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Polymorphism in the Promoter Region of Let-7 and Response to Doxorubicin Treatment in Egyptian Hepatocellular Carcinoma

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ABSTRACT

Objectives: This study was conducted to explore the potential role and clinical significance of rs 10877887 polymorphisms in the promoters of let-7 family and risk of HCC susceptibility, in addition to response to doxorubicin treatment in Egyptian patients. **Patients and methods:** We genotyped the single nucleotide polymorphism (SNP) in 100 patients with HCC and 50 healthy controls. Analysis of the rs10877887 was done using polymerase chain reaction-restriction fragment length polymorphism assay. **Results:** We found that the rs 10877887 genotype distribution and allele frequency did not associated neither with increased risk of developing HCC (adjusted OR=0.7178, 95% CI, [0.4409 – 1.1687], p1=[0.1825] P>0.05) nor with the response to doxorubicin treatment (adjusted OR=1.1874, 95% CI, 0.6683.-2.1096, P=0.5581). **Conclusion:** These findings indicate that the rs10877887 CC/CT may not play a role as a risk factor for the development of HCC. Also, it cannot be used to detect the response of Egyptian patients to doxorubicin treatment.

***Keywords**: Doxorubicin; Egyptian patients; Hepatocellular Carcinoma; Let-7; Polymorphism*

INTRODUCTION

Liver cancer is one of the most frequently diagnosed cancers worldwide. It is the second leading cause of cancer-death in men and the sixth leading cause of cancer-related death in women[1]. Hepatocellular carcinoma (HCC) is account for 70% to 85% of the primary liver cancer cases and rarely detected at its early stage, resulting in a short survival of few months[2]. Major treatment modalities of HCC are surgery, chemotherapy, regional therapies such as radiofrequency ablation, transarterial chemo-embolization (TACE) and molecular targeting therapies. In either systemic chemotherapy or TACE, Doxorubicin (DOX) is one of the most commonly used drugs with proven efficacy, but has serious side effects. In a previous study done by Ferlay et al.,[3] he found that among 475 patients who received DOX, a 16% response rate was documented, with a median survival time of 3–4 months. In significant grade 3 or above; hematologic and gastrointestinal toxicities were encountered in patients treated with DOX, including neutropenia (63%), febrile neutropenia (17%), thrombocytopenia (24%), elevation of transaminases

(13%), and diarrhea (7%)[4]. Therefore, it is imperative to find a marker which detect if there is a response to DOX treatment or not in order to reduce its toxicities while maintaining its efficacy.

MicroRNAs (miRNAs), a class of noncoding RNAs of ~22 nucleotides in length, have emerged as key posttranscriptional regulators of gene expression. It has been reported that many miRNAs are involved in human cancers, such as lung, breast, brain, leukemia, colorectal cancer, and liver functioning as oncogenes or tumor suppressor genes[5].

The let-7 miRNA plays a critical role in cell proliferation and differentiation; it is one of the earliest discovered miRNA and has continued to gain recognition in recent years[6]. Several studies have proved that let-7 miRNA participates in the tumorigenesis and metastasis of different types of cancer, such as breast and lung cancers[7-9]. The let-7 family has ten members: let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let7i, miR-98, and miR-202. Single nucleotide polymorphisms (SNPs) in some miRNAs or their targets are associated with risk of HCC. A research done by Xu et al.,[10] has linked polymorphisms of some let-7 family members to cancer, for example, the interaction of the pri-let-7a-1 rs10739971 polymorphism and the *ERCC6* rs1917799 polymorphism is significantly correlated with the risk of GC. The rs10877887 polymorphism in the promoter region of let-7 is significantly associated with prognosis of hepatocellular carcinoma[11].

Thus the aim of the current study was to investigate the potential role of a genetic polymorphism within the promoter region of Let-7 gene (rs10877887) with the risk of HCC. Besides, determine its role in detecting the response of HCC patients to Doxorubicin treatment.

SUBJECTS AND METHODS

Subjects

One hundred HCC patients (73 males and 27 females) recruited from National Cancer Institute (NCI), Kasr EL Einy, Cairo, Egypt. In addition to 50 healthy control volunteers (37 males and 13 females) did not suffer from any disease. Before inclusion, all patients were subjected to full history (age, gender, duration of disease, type of treatment and family history) and laboratory investigations to exclude any condition that may interfere with the studied parameters. All subjects were given written informed consent prior to participation in the study. The study was performed according to the regulations and recommendations of the Declaration of Helsinki.

A peripheral blood sample was collected in 0.5 M EDTA containing tubes from each subject; and stored at −20 °C for use in DNA extraction.

DNA extraction and genotyping

Genomic DNA was extracted using (the purelink® genomic DNA) kit provided by Invitrogen™ from EDTA-anticoagulated peripheral blood leukocytes. DNA was extracted according to the manufacturer's protocol.

Genotyping of one SNP (rs10877887) within the promoter region of Let-7 was performed by real-time polymerase chain reaction, using TaqMan SNP genotyping assay using ViiA™ 7 Real-Time PCR System

TaqMan probes were labeled with the fluorescent dyes VIC® and FAM® which were used to detect different possible genotypes (CC, CT and TT). Real time PCR was carried out in a total volume of 25 µL using the conditions recommended by the manufacturer. Analysis of data was performed and the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence using the Rotor gene Q specialized built-in integrated software for allele discrimination (QIAGEN).

Statistical analysis

Hardy–Weinberg equilibrium was assessed within patients and control by using a goodness-of-fit X2 test. Genotype and allele frequencies were compared between the studied groups by the X2 test with Yates correction or Fisher's exact test when necessary and binary logistic regression analysis was applied. The odds ratio (OR) and confidence intervals (CI) were provided using SPSS package version 22 of Windows (Chicago, IL, USA 2013). Unpaired student t-test was used to test the significance of results of quantitative variables where data were expressed as M ± SEM. Probability values< 0.05 were considered statistically significant. Graphs were plotted using Graphpad Prism 5 (For Windows, 1992–2007 Graphpad software Inc., V 5.01, USA).

RESULTS

The characteristics of all subjects included in this study are listed in (**Table 1**). The genotype and allele distributions of studied SNP in the HCC patients and controls were in Hardy–Weinberg equilibrium (**Table 2**). The assessment of the impact of genotype and allele frequencies of Let-7 gene polymorphism (rs10877887) on the risk of HCC showed that the frequencies of TT, CT and CC genotypes of let-7 gene polymorphism and that of T and C alleles were not significantly different between HCC patients and control groups at (p= 0.8131) and (p = 0.1818) respectively, the same results occurred between the responder and non-responder groups at (p= 0.4142) and (p= 0.558) respectively as shown in (**Table 3**).

Table 1. Demographic and clinical characteristics of HCC patients (responders and non responders) group and healthy control group

Parameters (Mean ± SEM)	Patients n=100		Control n=50	P value
	Responders n=50	Non responders n=50		
Age	**60.8** ± 0.6857	61.96 ± 0.8866	38.4 ± 0.882	**P< 0.05**
Range	30- 70 years	30- 70 years	30- 70 years	
Sex	10 (20 %) female 40 (80 %) male	17 (34 %) female 33 (66 %) male	13 (26%) female 37 (74%) male	**P> 0.05**
No. of lesions				
M	12	18		
1	27	18	–	
2	11	14		
Child class A	38	34		
Child class B	12	16	–	
Ps1	33	37		
Ps2	17	13	–	
Cirrhosis	50	50	_	
HBV	0	0	_	
HCV	50	50	_	

SEM: *standard error.* ***PS1:*** *performance status 1 (symptomatic but completely ambulatory).*
PS2: *performance status 2 (symptomatic <50% in bed during day).*
HBV: *Hepatities B virus.* ***HCV:*** *Hepatities c virus.*

Table 2. Genotypes frequencies distribution of Let-7geneT> C polymorphism between control and patients and in different disease pattern according to Hardy - Weinberg equilibrium

	Control		HCC patients					
			Responders		Non responders		Total	
Genotype	Observed (No)	Expected (No)	Observed (No)	Expected (No)	Observed (No)	Expected (No)	Observed (No)	Expected (No)
TT	19	15.13	22	21.13	18	18.61	40	39.69
CT	17	24.75	21	22.75	25	23.79	46	46.62
CC	14	10.13	7	6.12	7	7.61	14	13.69
X^2	4.9		0.3		0.13		0.02	
Allele frequency p. value	0.0638		0.0039		0.00169		0.00026	
T allele (P)	0.55		0.65		0.61		0.63	
C allele (q)	0.45		0.35		0.39		0.37	

Table 3. Distribution of Let-7 geneT-286 C polymorphism (rs 10877887) genotypes and allele frequencies in the studied subjects and in different disease pattern

Genotypes	rs10877887 of Let-7 gene			
	Control (50)	HCC patients (100)		
		Total	HCC Responder patients (50)	HCC Non responder patients (50)
TT	19 (38%)	40 (40%)	22(44%)	18(36%)
CT	17 (34%)	46 (46%)	21(42%)	25(50%)
CC	14 (28%)	14 (14%)	7(14%)	7(14%)
Significance level	P = 0.0965 X^2= 4.677 & df =2		P = 0.6880 X^2= 0.7478 & df =2	
OR [95%CI, P1]	0.9194[0.4578 –1.8463, p1 = 0.8132]		1.3968 (95% CI: 0.6256 -3.1190, p = 0.4148)	
TT	19 (38%)	40 (40%)	22	18
CT + CC	31(62%)	60 (60%)	28	32
Significance level	P = 0.8131 X^2= 0.05588 & df =1		P = 0.4142 X^2= 0.6667 & df =1	
T allel	55(55 %)	126 (63%)	65(65%)	61(61%)
C allel	45(45 %)	74 (37%)	35(35%)	39(39%)
Significance level	P = 0.1818 X^2= 1.783 & df =1		P = 0.5580 X^2= 0.3432 & df =1	
OR [95%CI, P1]	0.7178[0.4409 – 1.1687] , p1=[0.1825]		1.1874 (95% CI: 0.6683.-2.1096, p=0.5581)	

No: number. df: degree of freedom. p1: data obtained from chi square test.

Table 4. Odds ratios of Let-7 gene T>C polymorphism genotype and allele frequencies between control group and HCC patients group and in different disease pattern

	Control	HCC patients		
		Total	Responders	Non responders
Genotype				
TT	19(38%)	40(40%)	22(44%)	18(36%)
CT+CC	31(62%)	60(60%)	28(56%)	32(64%)
Odd ratio (OR)	0.9194		1.3968	
95%CI	0.4578 to 1.8463		0.6256 to 3.1190	
P value	0.8132		0.4148	
Alleles				
T allele	55(55%)	126(63%)	65(65%)	61(61%)
C allele	45(45%)	74(37%)	35(35%)	39(39%)
Odd ratio (OR)	0.7178		1.1874	
95%CI	0.4409 to 1.1687		0.6683 to 2.1096	
P value	0.1825		0.5581	

Table 5. Distribution of let-7 geneT-286 C polymorphism (rs 10877887) genotypes and allele frequencies between child class A and child class B in HCC patients and in different disease pattern

Genotypes	rs10877887 of Let-7 gene					
	HCC patients					
	Total		Responder		Non responder	
	Child class A HCC patients (72, 72%)	Child class B HCC patients (28, 28%)	Child class A HCC responder patients (38, 76%)	Child class B HCC responder patients (28, 28%)	Child class A HCC nonresponder patients (34, 68%)	Child class B HCC nonresponder patients (16, 32%)
TT	28 (38.9 %)	12 (42.9 %)	17(44.7%)	5(41.7%)	11(32.4%)	7(43.8%)
CT	33 (45.8 %)	13 (46.4 %)	16(42.1%)	5(41.7%)	17(50%)	8(50%)
CC	11 (15.3 %)	3 (10.7 %)	5(13.2%)	2(16.6%)	6(17.6%)	1(6.2%)
Total	72	28	38	12	34	16
Significance level OR [95%CI, P1]	**P =** 0.8266 **χ^2=** 0.3808 **& df** =2 **0.8485[0.3499 – 2.0576, p1 = 0.7162]**		**P** = 0.9512 **χ^2**= 0.1002 & **df** =2 1.1333[0.3046– 4.2163, p1 = 0.8519]		**P** = 0.4961 **χ^2**= 1.402 & **df** =2 0.6149[0.1813 –2.0858 p1 = 0.4352]	
TT	28 (38.9 %)	12 (42.9 %)	17(44.7%)	5(41.7%)	11(32.4%)	7(43.8%)
CT + CC	44 (61.1 %)	16 (57.1 %)	21(55.3%)	17(58.3%)	23(67.6%)	9(56.2%)
Significance level	**P =** 0.7161 **χ2=** 0.1323 **& df** =1		**P** = 0.8518 **χ^2**= 0.03489 & **df** =1		**P** = 0.4335 **χ^2**= 0.6134 & df =1	
T allel	89 (61.8 %)	37 (66 %)	50(65.8%)	15(62.5%)	39(57.4%)	22(68.8%)
C allel	55 (38.2 %)	19 (34%)	26(34.2%)	9(37.5%)	29(42.6%)	10(31.2%)
Significance level OR [95%CI, P1]	**P =** 0.5748 **χ^2=** 0.3148 **& df** =1 **0.8310[0.4350– 1.5875] , p1=[0.5750]**		**P** = 0.7683 **χ^2**= 0.08676 & **df** =1 1.1538[0.4451 – 2.9914] , **p1**=[0.7684]		**P** = 0.2757 **χ^2**= 1.188 & **df** =1 0.6113[0.2514 – 1.4866] , p1=[0.2777]	

No: *number.* ***df:*** *degree of freedom.* ***p1****: data obtained from chi square test.*
p2*: data obtained from logistic regression analysis.*

Binary logistic regression analysis showed that the difference between the CT+ CC genotypes and the TT genotype and allele frequencies were statistically non significant between HCC and control group at p= 0.8131with odd ratio = 0.9194 (95% CI: 0.4578 - 1.8463) and at P= 0.1825 with OR 0.7178 (95% CI: 0.4409 – 1.1687) respectively. Also the same results obtained between responder and non responder groups (**Table 4**).

The genotypes as well as alleles frequencies between Child class A HCC (No: 72) and child class B HCC (No: 28) showed no significant difference from controls at P= 0.8266 and P= 0.5748 respectively. Again, the same results obtained between responders and non-responders of the two child classes (**Table 5**).

DISCUSSION

An increasing amount of studies and consensus reported that miRNAs can act as oncogenes or tumor suppressors and play important roles in the occurrence and development of cancer[12,13]. However, the correlations of miRNA variants with the risk and prognosis of patients with different types of cancers remain to be explored.

Nowadays the miRNA *let-7* family members play an important role in several hallmarks of cancer, including repressing cellular proliferation, inducing cell apoptosis, suppressing aerobic glycolysis, inhibiting invasion and metastasis, and regulating tumor innate immune reactions by interacting with their

targets[14]. *Let-7*-related SNPs, which are located in the *let-7* gene region or in the target gene region, were reported to be associated with the development or prognosis of tumors.

This study was conducted to explore the associations of potentially functional SNP (re10877887) in the promoter region of let-7 with risk of HCC and also the effect of this polymorphism on the response to doxorubicin treatment in Egyptian patients. Results of this study showed that the individual carrying CT or CC genotype of rs10877887 is not more susceptible for developing HCC, suggesting that the genetic variant may not serve as a prognostic marker for the survival of HCC patients.

Our results came in agreement with a study done by Huang et al.,[15] who reported that the polymorphism in the rs 10877887 showed no statistical significance with susceptibility to HCC in Chinese population. This study itself –like our study-came in contrast with a study done by Sui et al.,[16] on the same Chinese population and reported that SNP in the rs10877887 in the promoter region of Let-7 are significantly associated with high risk to HCC compared to those with the wild TT type. Another study done by Xie et al.,[17] found that hepatocellular carcinoma patients carrying the C allele of rs10877887 in the promotor region of let-7i had a significantly increased death risk compared to patients with the TT genotype.

Also, a recent study done by Zhi-Fang Jia et al.,[18] observed no associations between polymorphism in the let-7 gene region (rs10877887) and the risk of development and the overall survival of GC. These inconsistent results, indicating that polymorphism in this SNP need further evaluation and studies, and this is one of the factors which support us to make the study on Egyptian patients.

Our results can be explained in part that the T to C exchange could be a recessive mutation and C allele is a recessive allele, and in the presence of T allele, the antagonizing effect of the mutated Let-7 will be compensated by the wild-type Let-7.

This study, for the first time studied the effect of the genetic polymorphism in the promoter region of Let-7 on the response of HCC patients to treatment with Doxorubicin as one of the well known drugs in this field and we found that there was no significant difference between non responder HCC patients with mutant SNP and Responder HCC patients with the same mutation. Suggesting that polymorphism in this rs10877887 will not modify the response of HCC patients to this drug, although a study done by Meng et al.,[19] on the miRNA Let-7 itself reported that inhibition of let-7 increases the chemosensitivity of hepatocellular CSCs to doxorubicin and sorafenib.

These result differences may be in part due to the effect of ethnicity difference on gene susceptibility among different population that caused by genetic heterogeneity and differences in environmental factors, such as socioeconomic factors as Manni et al.,[20] reported in their study on the Y-chromosome gene pool in the modern Egyptian population and confirmed the mixture of European, Middle Eastern and African genetic characteristics in Egyptians genetics. Analysis of Y-chromosome haplotypes demonstrated different distribution of different haplotype between Upper Egypt and Lower Egypt [21], i.e. different ethnic origins of both populations [22].

CONCLUSION

According to our knowledge, this is one of the first studies that demonstrate the effect of Let-7 gene polymorphism and response to Doxorubicin treatment, and the result of this study indicated that CT genotype and C allele of rs10877887neither significantly associated with the susceptibility to HCC nor with the response to treatment with doxorubicin in Egyptian patients. The frequencies of the genotypes and alleles showed no significant differences between HCC patients with various degrees of the disease according to Child Class (Child class A & Child class B) at rs10877887. Nevertheless, the crucial role played by the Let-7 family in the prognosis of many cancers nowadays necessitates further investigations for other polymorphisms within Let-7 gene to unveil their role of in HCC.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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