibit high frequency genomic alterations in human cancer", *Proc Natl Acad Sci USA*, Vol. 103, pp. 9136–9141, 2006.

87. Handley, D. A., "*Colloidal gold: principles, methods, and applications*", In: Hayat MA (editor), pp. 12–32, Academic Press, 1989.

88. Schaeffer, W. I., "Mammalian Cell Culture in Molecular Biology, University of Vermont", [*online*], http://www.uvm.edu/~wschaeff/BasicCulture1.html [retrieved 12 February 2012].

89. Griffiths-Jones,S., "miRBase: microRNA sequences and annotation", Curr.Protoc. Bioinformatics, Vol. 12, No.10, 2010.

90. Giljohann, D. A., D. S. Seferos, A. E. Prigodich, P. C. Patel, and C. A. Mirkin, " Gene Regulation with Polyvalent siRNA-Nanoparticle Conjugates", *J Am Chem Soc,* Vol. 6, No.131, pp. 2072–2073, 2009.

91. Oishi, M., J. Nakaogami, T. Ishii and Y. Nagasaki, "Smart PEGylated Gold Nanoparticles for the Cytoplasmic Delivery of siRNA to Induce Enhanced Gene Silencing", Chem,Lett. Vol. 9, No. 35, pp. 1046-1047, 2006.

92. Sevli, S., A. Uzumcu, M. Solak, M. Ittmann and M. Ozen, "The function of microRNAs, small but potent molecules, in human prostate cancer", *Prostate Cancer and Prostatic Diseases,* Vol. 3, No. 13, pp. 208-217, 2010.

93. Mead, E. A., and Z. Tu, "Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, Anopheles stephensi", *BMC Genomics,* Vol. 9, No.244, pp.244-249, 2008.

94. Lodes, M. J., M. Caraballo, D. Suciu, S. Munro, A. Kumar and B.Anderson, " Detection of Cancer with Serum miRNAs on an Oligonucleotide Microarray", *Journel pone,* Vol. 7, No. 7, pp. 1371-1389, 2009.

95. Nuovo, G. J., E. J. Lee, S. Lawler, J. Godlewski and D. Thomas, "In situ detection of mature microRNAs by labeled extension on ultramer templates", *Schmittgenio Techniques,* Vol. 46, pp. 115–126, 2009.