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Original Article

Presence and Interactions of *Entamoeba histolytica* Lectin and *p53* Protein in Colorectal Cancer

Leila Haghighi, *Abdolhossein Dalimi, Majid Pirestani, Fatemeh Ghaffarifar

Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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***Correspondence**
Email:
dalimi_a@modares.ac.ir

Abstract

Background: We aimed to analyze the presence of *Entamoeba histolytica* lectin light chain antigen and *p53* protein in colorectal cancer biopsies to uncover potential antigenic interactions between them.

Methods: Overall, 150 colorectal cancer biopsy samples were subjected to examination for the specific *E. histolytica* antigen and *p53* protein through the application of the immunohistochemical technique. To augment diagnostic precision, the western blotting method was employed and its results were compared with those obtained from immunohistochemistry (IHC).

Results: Among the 150 colorectal cancer biopsy samples examined, 100 cases tested positive for the *p53* protein, while 19 cases exhibited positivity for the *E. histolytica* light chain lectin antigen. Notably, only 14 cases demonstrated a positive presence for both the *p53* protein and the *E. histolytica* protozoan.

Conclusion: *Entamoeba histolytica*, a leading cause of parasitic infections, raises questions about its potential link to colorectal cancer, specifically in connection with the *p53* protein. The findings presented in this investigation signal the need for further research to comprehensively understand the implications of *E. histolytica* in colorectal cancer diagnosis and treatment strategies.

Introduction

Entamoeba histolytica globally ranks as the third leading cause of death from parasitic infections. Amoebiasis, a disease caused by this protozoan, remains pervasive in underdeveloped countries, claiming approximately 100,000 lives annually (1). Rosales-Encina and Kars (1987) reported the first isolation of a lectin from *E. histolytica*, a protein with the ability to agglutinate red blood cells (RBCs). This lectin displays specific recognition and binding to carbohydrates present on colon epithelial cells. (2). This lectin recogniz-

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es and binds to carbohydrates present in colon epithelial cells. Subsequent research highlighted its pivotal role as an adherence factor, enabling *E. histolytica* to bind to intestinal cells. The lectin, a carbohydrate-binding protein, is a distinguishing indicator of this protozoan from its subgroups, facilitating attachment to intestinal cells. *E. histolytica* lectin is recognized as an invasive factor, consisting of three subunits: heavy subunit (Hgl), light subunit (Lgl), and medium intermediate subunit (Igl) (2). Moreover, it can break down intestinal mucin polymerase by binding and secreting glycosides. As mucin on the intestinal cell surface decreases, the lectin attachment to intestinal epithelial cells strengthens, triggering the host's inflammatory response (3).

Lectin, a unique protein, specifically recognizes and binds to glycans on the cell surface without causing any change. In contrast, the sticky glycoproteins on the surface of bacteria, viruses, or parasites adhere to the host cells' surface modifications. This property enables *E. histolytica* to resist the complement system, while also aiding cell lysis through the activation of C5b-9 with the expression of CD59-like protein (4). Additionally, this protozoan can produce anti-inflammatory pentapeptides, acting as a monocyte inhibiting factor (MIF) (5). Consequently, Gal/GalNac lectin is considered a multifunctional protein (6). However, whether this lectin remains on the surface of IECs due to the immune system's effect is still unknown (7). This persistence contributes to the spread of inflammation, necrosis, and mega colon (8), prompting investigation into the antigenic structure's similarity with proteins on the surface of intestinal tumor cells, such as *p53*.Meanwhile, *p53*, frequently mutated in human cancers, plays a significant role in the adenoma-carcinoma transition (9). *E. histolytica* gene promoter sequences, exhibiting high homology to oli-*p53*, form complexes with nuclear proteins that are abrogated by oli-*p53*. *Ehp53* protein levels increase in UV-irradiated trophozoites.

However, O-GlcNAcylation changes between different tumor types lack a clear pattern(10). GlcNAcylation, a dynamic post-translational modification, involves the addition of N-acetyl glucosamine (11). Sugars are attached to serine and threonine residues in proteins by the enzyme O-Linked-N-acetyl glucosamine transferase (OGT), while the enzyme O-GlcNAcase (OGA) removes the modifications. It remains unclear how O-GlcNAc alterations in cells influence *E. histolytica*-induced cell death and which signaling molecules are involved in the deglycosylation process. Another study has shown that *E. histolytica* can bind to the *p53* protein and human DNA through its High-Mobility-Group Box (12). As mentioned, *E. histolytica* possesses Gal/GalNac lectin, a key factor in binding this protozoan to host cells. The pathogenic mechanism of *E. histolytica* and its cytolytic effect in host cells are not yet well understood. Notably, *E. histolytica* possesses a protein, Eh*p53*, similar to human *p53*, with the ability to bind to the human *p53* DNA sequence (oli-*p53*) (12). The potential impact of this protozoan on colorectal cancer incidence and its connection to *p53* remain largely unexplored.

We aimed to examine the presence of *E. histolytica* lectin light chain antigen and *p53* protein in colorectal cancer biopsies to identify any antigenic interaction between them.

Materials and Methods

Certificate of Medical Ethics

This study received approval from the ethics committee of Tarbiat Modares University, bearing the code number: IR.MODARES.REC.1402.004.

Sample Collection

Overall, 150 paraffin block biopsy samples of colorectal adenocarcinoma, sourced from Baqiyatollah al-Azam Hospital in Tehran, were subjected to immunohistochemical (IHC) staining to detect the presence of *E.*

histolytica antigen and *p53* protein. Sections measuring 3 to 5 microns were meticulously prepared on Poly-L-lysine slides.

For western blotting protein extraction, 15-micron sections of paraffin tissues were carefully placed in micro tubes, ensuring precision in the collection process.

Immunohistochemical staining

Immunohistochemical staining (13) was employed to assess the antigenic expression of *E. histolytica* lectin and *p53* protein. Following deparaffinization, dehydration, and peroxidase inhibition achieved with 3% hydrogen peroxide and methanol, slides underwent antigen retrieval in citrate buffer (pH 6) at 121°C under 15x pressure for 15 minutes in an autoclave. The slides were subsequently incubated with specific antibodies for light chain of *E. histolytica* (GeneTex, USA) and *p53* (Dako, Denmark), following a blocking step to enhance specificity. After the incubation with the secondary antibody (Envision; Dako, Denmark), reactions were visualized using diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin. This meticulous immunohistochemical staining protocol ensured the accurate detection and localization of *E. histolytica* lectin and *p53* protein within the biopsy samples.

Western blotting technique

To corroborate the findings obtained through IHC, the western blotting technique (14) was employed. Following deparaffinization, tissue lysates were prepared, and subsequent centrifugation facilitated the determination of protein concentrations using the Bradford method. Subsequently, SDS-PAGE electrophoresis separated the proteins, which were then transferred to PVDF membranes and probed with primary antibodies.

The visualization of proteins occurred post-incubation with secondary antibodies. The transfer of samples from the gel to paper was achieved through electric current. In the blocking step, a solution consisting of 2% non-fat dry milk in TBS-T buffer was applied to the paper to prevent non-specific reactions of the primary antibody. The blocking solution, comprising non-fat dry milk, was gently shaken on the PVDF paper for one hour and 15 minutes at room temperature.

Following the blocking period, the paper underwent incubation with a diluted β-actin primary antibody (sc-47778, 1:300) and *E. histolytica* lectin primary antibody (1:200) for 16 to 18 hours. Subsequent to this incubation, the paper was washed three times for 15 minutes each with TBS-T buffer. Anti-rabbit secondary antibodies at a concentration of 1:1000 for all primary antibodies were then applied to the paper, followed by another one-hour and 15-minute incubation at room temperature.

After this stage, the paper was washed three times for 15 minutes each with TBS-T buffer. Following blotting and the emergence of bands, the concentration of the extracted proteins was measured and normalized with the control sample. This rigorous western blotting protocol ensured the reliability and accuracy of the protein detection process.

Statistical analysis

The processed data were subjected to analysis using Microsoft Excel and the Statistical Package for the Social Sciences (SPSS) software, version 26 (IBM Corp., Armonk, NY, USA). To assess both statistical differences and agreement between *E. histolytica* lectin and *p53*, the McNemar test and kappa tests were employed. A significance level of $P<0.05$ was considered statistically significant, providing a robust framework for evaluating the observed associations and discrepancies between the two variables.

Results

Out of the 150 colorectal adenocarcinoma samples included in the study (73 women, 77

men), IHC revealed that 100 samples tested positive for the presence of the *p53* protein. Notably, 19 samples (12.67%) exhibited positive staining for *E. histolytica* lectin, with 14 of these samples co-expressing the *p53* protein (Table 1). These findings provide initial insights into the prevalence and co-occurrence of *E. histolytica* lectin and *p53* protein within the colorectal adenocarcinoma samples.

Table 1: The agreement between levels of *p53* variants and *Eh* -lectin in colorectal adenocarcinoma samples

Adenocarcinoma (No.=150)				
		Eh lectin		Total
		Positive	Negative	
p53	Positive	14	86	100
	Negative	5	45	50
Total		19	131	150

Confirmation of Positivity

All 19 samples that tested positive for *E. histolytica* lectin through IHC staining were further validated using the western blot method. This meticulous cross-verification revealed a remarkable 100% concordance between the two techniques, highlighting the robustness and reliability of the findings (Fig. 1, 2). This consistency fortifies the confidence in the detected presence of *E. histolytica* lectin within the colorectal adenocarcinoma samples.

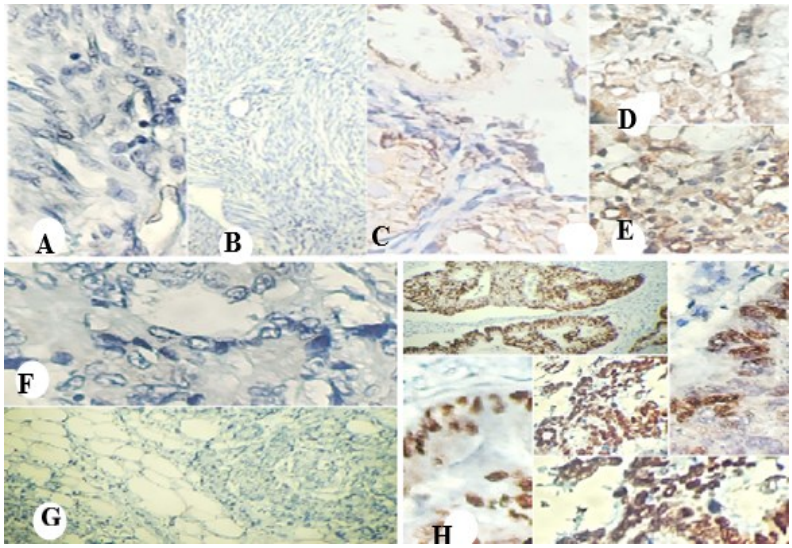

Fig. 1: IHC Staining Images
A, B: *E. histolytica* lectin negative staining images; C, D, and E: *E. histolytica* lectin positive staining images; F, G: Negative staining images for *p53*; H: Positive staining images for *p53*

The images in Fig. 2 illustrate the distinct staining patterns observed in the immunohistochemical analysis. Panels A and B represent negative staining for *E. histolytica* lectin, while panels C, D, and E showcase positive staining for *E. histolytica* lectin. Panels F and G display negative staining for *p53*, contrasting with panel H, which exhibits positive staining for *p53*. These visual representations offer a comprehensive view of the immunohisto-

chemical results, emphasizing the variability in staining between *E. histolytica* lectin and *p53*.

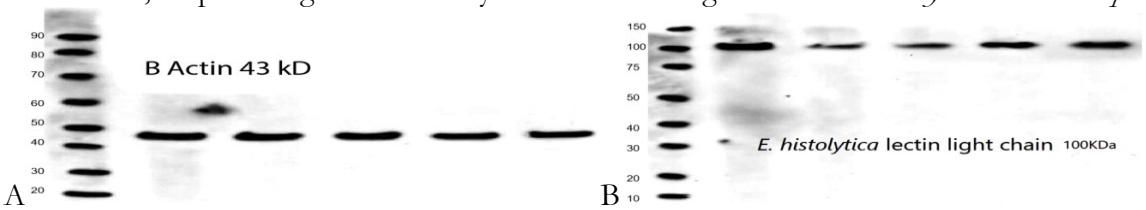

Fig. 2: Western blotting bands A) ß-actin 43KD; B) *E. histolytica lectin* 100KD

The combined analysis of immunohistochemical staining (Fig. 1), Western blotting (Fig. 2), and absorbance values (Table 2) collectively signifies the presence of the lectin light chain of the *E. histolytica* antigen within the colorectal adenocarcinoma samples. To enhance interpretability, a cutoff for absorbance values was determined at 0.68, designating values higher than this threshold as a positive indicator of the *E. histolytica* antigen.

In-depth insights into the relationship between *p53* expression levels and *E. histolytica* lectin are provided in Table 2. Significantly, a correlation was observed between these variables, reaching statistical significance at $P<0.05$. These findings contribute to a nuanced understanding of the interplay between *p53* and *E. histolytica* lectin expression in the context of colorectal adenocarcinoma.

Table 2: The amount of absorption obtained in terms of the lectin light chain of *E.histolytica* antigen in colorectal adenocarcinoma samples based on western blotting results.

Sample		Actin	*E.histolytica*		Ratio
Negative* (Mean)	1	10128.27	14740.7		0.687095
Positive	2	13172.50	14305.58		0.974051
Positive	3	13987.58	18023.72		0.802065
Positive	4	22104.55	23028.26		1.041788
Positive	5	17931.41	18475.79		1.030359
Positive	6	18084.12	25939.81		1.434397
Positive	7	15710.29	15010.48		1.046621
Positive	8	13434.45	14204.58		0.945783
Positive	9	16088.99	13988.65		1.150146
Positive	10	13170.45	13382.29		0.98417
Positive	11	16012.2	15505.46		1.032681
Positive	12	13141.85	12107.46		1.085434
Positive	13	14875.77	13274.51		1.120627
Positive	14	15394.87	11231.27		1.370715
Positive	15	8061.865	9985.267		0.807376

*** Values higher than 0.68, which is the average result of 131 negative cases, were considered as a cutoff.**

P53 positive (P53+) samples

Among the 100 samples that tested positive for *p53*, 14 samples also exhibited positive expression for *E. histolytica* lectin (*Eh*+). The disparity in *E. histolytica* lectin expression between these two groups was statistically significant, with a *P*-value less than 0.005. It is essential to note that this significant difference does not imply concordance between the *Eh*+ and *Eh*- samples within the P53+ group (Fig. 3).

P53 negative (P53-) samples

Within the subset of samples testing negative for *p53* (50 samples), 5 were identified as positive for *E. histolytica* lectin (*Eh*+). Conversely, 45 samples in this category did not express *E. histolytica* lectin (*Eh*-). The variation in *E. histolytica* lectin expression in this cohort was also statistically significant, with a *P*-value less than 0.005. This variation contradicts the expectation of a uniform antigenic structure in *Eh*- and *Eh*+ samples within the *p53*-negative group (Fig. 3).

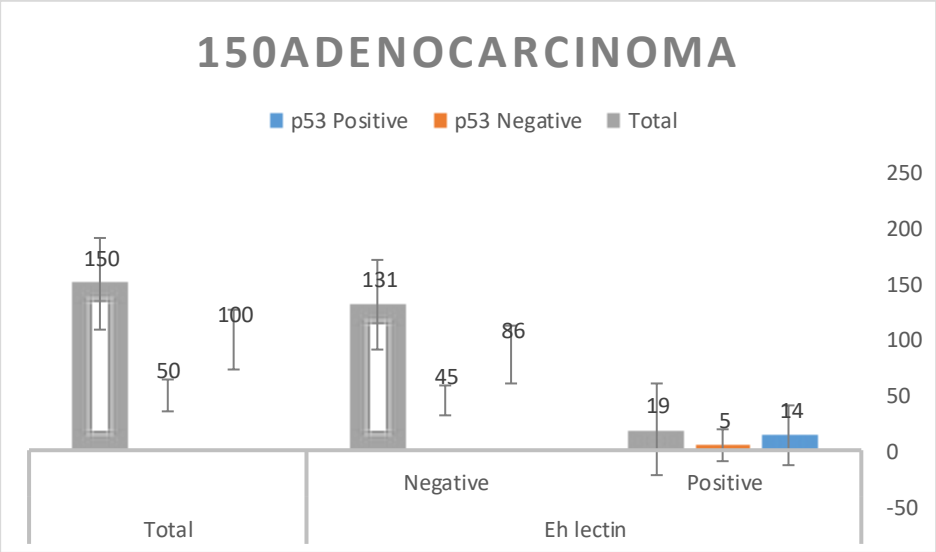

Fig. 3: 150 Adenocarcinoma Samples - Positive and Negative for *E. histolytica* Lectin Antigen in *P53* Positive and Negative States

The statistical analysis explored the differences and agreements between *P53* in both positive and negative states and *E. histolytica* (*Eh*) lectin in both positive and negative states. The McNemar test revealed a significant difference between *P53* and *Eh* lectin, with a p-value less than 0.001. The kappa test indicated a weak agreement (Kappa = 0.028) between *P53* and *Eh* lectin. These findings provide insights into the comparative expression patterns of *P53* and *E. histolytica* lectin in the examined adenocarcinoma samples.

Discussion

Amoebiasis, caused by *E. histolytica*, remains a considerable global health concern (15). Examining the significance of *p53* in cancer, particularly its role in maintaining genomic stability and acting as a tumor suppressor, provides a critical context. The introduction of Eh*p53*, a protozoan analog to mammalian *p53*, sets the stage for exploring potential antigenic similarities, aligning with the specific focus of our study.

Despite recent advancements in immunohistochemistry facilitating the detection of *E. histolytica* in fresh stool samples, challenges persist in diagnosing amebiasis, often requiring invasive procedures like colonoscopy and pathology-based assessments (16). Notably, the efficacy of certain diagnostic methods on paraffin-embedded samples remains a subject of investigation (17).

Earlier studies have hinted at potential antigenic mimicry between *E. histolytica* and tumoral cells in the colon. Notably, the immunohistochemical detection of *E. histolytica*

lectin antigen in colon adenocarcinoma samples has been reported (18). However, the exploration of antigenic similarity between *E. histolytica* and the tumor cell surface, particularly in comparison with the *p53* protein, has not been thoroughly examined (19).

Mutations in the *p53* gene are identified in a significant proportion of colorectal tumors. These mutations activated in response to DNA damage and oncogenic stress, *p53* regulates various cellular responses crucial for tumor suppression. It is widely recognized that *p53* plays a central role in growth, genome stability, aging, and tumor suppression. Mutations in the *p53* gene have been identified in 34% of proximal colon tumors and 45% of distal colorectal tumors. Activated by DNA damage and oncogenic stress, *p53* orchestrates a myriad of cellular responses essential for tumor suppression. The transcription factor *p53* induces the activation of MDM2, a primary E3 ubiquitin ligase targeting *p53* for proteasomal degradation, forming a negative feedback loop. DNA repair defects can induce genome instability, leading to cancer (20). The *p53* gene, mutated in over 50% of human cancers, is known as the "Guardian of the Genome" and "Guardian Angel Gene" for its critical role in genomic stability and tumor suppression through apoptosis induction, cell cycle arrest, and inhibition of angiogenesis (21).

The study introduces *Ehp53*, identified as the first *p53* analog in protozoans and speculated to be the evolutionary precursor of mammalian *p53*. The investigation aims to explore potential antigenic similarity between *E. histolytica* lectin and the *p53* protein in tumoral cells, presenting preliminary findings suggesting a tentative likeness (22).

Contrary to expectations, the study's results indicated that no discernible antigenic agreement between *E. histolytica* lectin and the *p53* protein in colorectal adenocarcinoma cells. The weak agreement observed is critically examined, and the potential reasons for the positive outcomes with western blotting and immunohistochemistry are highlighted.

According to the positive results in western blot and immunohistochemistry related to E. histolytica lectin light chain antigenic index, there is a need for further in-depth analysis. A potential role of a different monoclonal antibody, such as macrophage inhibitory factor (MIF), is suggested. Furthermore, the clinical implications of this antigen as a diagnostic biomarker, potentially influencing prognosis and therapeutic strategies, are suggested as avenues for future research.

There were no specific limitations that hindered the work.

Conclusion

Only 14/100 colorectal adenocarcinoma samples exhibited positivity for both the *p53* protein and the *E. histolytica* antigen. This observation suggests a modest likelihood of antigenic agreement between the lectin of *E. histolytica* and the *p53* antigen.

Further research is necessary to unravel the complexities of this relationship to provide potential insights for innovative diagnostic and therapeutic approaches. Suggesting continued research to comprehensively understand the effect of *E. histolytica* on colorectal cancer is crucial to advance our understanding of protozoan-cancer interactions in the scientific community.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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