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Research Article

Expression of Trx-1, HIF-1α, and Their Associations with Clinicopathological Parameters in Gastric Cancer

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Abstract

Objectives: Thioredoxin-1 (Trx-1) is a small redox protein that plays an important role in many biological processes. Although increased expression of Trx-1 in various solid tumors has been reported, its clinical significance and diagnostic value in human gastric cancer (GC) are not well defined. Hypoxia-inducible factor-1α (HIF-1α) is actively involved in the metabolism of many tumors; however, the relationship between its expression levels and clinical significance in GC still needs to be established.

Methods: In this study, the levels of HIF-1α and Trx-1 mRNA and protein in 20 freshly frozen GC and matched normal tissues were determined by quantitative PCR (qPCR). Additionally, an immunohistochemistry assay was performed to measure the protein levels of Trx-1 and HIF-1α in 162 GC samples.

Results: We observed that the Trx-1 mRNA and protein levels increased significantly in GC tissues, while that of HIF-1α also increased, and both were associated with a poor prognosis ($p<0.001$). Multivariate analysis revealed that Borrmann type, Trx-1, and HIF-1α were independent predictors of GC prognosis ($p<0.001$).

Conclusion: Our results indicate that both Trx-1 and HIF-1α may be promising prognostic indicators and therapeutic targets for GC patients.

Keywords: Gastric cancer, Trx-1, HIF-1α, prognosis

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Gastric cancer is the fifth most frequent malignancy and the third most common cause of cancer-related deaths, accounting for 5.6% of new cancers worldwide.[1] There are over 1 million new cases and 790,000 deaths from GC every year around the world.[2] Despite significant advances in surgical techniques and new medical technology, the outcome for GC patients with advanced or metastatic tumors remains poor, with a five-year survival rate of 5-20%.[3] Therefore, it is essential to identify new diagnostic and prognostic biomarkers to enable improved and accurate prediction of recurrence and to develop effective therapeutic targets for GC patients.

Hypoxia is one of the most typical characteristics of solid tumors, including gastric cancer. It is related to cell proliferation, anti-apoptosis, migration, and invasion in various tumor cells, and its overexpression often leads to poor prognosis.[4–6] HIF-1, as a transcription factor, comprises the HIF-1α and HIF-1β subunits and plays a key role in regulating the cellular hypoxic response. HIF-1α is a type of nuclear protein with transcriptional activity under hypoxic conditions, and it is overexpressed due to the rapid proliferation of tumor cells.[7] Normally, HIF-1α undergoes quick, proteasome-mediated degradation, but under hypoxic conditions, it is stabilized. It is overexpressed in various cancers, including those of the

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ovary, breast, uterus, and cervix, and its overexpression often leads to poor prognosis.[8] HIF-1α is favorably linked to phosphorylated AKT. Studies have also shown that the promotion of the AKT-HIF-1α-VEGF pathway, independent of hypoxia, aids in GC tumorigenesis and angiogenesis.[9]

Trx-1 is a member of the thioredoxin protein family and undergoes reversible NADPH-dependent reduction by selenocysteine-containing flavoprotein Trx-1 reductases. Trx-1, through its redox activity, regulates the activity of enzymes and increases the DNA binding and transactivating activity of transcription factors.[10] It is often upregulated in many human cancers, including those of the stomach, liver, and colon.[11,12] Its overexpression is associated with cancer cell proliferation, anti-apoptosis, tumor invasion, and poor prognosis in patients.[13] Trx-1 interacts with a number of transcription factors to regulate cell growth and survival, and it also binds to redox-sensitive enzymes to regulate their activity, including apoptosis signal-regulating kinase-1 and protein kinase C.[14] Welsh et al.[15] have shown that increased Trx-1 expression is associated with increased VEGF production and enhanced tumor angiogenesis.

However, the relationship between HIF-1α and Trx-1 has not been reported. In the present study, we evaluated the expression of HIF-1α and Trx-1, as well as the clinicopathological parameters in gastric cancer.

Methods

Patients and Tissue Specimens

In this study, 162 GC patients were retrospectively selected from the database of the Xuzhou Central Hospital, China, between 2015 and 2020. The 20 samples of tissue were taken from both cancer and macroscopically normal mucosa at least 5 cm away from the GC patients who underwent radical gastrectomy without preoperative chemoradiotherapy. The collected specimens were fresh-frozen in liquid nitrogen immediately and stored at -80°C until RNA extraction. Other specimens were fixed in 10% formalin and embedded in paraffin for pathology examination. The patients' clinical data were collected and are shown in Table 1. The study was conducted in accordance with the

Table 1. Relationship between Trx-1 and HIF-1α expressions and clinicopathologic features

	HIF-1α		p	Trx-1		p
	Positive	Negative		Positive	Negative	
Gender			0.185		0.687	
Male	63	24		53	34	
Female	47	28		48	27	
Age			0.729			0.681
>60	33	17		30	20	
<=60	77	35		71	41	
Borrmann type			0.071			0.029
I+ II	51	22		45	38	
III+ IV	59	20		56	23	
Differentiation			0.284			0.009
Well /Moderately	50	19		51	18	
Poorly	60	33		50	43	
Depth of invasion (T)			0.038			0.000
T1-T3	64	39		53	50	
T4	46	13		48	11	
Lymph node metastasis (N)			0.028			0.090
N1/ N2	70	42		65	47	
N3	40	10		36	14	
Gastrectomy			0.539		0.155	
Subtotal	41	22		35	28	
Total	69	30		66	33	
Tumor location			0.857			0.079
Middle third and Middle	47	23		49	21	
Lower third and whole	63	29		52	40	
Tumor size			0.816			0.353
<=5cm	72	35		64	43	
>5cm	38	17		37	18	

Declaration of Helsinki. All patients signed a written and informed consent form. The Institute Research Ethics Committee of Xuzhou Central Hospital reviewed and approved this protocol.

Immunohistochemistry

Fresh specimens were fixed in 4% neutral formalin and embedded in paraffin. Immunohistochemical staining was performed with an Immunohistochemical SP9000 kit according to the manufacturer's instructions. Briefly, 4-μm sections were cut from formaldehyde-fixed, paraffin wax-embedded tumor tissue blocks. All the slides were dewaxed and rehydrated. Endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min, the slides were microwaved in citrate buffer for 15 min for antigen retrieval, and then blocked with 5% bovine serum albumin for 30 min at room temperature. Immunostaining was performed with anti-HIF-1α and anti-Trx-1 rabbit polyclonal antibodies (1:100, 1:200). The slides were then incubated overnight at 4°C with the primary antibodies in a humid chamber. The slides were incubated with biotinylated secondary goat anti-rabbit antibody and avidin-biotin-peroxidase complex for 20 min at room temperature, followed by incubation with 3,3-diaminobenzidine (DAB). Finally, the slides were counterstained with hematoxylin.

Evaluation of Immunohistochemical Staining

The immunohistochemical results were independently evaluated by 2 investigators according to staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining), and the percentage of cells stained was assessed using a semiquantitative 4-point scale (0, <10% of cancer cells stained; 1, 10-20% of cancer cells stained; 2, 21-50% of cancer cells stained; 3, >50% of cancer cells stained). If the product of staining intensity and the percentage of positive cells was ≥3, it was deemed to be immunoreaction positive (+).

Extraction of Total RNA and Quantitative Real-Time PCR

Fresh frozen human GC tissue was used for the extraction of total RNA using TRIzol Reagent from Invitrogen (USA), and reverse transcription was carried out using the ReverTra Ace qPCR RT Kit from Toyobo (Japan) as per the instructions of the manufacturer. The Trx-1 and HIF-1α mRNA levels were determined by RT-PCR on the Sequence Detection System ABI 7900HT from Applied Biosystems (USA) using SYBR-Green dye from Toyobo (Japan) and the following primers: Trx-1, sense (5'-CAACCCTTTCTTTCATTCCCTCT-3') and antisense (5'-CACCCACCTTTTGTCCCTTCT-3'); HIF-1α, sense (5'-ATCCATGTGACCATGAGGAAATG-3') and antisense (5'-TCGGCTAGTTAGGGTACACTTC-3'); GAPDH, sense (5'-ATCAAGAAGGTGGTGAAGCAGG-3') and antisense (5'-CGTCAAAGGTGGAGGAGTGG-3').

Statistical Analysis

All statistical analyses were processed using statistical software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). For analyses of Trx-1 and HIF-1α levels and clinicopathological factors, the Chi-square test was applied. For univariate and multivariate analysis, the model of Cox's proportional hazards was used to identify independent prognostic factors. The statistically significant values were $p<0.05$.

Results

Expressions of HIF-1α and Trx-1 in Gastric Cancer Tissues

The expression of Trx-1 and HIF-1α in GC tissue was significantly higher than that in matched normal tissue and was mainly expressed in the cytoplasm of gastric cancer cells (Fig. 1). In gastric cancer tissues, the positive rates of HIF-1α and Trx-1 expression were 68% (110/162) and 62% (101/162), respectively. The expression levels of HIF-1α and Trx-1 were significantly higher in gastric cancer tissues compared with normal gastric tissues ($p<0.05$).

Trx-1 and HIF-1α Expressions in Fresh GC Tissue

qPCR was carried out to evaluate the Trx-1 and HIF-1α mRNA expressions in fresh GC and corresponding normal tissues. As shown in Figure 1, the expressions of Trx-1 and HIF-1α in GC tissues were upregulated compared with normal tissues (Fig. 2).

Clinical Significance of Trx-1 and HIF-1α Expression in GC

Table 1 lists the clinicopathological characteristics of Trx-1 and HIF-1α. The expression of HIF-1α significantly correlated with the depth of tumor invasion and lymph node metastasis. Likewise, enhanced Trx-1 expression correlated with Borrmann type, differentiation, and depth of tumor invasion.

Prognostic Significance of GC Patient Survival

We performed survival analysis of 162 patients using clinical follow-up results to evaluate the prognostic potential of Trx-1 and HIF-1α in GC, and the results are presented in Table 2. The overall 5-year survival rate was 33%, and mean survival was 60.27±2.52 months. The favorable clinical prognostic indicators of survival were Borrmann's classification, differentiation, depth of invasion, tumor location, Trx-1, and HIF-1α on univariate analyses ($p<0.05$) (Table 2).

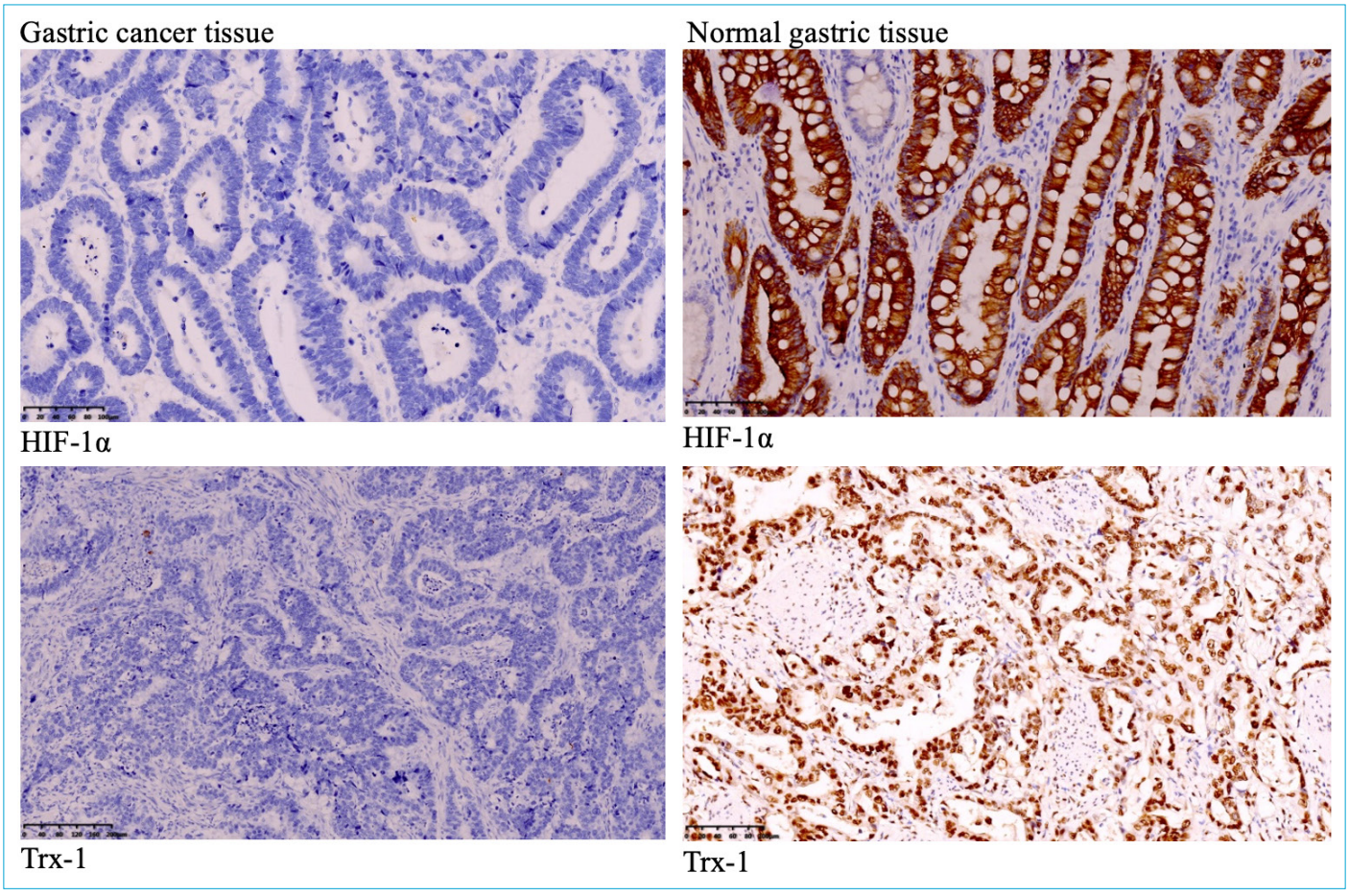

Figure 1. Expressions of HIF-1α and Trx-1 proteins in gastric cancer tissues and normal gastric tissues (IHC staining, ×20).

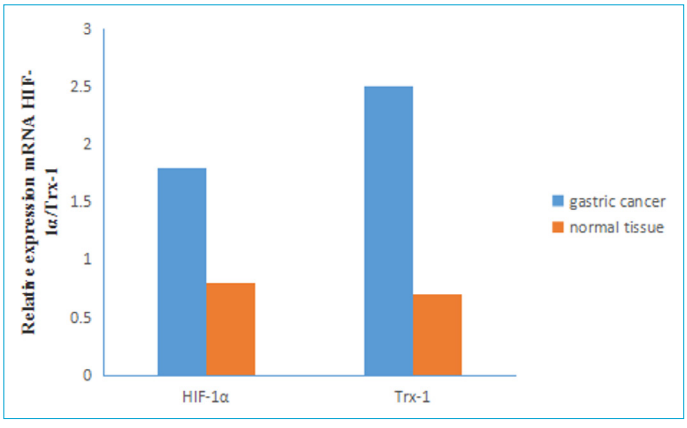

Figure 2. Expressions of HIF-1α and Trx-1 mRNA in gastric cancer tissues and normal gastric tissues.

The Cox proportional regression hazard model showed that Borrmann's classification, Trx-1, and HIF-1α were associated with postoperative survival (Table 3). Apparently, high expression of Trx-1 and HIF-1α was linked to poor prognosis for GC patients (Figs. 3, 4).

Discussion

In the present study, the expressions of HIF-1α and Trx-1 were significantly increased in gastric cancer tissues compared with normal gastric tissues. The HIF-1α expression was significantly associated with that of Trx-1, and we detected a positive correlation between the expression of Trx-1 in the 162 gastric cancer cases. The expression of HIF-1α significantly correlated with the depth of tumor invasion and lymph node metastasis. Likewise, enhanced Trx-1 expression correlated with Borrmann type, differentiation, and depth of tumor invasion.

Hypoxia is a common trait of solid tumors, and HIF-1α has important functions in the development of various tumors, especially solid tumors. A fast-growing tumor quickly outgrows its vascularization, which deprives the tumor cells of oxygen. This intra-tumor hypoxia inhibits the activity of prolyl hydroxylase domains, which can lead to stabilization and overexpression of HIF-1α, resulting in the upregulation of numerous genes, including vascular endothelial growth factor, phospholipase D2, survivin, etc.[16,17] In recent years,

Table 2. Univariate analysis of the correlation between clinicopathological factors and prognosis

	Median Survival	HR (95% CI)	Univariate p
Gender		1.062 (0.637-1.772)	0.818
Male	56.78		
Female	63.96		
Age		1.417 (0.867-2.317)	0.165
<=70	61.06		
>70	50.58		
Borrmann type		3.457 (2.072-5.770)	0.000
I+ II	67.05		
III+ IV	58.21		
Differentiation		2.087 (1.276-3.412)	0.003
Well /Moderately	66.12		
Poorly	51.74		
Depth of invasion (T)		2.222 (1.355-3.643)	0.002
T1-T3	67.16		
T4	50.35		
Lymph node metastasis (N)		1.889 (1.164-3.066)	0.010
N1/ N2	67.03		
N3	52.53		
Gastrectomy		1.563 (0.938-2.605)	0.087
Subtotal	61.78		
Total	56.97		
Tumor location		0.587 (0.363-0.950)	0.030
Middle third and Middle	56.56		
Lower third and whole	62.35		
Tumor size		0.808 (0.474-1.377)	0.432
<=5cm	64.92		
>5cm	56.55		
HIF-1α		0.576 (0.347-0.956)	0.000
Positive	43.62		
Negative	73.31		
Trx-1		0.165 (0.086-0.316)	0.000
Positive	44.29		
Negative	73.22		

Table 3. Multivariate analysis of the correlation between clinicopathological factors

Variables	B	S.E.	Wald	p	OR	95% CI	
						Lower	Upper
Borrmann type	0.881	0.295	8.905	.003	2.413	1.353	4.304
Differentiation	0.298	0.274	1.179	.278	1.347	0.787	2.304
T	0.348	0.317	1.205	.272	1.417	0.761	2.638
N	0.004	0.325	0.000	.991	0.996	0.527	1.885
Tumor location	0.470	0.266	3.125	.077	0.625	0.371	1.052
HIF-1α	0.822	0.354	5.392	.020	0.440	0.220	0.880
Trx-1	1.184	0.409	8.365	.004	0.306	0.137	0.683

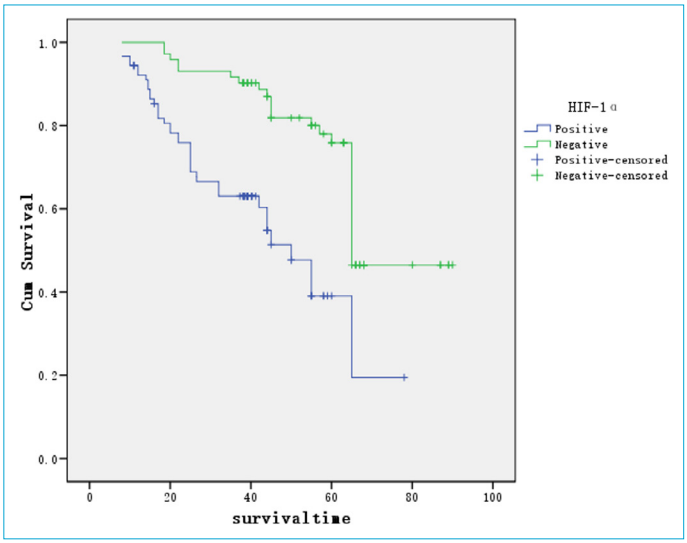

Figure 3. The Kaplan-Meier survival curves according to the level of HIF-1α expression in gastric cancer tissues.

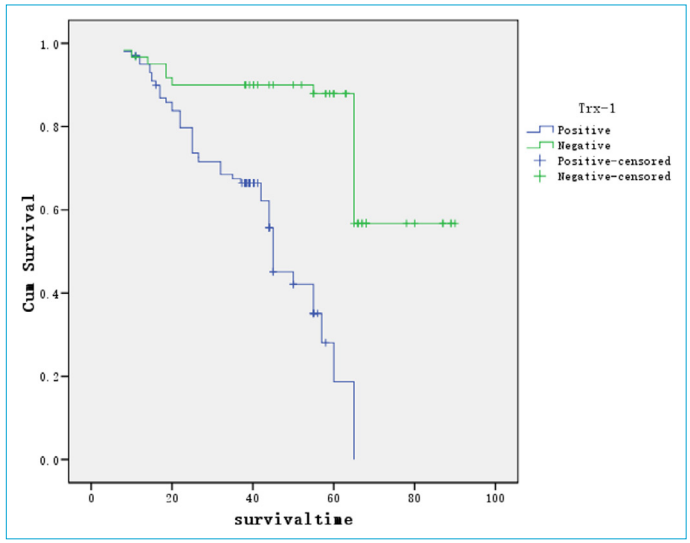

Figure 4. The Kaplan-Meier survival curves according to the level of Trx-1 expression in gastric cancer tissues.

the function of HIF-1α has been deeply probed, and overexpression of HIF-1α promotes the development of glioblastoma, gastric cancer, hepatocarcinoma, and colorectal cancer. There is much evidence that HIF-1α promotes a stem cell-like phenotype in many tumor cells.[18,19] Our results showed that HIF-1α mRNA and protein levels were significantly higher in tumor tissues than in normal tissues. The survival analysis also revealed a notably shorter survival time for GC patients with high HIF-1α levels compared with those with lower expression. Furthermore, increased HIF-1α expression also correlated with the depth of tumor invasion and lymph node metastasis. These results are corroborated by the multivariate Cox regression analysis and reveal that HIF-1α is a prognostic factor for GC.

It has been verified that Trx-1 plays a key role in cancer progression, metastasis, and multidrug resistance. It also plays an essential role in maintaining a reduced environment in the cells and protects against oxidative stress.[20] The loss of redox homeostasis is involved in the pathogenesis and development of many diseases, including GC. Although the increased expression of Trx-1 in various solid tumors has been reported, the prognostic significance of Trx-1 expression and function in human GC has not been extensively studied. Shang et al.[12] studied the high Trx-1 expression that predicted poor prognosis, and its expression was an independent prognostic factor for overall survival of GC patients. Knockdown of Trx-1 expression inhibited GC cell growth, migration, and invasion in vitro, as well as tumor growth and lung metastasis in vivo. Similarly, our results showed that both increased mRNA expression and protein expression of Trx-1 were associated with poor patient survival. We found that the mRNA expression of Trx-1 is increased in human GC tissues compared with the paired normal tissues. Patients with high Trx-1 mRNA expression levels had poor postoperative survival rates compared with patients with low Trx-1 mRNA expression levels. We also detected Trx-1 protein expression in 162 human GC tissues by immunohistochemistry. Kaplan-Meier analysis showed that patients with low expression levels of Trx-1 had longer overall survival than those with high expression levels. Increased Trx-1 expression was significantly correlated with aggressive clinicopathological characteristics, including Borrmann type, differentiation, and depth of tumor invasion. Moreover, multivariate analysis revealed that Trx-1 expression was an independent prognostic factor for the overall survival of GC patients.

Conclusion

The results of the present research suggest a significant role of Trx-1 and HIF-1α in the biology of gastric cancer. However, the mechanisms by which Trx-1 and HIF-1α mediate the regulation of GC progression need to be explored further, which may ultimately promote the development of new anti-cancer strategies. Therefore, large-scale further research is needed to elaborate on the specific pathway.

Disclosures

Ethics Committee Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Xuzhou Central Hospital.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – G.N.; Design – G.N.; Supervision – A.F. ; Materials – G.N.; Data collection and processing – G.N.; Analysis and interpretation – G.N.; Literature search – G.N.,; Writing – A.F.; Critical Review – G.N., A.F.

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