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Genotyping of rs12997 SNP in binding site of let-7b miRNA in colorectal cancer

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Abstract

The current study aims to Genotyping of rs12997 SNP in binding site of let-7b miRNA in ACVR1 gene using PCR-sequencing, the results found the percentage of deletion mutation in patient was 66.66% while in control group 3.7 % in highly significant differences, other study characteristic non- significantly association with rs12997 belong to the gender the deletion mutation was higher in male 41.66% than female 25%, the percentage of deletion mutation was a same percentage (10.41%) in both in Adenocarcinoma and Mucinous adenocarcinoma, in cancer grade the highest percentage was observed in well differentiated (37.5%) and moderate differentiated (29.16%), the deletion mutation appeared in all grades of lymph node metastases, in the N0 (31.25%), N1 (14.58 %) , N2 (8.33%), N1b (2.08%), N2b (6.25%) and N2a (4.16%). the highest percentage of deletion mutation observed in III and I (20.83%) , and in II ,IIA and IIIb (6.25%), According to metastasis the M1 deletion mutation was (4.16%) whlile in the Mx the deletion mutation was (2.08%) , non-sig assocation observed between genotypes (CT, CC, and TT) between patients and control (p 1.3333, 0.3811) and also non- significant association with cancer characteristic in current study. The findings concluded that the deletion mutation was strong association with colorectal cancer but genotypes didn't effecte in colorectal cancer characteristics.

Keywords

Genotyping, rs12997 SNP, binding site, let-7b miRNA, colorectal cancer.

The miRNAs classified in cancer by researchers to two types including oncogene (oncomiRNA) and tumor suppressor miRNA (TSmiRNA). The OncomiRNAs have an oncogenic features target tumor suppressor genes while TSmiRNAs have a tumor-suppressive role by oncogenes targeting. The excessive production of oncomiRNAs cusses' elevation cell proliferation and inhibition in apoptosis processing, meanwhile TSmiRNAs down regulation lead to tumor development acceleration (Iorio et al., 2012). The un controlled production of some miRNAs have been found to be associated with different types of cancer (Calin et al., 2002). Investigation found more than 11.5 million SNPs that found in every 100−300 bp that located in coding and non-coding sequences but in high percentage in the UTRs, with a minor allele frequency of >1% (MAF > 1%). SNPs can be effected in gene expression, types of proteins and in different gene regulation processing (Cargill et al., 1999; Altshuler et al., 2005; Guo et al., 2013; Sukhumsirichart et al., 2018). the gene expression can be affected by SNPs in the UTR by disrupting miRNA binding, protein− mRNA interactions and polyadenylation (Schwerk et al., 2015; Steri et al., 2018). The deregulation of genes are the main mechanisms by which cells can develop to malignancy, this is while a large proportion of genes that implicate in cancers are regulated by miRNAs that bind to 3-UTRs region of them (Skeeles et al., 2013). The current study aims to detect on of an important microRNA binding site of let-7 b and its

association with colorectal cancer in some Iraqi cases.

Methodology

A case control study was designed to study association binding site variation of microRNA let-7 b with some Iraqi colorectal cancer. 49 colorectal cancer cases and 27 healthy individuals were enrolled in current study. The Histopathology test and diagnosis were implemented in the Alhasanin lab by Dr. liwaa Al-Kelabi. The DNA was isolation from embedded tissue by DNA extraction kit, the rs12997 was detected by PCR-sequencing using f-AAATGTTGGCTGCGTACTCC and r-TGTCACGTGGGTAATGGCTA to produce 398 bp at TM 58 ϵC. then products were sequenced by genetic analyzer. Data analyzed using x2 test and odd ratio at p value less than 0.05.

Results and discussion

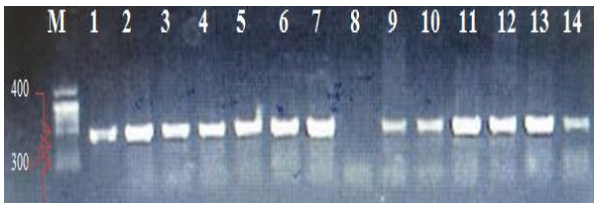

Figure (1) the electrophoresis pattern of rs12997 amplification in study groups, M DNA ladder (100-1000bp), lanes 1-6 amplification target SNP in patients, lanes 7-13 amplification target SNP in control group, sample 8 referred to deletion mutation in study groups. Amplification product 398 bp.

The results of rs12997 SNP genotyping showed that the Polymerase chain reaction product about (398 bp) for both cancer patients and control group as shown in Figure (1).

The result of rs12997 genotyping shows three genotyping, the wild type homozygotes CC and TT, and the mutant heterozygote genotyping CT, as shown in Figure (2).

According to the projection, the genome contains up to 11.5 million SNPs, most of which are located in UTRs. These SNPs are predicted to occur every 100−300 base pairs and have a minor allele frequency (MAF) of >1% (MAF > 1%). There are two types of SNPs in the coding region: synonymous and non-synonymous, which do not change the order of amino acids. Non-coding regulatory SNPs (rSNPs) can affect gene expression by modifying mRNA splicing/degradation and promoter function (Sukhumsirichart et al., 2018; Altshuler et al., 2005; Guo et al., 2013) . As a result, each specific SNP in a gene may have a unique impact on pathophysiology. Given that genetic variants and SNPs in the 3′-UTR can alter gene expression by interfering with miRNA binding, polyadenylation, and protein-mRNA interactions, the functional impact of these SNPs has received excessive attention (Steri et al., 2018).

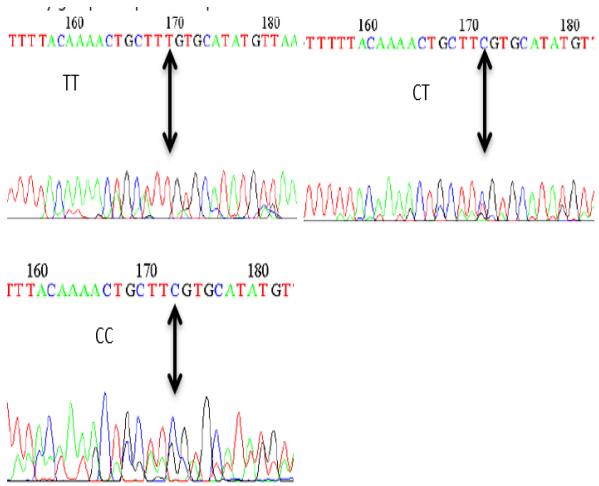

Figure (2) the histograms of rs12997 sequences in study groups (homozygotes CC and TT, heterozygote CT)

The deletion mutation was observed in both patient and control group, the percentage of deletion mutation in patient was 66.66% while in control group 3.7 % the remain sample that observed the normal gene without deletion was 33.33% in patient and 96.29% in control group, there is statistically differences between normal and deletion mutation in the study group. Figure (3)

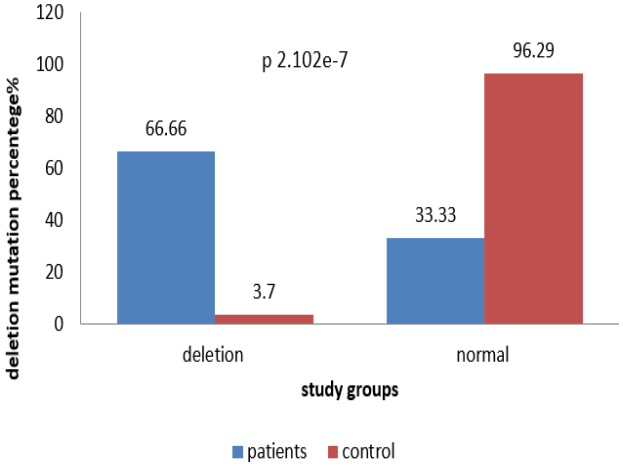

Figure (3) the percentage of deletion mutation (rs12997) in study group (X2, p <0.05).

Numerous tumors have been linked to the abnormal expression of miRNAs, it has been shown. For the first time, Calin et al. showed in 2002 that down regulation of MIR-15a/16-1 is related to chronic lymphocytic leukemia (Calin et

al., 2002).

The result shows the distribution of deletion mutation in rs12997 according to the gender deletion mutation was higher in male 41.66% than female 25%, while the normal also higher in male 22.91% than female 10.41%, and there are not significantly differences in the study group, Figure (4).

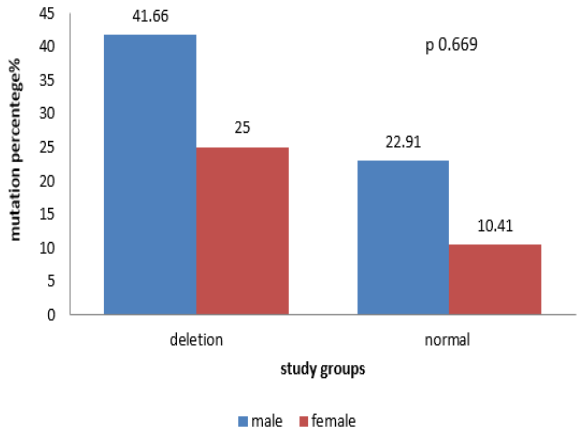

Figure (4) the percentage of deletion mutation (rs12997) in study group according to sex

The identification of an mRNA by a miRNA is mostly based on its seed sequence, which consists of nucleotides 2−7. A number of studies have also demonstrated that SNPs in the 3′-UTR of an mRNA can affect the functions of miRNAs by altering the secondary structure of the 3′-UTR and thermodynamic characteristics of the hybridization site, upsetting miRNA recognition elements (MREs), reducing binding yield, and possibly creating new sites or increasing binding efficiency between miRNA and the target site (Landi et al.,2008). A miRNA's 3′-UTR is also released when a binding site is broken, which increases the miRNA's bioavailability and causes it to bind to each of its 200 anticipated targets. Numerous studies have demonstrated that 3′- UTR-located SNPs can be interesting candidates for the assessment of disease susceptibility, the development of a precision medicine strategy, and the observation of cancer patients in clinical settings (Ding et al., 2018; Ryan et al., 2010). We describe the miRNA binding site polymorphism in inflammatory genes linked to CRC in the following from two perspectives: a detailed look at the past with a literature review and a peep at the future with a bioinformatics analysis to recommend miRSNPs for subsequent investigations.

The two sub- types of colon cancer were investigated in this study the Mucinous adenocarcinoma and Adenocarcinoma, according to the result of analysis the percentage of deletion mutation was same percentage (10.41%) in both in Adenocarcinoma and Mucinous adenocarcinoma, while in Adenocarcinoma the normal region without deletion was higher (56.25%), in the Mucinous adenocarcinoma the normal region was (22.91%) and there were no significant differences among two type, Figure (5).

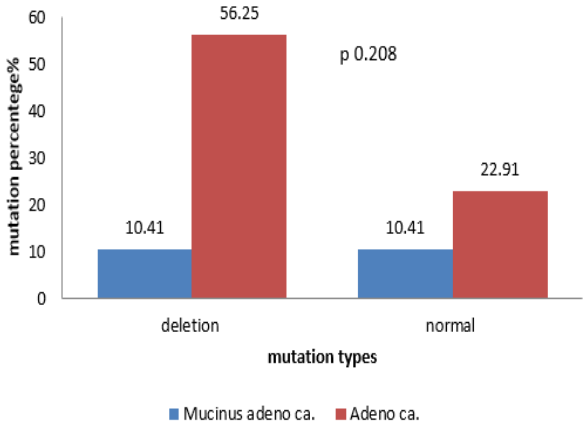

Figure (5) the percentage of deletion mutation (rs12997) in patients group according to cancer sub-type

The result of distribution of deletion mutation in cancer grade, in the well differentiated the percentage of normal (without deletion) was 18.75%, while the percentage of deletion mutation was 37.5%, in the midrate differentiated the percentage of normal 10.41% while the percentage of deletion mutation was 29.16%, in both Poor differentiated and high grad of colon cancer the deletion mutation disappeared and the normal percent was appearance in the same percentage 2.08% and there were no significant differences observed among grade of colon cancer , as shown in (Figure 6), by comparing the percentage of deletion mutation in all grade of colon cancer the percentage of deletion mutation from highest percentage was observed in both well differentiated(37.5%) and modrate differentiated(29.16%) and absent in both poor differentiated and high grade respectively .

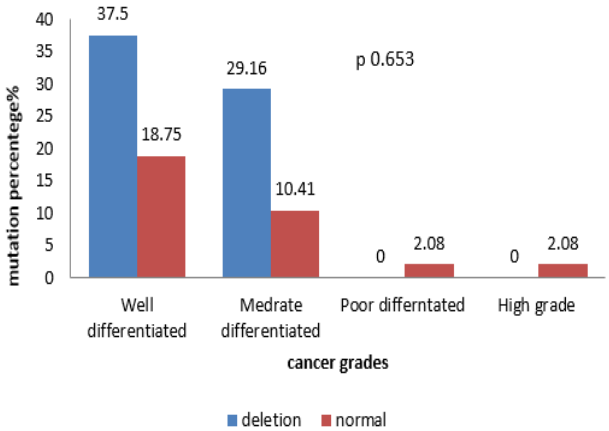

Figure (6) the percentage of deletion mutation (rs12997) in patients group according to cancer grade

The metastases of lymph node, the deletion mutation appeared in all grade of lymph node metastases, in the N0 the percentage of deletion mutation was 31.25% and 18.75% is normal, in the N1 14.58 % was deletion mutation and 6.25% was normal, in the N2 8.33% was deletion and 4.16% was normal, in N1b the same percentage was observed in both deletion and normal (2.08%), in the N2b the deletion mutation was 6.25% and 2.08% was normal, in the N2a deletion mutation was 4.16% and the normal absent , by compare the percentage of deletion mutation in lymph node the highest percentage was in N0 while deletion mutation not observed in N2a , and there is no significantly differences among study group p-value (p≥0.050) Figure (7) .

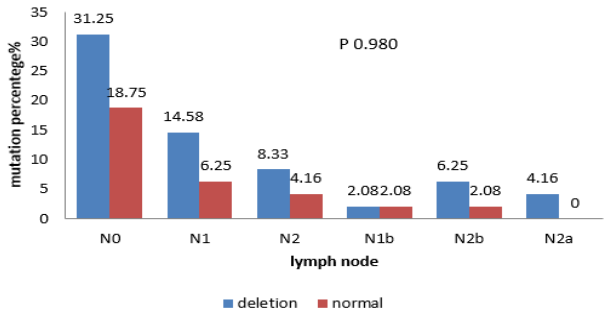

Figure (7) the percentage of deletion mutation (rs12997) in patients group according to lymph node

Activin A's type I receptor, commonly known as ALK2's, is ACVR1 (Renlund et al., 2007) Activins are structurally related signaling proteins that are dimeric growth and differentiation factors that are members of the TGF-superfamily. A heteromeric complex of receptor serine kinases, including at least two type I (I and IB) and two type II (II and IIB) receptors (Wu et al., 1994). These receptors are all transmembrane proteins that contain a cytoplasmic domain with predicted serine/threonine specificity, a transmembrane domain, and an extracellular domain that binds ligands and has a cysteine-rich region (Donaldson et al., 1992).

Type II receptors are necessary for binding ligands and for the production of type I receptors, whereas type I receptors are crucial for signaling. After ligand binding, types I and II receptors come together to form a stable complex, which causes type II receptors to phosphorylate type I receptors. Together with activin type II receptors, the activin A type I receptor encoded by ACVR1 signals a specific transcriptional response (Renlund et al., 2007). The majority of earlier investigations on ACVR1 revealed that this gene's mutations have been linked to the prevalence of progressive fibrodysplasia ossificans (Eresen et al., 2013). The connection between ACVR1 and cancer was only noted in two papers. Recurrent copy number increases of ACVR1 and accompanying transcript overexpression have been linked to survival in head and neck squamous cell carcinomas, according to Ambrosio et al (2011). According to Wiley et al. (2011), the mouse lens's Acvr1 BMP receptor functions as a tumor suppressor. In the current investigation, we hypothesized that the mutation rs12997 on ACVR1 could alter its binding to miR-330-3p and result in the loss of regulation. As a result, ACVR1 with the G allele may express differently from the reference allele, indicating a relationship with the risk of CRC. According to a study, the gene ACVR1 may be crucial for tumor development (Gong et al., 2014).

The distribution of deletion mutation in the cancer stage, the highest percentage was the same in both III and I (20.83%) , and also the same percent in both II ,IIA and IIIb was (6.25%), equal percentage of deletion mutation appearances in IV, IIIC and IIC as shown in Figure (8).

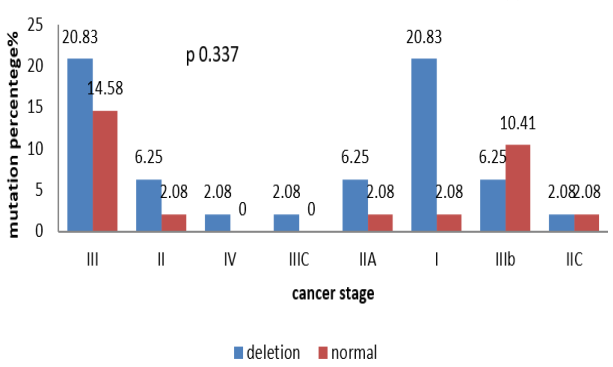

Figure (8) the percentage of deletion mutation (rs12997) in patients group according to stages of cancer

The deletion of particular microRNA types, such as the miR-15 and miR-16-1 cluster in mice, successfully highlighted the crucial function of these two miRNAs in tumor suppression by accurately recapitulating the symptoms associated with chronic lymphocytic leukemia seen in humans (Klein et al., 2010). The miRNA expression is dysregulated in cancer, and its signatures could be exploited for tumor categorization, diagnosis, and prognosis, according to miRNA profiling and deep sequencing results in the years that followed.

According to metastasis of cancer the result shows that in the M1 stages the higher normal region with percent (62.5%) and deletion mutation was (4.16%) whlile in the Mx the deletion mutation was (2.08%) and the normal was (31.25%) and no significant obseved amoung metastasis of cancer , Figuer (9).

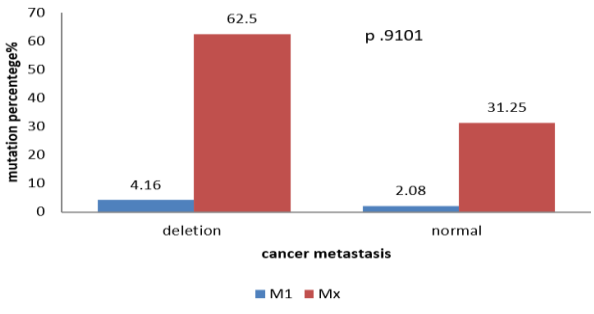

Figure (9) the percentage of deletion mutation (rs12997) in patients group according to metastasis of cancer

Changes in genomic miRNA copy numbers and gene positions are frequently implicated as the cause of abnormal miRNA expression in malignant cells relative to normal cells (amplification, deletion or translocation). The loss of the miR-15a/16-1 cluster gene at chromosome 13q14, which is frequently seen in people with B-cell chronic lymphocytic leukemia, is the earliest identification of a miRNA gene position alteration (Calin et al., 2002). MiR-143 and miR-145's home on 5q33 are frequently deleted in lung cancer, which lowers the expression of both miRNAs (Calin et al., 2006). In contrast, miR-17-92 cluster gene amplification was seen in B-cell lymphomas (Tagawa et al., 2005) and lung cancers (Hayashita et al., 2005), as well as T-cell acute lymphoblastic leukemia (Mavrakis et al., 2010). These translocations of this cluster gene result in overexpression of these miRNAs in these tumors. High-resolution array-based comparative genomic hybridization on 227 specimens from human ovarian cancer, breast cancer, and melanoma confirmed the high frequency of genomic changes in miRNA loci (Zhang et al., 2006).

Additional genome-wide analyses showed that many miRNA genes are situated in genomic areas linked to cancer. These regions may include fragile sites or common breakpoint regions, minimal regions of loss of heterozygosity, which may contain tumor suppressor genes, minimal regions of amplification, which may also contain oncogenes (Peng and Croce, 2016).

The result of rs12997 genotyping shows three genotyping the mutant heterozygote (CT) and homozygotes (CC) and (TT) , the mutant heterozygote (CT) was higher frequency in control (20) than patient (12) while the distribution of homozygotes (CC) was in patient (1) and absent in control groups, the homozygotes (TT) higher in control (5) than patient group (3) , no significant observed between genotyping and gender as shown in Table (1).

Table (1) distribution of study groups according to rs12997 genotyping

Genotyping Of rs10889677	Patients	Control	Odd ratio (CI95%)	P
CT	12	20	1.3333 0.2984 to 5.9575	1.3333
CC	1	0	4.7143 0.1467 to 151.4895	0.3811
TT	3	5		

The result of rs12997 genotyping distribution of sub type shows that the heterozygotes AT was more frequant in Adenocarcinoma (7) while homozygotes CC was (1) and TT was (3), in another subgroup Mucinous adenocarcinoma also the heterozygotes AT was more frequant (5) while homozygotes CC and homozygotes TT was not appeared (0), and there are no significant differences among sub-type, In the differentiated the mutant heterozygotes AT was most common popular ,in the stages well differentiated was (7) while in the Moderate differentiated (4) and in the high grad (1) and not appeared in Poor differentiated.as shown in Table (2).

In the colon cancer stages, the mutant heterozygotes AT was appeared in similar frequency (1) in both II, IIA, IIC and I while high frequency in both III with (5) and IIIB with(3) and absent in both IIIC and IV , the homozygotes CC was absent in all stages expect IIIB stages with (1) frequent , the homozygotes TT appeared with (2)frequent in III and also appeared with less frequent (1) in IIIB stage, and disappearances in each II, IIIC ,IIA, IIC ,I and IV, In the case of Metastases of Lymph node the distribution of mutant heterozygotes AT was higher in N0 with (7)frequent , in N1 and N2 was similar frequent, and (1) frequent in N1b and disappeared in both N2b and N2a , the frequencies of homozygotes CC despite lymph node Metastases was absent in all stages expect N1 with (1) frequency and the homozygotes TT was appeared in both N0 with (2) frequency and in N2b with (1) frequent and absent in all other stages .

In the two stages of Metastases in the Mx stages the heterozygotes AT was (10) while the homozygous CC was (1) and TT was (3) while the M1 stages only the heterozygotes AT appeared (2) and the homozygous CC and TT was absent , the tumor growth in colon cancer mutant heterozygotes AT was in T2 with (4) , the homozygous CC and TT was absent , in the T3 mutant heterozygotes AT was more popular (7) , the homozygous CC was (1) and TT was (3) , In development stages T4 only the mutant heterozygotes AT was appeared (1) while the homozygous CC and TT was absent . In all distribution of genotyping with the sub-groups of colon cancer the result shows no significant differences among rs12997genotyping as shown in Table (2).

Low levels of Dicer expression or its disruption in colorectal and breast cancer encourage epithelial-to-mesenchymal transition (EMT), metastasis formation, and the development of cancer stemness (Martello et al., 2010; Iliou et al., 2014). Along with its role as a haplo-insufficient tumor suppressor, Dicer has also been linked in recent studies to the promotion of CSC and metastatic activity in a variety of cancer cell lines and human tumors. HIF-1 suppresses Dicer expression

by encouraging its ubiquitination and autophagy-mediated degradation, which prevents the maturation of known tumor suppressor miRNAs like let-7 and miRNA-200b (Lai et al., 2018).

Table (2) distribution of patient sub- groups according to rs12997 genotyping

Subjects	CT	CC	TT	P
Sub-types				
Mucinus adeno carcinoma	5	0	0	0.1771
Adenocarcinoma	7	1	3	
Differentiation				
Well differentiated	7	1	1	0.2692
Moderate differentiated	4	0	1	
Poor differentiated	0	0	1	
High grade	1	0	0	
Stage				
III	5	0	2	0.9555
IIIB	3	1	1	
II	1	0	0	
IIIC	0	0	0	
IIA	1	0	0	
IIC	1	0	0	
I	1	0	0	
IV	0	0	0	
Lymph node				0.2599
N0	7	0	2	
N1	2	1	0	
N2	2	0	0	
N1b	1	0	0	
N2b	0	0	1	
N2a	0	0	0	
Metastases				0.6832
Mx	10	1	3	
M1	2	0	0	

A subset of RBPs, as opposed to Dicer1, DGCR8, and AGO RBPs, can compete with or inhibit miRNA-mediated mRNA silencing by binding sequences at or close to miRNA recognition elements. Let-7 (and other miRNAs) maturation and subsequent silencing of target mRNAs are mediated by let-7 (and other miRNAs) RBPs (MREs) (van Kouwenhove, et al., 2011) The suppression of let-7 expression and function, as well as how Lin28A and Lin28B (together known as Lin28) affect stem cell maintenance, metabolism, and cancer, are the most well-known effects of these (Viswanatha et al, 2009; Cho et al., 2012; Shyh-Chang et al., 2013). Two closely similar RBPs and proto-oncogenes called Lin28A and Lin28B bind to the let-7 precursor (pre-let-7) and prevent the miRNA-processing machinery from cleaving it, hence inhibiting let-7 synthesis through different methods (Newman et al., 2008; Piskounova, et al., 2011; Michlewski et al., 2019). Pre-let-7 is bound by Lin28B, which hinders miRNA processing in the nucleus, but Lin28A functions in the cytoplasm to attract TUT4 or TUT7, which adds a short oligo (U) stretch to the 3′-end of pre-let-7, preventing Dicer from processing it, causing pre-let-7 breakdown (Michlewski et al., 2019; Heo et al., 2009) Activation of either Lin28A or Lin28B causes the worldwide post-transcriptional down regulation of let-7 miRNAs in many cancers, which is connected to advanced tumor stages and poor patient outcomes. Lin28A and Lin28B are aberrantly expressed in a variety of tissue malignancies (Viswanathan et al., 2009). Overall, these results imply that particular genomic areas enclosing miRNA genes may be amplified or deleted, leading to aberrant miRNA expression in malignant cells.

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