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Synergistic effect of Antagomir molecule of Let7a transfection and CGIs-methylation on colorectal cancer progression

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ABSTRACT

Colorectal cancer is currently most frequent cancer type in oncologic pathology. It is a prevalent disease among the elderly. siRNAs are double stranded RNA molecules with a length of 21-23nucleotides. The miR was given the nomenclature lethal7, Let7. Twelve genetic alleles of Let7 were described. Reduced serum let7 levels have also been documented in numerous tumors. The human colorectal adenocarcinoma(CRC) cell lines HT29(ATCC® HTB-38), and normal lung fibroblast cell line (ATCC® CCL-75) were utilized in this research for Let7a antagomir molecule transfection and CpGs-islands methylation. Cell counting was achieved for 6-well plate and 24-well plate prior to transfection using hemocytometer. The two cell lines were trans-fected with Let7a antagomir moleculer. Transfection efficiency, cell viability and target miRNA expression was conducted, then CGIs-methylation was achieved by bisulfate conversion. The present outcomes showed un-regula-tion of oncogene and down regulation of TSGs, in addition CpG-islands methylation showed significant methylation of TSGs promoter region and unblocking of oncogene promoter region. The researchers concluded that analysis of interperetation outcomes of Let7a(antagomir transfection)-CGIs-methylation led to exhibited of H29 cell line prolife-ration.

1. INTRODUCTION

Colorectal cancer is currently most frequent cancer type in oncologic pathology [11]. It is a prominent malignant mass of the gastro-intestinal tract, and it is the second leading reason of mortality in both developed and developing countries. It is a prevalent disease among the elderly [24], Women have a higher incidence than men five times. According to the Incidence, Epidemiology, and Final Results program, 132,700recent cases of colorectal cancer were diagnosed in the Northern America in 2015. This is equivalent to 8% of all newly diagnosed cancers [25].

Since its discovery in 1998, gene silencing by RNA interference (RNAi) has greatly evolved in a very short period of time [22]. Sm-all interfering RNA(siRNA)molecules mediate this extremely conserved process of

post-transcrptional gene silencing. siRNAs are double stranded RNA molecules with a length of 21-23nucleotides [4]. The miR was given the nomenclature lethal7, Let7 [1]. Twelve genetic alleles of Let7 were described [14]. Reduced serum let7 levels have also been documented in numerous tumors [3], [23]. When compared healthy controls to individuals associated with colorectal cancer have lower amounts of let-7in stool speci-mens, providing a less invasive technique for diagnosis [16]. Utilizing of let7 as a therapeutic tool has been demonstrated by successful in vivo and ex vivo studies [3]. whereas it decreases cellular multiplication and invasion [7], [26]. Small RNA duplexes are eventually deposited upon AGO proteins, which unwinding them to construct an RNA induced silencing complex(RISC), which is the operational element that identifies target mRNA molecules. Typically, the strand with the most unstable terminal on the5'side is chosen [12].

The covalent attachment of a methyl group to the cytosine ring of a CpG dinucleotide is known as DNA methylation Although CpG dinucl-eotides are found throughout eukaryotic species genomes, CpG-rich areas are particularly abundant in gene promoter regions [19]. Cancer is characterized by genome wide hypo methylation and promoter specific hyper methylation. The first epigenetic change discovered in malig-nancies was the loss of DNA methylation at CpG islands on a global scale. Hypo-methylation at repeated sections in the genome, is linked to genomic instability [8].

Recently there has been no research done locally in the subject of gene silencing using ncRNA transfection in cell line. The focus of this research was to undertake a new strategy at the molecular scale in order to understand the biological activity of ncRNA molecule in colorectal cancer progression.

2. Material and methods

2.1 Cell lines preparation for transfection

The human colorectal adenocarcinoma(CRC) cell lines HT29(ATCC® HTB-38), and normal lung fibroblast cell line (ATCC® CCL-75) were grown in DMEM supplemented with 10% fetal calf serum, in addition to penicillin/ Streptomycin, L-Glutamine. Before transfection with hsa-let-7a antagomir sequence, the 2 cell lines were detached from T25-flask, the medium was removed from flask and wash cells with 1X PBS to remove any remaining medium, then transferred to 24-well for upcoming expression and XTT assays.

2.2 Cell counting

The cells were transferred to 6-well and 24-well prior to transfection. For counting, the cells were detached from T25-flask by removing the medium from flask and wash them with 1X PBS to remove any remaining medium, three ml of trypsin was added to flask of cell line and incubated until cells detach, then two ml of DMEM medium was added to inactivate trypsin and the cells were collected from flask by centrifugation of cell suspension at 1500 rpm for 5min,the supernatant was removed without touching cell pellet and 1ml of fresh medium was added for cell counting. Hemocytometer was used for cell counting.10µl of cell suspension and 10µl of trypan blue were loaded on grid-lined part of hemocytometer, from 16 squares, 4 of them were counted and an average of 4squares were taken, then they were multi-plied with 250000 to get cell number in 1ml. For 6 well-plate average3x 10^5 and for 24 well-plate average of 5x 10^4 cell were seeded to each well and volume completed to 500µl with growth medium.

2.3 XTT protocol

XTT assay for transfected 24-well plates were performed by using XTT Cell Viability Kit (RIBOBIO, China). According to manufacturer protocol working solution was prepared immediately before use and heated to 37°C and swirled gently until a clear solution was obtained. For each 24-well plate to be tested, 25µl activation

reagent was mixed with 5ml of XTT solution to derive activated XTTsolution.50µl activated XTT solution was added to wells, then the plates were incubated in an incubator for 4hours.After that plates were shaken gently to distribute the dye in the wells. Finally absorbance were measured with HEALES MB-530 ELISA Reader at a wave length of 450-500nm.Back ground absorbance was measured at a wave length between 630-690. Back-ground absorbance were subtracted from signal absorbance to obtain normalized absorbance values.

2.4 Expression of target miRNA

2.4.1 RNA extraction and Complementary DNA (cDNA)synthesis

MiRNA isolation kits was supplied by Gene All (S.Korea).A specific "stem-loop primer" was designed for hsa-Let-7a was 5p-GAAAGAAGGCG AGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCGCC TTCTTTCAACTATAC and snRNA-RNU6-CGCTTCACGAATTTGCGT GTCAB(Ella biotechnology, Germany), which was used in cDNA synthesis. The master mix was supplied by Wizbio (S.Korea), which included 10X reaction Buffer(2µl),primer(2µl),20X dNTP mix(1µl),reverse transcriptase (1µl), RNase Inhibitor 0.5µl and RNase free Water3.5µl miRNA template 10µl to complete the final volume as 20µl.Amplification conditions were included three steps,25(°C) for10min.,37°C for120 min, 85(°C) for 5 min. and finally 4(°C) as cooling temp.

2.4.2 MiRNA expression

RT-qPCR primers for hsa-let-7a-5p was provided by Ella biotech (Germany), were designed in this study which included F-TGAGGTAGT AGGTTGTATAGTT and R-CGAGGAAGAAGACGGAAGAAT, in addi tion to snRNA-RNU6-F-GCTTCGGCAGCACATATACTAAAAT and sn RNA-RNU6-R-CGCTTCACGAATTTGCGTGTCAT as universal primer set. The miR-qGreen-ROX master mix was supplied by wizbio biotech (S. Korea), which included Wiz Bio miRqGreen-ROX master mix10µl ROX Dye(20X), and additional materials were 1µl miRNA specific forward Pri-mer1µl(5µM), miRNA universal reverse primer1µl(5µM), miRNA-cDNA template(2µl) and RNase free distilled water(5µl). Amplification conditions were initial denaturation 95 for 300sec, 40 cycles of denature 95 for 10-30 sec and anneal 60 for10-60 sec and melting curve 65-95 for 2-5sec.

2.5 Expression of interested genes

2.5.1 Target mRNA Isolation and mRNA-cDNA Synthesis

For mRNA isolation, RiboEx and Hybrid-R was provided by GeneAll (S.Korea). HyperScript™ First strand synthesis kit was supplied by GeneAll (S.Korea).The amplification components used for mRNA-cDNA thynsis were involved mRNA template(2µl), hexamer primer1µl(5µM) dNTP mix (1µl),10X RTase reaction buffer(2µl) 0.1M DTT(2µl),reverse transcriptase (1µl) and RNase inhibitor(1µl)and RNase free distilled water(10µl). Comp-lementary DNA synthesis reaction conditions were included55(°C) for 60 min and 85(°C) for 5min. The obtained cDNA samples were frozen at-80°C.

2.5.2 Target mRNA expression

Real Amp™SYBR qPCR Master mix was provided by GeneAll (S. Korea). Target primers were designed in this study listed in table (1). Amplification mixture were included 2X SYBR green Master mix(10µl), ROX Dye(1µl), forward Primer(10µM)1µl, reverse Primer(10µM) 1µl, cDNA template (4µl) and RNase free distilled water (3µl).

Table (1): Primers were used in Real Time PCR for detection Target mRNA

Primer	Sequence(3`-5`)			
ACTB	F	CATGTACGTTGCTATCCAGGC	R	CTCCTTAATGTCACGCACGAT
KRAS	F	ACAGAGAGTGGAGGATGCTTT	R	TTTCACACAGCCAGGAGTCTT
AKT1	F	AGCGACGTGGCTATTGTGAAG	R	GCCATCATTCTTGAGGAGGAAGT
TP53	F	CAGCACATGACGGAGGTTGT	R	TCATCCAAATACTCCACACGC
KEAP1	F	CTGGAGGATCATACCAAGCAGG	R	GGATACCCTCAATGGACACCAC
TNFa	F	CCTCTCTCTAATCAGCCCTCTG	R	GAGGACCTGGGAGTAGATGAG
IL6	F	ACTCACCTCTTCAGAACGAATTG	R	CCATCTTTGGAAGGTTCAGGTTG
NFKB	F	AACAGAGAGGATTTCGTTTCCG	R	TTTGACCTGAGGGTAAGACTTCT
VCAM	F	GGGAAGATGGTCGTGATCCTT	R	TCTGGGGTGGTCTCGATTTTA

2.5.3 Methylation experiment

For the isolation of DNA, Cell SV mini kit was supplied by GeneAll (S. Korea). DNA Methylation-Gold™ Kit was supplied by ZYMO RESEARCH(China). For DNA methylation protocol,130µl of the CT (900 µl water,300 µl of M-Dilution Buffer, and 50µl M dissolving buffer to a tube of CT Conversion reagent) Con version Reagent was add to 20µl of DNA sample in a PCR tube that were place in a thermal cycler to perform the following steps (table 2):

Table (2): Bisulfate conversion reaction conditions

Step	Temp (°C)	Time	Cycle
Initial Denaturation	98	5 min	1
CT reaction	64	2.5 hours	1
Hold	4	20 min	1

Then 600µl of M-Binding Buffer was add to a Zymo-Spin™ IC that placed into collection tube. Centrifuged at full speed (>10,000xg) for 30sec. Discard the flow through, then 100µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds after that add 200µl of M-Desul-phonation buffer to the column and let stand at room temperature (20-30°C) for 15-20min. After the incubation, centrifuge at full speed for 30sec, 200µl of M-Wash Buffer was add to the column and re-centrifuge at full speed for 30 seconds. then 200µl of M-Wash Buffer was add and centrifuge for an additional 30sec. Finally 10µl of M-Elution Buffer was add directly to the column matrix and centrifuge for 30sec at full speed to elute the DNA.

2.5.4 Methylation-Specific PCR(MSPCR) and conventional PCR

At this stage, primers designed in this study according to the promoter regions of target genes for DNA with bisulfite conversion and provided by Ella biotechnology, Germany (table 3).

Table (3): Oligonucleotides primers for methylated and un-methylated target sequences

Primer	Sequence(3`-5`)	
	Forward	Reverse

U-KRAS	GGATGTGTGTTAGTATAGGTTGAT	ACCTAACCACAAAACTATCCCC
U-AKT1	TGGGTTATTTTATAGATGGGG	ACTAAAAAACAACTCCCAACAACC
U-TP53	GTATAAAGTGGTTGGTATGTGGTA	ATCATAAAACAAAAAAAACAAACCC
U-KEAP1	GAGGAAGTGATTTTATTGTGG	AAACAACACAACTCTTAAAATAACCC
M-KRAS	GATGCGCGTTAGTATAGGTCG	CTAACCGCAAAACTATCCCC
M-AKT1	TCGGGTTATTTTATAGACGGG	AACTAAAAAACAACTCCCGACG
M-TP53	GTATAAAGTGGTCGGTACGC	CGTCGTAAAACGAAAAAAACG
M-KEAP1	AGGAAGTGATTTTATCGCGG	GACGCAACTCTTAAAATAACCC

Master mix was provided by Wiz™(S.Korea). Amplification mixture components used in conventional and MS-PCR were included,2X master mix(10µl), forward Primer(10µM) 0.5µl, reverse Primer(10µM) 0.5µl and DNA template (5µl). Amplification condition were included. Amplification conditions were included, Initial Denaturation95°C for 5 min, 30cycles of denature 95°C for 30 sec. anneal 60°C for 30 sec, extend72°Cfor 30secand final extension72°C for 5 min.

2.6 Statistical Analysis

Gel documentary software (G:BOX Chemi XX6), was used for detection the size and density of methylated and un-methylated amplicon of target genes. Excel was used for result analysis, were included: Detection of stand- ard deviation for cell viability and mean of standard error for triplicate gene expression and analysis of gene expression for detection of CT values and fold change.

3. Results

3.1 Transfection efficacy

The current outcomes of transfectamine mixture penetration for anta- gomir miRNAs revealed different values of fold change were documented with WI38 and HT29 cell lines after transfection period (table4). Antagomir showed an increased in HT29 cell line in comparison with WI38 cell lines.

Table (4): Transfection efficiency of antagomir of let7a associated with WI38 and HT29 cell lines.

	WI 38	**HT29**
	Fold Change	**Fold change**
Control	1	1
Let7a	0.001015979	52.39104734

3.2 Cell lines viability

After transfection, XTT results showed different values in growth of transfected cell lines with let-7a and led to increase both of HT29 cell line proliferation value and WI38 cell line (table 5).

Table (5): Cell lines HT29andWI38 cell lines viability, after transfection of Let7a antagomir sequences.

WI38 Cell line	**Cell Viability Mean(%)**	**Cell Viability SD(%)**	**HT29 Cell line**	**Cell Viability Mean(%)**	**Cell Viability SD(%)**

Antagomir	Control	100	3.478105132	Control	100	5.128290951
	Let7a	105.8500690	6.587013705	Let7a	101.9396232	3.350824459

Many studies over all the word were focused on ncRNA. study done by [17], showed that transfection of Let7 antagomir sequence played a critical role in exhibiting of colorectal cancer cells expansion. Dur- ing past decade, ncRNAs are widely utilized as a clinical indicator of cancer, in addition they have high therapeutic index in cancer declining in vivo and ex vivo [9]. Let7a Transfection Let7a antagomir molecules showed high expression values of KRAS,TNF, IL6 andVCAM1 genes after transfection of HT29 cell line, while low expre- ssion values of TP53,KEAP1 and NFκB genes were observed. On the other hand, the same effects on HT29 cell line associated with WI38 cell transf- ected with antagomir molecules.

The present study revealed that antagomir molecules of Let7a worked as oncomiR after transfection of both cell lines under study and led to down regulating of tumor suppressor genes and up regulating of oncogenes and metastatic mediator gene. Overall, that Let7a antagomir oligonucleotide played a role in HT29 cell line progression (figures 1 and 2).

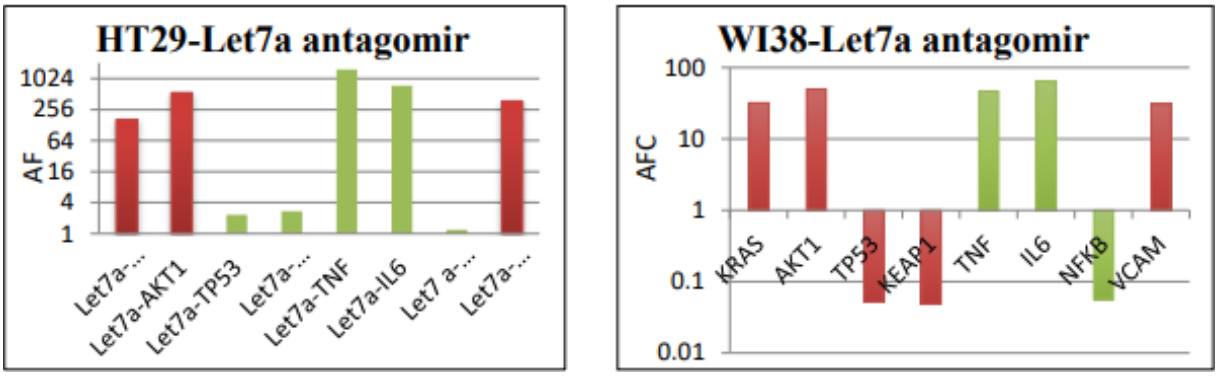

Figure (1): Transfection efficiency of *Let7a* molecule. HT29 (left) and WI38 cell lines(Right) transfected with *Let7a* antagomir molecule. AFC, Amplification.

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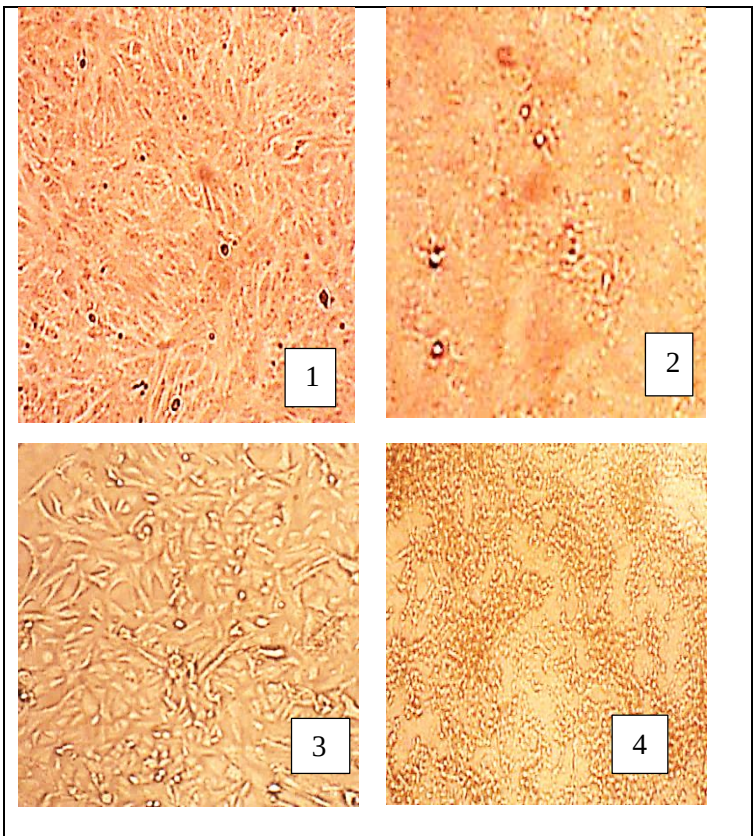

Figure (2): HT29 and WI38 cell lines transfected with Let7a antagomir molecules.1.HT29control,2.HT 29-Let7a-antagomir transfection. 3.WI38 control,4.WI 38-Let7a anta-gomir transfection.(Under magnification power 40X lens of inverted-fluoresce micro scope)

Antagomir molecule worked as cancer cell exhibitor, this indicated that down regulation of Let7a mimic molecules was occurred and led to increase HT29cell line proliferation. In addition the phenotypic results agreed to investigation of target mRNA expression after transfection with Let7a antag-omir molecules. Let7a currently have more than twenty eight alleles, all of them have high similarity in their sequencing and share with GAGGUAG as a motif region that have a key function in RNA induced silencing complex [5]. This molecules influenced on colorectal adenocar-cinoma expansion by suppressing or exhibiting some significant oncogenes like KRAS and C-myc translation [13]. [18] found that this molecule influences on mRNA translation of the RhoA gene that responsible of cell migration through the serum, which has a substantial impact on CRC regression. Other study carried out by [15]. showed that Let7a expression level elevated in CRC patients serum.

3.3 DNA Methylation

3.3.1 KRAS - CGIs Methylation

Let7a-antagomir-CpGs islands methylation showed unblocking effect of KRAS gene promoter, low density of methylated-amplicon observed at 190 bp. In both HT29 and WI38 cell lines(figures2).

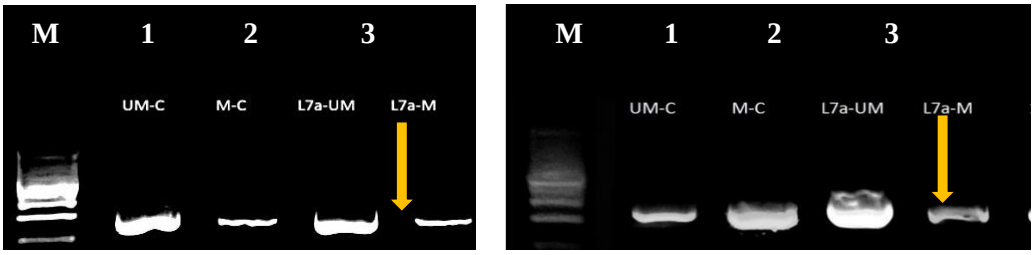

Figure (3): Amplification products of MS-PCR of KRAS gene(amplicons,190bp.) in HT 29(left)and WI38(right)cell lines as they were labeled. Line1,molecular marker(100 bp.). Line 4,Let7a-Methylation. Green arrows referred to significant result.

3.3.2 AKT1-CGIs Methylation

Interpretation of Let7a antagomir molecule transfection and CGIs-methylation showed an non-significant blocking effect of AKT1 as TSG promoter region at amplicon size 190bp.In both of HT29 and WI38 cell lines (figure 4).

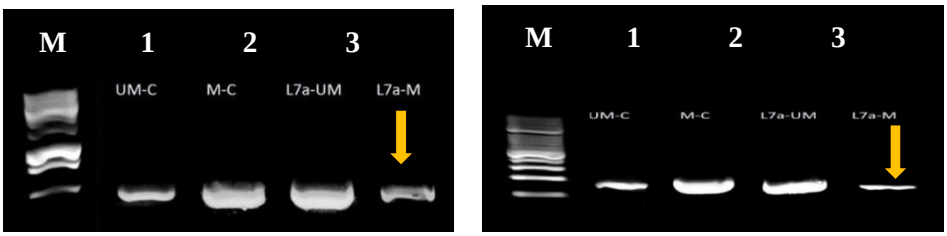

Figure (4): Amplification products of MS-PCR of AKT1 gene (amp-licons,190bp.) in HT29(left) and WI38(right) cell lines as they were labeled. Line1, molecular marker(100bp.). line4,let7a-M.Electrophoresis conditions 5v/cm and1.8% agarose gel concentration. Green arro-ws referred to significant result.

3.3.3 TP53 - CGIs Un-Methylation

Synergistic effect of Let7a antagomir molecule transfection and CGIs-methylation showed high density in blocking of TP53 as TSG promoter region and low density of un-methylated amplicon in both of HT29 and WI-38 cell lines (figure5).

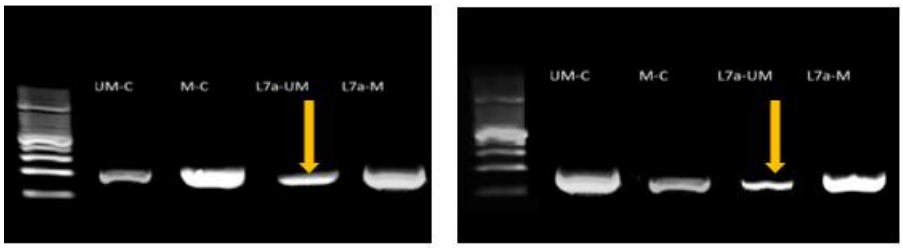

Figure (5): Amplification products of MS-PCR of AKT1 gene(amplicons,190bp.) in HT 29(left)and WI38(right)cell lines as they were labeled.Line1,molecular mar-ker(100bp.). 2, UM,un-methylation control,Line3,M, methylation control, Line3, Let7a- UM and Line 4,let7a-M.Electrophoresis conditions 5v/cm and 1.8% agarose gel concentration. Green arrows referred to significant results.

3.3.4 KEAP1-CGIs Methylation

Interpretation of Let7a antagomir molecule transfection and CGIs-methylation revealed a critical blocking effect of KEAP1 gene promoter region as TSG in both of TH29 and WI38 cell lines (figure 6).

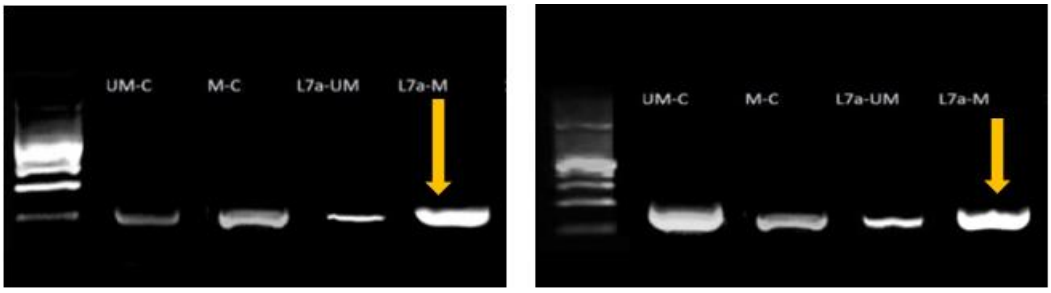

Figure (35): Amplification products of MS-PCR of KEAP gene(ampli-cons,190bp.) inHT29(left)and WI38(right)cell lines as they were label-ed.Line1,molecular marker (100bp.).2,UM,un-methylation control,Line 3,M,methylation control, Line 3,UM and Line 4,M.Electrophore sis conditions 5v/cm and 1.8% agarose gel concentration. Green arrows referred to significant results (Let7a-CpG-unmethy-lation, antagomir).

4. Conclusion

According to present results, synergistic relation between transfection and CGIs un-methylation noticed with antagomir molecule transfection. Cell line that transfected with antagomir molecule showed an increasing in 'switching on' of CGIs in promoter region of oncogenes and 'switching off of TSG-CGIs promoter region methylation, this was a critical indicator for synerg-istic effect of transfection of some ncRNA molecules in a decreasing of methylation efficiency, due to the role of miRNA in up or down regulation of some regulatory genes that have an essential role in cancer cell prolife-ration [21]. Recently discovered that some of miRNAs behave as epi-miRNA that play a role in epigenetic regulation. Epi-miRNAs are subclass of miRNAs that target epigenetic regulators., they lead to down regulation the express of some genes responsible of DNA methyltransfer ase, proteins that bind to methylated cytosine and prevent demethylase gene expression. The expression of epigenetic regulators and upstream epi-miR-NAs that target tumor development genes must be uncontrolled. Therefore, restoring of this regulatory system in cancer cells would be regarded as a therapeutic strategy in cancer therapy [20].

DNA damage increasing chromatin de-condensation, and chromosomal instability are all related with this event. Long and short interspersed nuclear components, as well as classical satellites, are typically highly methylated. Cells with aberrant histology occur as a result of aging or cancer, and frequently exhibit the reduction of DNA methylation in these locations [6].

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