Original Article

ANALYSIS OF DIFFERENTIAL EXPRESSION OF CLAUDIN-1 IN RELATION TO VEGF IN HEPG2 AND CACO-2 CELLS USING FLOW CYTOMETRY

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ABSTRACT

Background: The sustained polarity of epithelial cells, differentiation, and cell signaling are underpinned by Claudins, which are the principal proteins of tight junctions, essential for vital cell-to-cell adherence. Claudins play a significant role in both physiology and pathogenesis, as numerous studies have depicted their involvement in these processes. These proteins have an impact on normal body functions and disease development, including tumors. Due to their cell-specific expression, Claudins have been associated with the development of various tumors, suggesting their potential use as diagnostic or prognostic markers. It can be suggested that could serve to predict or diagnose the development of possible cancers. The objective of this study was to investigate the expression of Claudin-1 associated with VEGF in the liver and colon cancer cell lines namely HepG2 and Caco-2 respectively.

Materials and Methods: In-vitro study of Claudin-1 expression linked to VEGF in HepG2 and Caco-2 cell lines This study incorporates HepG2 and Caco-2 cell lines. Expression of Claudin-1 in HepG2 and Caco-2 cells was determined after flow cytometry analysis.

Results: Claudin-1 showed different expression patterns in HepG2 and Caco-2 cells underscoring its significant involvement in tumorigenesis and tumor progression.

Conclusion: The study evaluates the relationship between Claudin-1 and VEGF in cancer. It highlights Claudin-1 as a key indicator to identify and predict cancer. This might open doors to new treatment approaches.

Keywords: Biomarkers, Tumors, Claudins, Diagnosis, Cell lines, Epithelial Cells

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INTRODUCTION

Different types of claudin proteins make up tight junctions between cells where some members form closure into intercellular spaces and others create paracellular channels. Ions move through the pore pathway located at these channels. Among both tight junction development factors and structural elements, Claudin proteins create the paracellular channels that act as ion-selective pores, hence are required for barrier functionality. The leak route gets regulated by both Zonula occludens proteins (ZO) family members and essential membrane proteins with the MARVEL protein family (TAMP) controlling the leakage mechanism. The tight junction leak route is under stringent control through the ZO-1 actin-binding domain which operates through the peri-junctional actomyosin ring.[1]

The main claudin that creates the paracellular barrier at tight junctions in the epidermis is encoded by Claudin-1 (CLDN1), a gene that is believed to be a major contributor to human skin disorders.[2] The cellular entry of HCV depends primarily on four host determinants known as

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occludin (OCLN), claudin-1 (CLDN1), scavenger receptor class B type I (SR-BI) and cluster of differentiation 81 (CD81). Multiple host factors which confer cell susceptibility to HCV infection exist because their expression leads to infection readiness. CD81 and OCLN independently determine HCV tissue tropism in humans but the viral factors do not confer tissue tropism alone. The process of HCV attachment (attachment/binding factors) together with internalization/fusion (co-variables) depends on various host elements that go beyond these four fundamental entry components.[3]

All areas of study maintain a focus on creating protein-free media as a purification method and to secure biologically secure raw materials. The manufacturing costs and material production need continuous optimization especially during the development of viral vectors for gene therapy and recombinant proteins and antibodies synthesis. Cell culture methods for recombinant protein production must adapt to competing technology advances including those based on transgenic animal systems. All mammalian cells end up producing viruses as a natural result of gene therapy and vaccination development. The field of cultured mammalian cells has recently started using re-transplantation methods and investigators widely employ cultured cells for toxicity assessment.[4] The development of efficient mAb-based assays and diagnostic imaging techniques used to detect antigens and small chemicals from malignant cells will produce big improvements in modern cancer diagnostic medicine. The development of recombinant antigen synthesis combined with antibody creation methods has expanded mAb technology beyond its current state as an immature scientific field. Precise quantitative geographical and temporal measurements exist for disease diagnosis in mAb-based assays when compared to other alternative procedures.[5]

The defective tight junctions cause patients to experience volume loss and hypercalciuria leading to polyuria with polydipsia from excess sodium (Na^+) together with hypomagnesemia and its related symptoms and infantile nephrocalcinosis. Patients who have CLDN19 mutations show additional eye-related defects apart from their kidney symptoms. The poor renal projection affects both versions since chronic kidney disease typically requires renal replacement treatment during the patient's second or third life decade. Medical experts recognize nephrocalcinosis as a potential contributor to CKD (chronic kidney disease) development yet they have not identified its fundamental cause.[6]

Healthcare professionals identify major differences between malignant tumor blood vessel characteristics and those of regular blood arteries. Angiogenesis gets its main control from vascular endothelial growth factors also known as VEGFs. VEGFs use binding with vascular endothelial growth factor receptors (VEGFRs) to improve vascular permeability as well as vascular endothelial cell survival, migration, proliferation, tube formation and angiogenesis. The manifestation of VEGFs occurs because of three pathologic events including poor blood supply along with oxygen deprivation and malignant tumor growth. Secreted VEGFs enhance vascular permeability thus enabling plasma proteins to easily reach the extracellular matrix for aiding vasculogenesis while providing temporary support to entering endothelial cells.[7]

Cell analysis in solution becomes possible quickly through flow cytometry because of its multi-parametric abilities. The main function of lasers in flow cytometers involves the generation of scattered and fluorescence signals that photomultiplier tubes or photodiodes convert into readouts. A computer records the electrical signals derived from light impulses into data files. Flow cytometers use cell population testing by fluorescence and light scattering properties to conduct both evaluations and purification processes. Various fluorescent reagents serve purposes in flow cytometry systems. The system employs fluorescent antibodies as well as expression proteins and nucleic acid dyes as well as viability indicators and ion sensory agents.[8]

The basic flow cytometer contains four major components which include a fluid control

system that manages particle movements, an optical laser source, several capture components such as filters and detectors along with electronic data acquisition systems to analyze the results. The identification of cellular functions through fluorochrome-labeled probes along with natural cell detection has served as the primary basis for flow cytometry traditionally.[9]

MATERIALS AND METHODS

As it is done on cells study IRB is not required. Caco-2 and HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO_2. The cells were incubated for three days before further processing. Three cell treatment conditions were defined: untreated cells (control group), VEGF-treated cells, which received VEGF treatment for 48 hours at a concentration of 30 ng/ml, and VEGF + anti-VEGF antibody-treated cells, which received VEGF treatment (30 ng/ml) for 48 hours while also receiving an anti-VEGF monoclonal antibody (120 ng/ml for 48 hours). After adding 0.25%trypsin-EDTA (ethylenediaminetetraacetic acid) to the culture flasks, the cells were trypsinized and incubated at 37°C until they separated. The cell suspension was collected, and trypsin was neutralized with complete media. The cells were then centrifuged at 300 x g for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended in 1 ml of PBS (Phosphate Buffered Saline). Cells were counted using a hemocytometer. An aliquot of 10 µl of the cell suspension was mixed with an equal volume of trypan blue solution (0.4%), and 10 µl of the mixture was loaded onto a hemocytometer. Viable cells were counted under a light microscope, and the cell concentration was calculated. The counting was done by laboratory personnel prior to staining. Cells were prepared for staining using an indirect staining technique to identify protein expression.[10,11]

Aliquots of 1 x 10^6 cells were taken for each condition. Cells were labeled as untreated cells, VEGF-treated cells, and VEGF + anti-VEGF antibody-treated cells. Negative and positive control samples were also prepared. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, then washed twice with PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 minutes and washed again. Primary antibodies against Claudin-1 (rabbit anti-Claudin-1) and VEGF (mouse anti-VEGF) were added to the experimental samples and incubated for 1 hour at 4°C. Cells were washed three times with PBS and incubated with fluorophore-conjugated secondary antibodies for 30 minutes at 4°C in the dark: Anti-rabbit IgG Alexa Fluor 488 (green channel) for Claudin-1, Anti-mouse IgG Alexa Fluor 594 (red channel) for VEGF. Following staining, cells were washed, resuspended in 500 µl of PBS, and stored at 4°C until analysis.[12] Cells were analyzed using fluorescence-activated cell sorting by BD FACS Canto II (Becton, Dickinson and Company - BD Biosciences). The samples were run on a flow cytometer with appropriate settings for detecting Alexa Fluor 488 (green) and Alexa Fluor 594 (red). Data acquisition and analysis were conducted using BD FACS Diva Software.

RESULTS

Three distinct samples—untreated, VEGF-treated, and VEGF in the presence of monoclonal anti-VEGF-treated cells—were subjected to flow cytometric analysis of HepG2 and Caco-2 cells. The degree of Claudin-1 expression in connection to VEGF varied, according to the data. Relative Fluorescent Units (RFU) were used to express the values. The FACS machine was used to get all of the values, including those for the positive and negative controls. Both HepG2 and Caco-2 cells exhibit elevated Claudin-1 expression when used as positive controls. Green fluorescence, as detected by the FACS machine, revealed this (refer to table 1). Comparatively less fluorescence was seen in negative controls. The outcomes of untreated HepG2 and Caco-2 cell

samples were nearly identical. The values were essentially the same when VEGF was administered to both cells. In contrast to VEGF in the presence of monoclonal anti-VEGF antibody-treated HepG2 cells, VEGF in the presence of monoclonal anti-VEGF antibody-treated Caco-2 cells exhibited a higher fluorescence value and, consequently, higher manifestation of Claudin-1.

Table 1: Shows the average of value of fluorescence and therefore the average Claudin-1 expression in the HepG2 and Caco-2 Cells

Samples	Relative Fluorescent Unit (RFU)	Average
Positive HepG2 Cells	1029, 1004, 963	998.7
Positive Caco-2 Cells	1006,1017,1073	1032
Negative HepG2 Cells	244.1, 264, 198	235.4
Negative Caco-2 Cells	104,101,123	109.3
Untreated HepG2 Cells	507.4,511,578, 446,478,564	514.1
Untreated Caco-2 Cells	514.6,532.2,567, 553,499,561	537.8
VEGF HepG2 Cells	732,741,766,843,900.3, 774,737,812,754	784.4
VEGF Caco-2 Cells	831,813,821.5,881,772, 668,737,699,772	777.2
VEGF+AB HepG2 Cells	307,312,344,216, 294.2,319	298.7
VEGF+AB Caco-2 Cells	416.9,400,338.8, 418.9,373,330	379.6

Relative fluorescence averages for each of the samples are displayed. From each sample's three experimental readings, the average value has been determined (refer to table 1).

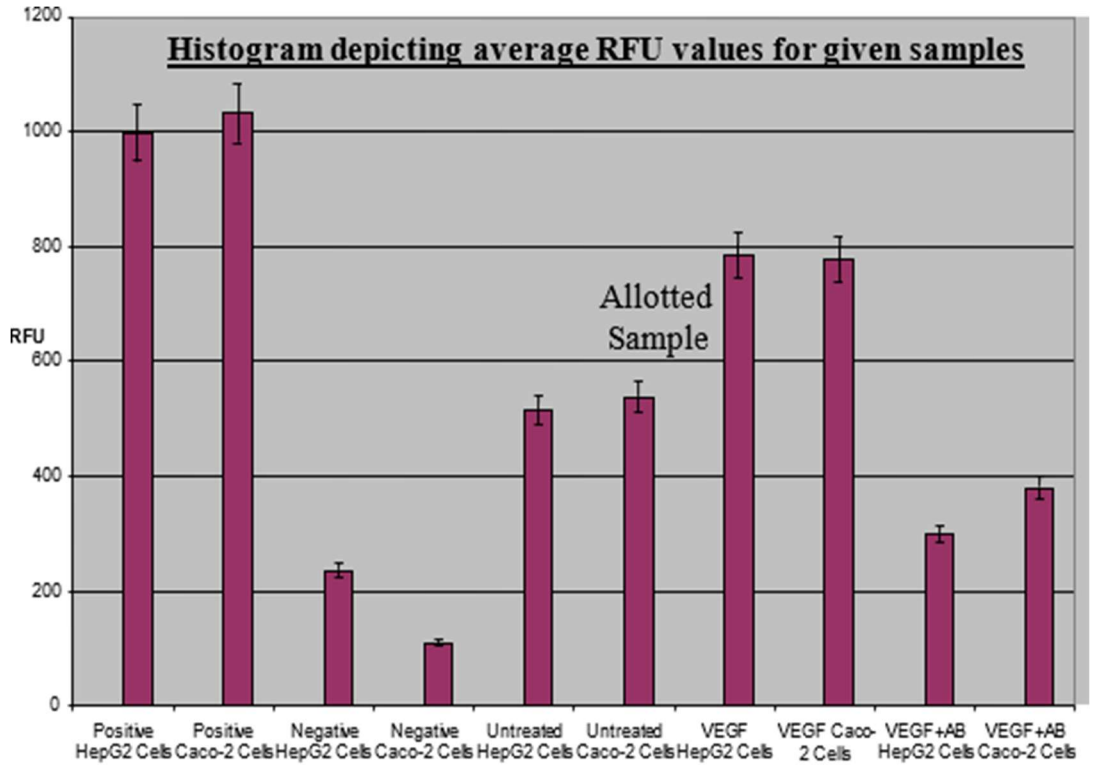
Table 2: Comparison of fluorescence values in Untreated Caco-2 Cells against Positive and Negative Control

Samples	Relative Fluorescent Unit (RFU)	Average
Negative Control (Caco-2 Cells)	104,101,123	109.3
Positive Control (Caco-2 Cells)	1006,1017,1073	1032
Untreated Caco-2 Cells	**553,499,561**	**537.7**

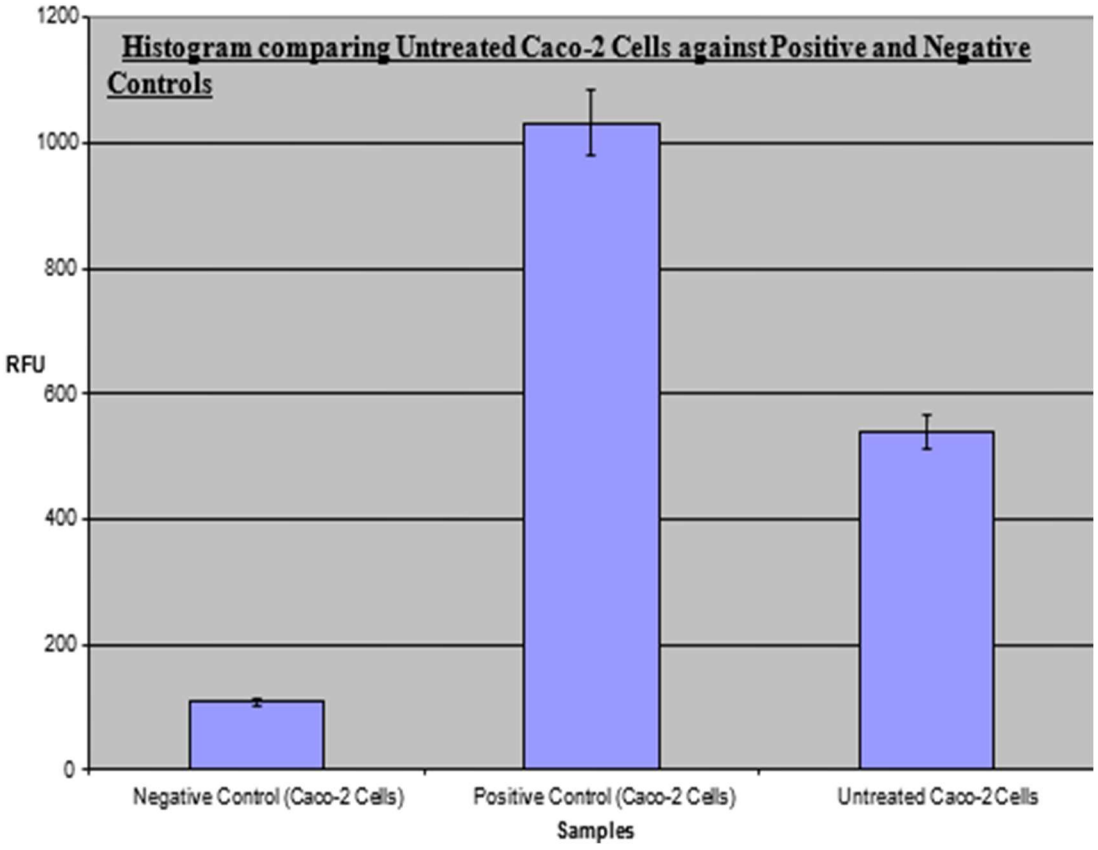
Fluorescence levels of the designated experimental sample, which includes both positive and negative controls and untreated Caco-2 cells. Untreated Caco-2 cells exhibit optimal claudin-1 protein expression when their average relative fluorescence value is lower than that of the positive control (refer to table 2).

The RFU value of VEGF-treated cells (both HepG2 and Caco-2 cells) significantly increases, suggesting that Vascular Endothelial Growth Factor is up-regulated. HepG2 and Caco-2 untreated cells exhibit respectable fluorescence levels (although lower than those of VEGF-treated samples), indicating claudin-1 expression (Graph 1).

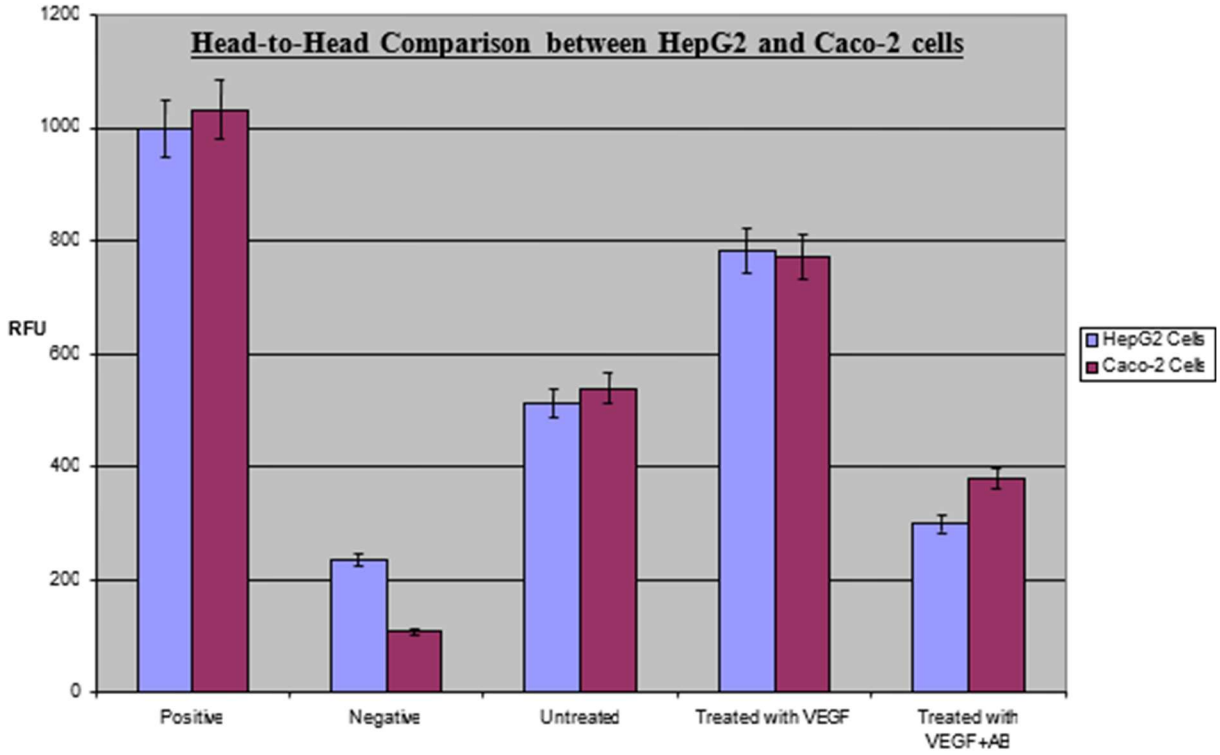
Untreated Caco-2 cells have demonstrated a significant fluorescence value when compared to both positive and negative controls.(Graph 2). HepG2 and Caco-2 cells, along with their corresponding positive and negative controls, have been directly compared (Graph 3). The RFU value for Caco-2 cells under positive control is somewhat higher than that of HepG2 cells. In the negative control, HepG2 cells exhibit greater fluorescence than Caco-2 cells, indicating higher claudin-1 expression. Compared to HepG2 cells, untreated Caco-2 cells express claudin-1 at a comparatively higher level. Both cell types exhibit nearly comparable fluorescence levels in untreated, VEGF-treated, and VEGF + Anti-VEGF monoclonal antibody-treated samples, with only minor differences in claudin-1 expression and VEGF regulation.



Graph 1: Indicating average values of fluorescence in untreated, VEGF treated and VEGF + antibody treated HepG2 and Caco-2 cells



Graph 2: Comparing the fluorescence value of untreated Caco-2 cells against positive and negative control



Graph 3: Direct Comparison between all samples of HepG2 and Caco-2 along with Controls

DISCUSSION

The human body protects itself from chemical entry through the protective function of the gut barrier. The proper functioning of this barrier preserves organisms at equilibrium state. The proteins known as claudins enable strong cellular bond formations between epithelial cells. The failure of the intestinal barrier worsens both celiac disease and inflammatory bowel disease along with other gastrointestinal disorders. Physiological disruptions of epithelial connectivity along with permeability reakdown from these pathogens cause numerous pathological diseases which potentially lead to cancer development. Tight junctions that connect cells together are formed through the proteins which belong to the claudin family.[13] Scientific studies have extensively researched CLDN-1 as the primary claudin protein because researchers have observed links between cancer cell initiation and spread through this protein. Scientific research shows that this particular protein suppresses cancer development in select cancer types. CLDN-1 produces tumor-promoting or tumor-suppressive activities as an individual factor or in combination with multiple molecules. Cancer progression is determined by CLDN-1 transport between cell membranes and cytoplasm as well as the nucleus. CLDN-1 engages many signaling pathways which represent an essential component in its overall significance.[14] Every HepG2 and Caco-2 cell sample that was sent to each group, together with the positive and negative controls for both cancer cell types, had its fluorescence levels measured using a FACS machine, yielding three readings for each sample. All of the readings were fairly close to each other within the experimental error range. Cancer invasion and metastasis require claudins to perform the functions of ion exchange and cell motility alongside epithelial-to-mesenchymal transition Scientists have started using claudins as

therapeutic targets to improve the outcomes of cancer patients. Scientists have proven that CLDN expression modifications occur with NA methylation DNA changes. DNA hypermethylation causes CLDN1 and CLDN7 downregulation in breast cancer cells as well as CLDN11 downregulation in gastric cancer cells. Colon cancer cells were shown to use histone-deacetylase enzymes for modifying mRNA stability which affects CLDN1 expression.[15] Mucosal tissue preservation requires the presence of Claudin-1 for proper physiological operations. Various research links claudin-1 to colorectal cancer yet the predictive significance remains questionable. This meta-analysis assessed the clinical significance along with predictive value of claudin-1 in CRC evaluation.[16] There is a remarkable boost in the proliferation of colon cancer cells caused by upregulation and desensitization of claudin-1 following EGFR/PKC/CLDN1 signaling pathway.[17] It has been reported that signaling pathways resulting in the invasion and migration of cells involve claudin-1.[18] The untreated samples displayed some fluorescence in the green channel. Auto-fluorescence can be assumed because there was no antibody present. Using the FACS apparatus, particular fluorescence patterns were seen following the samples' probing with fluorescent antibodies. Compared to Caco-2 stomach cancer cells, HepG2 liver cancer cells exhibited more auto-fluorescence. Green positive control cells at the time showed undeniable amounts of Claudin-1 articulation. Auto-fluorescence was unquestionably present in the fluorescence of the negative control cells. HepG2 and Caco-2 cells treated with VEGF exhibit remarkable fluorescence, according to earlier research (graph 1). Hepatocellular polarity and tight junction integrity are regulated by VEGF. The concept that cytokine or growth factor-induced alterations in hepatocyte permeability will promote HCV entry is supported by the fact that hepatoma polarization restricts the availability of basolaterally expressed tight junction protein viral receptors. Without a doubt, the VEGF-treated HepG2 significantly raised the HCV level.[19] In cancer, a proangiogenic state arises from an imbalance of stimulatory and inhibitory elements that seem to be driving the move to angiogenesis. The result of somewhat insufficiently blood supplied hyperplasia turning into an uncontrolled new vascular development is the advance of malignant tumors. When this balance is perturbed, angiogenesis increases and tumor growth gets out of control. Angiogenesis is strongly controlled by vascular endothelial growth factor A (VEGFA) whether one is healthy or suffering. Proangiogenic imbalance often develops at the gene level from the activation of oncogenes or the inactivation of tumor suppressor genes to cell environmental variables like hypoxia, hypoglycemia, cellular nutritional deficiencies, and metabolic acidosis.[20] The average fluorescence value and, thus, the average Claudin-1 expression in each cell sample showed fairly similar results. The auto-fluorescence phenomenon was illustrated by comparison with the positive and negative controls. Compared to Caco-2 cells, HepG2 cells showed a significantly higher level of autofluorescence.

CONCLUSION

This study discussed the distinct expression patterns of claudins in HepG2 and Caco-2 cells, in relation to VEGF, affirm their potential as diagnostic and prognostic markers for various tumors. This study has paved the way for future investigations into targeted therapies and precision medicine approaches that leverage claudins as biomarkers.

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ETHICAL APPROVAL

The conducted research is not related to either human or animal use.

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CONFLICT OF INTEREST

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

AUTHOR's CONTRIBUTION

SA: Study Design, Analysis and Results Interpretation, Manuscript Writing
MR: Data Collection, Manuscript Writing
AH: Conceptualization, Critical Review
CM: Critical Review

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