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Circulating MicroRNAs: new Diagnostic Approach for hepatocellular Carcinoma in Viral Hepatitis in Egyptians.

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ABSTRACT

Incidence of HCC in Egypt is increasing due to the high incidence of hepatitis C infection. HCC started as early invasive cancer with mild clinical and pathological deviation from chronic liver disease. Detection of specific and sensitive marker to help early diagnosis and treatment was our aim. 120 subjects were enrolled in the study, 40 patients suffering from chronic viral hepatitis,40 developed hepatocellular carcinoma and 40 healthy controls. Liver functions, αFP, HSP 70,GP73 were done for all the subjects, MicroRNAsRQ26a and RQ27a were done using the RT-PCR method. α Feto-protein, GP73 and HSP70 results showed significant difference between each of HCV & HCC group and the controls ($P<0.05$), while the difference between HCC and HCV group was highly significant ($P<0.001$). Results of RQ26a and RQ27a was down regulated in HCC group when compared with HCV and control groups. There was an inverse correlation between (RQ26a and RQ27a) results and each of GP73, α FP and HSP70 within the HCC group. MicroRNAs (RQ26a and RQ27a) panels, either alone or in combination with classical biomarkers, might eventually be used to classify samples with respect to liver function and progression to HCC ,helping to reach treatment decisions and establish prognosis.

Key words: viral hepatitis; hepatocellular carcinoma; MicroRNAs; Golgi 73; heatshock protein 70; αFeto protein.

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INTRODUCTION

Hepatitis C virus (HCV) is a major health disease with prevalence approximately 3% of world population [1]. This serious viral infection could be progress into chronic hepatitis, liver cirrhosis and hepatocellular carcinoma(HCC)[2]. In Egypt, chronic HCV accounted for 94% of HCC cases which constitutes 13% of all cancers in 2010 and is the second most frequent cancer in men with 6000–7000 deaths/year [1].

The diagnosis of HCC is confirmed using defined criteria provided by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD). These criteria mainly depend on typical radiological characteristics in dynamic contrast-enhanced imaging [3]. Biomarkers may be used as diagnostic tool in the presence of a suspicious lesion in a patient with liver cirrhosis [4]. In the past, increased serum α-fetoprotein (αFP) to significant level in liver cirrhosis patient with a suspicious liver mass >2 cm, was sufficient to diagnose HCC [5]. Recently, this rationale has been cancelled because αFP has shown low sensitivity and specificity. Moreover,level of αFP didn't increase in almost 80% of early HCCs [3]. Thus, researchers are looking for novel and non- invasive biomarkers that could detect early HCC and improve the management of these patients [2].

Heat shock protein 70 (HSP70) and Golgi protein 73 (GP73) have intensively been studied in diagnosis of HCC [6].HSP 70 is stress response protein. It can protect cells and enhance their damage repair. It is expressed under physiological and different studies have reported its altered level under stress conditions, including carcinogenesis. Overexpression of HSP70 in HCC leads to promoting tumor growth and metastasis [7]. GP73 is a type II Golgi-specific membrane protein[8] that is reported to be elevated in various types of cancer, such as seminomas [9], lung adenocarcinoma [10] and renal cell cancer[11]. However, GP73 is closely associated with chronic liver diseases, particularly HCC [12,13].

In addition to previous potential biomarkers, growing attention has been directed to non-coding RNA especially microRNA (miRNA) over the past ten years[14]. MiRNAs are small non-coding RNA molecules that act as regulators of mRNA expression [15].

In addition, microRNAs can do their function either intracellularly by regulating the expression of a target molecules or extracellulary after being released from the original cell in the form of free molecules or protein bound molecules[16]. Researchers have reported several advantages of miRNAs over other types of RNA including their relative stability against degradation, representation of the original cell and easily detectability in all types of human body fluids [17, 18]. Various studies have linked altered levels of miRNAs in different human diseases including HCC [19]. Defined miRNAs including miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801 have provided the highest diagnostic accuracy for the HCC especially on top of viral hepatitis from these miRNAs [19]. Some of these miRNAs acting as oncogenes and others are acting as tumor suppressors , mir 26a & 27a are tumor suppressors[20]

In our study we aimed to evaluate miR26a and 27a in liver cirrhosis and HCC and correlating their expression with other routine and novel diagnostic biomarkers including HSP70 and GP73.

METHODOLOGY

Subjects participated in this case control study were recruited from the outpatient clinic of both the National Research Center and the National hepatology and tropical medicine research institute in Cairo. A written consent was taken from each subject enrolled in the study according to the ethical committee of National Research Centre The study was part of the project |No.10010104 (10^{th} plane of NRC).

The inclusion criteria included: both sexes with age ranges from 18 to 70 years, cases of primary HCC and cases with liver cirrhosis caused by HCV. While the exclusion criteria included: ages below 18 or above 70, patients with metastatic malignancies in the liver, patients presented with tumors in any organs other than liver and Patients with chronic debilitating diseases.

The study included 120 subjects who were classified into three groups:

first group consists of 40 patients diagnosed with HCC (22 males and 18 females) their ages ranged from 50 to 63 (mean= 56.4) all of cases had chronic HCV infection,

Second group consists of40 patients with liver cirrhosis second to chronic HCV infection (22 males and 18 females), their ages ranged from 50 to 76 (mean= 59.4).

Third group consists of 40 healthy controls with no apparent disease (21 males and 19 females) their ages ranged from 36 to 70 (mean= 53.9).

All patients and controls were subjected to full clinical assessment and laboratory investigations including:

Clinical Examination: A detailed history taking including history of smoking, alcohol intake, occupational exposure to chemicals, drug intake, previous diseases etc. A thorough clinical assessment and total body examination with abdominal ultrasound and/or CT scan when indicated to detect tumor size and extension.

Laboratory Investigations: A blood sample was withdrawn from each subject and was divided, processed and stored according to the requirements and precautions of each test. The following investigations were done for all subjects of the study:

1-Routine laboratory tests: including conventional liver biochemical tests aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, serum albumin and prothrombin time.

2-Estimation of αFP, HSP 70 and GP 73 were assessed by enzyme immune assay technique (Eliza) following instructions on kits purchased from Immunospec Co for αFP and Glory Science Co. for HSP 70 and GP73.

3- MicroRNA was extracted from serum using miRNA assay Serum/Plasma Kit (Qiagen, Hilden, Germany). All serum RNA preparations were quantified by NanoDrop 1000 (Nanodrop, Wilmingtion, Delaware, USA). Measurement of serum microRNA levels was done using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) and microRNA-specific stem-loop primers (part of the TaqMan microRNA Assay Kit; Applied Biosystems). Real-time Quantitative PCR was performed using the Quantistudio 12Kflex Real-Time PCR System (Applied Biosystems), and the results were analyzed using the RQ manager software (Applied Biosystems).

The formula $2^{-\Delta Ct}$ was used to calculate the miRNA levels in serum, where ΔCt =mean (Ct of internal references) − Ct of target miRNA. The relative expression levels of miR-26a and miR-27a were calculated and normalized to miR-16 (Applied Biosystems, Foster City, CA) [21] using the comparative ΔCt method and the equation $2^{-\Delta\Delta CT}$, as described previously [22].

Statistical analysis of the results was performed using Microsoft Excel 2010 and statistical package for social science (SPSS version 24.0) for windows (SPSS IBM., Chicago, IL). Simple t- test and Pearson correlation were done according to Hirsh and Riegl [23]. All reported P- values were two-tailed, value <0.05 was considered significant.

RESULTS

Statistical analysis of our results indicated that liver functions were significantly deteriorated in HCV group when compared with the control group (P=0.001). HCC group showed a higher significant difference in liver functions when compared with control group: bilirubin (P=0.0001), albumin (P=0.0001) and ALT(P=0.005), while the difference in AST level was less significant (p=0.01). The differences between HCC and HCV groups about ALT, AST and bilirubin were also significant (p=0,006, 0.01and 0.04) respectively while albumin results showed no difference between both groups, [table 1].

Table [2] illustrates the t-test results of each of α FP, GP 73 , HSP 70 and MiRNAs (RQ26a& RQ27a). α FP result showed significant difference between each of HCV & HCC groups and the control group (P=0.01 and P= 0.001) respectively, also the difference between HCC and HCV was highly significant (P=0.001).

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The same table showed that Serum GP73 was significantly increased in HCV group more than controls (p=0.04), where the difference was highly significant between HCC and control (p=0.001).

In addition, no significant difference was found between HCV and HCC groups (p= 0.5). Concerning HSP70 Results , statistical analysis denoted a high significant difference in between the three groups.

Comparison of the molecular parameters RQ26a and RQ27a mean values between studied groups showed that there were significant differences between the three groups with down-regulation in HCC group less than HCV and control, fig [1], table [2].

Correlation of the results within HCC group showed that αFP was positively correlated with GP 73 (r= 0.431; P < 0.001), and was negatively associated with RQ 26a (r= -0.303; P < 0.001).

GP 73 results showed an inverse association with each of HSP 70 (r= -0.325; P < 0.001) fig [2], and RQ 26a (r= -0.253; P < 0.01) fig [3], while a direct correlation was found with RQ 27a (r= 0.28; P < 0.002). In addition, HSP 70 was directly associated with RQ 27a (r= -0.185; P < 0.04). A positive association was found between RQ 26a and RQ 27a (r= 0.204; P < 0.02) fig[4].

Table [1] Comparison of age, sex and liver functions between the studied groups

Descriptive parameters			Control N=40	HCV N=40	HCC N=40	P. value		
						Control & HCV	Control& HCC	HCV & HCC
Demographic Data	Age	Range	36.0 – 70.0	50.0 – 76.0	50.0 – 63.0	0.01*	0.1	0.01*
		Mean ± SD	53.97 ± 8.8	59.4 ± 6.06	56.4 ± 4.04			
	Sex	Female	19 (47.5%)	18 (45.0%)	18 (45.0%)	0.8		
		Male	21 (52.5%)	22 (55.0%)	22 (55.0%)			
Biochemical parameters	ALT	Range	22.0 – 38.0	22.0 – 110.0	36.0 – 6214.0	0.001**	0.005**	0.006**
		Mean ± SD	31.5 ± 4.9	59.23 ± 23.0	764.0 ± 1567.2			
	AST	Range	20.0 – 35.0	22.0 – 133.0	36.0 – 8813.0	0.001**	0.01*	0.01*
		Mean ± SD	28.9 ± 4.37	66.65 ± 29.1	981.25 ± 2232.7			
	Bilirubin	Range	0.4 – 0.9	0.8 – 2.7	0.64 – 2.9	0.001**	0.0001**	0.04*
		Mean ± SD	0.61 ± 0.16	1.2 ± 0.44	1.5 ± 0.63			
	Albumin	Range	3.8 – 5.0	1.8 – 4.5	2.4 – 4.2	0.001**	0.0001**	0.9
		Mean ± SD	4.36 ± 0.38	3.3 ± 0.61	3.34 ± 0.46			

** is a highly significant at the p<0.001
* is significant at the p<0.05

Table [2] Comparison of α FP, GP73, HSP 70, RQ26a and RQ27a between the studied groups

Biomarker&Molecular parameters			Control N=40	HCV N=40	HCC N=40	P. value		
						Control & HCV	Control & HCC	HCV & HCC
Biomarker parameters	α Feto-protein	Range	2.30 -15.0	1.4 - 46.0	2.0 - 2384.0	0.01*	0.001**	0.001**
		Mean ± SD	3.4 ± 2.72	7.85 ± 9.55	564.5 ± 900.9			
	Human golgi73	Range	1.3 - 5.2	7.2 - 95.4	8.3 - 36.5	0.001**	0.001**	0.5
		Mean ± SD	3.67 ± 1.12	15.6 ± 14.77	13.9 ± 6.08			
	HSP 70	Range	60.7 - 96.2	32.0 - 113.9	50.0 - 96.2	0.001**	0.002**	0.005**
		Mean ± SD	75.67 ± 11.43	59.67 ± 14.46	67.89 ± 10.66			
Molecular parameters	RQ26a x 10^2	Range	20.0 - 262.0	3.0 - 235.0	7.0 – 66.0	0.03*	0.01*	0.01*
		Mean ± SD	93.99 ± 77.37	59.66 ± 68.39	29.15 ± 18.09			
	RQ27a	Range	0.08 - 356.0	0.10 - 78.08	0.04 - 15.61	0.02*	0.01*	0.01*
		Mean ± SD	18.98 ± 78.34	9.7 ± 17.41	3.13 ± 4.88			

** is a highly significant at the p<0.001
* is significant at the p<0.05

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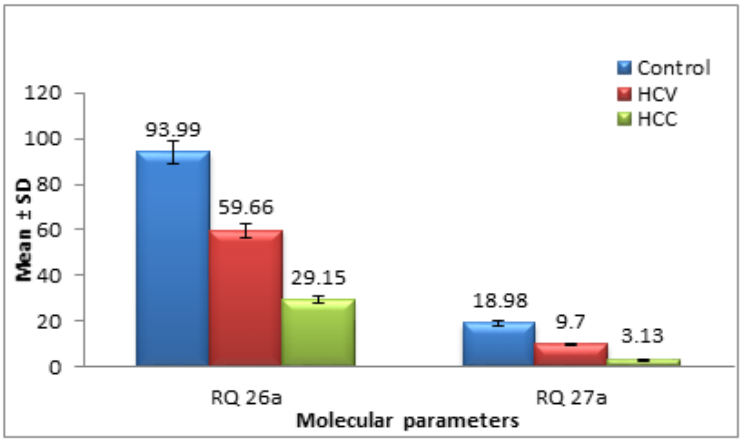

Figure [1] Mean of Molecular Parameters in the Study Groups

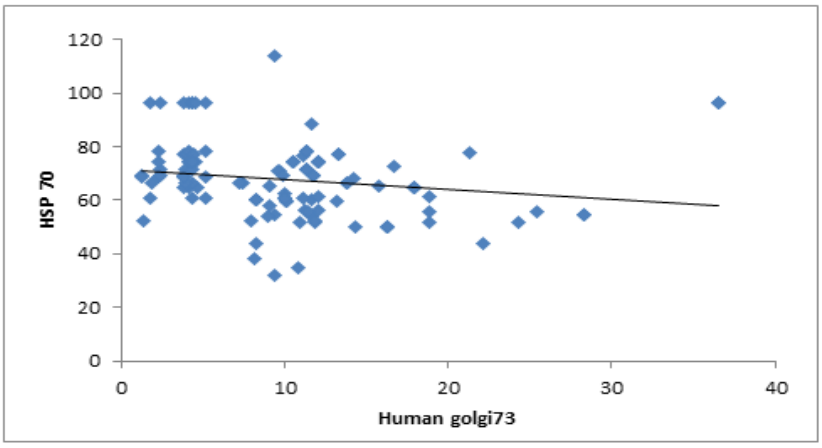

Figure [2] Correlation betweenGP73 and HSP 70 within HCC group

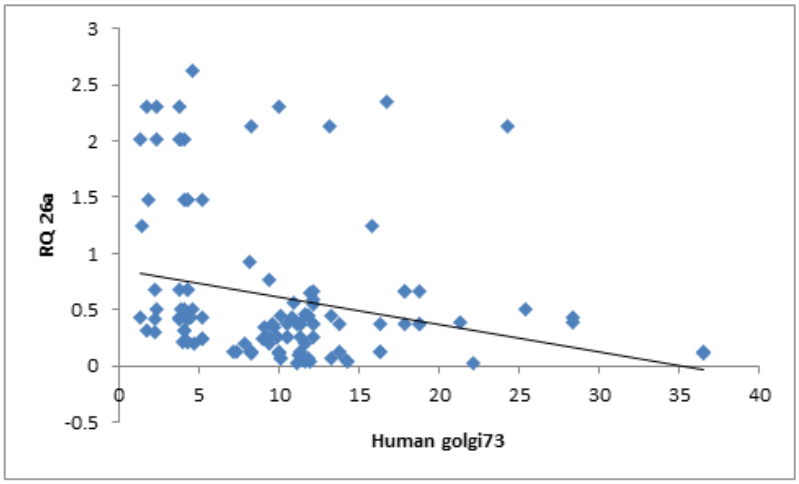

Figure [3] Correlation between GP 73and RQ 26a within HCC

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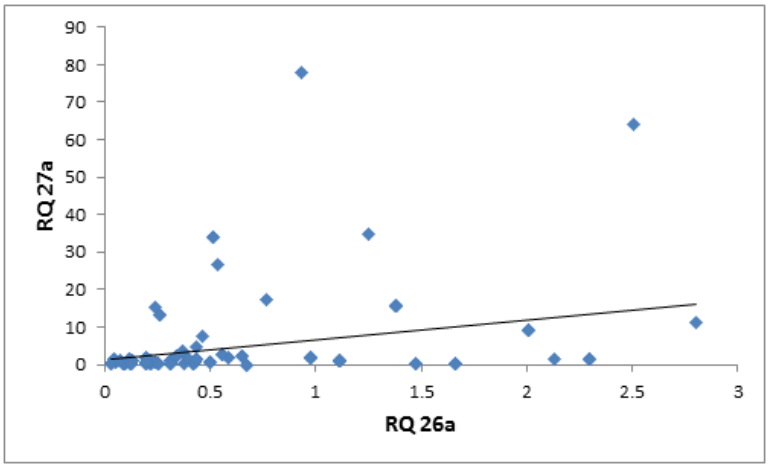

Figure [4] Correlation between RQ 27a and RQ 26a in HCC group

DISCUSSION

HCC is the second cause of cancer-related morbidity all over the world[24].It almost follows liver cirrhosis because fibrosis and cirrhosis modulate the interactions between cancer cells and extracellular matrix in HCC as reported by many researchers [25].

Results of this study indicated that the liver functions of both patient groups were deteriorated as compared with controls. It is well known that inflammation, fibrosis, and cirrhosis lead to liver failure which fulminate in HCC. These results were recorded by Waleed and coworker (2016) who stated that there was a highly significant difference regarding all measured liver function tests as well as αFP between healthy control group and HCC group ($P < 0.001$) [26].

On the other hand, our study revealed that αFP results showed that the difference between HCC and HCV was highly significant (P=0.001). These results indicated that αFP could be considered as signal for incidence of carcinoma. αFP is currently widely used to distinguish between HCC and benign liver lesions. Diagnosis of HCC without pathologic confirmation can be achieved by assessing serum αFP level combined with imaging techniques, including ultrasonography, magnetic resonance imaging , and computerized tomography [27,28]. However, Filmus and colleges (2004) found that the sensitivity of this marker is limited (41-65%) [29].

On the other hand, serum GP73 results of this study were significantly higher in HCV group than controls (p=0.04), while the difference was highly significant between HCC and controls (p=0.001) . However, the difference between both HCV and HCC groups was not significant. These results denoted that only affected hepatocytes do express GP73 protein, and its expression is significantly increased in liver diseases such as HCC. The same conclusion was reached by Kladney et al., (2002) [30]. In addition, Riener et al.,(2009) [31] and Mao et al., (2010) [32] considered serum GP73 as a valuable biomarker for patients with HCC.

Kai and coworker (2013) concluded from their study that GP73 protein level is significantly increased in patients with HCC compared with healthy controls, decreased following surgical resection of HCC lesions and increased with tumor recurrence[33]. The present study showed that there is a positive correlation between αFP and GP 73 ($r= 0.431$; $P < 0.001$), a result which help in establishing diagnosis of HCC.

HSP70 was increased in our HCC group more than control and HCV groups (p=0.002 & P=0.005) respectively. The same results were reached before by Sakamoto and his colleges(2009), who recorded that by gene-expression, profiling from approximately 12 600 analyzed genes, HSP70 is abundantly upregulated in early HCC[34]. HSP70 expression is not observed in non-malignant nodules or other benign nodular lesions, hepatocellular adenoma and focal nodular hyperplasia. This makes HSP70 a useful histological marker to distinguish early HCC from precancerous lesions and to differentiate benign and malignant liver nodules.

In addition, Shin E et al., (2011) stated that HSP70 may be used as an indicator of prognosis for HCC. Its conclusion was established in 282 out of 392 HCC cases (71.9%), whereas only 14 of 115 non-neoplastic liver tissues expressed HSP70 (P<0.001) [35]. Whereas Tremosini Set al., (2012) specified that the sensitivity and specificity in detecting HCC of HSP70 were identified as 57.5% and 85%, respectively [36]. The same results were reached by Gehrmann M et al., (2014) who concluded that serum HSP70 levels are consecutively increased in patients with HCV and HCC and thus might have a prognostic value [37].

MicroRNAs (miRNAs) are evolutionarily conserved small no coding RNAs involved in the regulation of gene expression and protein translation.

Many studies have shown that they play a crucial role in driving organ and tissue differentiation during embryogenesis and in the fine-tuning of fundamental biological processes, such as proliferation and apoptosis [38]. Results of the present study showed that MiRNAs level (RQ26a & RQ27a) were significantly down regulated in both HCC and HCV groups less than controls, where the lowest level was found in HCC cases . Furthermore, our study revealed that RQ26a level was directly associated with RQ 27a in HCC group (r= 0.204; P < 0.02). Nan Zetal., (2016) specified that MiR-27a-3p was reported to act as a tumor suppressor in other types of cancer [39]. Nelson H and Kazuaki C., (2016) stated that growing evidence indicates that their deregulation plays an important role in cancer onset and progression as well, where they act as oncogenes or onco-suppressors [40]. The same conclusion was reached by Thomas etal., (2016) who mentioned that miRNAs (miR-26a, miR-122, and miR-130a) were down-regulated in HCC, and their up-regulated gene targets are primarily associated with aberrant cell proliferation that involves DNA replication, transcription and nucleotide metabolism [41]. On the contrary, Shujie He et al., (2016) demonstrated that the expression levels of miR-27b were significantly increased in HCC cell lines, as compared with in normal human liver cells. In addition, miR-27b was frequently up regulated in HCC tissues, as compared with in normal adjacent tissues. Furthermore, elevated miR-27b expression levels were significantly correlated with tumor differentiation, Tumor Node Metastasis stage and vascular invasion (P<0.05). Knockdown of miR-27b expression inhibited HCC cell migration and invasion [42]. Plasma miRNA panel had considerable clinical value for the early diagnosis of HCC and could help patients who might have otherwise missed the curative treatment window benefit from optimal therapy [43]. Scientists identified a miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) that provided high diagnostic accuracy for discriminating patients with HCC from the healthy population [33]. Also Zhao et al 2015 suggest that loss of tumor suppressor miR-26a are specific indicators of HCV infection-associated HCC while altered expression of miR-181, miR-122 and miR-23a appears to be a less specific indicator of HCV infection-associated HCC[44].

A meta-analysis done by Annacarmen et al 2016 on large datasets of microarray-based transcriptional profiles of HBV- and HCV-associated HCC showed inhibited state for several miRs. In particular, specific inhibition of miR-16, miR-146 and the let-7 family of miRs in HCC while miR-26, miR-124 and miR-155 were inhibited in HBV-associated HCC. miR-24, miR-29, miR-124 and miR-291 were inhibited in HCV-associated HCC[45].

The prognosis of HCC patients does depend only on tumor size and number but also affected by a complex interplay between different genetic, epigenetic and environmental factors [46]. Thus, the ability to predict patient prognosis is complex.

However, results reported by Shi and his colleagues showed that HCC patients with low levels of miR-26 have better response to interferon-alpha therapy compared to patients with high levels, suggesting that expression of miR-26 can be considered as good indicator & predictor of the response to interferon-alpha therapy [47].

In conclusion,miRNAs26a and 27a expression altered in HCC patients and can be used either alone or in with classical biomarkers to diagnose early HCC. This help to improve HCC management and reach best treatment decisions for sake of the patients. Limitation of the study was the small sample size & detection of only 2 miRs. Further studies on a larger number of patients are needed to confirm these findings & clarify their role in disease staging and prognosis.

Ethical approval: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

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