Original Article

Death Receptor 4 (DR4) Polymorphism and Susceptibility to Hepatocellular Carcinoma in Egyptian Patients.

Ghada E Hamoda[1], Mohamed A Korany[2], Warda Othman[3]

Medical Biochemistry[1], Internal Medicine[2], and Hepatology Departments, Faculty of Medicine[1,2], National Liver Institute3, Menofia University

Abstract:

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Genetic polymorphisms have been reported to play a role in susceptibility to HCC. The present study aimed to study the possible association between DR4 gene polymorphisms and susceptibility to HCC in Egyptian patients. The study was carried out on 160 subjects divided into 3 groups: Group (A) included 78 HCC patients, group (B) included 42 chronic hepatitis C patients (HCV) and group (C) included 40 age and sex matched healthy control. All subjects were submitted to full history taking, liver function tests and DR4 gene polymorphisms by (PCR- RFLP). This study found a significant difference between HCC and each of HCV and control group, while there is no significant difference between HCV group and control group as regarding DR4 genotyping. The rs20575 polymorphism CC and CG genotypes occurred at increased frequencies in patients with HCC in relation to other groups and the risk of HCC was linked to carriage of rs20575 C allele (OR:1.66) when compared to HCV group and (OR:2.05) when compared to controls respectively.

The present study reported that carriage of DR4 rs20575 CC, CG genotypes have a role in susceptibility to HCC and we recommend performance of this work on a large scale to confirm these results.

Keywords: DR4 / HCC

Introduction:

Hepatocellular Carcinoma (HCC) is the third most common cause of cancer-related death world-wide and each year approximately 750.000 new cases are diagnosed (Jemal et al., 2011).

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alfatoxin B_1, alcohol and non alcoholic steatohepatitis are regarded as the main carcinogenic factors (Kew., 2014).

However, not all individuals infected with HBV/HCV develop HCC indicating implication of other environmental and genetic risk factors in multistage process of this complex disease (Li et al., 2009).

Tumor development is normally prevented by the immune system, which eliminated transformed cells via induction of apoptosis by tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Mellier et al., 2010).

TRAIL induces apoptosis by binding to death receptors (DR) and subsequent activation of the apoptotic cascade (Horak et al., 2005), and it induces apoptosis in a variety of transformed or tumor cells but not normal cells, making it an attractive agent for cancer therapy (Chen et al., 2009).

Death receptor 4 (DR4) was the first DR for TRAIL to be identified and it is located on chromosome 8 p21 (Hazra et al., 2003).

DR4 is type 1 membrane protein receptor which contains 486 amino acids which form two extra cellular cystein rich ligand binding pseudo repeats (50s and 90s loops), a single transmembrane helix and a cytoplasmic death domain (ectodomain) which provokes apoptosis upon TRAIL binding (Frank et al., 2005) .

Human death receptor gene consists of 10 exons and 9 introns in the coding regions. Exon sizes vary form 32 bp to 320 bp and intron sizes vary from 86 to 4700 bp and the principle elements of the DR4 ligand- binding domain are encoded by exons 3 and 4 (Fisher et al., 2001).

DR4 gene is highly polymorphic and DR4 mutation have been described in different human cancers (Tastemir-KorKmaz et al., 2013) However, the most studied polymorphisms are rs20575 with C to G substitution at position

626 (C626G) in the ectodomain of DR4 and rs20576 with A to C substitution at position 683 (A683C) in the extracellular cystein- rich domain of DR4 (Chen et al., 2014).

The present study aimed to study the possible association between DR4 gene polymorphisms namely rs20575 (C626G) with susceptibility to HCC patients in Egyptian patients.

Subjects and Methods:

This study was carried out on 160 subjects, 78 patients with HCC, 42 with chronic hepatitis C virus infection and 40 age and sex matched healthy controls, the patients were selected from National Liver Institute and Internal Medicine, Menofia University Hospitals in the period from Jan 2014 to July 2014.

The subjects were divided into 3 groups:

Group (A): It included 78 HCC patients (61 males and 17females). Their mean age was (44.53 $\pm$ 4.63 years). All patients were Hepatitis C antibody positive and Hepatitis B surface antigen negative, cancer was on top of cirrhosis. This group was diagnosed according to (Barcellona class), which depend on the combination of CT and ultrasound (US) results or on the combination of Alfa fetoprotein and either of CT or US results.

Group (B): It included 42 chronic hepatitis C virus patients HCV group (35 males and 7females). Their mean age was (43.66 $\pm$ 5.30 years).

Group (C): It included 40 age and sex matched healthy controls (32 males and 8 females). Their mean age was (42.40 $\pm$ 5.60 years). All controls are HCVAb and HBsAg negative.

Exclusion criteria included patients with autoimmune liver disease, suspected drug- induced cirrhosis or those with cryptogenic cirrhosis.

All Subjects were submitted to full history taking, clinical examination, ultrasonography, CT scan., laboratory investigation including liver function tests, complete blood picture, HBsAg, HCVAb, alpha fetoprotein (αFP) and assessment of DR4 rs20575 gene polymorphisms using PCR- RFLP technique.

Sample collection and assay:

10 ml of venous blood were divided into 2 parts, five ml were transferred into a plain tube, the clear supernatant serum was used for determination of liver function tests using fully automated auto analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA), viral markers HBsAg and HCVAb were determined by eletrochemiluminecence immunoassay using Cobase immunoassay analyzer (Roche Diagnostic, Germany).

The remaining 5 ml blood were transferred into EDTA containing tube and used for lymphocyte separation for further molecular analysis, then lymphocytes was mixed with phosphate buffer solution and stored in cryotubes at -80^{o}C till analysis (Bio test AG, Dreieich, Germany). DNA from lymphocytes samples was isolated using the QIAGEN extraction kit (Hilden, Germany) and eluted DNA was stored at-20^{o}C for PCR - RFLP.

PCR for the DR4 gene was carried out to a total volume of 25 ul, containing 10 ul genomic DNA, 0.25 ul of each primer (50μM)(Midland, Texas), 2.5 ul of 10x Taq polymerase buffer, 1.5 ul of 25 mM Mg Cl_2 (Qiagen Hiden, Germany), 0.25ul of ampil taq DNA polymerase (5 units / ul) (Qiagen Hiden, Germany), 0.5 ul of dNTPs (10mM) (Qiagen Hiden, Germany), 3ul of DMSo and 6.75 ul of H_2o.

The DR4 gene polymorphisms were analyzed using the following primers:

Polymorphism	Primers	RE	PCR products
rs20575 exon 4 [C626G]	Forward 5`-AAGGTCAAGGGAC ACGT CAGG-3` Reverse 5`-GCTTCTGT GG TTTCTTT GAGG-3`	DraIII	Allele C 164 bp, 56 bp Allele G: 220 bp

PCR amplification for these polymorphisms were performed in a programmable perkin elmer thermal cycler 2400 (USA), at 94°C for 5 min, followed by (35 cycles) at 94°C for 30s , 62°C for 30s (for A683C, annealing temperature is 58°C), 72°C for 30s and one final cycle of extension at 72°C for 7 minutes.

Then the amplification products were separated by electrophoresis through 3% agarose gel stained with ethidium bromide, one band was observed 220 bp for rs20575 (Tastemir-Korkmaz et al., 2013).

The DR4 genotyping using restriction fragment length polymorphism:

The PCR product of the rs20575 of DR4 gene was digested by DraIII restriction enzyme (RE) (provided by fermentas), the digestion products were resulted in 164 bp and 56 bp for CC genotype and 164 bp, 56 bp and 220 bp for CG genotype and 220 bp for GG genotype.

The reaction conditions were, 17ul nuclease- free water, 2ul 10x buffer, 10 ul DNA and 1 μl (2units) restriction enzyme. The mixture was incubated for 2 hours at 37°C then 10 ul of the products were loaded into 3% agarose gel containing ethidium bromide for electrophoresis.

Statistical Analysis:

Results were collected, tabulated, statistically analyzed by IBM personal computer and statistical package SPSS version 16 (SPSS inc. Chicago, Illinois, USA). All data were expressed as Mean ± standard deviation and number and percent. P-value of <0.05 was considered statistically significant.

Results:

Table (I): Shows no significant difference among the three studied groups regarding age and gender.

Table (II): Shows a significant difference among the three studied groups as regarding liver function tests, there is a significant increase of ALT, AST, ALP, TBil, while there is a significant decrease as regarding TP, ALB, PC% in each of HCC and HCV groups when compared to control group. There is a significant increase of TBil, αFP, ALP while there is a significant decrease as regarding PC% in HCC carcinoma group when compared to HCV group while there is no significant difference as regarding other parameters.

Table (III): Shows a significant difference between HCC group and HCV group as regarding DR4 genotyping and allele distribution. In the rs20575 polymorphism the frequency of CC and CG genotypes were associated with 3.12 and 3.01 times higher risk of HCC than GG genotype respectively and frequency of C allele was associated with 1.66 times higher risk of the HCC than G allele.

Table (IV): Shows a significant difference between HCC group and control group as regarding DR4 genotyping and allele distribution. In the rs20575 polymorphism the frequency of CC and CG genotypes were associated with 4.80 and 3.24 times higher risk of HCC than GG genotype respectively and frequency of C allele was associated with 2.05 times higher risk of the HCC than G allele.

Table (V): Shows no significant difference between HCV group and control group as regarding DR4 genotyping and allele distribution.

Table (I): Statistical comparison of demographic criteria between the three studied groups.

Criteria	Studied groups						P value
	Controls (n=40)		HCV patients (n=42)		HCC patients (n=78)		
Age (years):	42.40 ± 5.60		43.66 ± 5.30		44.53 ± 4.63		0.09
Sex:	**No.**	**%**	**No.**	**%**	**No.**	**%**	
Male	32	80.0	35	83.3	61	78.2	0.79
Female	8	20.0	7	16.7	17	21.8	

Table (II): Statistical comparison between the studied groups regards laboratory parameter rs.

Laboratory parameters	Studied groups			P value
	Controls (n=40)	HCV patients (n=42)	HCC patients (n=78)	
Prothrobin concentration (PC%)	99.80 ± 0.41	93.09±6.38	71.47±11.48	P1 0.001 P2 0.001 P3 0.001
Alanine aminotransferase (ALT U/L)	26.77±5.21	63.43±6.10	70.23±25.23	P1 0.001 P2 0.08 P3 0.001
Aspartate aminotransferase (AST U/L)	28.45±5.95	62.35±10.70	68.14±17.17	P1 0.001 P2 0.07 P3 0.001
Total bilirubin (T Bil mg/dl):	0.88±0.15	1.17±0.25	2.33±0.95	P1 0.001 P2 0.001 P3 0.05
Alfa fetoprotein (αFP ng/ml)	2.41±0.75	2.60±0.63	34.14±12.83	P1 0.001 P2 0.001 P3 0.52
Total protein (TP gm/dl)	6.68±0.46	5.66±0.58	5.38±0.92	P1 0.001 P2 0.12 P3 0.001
Seurm Albumin (ALB mg/dl)	3.77±0.38	3.04±0.22	2.91±0.58	P1 0.001 P2 0.15 P3 0.001
Alkaline phosphatase (ALP U/l)	54.65±10.15	59.85±7.32	91.19±12.86	P1 0.001 P2 0.001 P3 0.03

P 1 (HCC versus controls)
P2 (HCC versus HCV)
P3 (HCV versus controls)

Table (III): Distribution of DR4 genotypes and alleles between HCV and HCC patients.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	HCV patients (n=42)		HCC patients (n=78)				
rs20575 (C626G):							
GG	13	30.9	10	12.8			
CG	19	45.3	44	56.4	5.08	0.02	3.01 (1.12–8.06)
CC	10	23.8	24	30.8			3.12 (1.03–9.43)
rs20575 (C626G):							
G allele	45	63.6	64	41.0	3.47	0.05	1.66 (0.97-2.83)
C allele	39	46.4	92	59.0			

Odds ratio (OR) at 95% confidence interval (CI)

Table (IV): Distribution of DR4 genotypes and alleles between HCC patients and controls.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	Controls (n=40)		HCC patients (n=78)				
rs20575 (C626G):							
GG	14	35.0	10	12.8			
CG	19	47.5	44	56.4	8.6	0.014	3.24 (1.22–8.58)
CC	7	17.5	24	30.8			4.80 (1.49–15.46)
rs20575 (C626G):							
G allele	47	58.7	64	41.0		0.009	
C allele	33	41.3	92	59.0	6.67		2.05 (1.18-3.54)

Table (V): Distribution of DR4 genotypes and alleles between HCV patients and controls.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	Controls (n=40)		HCV patients (n=42)				
rs20575 (C626G):							
GG	14	35.0	13	30.9			
CG	19	47.5	19	45.3	0.518	0.077	0.93 (0.35–2.49)
CC	7	17.5	10	23.8			0.65 (0.19–2.21)
rs20575 (C626G):							
G allele	47	58.7	45	53.6	0.45	0.50	0.81 (0.44-1.50)
C allele	33	41.3	39	46.4			

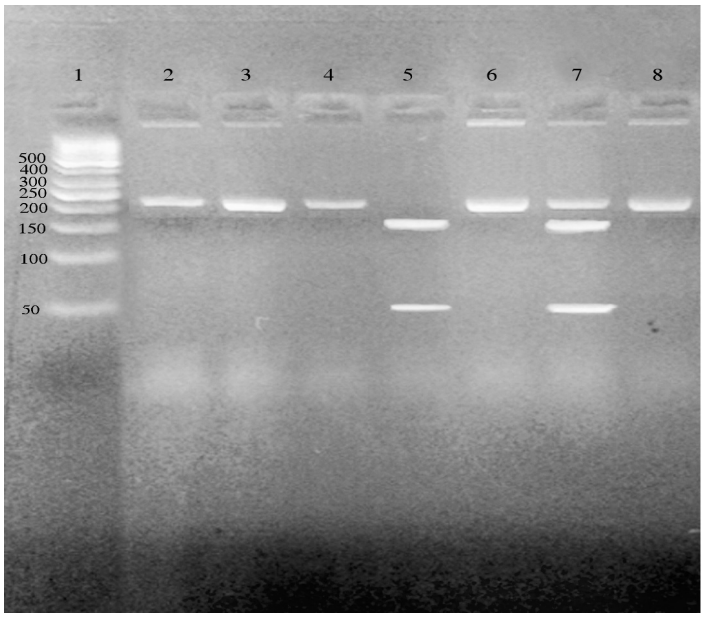

Figure (1): Shows the agarose gel electrophoresis for DR4 rs20575 polymorphism after digestion by Dra III

Lane 1 indicate ladder (50bp)
Lanes 5 indicate CC genotype (164bp & 56bp)
Lanes 7 indicate CG genotype (164bp, 56bp & 220bp)
Lanes 2,3,4,8 indicate GG genotype (220bp)

Discussion:

Death receptor-4 is a tumor suppressor gene and plays as an important mediator of apoptosis. Binding of these rec eptors to their cognate ligands lead to receptor aggregation and recruitment of adaptor proteins forming death inducing signaling complex which in turn activate Caspase 8 and 10 for the triggering of apoptosis (Shirley et al., 2011). Polymorphism in DR_4 gene may reduce apoptotic capacity and provoke proliferation of cell and cancer (Mittal et al., 2015).

Normal variations within the sequence of the apoptotic genes are suggested to lead suboptimal apoptotic capacity finally increasing cancer risk (Geoge et al., 2012).

This study found that, there is a significant increase of AST, ALT, total bilirubin, while there is a significant decrease as regarding serum albumin, total protein and prothrombin concentration in HCC and HCV groups when either of them is compared to the control group.

These results are matched with (Awadellah et al., 2011) who reported a significant deterioration of liver function in HCC and HCV patients when compared to controls.

The present study found a significant increase of α FP in HCC group when compared to HCV group and controls. These results are in accordance with (Spadaro et al., 2005).

This study detected a significant difference among the three studied groups as regarding DR4 genotyping, there is a significant difference between HCC group when compared to either HCV group or control group, while there is no significant difference between HCV group and the controls. On comparing HCC & HCV groups, as regarding rs20575 polymorphism, the frequency of CC and CG genotypes are associated with 3.12 and 3.01 times higher risk of HCC than GG genotype and C allele was associated with 1.66 higher risk of HCC than G allele While on comparing HCC to controls as regarding rs20575 polymorphism, the frequency of CC and CG genotypes are associated with 4.80 and 3.24 times higher risk of HCC than GG genotype and C allele was associated with 2.05 higher risk of HCC than G allele,

These results are in agreement with (Komer et al., 2012) who found that TRAIL receptor genetic variants are associated with an increased risk of HCC in patients with chronic hepatitis C. Lan et al., 2008, found that the increased HCC risk in HCV infected patients carrying the C626G risk variants of TRATL receptor 1 reflects less efficient immune control over HCV infection via TRAIL mediated mechanisms, the genetic variants in DR4 alter its affinity for TRAIL so, TRAIL- DR4 signaling is less efficient in these patients and reduced susceptibility of hepatocytes towards TRAIL –induced apoptosis facilitating HCC development. (Komer et al., 2012).

However previous studies investigating the role of DR4 gene polymorphism in Cancer, Golvez et al, 2014 suggested a possible role of TRAIL receptor, polymorphism in B cell lymphoma genesis.

Rai et al., 2014 concluded that DR4 haplotypes especially rs20575C and rs20576A significantly increased gallbladder cancer risk and this haplotype may change the apoptotic signals and modulate cancer susceptibility by promoting tumor cells survival and tumor growth rather than initiating tumor formation.

Harza et al., 2003 showed an increased risk of C to G transition in 626 position of exon 4 of DR4 gene in bladder cancer and meta

analysis by (Chen et al., 2014) indicated that C626G polymorphism in DR4 gene is associated with cancer susceptibility. On the other hand, (Martinez- Ferrandia et al., 2007) found no association between DR_4 polymorphism and breast cancer in spanish women and also in Turkish population with lung cancer (Tastemir- Korkmaz et al., 2013).

Conclusion:

The present study reported that carriage of rs20575CC, CG genotypes have a role in susceptibility to HCC and we recommend performance of this work on a large scale to confirm these results.

References:

1. **Awadallah A, Issa H, and Soliman M (2011):** Evaluation of serum chromogranin A as a useful tumor marker for diagnosis of hepatocellular carcinoma. Journal of American Science; 7: 999-1004.
2. **Chen B, Liu S, Wang X, et al. (2009):** TRAIL- R1 Polymorphisms and cancer susceptibility: an evidence based meta analysis. EJC; 45 :2598-2605.
3. **Chen W, Tang W, Zhang M, et al. (2014):** Association of DR4 (TRAIL- R1) polymorphism with cancer risk in Caucasians: and updated Meta- analysis. Asian Pac J Cancer Prev; 15: 2889- 2892.
4. **Fisher M, Virmani A, Wu L, et al. (2001):** Nucleotide substitution in the ectodomain of TRAIL receptor is associated with Lung cancer and Head and Neck cancer. Clin Cancer Res; 7:1688-1697.
5. **Frank B, Hemmink K, Shanmugam K, et al. (2005):** Association of death receptor haplotype 626C- 683C with an increased breast cancer risk. Carcinogenesis; 26: 1975-1977.
6. **Geoge G, Mandal R, Kesanwanip, et al., (2012):** Polymorphisms and haplotypes in caspases 8 and 9 genes and risk for prostate cancer. Urol Oncol; 30: 781- 789.
7. **Golvez H, Cosano R, Moreno T, et al. (2014):** Association of polymorphisms in TRAIL and TRAILR genes with susceptibility to lymphomas. Ann Hematol; 2: 243- 247.
8. **Hazra A, Chamberlain R, Grossman H, et al. (2003):** Death receptor 4 and bladder cancer risk. Cancer Res; 63: 1157- 1159.
9. **Horak P, Pils D, Roesster M, et al. (2005):** common death receptor 4 (DR4) polymorphisms don't predispose to ovarian cancer. Gynecologic Oncology; 97: 514- 518.
10. **Jemal A, Bray F, Center M et al. (2011):** Global cancer statistics. CA Cancer J Clin; 61: 69-90.
11. **Kew M (2014):** Heptocellular carcinoma: epidemiology and risk factors. Journal of hepatocellular Carcinoma; 1: 115- 125.
12. **Komer C, riesner K, Kramer B, et al. (2012):** TRAIL receptor 1 (DR4) polymorphisms C626G and A683C are associated with an increased risk for hepatocellular carcinoma (HCC) in HCV-infected patients. BM C Cancer; 12: 85- 92.
13. **Lan L, Gorke S, Rau S, et al. (2008):** Hepatitis C virus infection senitizes human hepatocytes To TRAIL induced apoptosis in a caspase dependent manner. J Immunol; 181; 4926- 4933.
14. **Li R, Shugarty, Zhou W, et al. (2009):** Common genetic Variations of the cytochrorne P450 gene and risk of hepatocellular carcinoma in a Chinese population. Eur J Cancer; 45:12 39-12 74.
15. **Martinez- Ferrandia J, Rodriguez Lovevpez R and Milnece R (2007):** Polymorphism in TRAIL receptor genes and risk of breast cancer in Spanish women. Cancer Biomark; 3: 89- 93.
16. **Mellier G, Huang S, Shenoy Kand Pervaiz S (2010):** TRAILING death in cancer mol Aspect Med; 31: 93-112.
17. **Mittal R, Mandal R, Singh A et al. (2015):** Death receptor 4 variants enhanced prostate cancer risk in North Indian population. Tumor Biology 36 (7) 5655- 61 doilo.1007/s 13277-015-3239-z.

18. **Rai R Sharma K, Sharmas, Misra S, et al. (2014):** Death receptor (DR4) Haplotypes are associated with increased susceptibility of gall bladder carcinoma in North Indian population. Plos one Feb 2014, vol: 9 issue 2, P1.

19. **Shirely S, Morizot A and Micheau O (2011):** regulating TRAIL receptor induced cell death at the membrane. Recent Pat Anticancer Drug Discov; 6: 311- 323.

20. **Spadaro A, Ajello A, Morace C, et al. (2005):** Serum chromogranin A in hepatocellular carcinoma diagnostic utility and limits. World J Gastroenterol; 11: 1765- 1775.

21. **Tastemir- Korkmaz D, Demirhan O and Kulecis (2013):** There is no significant association between death receptor 4 (DR4) gene polymorphisms and lung cancer in Turkish population. pathol Oncol Res; 19: 779- 84.