Journal of Cancer Science and Research

Ahmad et al., J Can Sci Res 2016, 3:S2
DOI: 10.4172/2576-1447.1000S2-003

Review Article Open Access

# Decreased Expression of Claudin 1, 3, 4, 5 and 7: A New Prognostic Marker in Colon Carcinoma

**Abrar Ahmad**[1], **Rahel Befekadu**[2], **Shlear Askari**[3] **and Victoria Hahn-Strömberg**[2,4*]

[1]*Department of Clinical Medicine, Örebro University, Örebro, Sweden*

[2]*Department of Laboratory Medicine, Section for Transfusion Medicine, Örebro University Hospital, Örebro, Sweden*

[3]*Department of Laboratory Medicine, Section for Clinical Chemistry, Örebro University Hospital, Örebro, Sweden*

[4]*Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden*

[*]**Corresponding author:** Victoria Hahn-Strömberg, Senior Investigator, Department of Clinical Medicine, Orebro University, Örebro, Sweden, Tel: +46723281284; E-mail: victoriastromberg@hotmail.com

**Received date:** April 07, 2016; **Accepted date:** May 03, 2016; **Published date:** May 11, 2016



## Abstract

**Background and objective:** Claudins (CLDNs) are members of a large family of tissue specific adherent proteins which have been suggested as tumor markers. In this study we analyzed the protein expression of CLDN 1, 3, 4, 5 and 7, their clinical significance and association with tumor growth pattern in colon carcinoma.

**Methods:** Immunohistochemical staining was used to detect the expression of CLDN 1, 3, 4, 5 and 7 in samples diagnosed with colon carcinoma as well as the adjacent normal mucosa. Complexity Index (CI) was calculated using images of cytokeratin-8 stained slides of the tumours using Photoshop CS, Fovea Pro, and Image J computer programs. The results from the Immunohistochemistry (IHC) and CI were correlated to clinicopathological parameters as well as 5-years survival of the patients diagnosed with colon carcinoma.

**Results:** Significantly high staining intensity was observed in normal mucosa as compared to colon cancer tissue for CLDN 4 (p=0.031) and 7 (p=0.011) and number of stained cells for CLDN 4 (p=0.001). A significant association was also observed between weaker expression at the invasive front of the tumor tissue and heterogenous expression of CLDN 1 (p=0.000), CLDN 3 (p=0.003), CLDN 5 (p=0.001) and CLDN 7 (p=0.000). There was no significant correlation between the expression of CLDNs, CI and clinicopathological parameters. Similarly, no significant association was found between CLDN expression and 5-years survival of the patients diagnosed with colon carcinoma.

**Conclusion:** Altered expression of CLDN 1, 3, 4, 5 and 7 in colon carcinoma cells may play a promoting role in colon carcinoma development and is inversely proprtional to higher expression at invasive front of the tumor. CLDN protein expression can be used as a tumor marker since the expression generally is weaker in tumor tissue compared to the normal mucosa.

**Keywords:** Tight junctions; Complexity index; Immunohistochemistry; Heterogenous expression

## Abbreviations:

CLDN: Claudin; IHC: Immunohistochemistry; CI: Complexity Index; TJ: Tight Junction; FFPE: Formalin Fixed Paraffin-Embedded.

## Introduction

Intestinal epithelium cells are joined together to form Tight Junction (TJ) complexes which control the paracellular diffusion between the cells. These complexes play an important role in maintaining the concentration differences of small molecules across epithelial cell sheets by sealing the plasma membranes of adjacent cells at the apical surface. This results in the creation of a continuous impermeable or semi permeable barrier for diffusion across the cell sheets [1-3].

The CLDN name originates from the Latin word "claudere" ("to close"), which indicates the barrier role of those proteins. This family of proteins consists of 27 known CLDN members with a size ranging from 20-27 kDa [4,5]. They are essential for the formation of TJs between epithelial cells together with other adherent proteins called occludins [6]. Several studies have reported altered expression of CLDNs as prognostic marker in various human cancers such as tumors of the gastrointestinal tract, renal, ovary, pancreas, breast, melanoma, liver, lung, uterus and prostate [6,7].

A study by Bello, et al. shows up regulation of CLDN 3 and CLDN 4 in ovarian cancer as well as high expression of CLDN 7 as an early event in carcinoma of the tongue [8]. Furthermore, CLDN 1 has also been found to be down regulated in colorectal carcinoma suggesting a probable target of beta catenin/Tcf signalling [1]. According to previous studies, some reports have presented decreased expression of CLDN 5 in hepatocellular and renal carcinomas [8-10].

There are relatively few reports on the protein expression of CLDNs in colon carcinoma and most of the studies have determined the expression of one or two CLDNs [11-13]. We aimed this study to evaluate the expression and significance of CLDN 1, 3, 4, 5 and 7

proteins in paired normal and tumor tissue samples from 61 patients diagnosed with colon carcinoma and to uncover any possible association between CLDN expression and growth pattern of tumor.

To analyze a tumor, its size and growth pattern are important variables as tumor progression in surrounding tissues shows different patterns depending upon the, invasive margin, number and distribution of tumor cells. Infilterative and expensive are two different categories of tumor growth in which former has irregular and coarse invasive front and considered as responsible for worst prognosis [14,15]. Multiple scoring systems have been introduced to describe the tumor growth in different carcinomas [16]. In 2008, a computer software based technique was introduced by Franzen and Hahn-Strömberg in which the tumor invasive front was graded quantitatively from 1-5, called CI where 1 represents the smooth and regular invasive front while tumors with 5 CI score have highly irregular invasive front with separated tumor cells and clusters [17]. This classification was based upon the fractal dimensions, and number of tumor cells. The proteins, which are involved in the intercellular adhesions are important in maintaining the morphology of the tumor and affect the invasion and metastasis [18,19]. Being a part of TJ complex, CLDNs have a significant role in cell adhesion [20]. So we hypothesize that there is a correlation between tumor progression, tumor growth pattern and adhesive protein expression.

The aim of our study was to compare the expression patterns of 5 different CLDN proteins (CLDN 1,3,4,5 and 7) in normal and paired human colon carcinoma tissue samples and to correlate these results with 5-year survival data of the patients, growth pattern of tumor and clinicopathological parameters of the patients diagnosed with colon carcinoma.

## Material and Methods

### Sample selection

From the pathology archives, 61 samples diagnosed with colon carcinoma from 2000 to 2009 at Örebro University Hospital were randomly selected. The age of the selected patients at diagnosis varied from 51 to 90 years for both men and women. Equal numbers of control samples were selected from the same patients to compare with tumor samples. Rectal carcinoma samples were excluded since these are often treated with radiotherapy prior to surgery which may result in altered morphologic characteristics. Uppsala University Ethical Committee, Uppsala, Sweden, approved this study.

### Immunohistochemistry (IHC) staining

4 µm thick sections were cut from Formalin Fixed Paraffin-Embedded tissue (FFPE) blocks using a Leica microtome (Buffalo Grove, USA) and the sections were placed on super-frost slides and incubated for 60 min at 62°C. Sections were pretreated for 1 hour and 20 min with EDTA buffer saline solution pH of 9.3 at 97°C.

Primary antibodies anti- CLDN 1 and anti- CLDN 7 were purchased from Invitrogen (California, USA) and anti- CLDN-3,-4, and 5 from Abcam (Cambridge, UK). All antibodies were polyclonal rabbit primary antibodies designed specifically for IHC of FFPE tissue. IHC staining was performed according to manufacturer's protocol (Dako cytomation) with incubation of the primary antibodies for 30 minutes at room temperature with working dilutions of 1:200, 1:400, 1:300, 1:200 and 1.1000 for anti- CLDN 1, 3, 4, 5 and 7 respectively. Staining was performed using an Autostainer from Dako (Dako Produktionsvej, Glostrup, Denmark).

### Scoring of slides

Slides were scored in accordance with earlier publications on intensity of staining as well as number of stained cells, with intensity scores of 0, 1, 2 and 3 where 0=no staining, 1=weak, 2=moderate and 3=strong staining. The number of stained cells was scored accordingly with grade 0 <10%, grade 1=10-50%, grade 2=50-80% and grade 3>80% stained cells.

### Complexity Index (CI)

40 samples from the patients diagnosed with colon carcinoma were selected randomly for computer image analysis from the same samples used for CLDN expression study. Sectioning, staining, and image processing was performed using the same method described by Franzen, et al. [1,17]. In short, images from the invasive front of the tumor area were taken by using a Leica DC200 digital camera mounted on a Leica DMRXE microscope with a 10X objective lens (Leica Microsystems GmbH, Wetzlar, Germany). From each specimen on average of 8 (4-12) images were captured for every tumor specimen. The number of images depended upon the length of the tumor-stromal area. Images were thresholded into white and black where black was the tumor area with white background. The black area was then removed so that only the outline of the tumor was left. The number of tumor cells, tumor cell clusters and fractal dimensions were calculated using the tumor outline image. The results were translated into a CI value ranging from 1-5.

### Statistical analysis

Continuous variables were measured by mean and standard deviation and categorical variables by frequencies. Significance of association between colon carcinoma and adjacent normal tissue CLDN proteins expression was measured statistically by McNemar test. To determine any statistical association between expression of CLDNs in colon cancer tissue and possible explanatory variable, Pearson's Chi-squared and Fisher exact test were applied appropriately. To determine a correlation between CI, CLDN expression and clinicopathological parameters, Spearman's correlation coefficient (Rho) test was used. Pearson's Chi-squared test was used to observe heterogeneity in expression. Kaplan Mayer's test was performed to determine any association between CLDN expression and 5-years survival of the patients. Two-sided p-value of ≤0.05 was taken as significant. SPSS version 20 (SPSS Inc., Chicago, IL, USA) and Stata (version SE11.1) were used for statistical analysis.

## Results

### Clinicopathologic study

To determine the protein expression of CLDN 1, 3, 4, 5 and CLDN 7 in colon cancer and in adjacent normal mucosa tissue samples, a random selection of 61 patients' samples were analyzed using IHC. The age of the patients ranged from 51 to 90 years. There were 34 samples from men and 27 from women. 34 of the tumor samples were from right colon and 27 from the left side of colon. Different clinical and pathological parameters (gender, tumor wall penetration (T), lymph noded metastasis (N), distant metastasis (M), Dukes stages,

differentiation and localisation of the patient tumors) are provided in Table 1.

| Characteristics | Number (%) |
|---|---|
| **Gender** | |
| Male | 34 (56%) |
| Female | 27 (44%) |
| **T Stages** | |
| T1 | 1 (1%) |
| T2 | 22 (36%) |
| T3 | 32 (52%) |
| T4 | 7 (11%) |
| **LN metastasis** | |
| No | 38 (63%) |
| N1 | 10 (16%) |
| N2 | 13 (21%) |
| **Distant metastasis** | |
| Mx | 17 (27.8%) |
| M0 | 38 (62.3%) |
| M1 | 6 (9.8%) |
| **Differentiation** | |
| High | 6 (10%) |
| Med | 37 (60%) |
| Low | 18 (30%) |
| **Dukes** | |
| A | 14 (23%) |
| B | 23 (38%) |
| C+D | 24 (39%) |
| **Localisation** | |
| Right colon | 34 |
| Left colon | 27 |
| LN metastasis: Lymph node metastasis | |

**Table 1:** Clinical and pathological characteristics of the patient data.

## Immunohistochemical study

Differential expression of CLDN 1, 3, 4, 5 and 7 between normal, highly differentiated and moderately differentiated tumor tissue cells are shown in Figure 1.

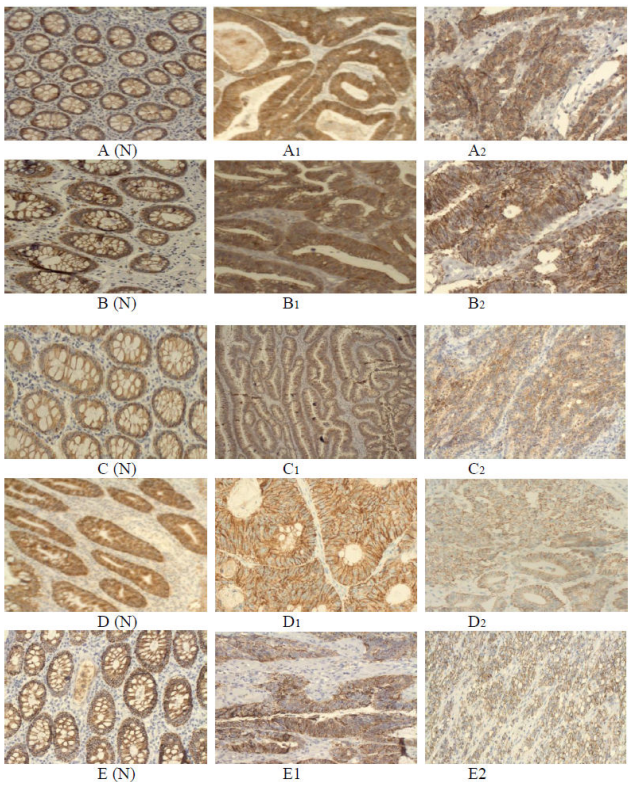


**Figure 1:** IHC staining for claudin 1 (A), claudin 3 (B), Claudin 4(C), Claudin 5 (D), Claudin 7(E) and Normal mucosa (N). Intensity score strong can be seen in A1-E1 and intensity score moderate can be seen in A2-E2.

Both the tumor and adjacent normal colonic mucosa were separately scored. The normal colon mucosa showed high staining intensity for all of these five CLDNs. The numbers of samples with cytoplasmic CLDNs staining were very low, only two cases showed protein expression for CLDN 1, 4 and 7 and one case for CLDN 3 and 5. All samples with cytoplasmic expression were from tumor tissue and were at T4 stage.

All the studied CLDN proteins show moderate to high staining intensity for normal as well as tumor samples (Table 2), however there was a significant difference in staining intensity between tumor and normal tissues for CLDN 4 (p=0.031) and 7 (p=0.011).

| Claudin | Type of tissue | Number of stained cells | | | Intensity of staining | | |
|---|---|---|---|---|---|---|---|
| | | High (N) | Medium (N) | p-value | High (N) | Medium (N) | p-value |
| Claudin 1 | Tumor | 32 | 29 | 0.131 | 25 | 36 | 0.582 |
| | Normal | 59 | 2 | | 39 | 22 | |
| Claudin 3 | Tumor | 37 | 24 | 0.963 | 28 | 33 | 0.803 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Normal | 51 | 10 | | 53 | 8 | |
| Claudin 4 | Tumor | 47 | 14 | 0.001 | 31 | 30 | 0.031 |
| | Normal | 52 | 9 | | 46 | 15 | |
| Claudin 5 | Tumor | 49 | 12 | 0.052 | 35 | 26 | 0.739 |
| | Normal | 40 | 21 | | 58 | 3 | |
| Claudin 7 | Tumor | 55 | 6 | 0.635 | 39 | 22 | 0.011 |
| | Normal | 59 | 2 | | 55 | 6 | |
| High=High expression, Medium= Medium expression, N= Number of samples | | | | | | | |

**Table 2:** Claudin expression in normal vs tumor samples from the patients diagnosed with colon carcinoma.

Staining intensities of CLDN 1, 3, 4, 5 and 7 expressions in tumor tissues were also compared with different clinical and pathological variables (Table 1). For the tumor wall penetration, proportion of tumors with lower CLDN 1, 4, 5 and 7 expressions were higher at T3+T4 stages then with higher expression, but CLDN 3 expression was observed reverse. In case of lymph node metastasis, variable expression was observed for different CLDNs such as, for CLDN 3, 4 and 5 proportion of tumor with lower expression and lymph node metastasis was comparatively higher to increased expression and lymph node metastasis, but opposite results were found for CLDN 1 and 7. Similar results were observed between CLDNs expression and other clinicopathological parameters such distant metastasis, Duke Stages and differentiation. However, in either case, none of the parameter was significantly associated with either CLDN that we determined in current project. Though, a trend was observed between CLDN 3 expressions and medium tumor differentiation (p=0.087), and also between CLDN 5 and lymph node metastasis (p=0.088).

We also compared the number of stained cells between normal and tumor samples. Only CLDN 4 has significantly low number of stained cells in tumor samples as compared with normal samples (p=0.001), while an opposite trend was observed for CLDN 5 (p=0.052). No significant association could be recognized for number of stained cells between tumor and normal tissues in CLDN 1, 3 and 7 (p>0.05) (results not shown).

Additionally, CLDN 1, 3, 4, 5 and 7 were further studied for heterogeneous expression, weak CLDN expression at invasive border and cell membranes of the tumor cells. Heterogeneity of CLDN expression was compared with clinicopathological parameters of the patients (TNM, tumor differentiation), weaker expression of CLDNs at membrane and invasive front of tumor to find any significant association. Heterogeneity in expression was significantly associated with weak expression at invasive border of tumor for CLDN 1 (p<0.001), CLDN 3 (p=0.003), CLDN 4 (p=0.026), CLDN 5 (p=0.001) and CLDN 7 (p<0.001) (Table 3).

| **Claudin** | **p-value** | | | | | |
|---|---|---|---|---|---|---|
| | **Weak exp inv front** | **Weak memb exp** | **T** | **N** | **M** | **Differentiation** |
| Claudin 1 | 0 | 0.845 | 0.453 | 0.73 | 0.953 | 0.635 |
| Claudin 2 | 0.003 | 0.717 | 0.244 | 0.479 | 0.903 | 0.811 |
| Claudin 4 | 0.026 | 0.905 | 0.244 | 0.479 | 0.903 | 0.398 |
| Claudin 5 | 0.001 | 0.264 | 0.453 | 0.73 | 0.953 | 0.635 |
| Claudin 7 | 0 | 0.462 | 0.453 | 0.73 | 0.953 | 0.635 |
| Weak exp inv frornt= weak expression at invasive border of the tumor<br>Weak memb exp= weak expression at cell membrane | | | | | | |

**Table 3:** Correlation between heterogeneous claudin expression and clinicopathological data of the patients diagnosed with colon carcinoma.

## Claudins (CLDNs) and Complexity Index (CI)

We also analyzed the CI value of each specimen with expression of CLDN proteins (CLDN 1,3,4,5 and 7) and clincopathological parameters (including tumor wall penetration (T), lymph node metastasis (N), distant metastasis (M) and differentiation) of the patients. Results indicate a trend for significant association between CLDN 5 expression and CI (p=0.06). Similar results were seen within tumor wall penetration and high value of CI (p=0.08). CI was not significantly associated with other studied CLDN expression or clinopathological parameters of the patients. Results are shown in Table 4.

| **Parameters** | **Correlation with Complexity Index (CI)** |
|---|---|
| Claudin 1 | -0.23 |
| Claudin 3 | -0.04 |
| Claudin 4 | 0.26 |
| Claudin 5 | 0.06 |

| | |
|---|---|
| Claudin 7 | -0.09 |
| T | 0.08 |
| N | -0.13 |
| M | 0.18 |
| Differentiation | -0.4 |
| T=Tumor wall penetration, N=lymph node metastasis, M= distant metastasis | |

**Table 4:** Correlation of CI with Claudin expression and clinicopathological parameters of the patients.

To investigate any possible association between CLDN expression (CLDN 1, 3, 4, 5 and 7) and 5-years survival of the patients, we performed Kaplan-Meier analysis (Figure 2). No significant relationship was observed between CLDN expression pattern and 5-years surival of the patients diagnosed with colon carcinoma. Statistical results for association between CLDN expression and survival are p=0.612, p=0.358 p=0.533, p=0.846 and p=0.538 for CLDN 1, CLDN 3, CLDN 4, CLDN 5 and CLDN 7 respectivley (Table 5).

| Protein | Kaplan-Meier test (p-value) |
|---|---|
| Claudin 1 | 0.612 |
| Claudin 3 | 0.358 |
| Claudin 4 | 0.533 |
| Claudin 5 | 0.846 |
| Claudin 7 | 0.538 |

**Table 5:** Relationship between claudin expression and survival of the Patients diagnosed with colon carcinoma.

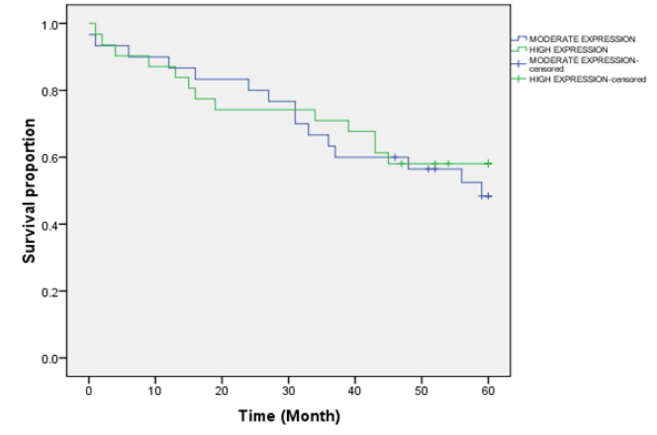


**Figure 2:** A survival curve, Kaplan Meier, showing relationship between claudin 1 expression and 5-year survival of patients diagnosed with colon carcinoma.

## Discussion

TJs are apical intercellular junctions in epithelial and endothelial cells. The two major functions defined for TJs are regulation of paracellular permeability and maintenance of the cell polarity through their barrier functions. CLDNs are among the proteins, which form TJs, assist as barrier protein and regulate the permeability of blood vessels and epithelium in different types of tissues [21-24].

Considering the involvement of TJ proteins in the regulation of epithelial proliferation and their potential usefulness as novel tools in cancer diagnosis, prognosis and treatment, we aim our study was to evaluate the expression and significance of CLDN 1, 3, 4, 5 and 7 proteins in paired normal and tumor tissue samples from 61 patients diagnoses with colon carcinoma and correlate the expression with growth pattern of the tumor. To address this issue, IHC was used to determine the patterns of distribution and intensity of expression of CLDNs between normal and adjacent tumor cells.

In our study, we found that most of the normal tissue cells showed a strong CLDN expression compared to the corresponding cancer tissue (Figure 1). Association between normal and paired cancer tissues was very high for CLDN 4 and 7 (Figures B1 and E1). Significant difference was observed between tumor and normal tissue staining intensities for CLDN 4 (p=0.031) and 7 (p=0.011) as there was high expression in normal samples as compared to tumors. Similar results were observed by Ersoz, et al. in colorectal cancer and Jung, et al. in gastric cancer cancer [25,26]. Recently, Suren, et al. reported a correaltion between CLDN 4 and aggressive behavior in colorectal carcinoma [27]. On the other hand, opposite results were found by Hwang, et al. and De Oliveira, et al. where CLDN 4 was upregulated in gastric and colorectal cancer respectively [28,29]. However, the complete correlation between CLDN 4 overexpression and invasive capacity of cancers have not been fully explained. Further studies have been warranted to explore the correlation between CLDN 4 and various types of cancers.

CLDN 7 was studied by Komensky, et al. in breast cancer and he reported low expression as compared with normal breast epithelium which is similar to our findings of CLDN 7 in colon carcinoma [30]. Sauer, et al. reported the reduced expression of CLDN 7 correlated with metastatic disease and higher tumor grade [31]. Similarly, low expression of CLDN 7 was reported in prostate and oesophageal cancers [7]. Regarding CLDN 1, 3 and 5, however, we did not find any significant association between expression intensities of (p>0.05).

We further explore the correlation between number of stained cells in tumor and normal samples. CLDN 4 (p=0.001) has significantly low number of stained cells in tumor samples as compared with normal samples but the opposite was seen for CLDN 5 (p=0.052). No significant association could be recognized for number of stained cells between tumor and normal tissues in CLDN 1, 3 and 7 (p>0.05). Previously, Caruso, et al. and Bujko, et al. reported the elevated levels of CLDN 1 in colorectal cancer [32]. Similarly, CLDN 7 was significantly associated with higher risk of distant metastasis in colorectal cancer [27,33].

Role of CLDN family in tumors is controversial [4,34]. According to a study Seo, et al. a decrease expression of CLDN 1 is correlated to prostate carcinoma and was recommended as an important marker for lung adenocarcinoma [35,36]. Initially it was considered that CLDN 5 is expressed only in endothelial cells, but later on it was found to be expressed in various other cancer cell types as in hepatocellular and renal carcinomas, lower levels of CLDNs 4 and 5 have been determined [37]. Similarly, a decreased expression of CLDN 3 has been associated

with ovarian epithelial carcinoma [38,39]. The possible reason for discrepancies could be the tumors selected in different studies were at different stages of progression, location of the tumor within the organ and also evaluation of expression at different parts of tumor such as invasive front and centrally located tumor cells. In this study, rectal samples were excluded as they undergo radiation therapy which could possibly have effects on the final results. However, the molecular mechanism responsible for the differential expression of CLDNs in cancer still remains unclear. A much better knowledge of the particular functions of CLDNs, elucidation of mutations responsible for expression and regulation in cancers could provide the important information for future therapeutic interventions.

Intensities of CLDN 1, 3, 4, 5 and 7 expressions in tumor tissues were also compared with different clinical and pathological variables. For the tumor wall penetration, proportion of tumors with lower CLDN 1, 4, 5 and 7 expressions were higher at T3+T4 stages then with higher expression, but CLDN 3 expression was observed reverse. In case of lymph node metastasis, variable expression was observed for different CLDNs such as, for CLDN 3, 4 and 5 proportion of tumor with lower expression and lymph node metastasis was comparatively higher to increased expression and lymph node metastasis, but opposite results were found for CLDN 1 and 7. Similar results were observed between CLDNs expression and other clinicopathological parameters such distant metastasis, Duke stages and differentiation. However, in either case, none of the parameter was significantly associated with either CLDN that we determined in current project. Though, a trend was observed between CLDN 3 expressions and medium tumor differentiation (p=0.087), and also between CLDN 5 and lymph node metastasis (p=0.088).

A 5-years survival analysis did not show any statistically significant association between CLDN expression in patients diagnosed with colon carcinoma. A possible explanation for these results could be small sample size and different stages of colon carcinoma of selected patients in our study.

IHC expression of CLDNs was also examined to expose any possible association between heterogeneity of expression, weaker expression at cell membrane and invasive border of the tumor and clinicopathological parameters of the patients diagnosed with colon carcinoma. A significant correlation was observed between heterogeneity and weaker expression of CLDNs at invasive front of the tumor. This reveals the fact that when CLDNs are not homogenous in expression, there is weaker expression at invasive border which may leads to low proliferation and metastasis of the tumor and vice versa. Studies of Ozerhan, et al. and Suzuki, et al. show that when there is high expression at invasive border of the tumor, tumor is more likely to metastize to other organs [40,41]. Further studies with large number of samples are required to investigate the relation between homogenous expression, invasive border expression and metastasis of tumor which may be an important marker in the selection of patients diagnosed with colon carcinoma for adjuvant therapy.

CI of 40 samples was calculated and graded between 1-5 by measuring fractal dimensions of the invasive front and number of tumor cells/clusters. Results were correlated with expression of CLDN proteins and clinicopathological parameters of the patients.

The results do not show any significant correlation with any of the compared clinical parameters. However a trend was detected between CLDN 5 expression and CI (p=0.06). It suggests when this protein is highly expressed, CI value of the tumor increases and high value of CI illustrate that tumor has irregular border and even separated into cells and clusters at grade 5. This result indicates that it is not only CLDN 5, which is responsible for maintaining the morphology of cells and there are many other factors which are involved in establishing the integrity of tissues. Another tendency was observed between CI value and tumor wall penetration (p=0.08). Similar findings was seen in a previous study by Mannan and Hahn Stromberg, which supports the CI theory that when the tumor has an irregular border, its CI value is high, thus the penetration capability is higher in tumors with high CI value than those having low CI value and smooth borders [42]. A significant association was seen between CI and tumor wall penetration by Hahn-srömberg, et al. when they observed growth pattern of the tumor and their relation to adhesion proteins and clinicopathological parametres [15]. A negatively significant association was seen between CI value and CLDN 3 expression which indicates that when this protein is expressed, tumor invasive front is regular and vice versa. This finding can be used in further studies to explain this association which can be a useful prognostic marker for colon carcinoma patients. We cannot perceive any significant correlation or trend of all other parameters considered to the CI value which is similar to the previous studies indicating that there may be some proteins other than CLDNs that also have a role in distorted growth pattern in colon carcinoma [1,20].

## Conclusion

In conclusion, the expression of CLDN proteins (CLDN 1, 3, 4, 5 and 7) was found significantly less in colon carcinoma tissue compared to the adjacent normal mucosa. We also observed that expression of CLDN (CLDN 1, 3, 4, 5 and 7) is not related to the growth pattern of the tumor as well as 5-years survival of the patients. However, protein expression and CI, both are independent prognostic markers in CRC. There is need of comparatively larger studies containing both gene and protein expression to assess the role of CLDN in colon carcinoma as well as other types of carcinoma.

## Authors' Contribution

Rahel Befekadu carried out the immunohistochemical staining as well as drafting the manuscript. Abrar Ahmad carried out the image analysis and the statistical analysis and contributed a lot to the drafting of the manuscript. Shlear Askari assembled the samples, performed the sectioning for immunohistochemical staining and helped with the staining. Victoria Hahn-Strömberg conceived of the study, design, coordination, funding and helped draft the manuscript. All authors read and approved the final manuscript

## Acknowledgement

We thank Nyckelfonden, Örebro University hospital, Örebro, Sweden and Lions Cancer research foundation, Uppsala, Sweden, for grants funding this study.

## References

1. Hahn-Stromberg V, Edvardsson H, Bodin L, Franzen L (2008) Disturbed expression of E-cadherin, beta-catenin and tight junction proteins in colon carcinoma is unrelated to growth pattern and genetic polymorphisms. APMIS 116: 253-262.
2. Weber CR, Nalle SC, Tretiakova M, Rubin DT, Turner JR (2008) Claudin-1 and claudin-2 expression is elevated in inflammatory bowel

disease and may contribute to early neoplastic transformation. Lab Invest 88: 1110-1120.

3. Darido C, Buchert M, Pannequin J, Bastide P, Zalzali H, et al. (2008) Defective claudin-7 regulation by Tcf-4 and Sox-9 disrupts the polarity and increases the tumorigenicity of colorectal cancer cells. Cancer Res 68: 4258-4268.
4. Singh AB, Dhawan P (2015) Claudins and cancer: Fall of the soldiers entrusted to protect the gate and keep the barrier intact. Semin Cell Dev Biol 42: 58-65.
5. Findley MK, Koval M (2009) Regulation and roles for claudin-family tight junction proteins. IUBMB Life 61: 431-437.
6. Tsukita S, Furuse M (2000) The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci 915: 129-135.
7. Kwon MJ (2013) Emerging roles of claudins in human cancer. Int J Mol Sci 14: 18148-18180.
8. Bello IO, Vilen ST, Niinimaa A, Kantola S, Soini Y, et al. (2008) Expression of claudins 1, 4, 5, and 7 and occludin, and relationship with prognosis in squamous cell carcinoma of the tongue. Hum Pathol 39: 1212-1220.
9. Paschoud S, Bongiovanni M, Pache JC, Citi S (2007) Claudin-1 and claudin-5 expression patterns differentiate lung squamous cell carcinomas from adenocarcinomas. Mod Pathol 20: 947-954.
10. Miettinen M, Sarlomo-Rikala M, Wang ZF (2011) Claudin-5 as an immunohistochemical marker for angiosarcoma and hemangioendotheliomas. Am J Surg Pathol 35: 1848-1856.
11. Bhat AA, Pope JL, Smith JJ, Ahmad R, Chen X, et al. (2015) Claudin-7 expression induces mesenchymal to epithelial transformation (MET) to inhibit colon tumorigenesis. Oncogene 34: 4570-4580.
12. Pope JL, Ahmad R, Bhat AA, Washington MK, Singh AB, et al. (2014) Claudin-1 overexpression in intestinal epithelial cells enhances susceptibility to adenamatous polyposis coli-mediated colon tumorigenesis. Mol Cancer 13: 167.
13. Hahn-Stromberg V, Askari S, Befekadu R, Matthiessen P, Karlsson S, et al. (2014) Polymorphisms in the CLDN1 and CLDN7 genes are related to differentiation and tumor stage in colon carcinoma. APMIS 122: 636-642.
14. Jass JR (1987) The pathological classification of colorectal cancer. Ann Acad Med Singapore 16: 469-473.
15. Jass JR, Atkin WS, Cuzick J, Bussey HJ, Morson BC, et al. (2002) The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. Histopathology 41: 59-81.
16. Bylund JR, Gayheart D, Fleming T, Venkatesh R, Preston DM, et al. (2012) Association of tumor size, location, R.E.N.A.L., PADUA and centrality index score with perioperative outcomes and postoperative renal function. J Urol 188: 1684-1689.
17. Franzen LE, Hahn-Stromberg V, Edvardsson H, Bodin L (2008) Characterization of colon carcinoma growth pattern by computerized morphometry: definition of a complexity index. Int J Mol Med 22: 465-472.
18. Wang X, Wan F, Pan J, Yu GZ, Chen Y, et al. (2008) Tumor size: a non-neglectable independent prognostic factor for gastric cancer. J Surg Oncol 97: 236-240.
19. Ohene-Abuakwa Y, Pignatelli M (2000) Adhesion molecules as diagnostic tools in tumor pathology. Int J Surg Pathol 8: 191-200.
20. Hahn-Stromberg V, Edvardsson H, Bodin L, Franzen L (2009) Tumor volume of colon carcinoma is related to the invasive pattern but not to the expression of cell adhesion proteins. APMIS 117: 205-211.
21. Hewitt KJ, Agarwal R, Morin PJ (2006) The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer 6: 186.
22. Kojima F, Ishida M, Takikita-Suzuki M, Hotta M, Katsura K, et al. (2010) Claudin expression profiles in Epstein-Barr virus-associated nasopharyngeal carcinoma. Oncol Rep 23: 927-931.
23. Nemeth J, Nemeth Z, Tatrai P, Peter I, Somoracz A, et al. (2010) High expression of claudin-1 protein in papillary thyroid tumor and its regional lymph node metastasis. Pathol Oncol Res 16: 19-27.
24. Wu P, Wu D, Ni C, Ye J, Chen W, et al. (2014) γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. Immunity 40: 785-800.
25. Jung H, Jun KH, Jung JH, Chin HM, Park WB (2011) The expression of claudin-1, claudin-2, claudin-3, and claudin-4 in gastric cancer tissue. J Surg Res 167: e185-e191.
26. Ersoz S, Mungan S, Cobanoglu U, Turgutalp H, Ozoran Y (2011) Prognostic importance of Claudin-1 and Claudin-4 expression in colon carcinomas. Pathol Res Prac 207: 285-289.
27. Suren D, Yildirim M, Kaya V, Alikanoglu AS, Bulbuller N, et al. (2014) Loss of tight junction proteins (Claudin 1, 4, and 7) correlates with aggressive behavior in colorectal carcinoma. Med Sci Monit 20: 1255-1262.
28. De Oliveira SS, De Oliveira IM, De Souza W, Morgado-Diaz JA (2005) Claudins upregulation in human colorectal cancer. FEBS Lett 579: 6179-6185.
29. Hwang TL, Changchien TT, Wang CC, Wu CM (2014) Claudin-4 expression in gastric cancer cells enhances the invasion and is associated with the increased level of matrix metalloproteinase-2 and -9 expression. Oncol Lett 8: 1367-1371.
30. Kominsky SL, Argani P, Korz D, Evron E, Raman V, et al. (2003) Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 22: 2021-2033.
31. Sauer T, Pedersen MK, Ebeltoft K, Naess O (2005) Reduced expression of Claudin-7 in fine needle aspirates from breast carcinomas correlate with grading and metastatic disease. Cytopathology 16: 193-198.
32. Caruso M, Fung KY, Moore J, Brierley GV, Cosgrove LJ, et al. (2014) Claudin-1 Expression Is Elevated in Colorectal Cancer Precursor Lesions Harboring the BRAF V600E Mutation. Transl Oncol 7: 456-463.
33. Bujko M, Kober P, Mikula M, Ligaj M, Ostrowski J, et al. (2015) Expression changes of cell-cell adhesion-related genes in colorectal tumors. Oncol Lett 9: 2463-2470.
34. Ricardo S, Gerhard R, Cameselle-Teijeiro JF, Schmitt F, Paredes J (2012) Claudin expression in breast cancer: high or low, what to expect? Histol Histopathol 27: 1283-1295.
35. Das P, Goswami P, Das TK, Nag T, Sreenivas V, et al. (2012) Comparative tight junction protein expressions in colonic Crohn's disease, ulcerative colitis, and tuberculosis: a new perspective. Virchows Arch 460: 261-270.
36. Chao YC, Pan SH, Yang SC, Yu SL, Che TF, et al. (2009) Claudin-1 is a metastasis suppressor and correlates with clinical outcome in lung adenocarcinoma. Am J Respir Crit Care Med 179: 123-133.
37. Seo KW, Kwon YK, Kim BH, Kim CI, Chang HS, et al. (2010) Correlation between Claudins Expression and Prognostic Factors in Prostate Cancer. Korean J Urol 51: 239-244.
38. Amasheh S, Schmidt T, Mahn M, Florian P, Mankertz J, et al. (2005) Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. Cell Tissue Res 321: 89-96.
39. Takala H, Saarnio J, Wiik H, Soini Y (2007) Claudins 1, 3, 4, 5 and 7 in esophageal cancer: loss of claudin 3 and 4 expression is associated with metastatic behavior. APMIS 115: 838-847.
40. Ozerhan IH, Ersoz N, Onguru O, Ozturk M, Kurt B, et al. (2010) Fascin expression in colorectal carcinomas. Clinics (Sao Paulo) 65: 157-164.
41. Suzuki H, Masuda N, Shimura T, Araki K, Kobayashi T, et al. (2008) Nuclear beta-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. Anticancer Res 28: 1821-1830.
42. Mannan A, Hahn-Stromberg V (2012) K-ras mutations are correlated to lymph node metastasis and tumor stage, but not to the growth pattern of colon carcinoma. APMIS 120: 459-468.

This article was originally published in a special issue, entitled: **"Gastrointestinal Cancer and Stromal Tumors",** Edited by Jilin Cheng