

**Immunohistochemical Expression of Caspase-3 in Colorectal Carcinoma****Sheren FM Ahmed<sup>a</sup>, Christeen NB Shaheed<sup>b\*</sup>, Eman MS Muhammad<sup>a</sup>**<sup>a</sup>Department of Pathology, Faculty of Medicine, Sohag University, Sohag, Egypt.<sup>b</sup>Department of Pathology, Oncology Sohag Center, Sohag, Egypt.**Abstract****Background:** Apoptosis is an important factor in the development of tumors especially colorectal carcinomas (CRC). Caspase-3 is considered an ideal marker for measuring apoptosis in these tumors.**Objectives:** To evaluate the Caspase-3 expression and its correlation with the available clinicopathological criteria in patients with CRC.**Patients and methods:** Fifty cases of CRC removed by colectomy were enrolled in this study. Caspase-3 expression was evaluated by immunohistochemistry (IHC) using streptavidin-biotin-technique.**Results:** Caspase-3 appeared as brownish nuclear staining and was expressed in all cases of CRCs. Caspase-3 showed positive significant correlation with age ( $p=0.046$ ), histological variant ( $p=0.02$ ), and negative correlation with tumor grade ( $p<0.001$ ), depth of tumor invasion; T stage ( $p<0.001$ ) and lymph node status ( $p<0.001$ ).**Conclusion:** High expression of Caspase-3 in CRC patients related to favorable clinicopathological features and could be a potential independent prognostic factor. Caspase-3 may act as a tumor suppressor in CRC.**Keywords:** Colorectal carcinoma, apoptosis, immunohistochemistry, Caspase-3.**Introduction**

CRC is considered a major health problem as it is the 3<sup>rd</sup> most common cancer worldwide (Bray et al., 2018) and the 4<sup>th</sup> most common cancer cause of death globally (Haraldsdottir et al., 2014). CRC is the seventh most frequent cancer in Egypt; representing 3.47% of male cancers and 3% of female cancers (Ibrahim et al., 2014).

CRC is a frequent malignancy being associated with high morbidity and mortality and because of its association with metastases. The incidence increases with increasing age and is low at ages younger than 50 years (Brenner et al., 2014).

In many countries, prognosis of patients with CRC has slowly but steadily improved during the past decades in many countries. The five-

year survival rate of patients dramatically declines from approximately 90% in early-stage non-metastatic tumors to about 5% in cases with distant metastasis (Pizzini et al., 2013). One of the hallmarks of cancers is their ability to accumulate mutations that provide progressive and survival advantages as well as resistance to cell death mechanisms “evading apoptosis” (Hector and Prehn, 2009).

Apoptosis is a mode of programmed cell death that helps to remove cells that are no more required; it is coordinated by members of the Caspase family of cysteine proteases (Walsh et al., 2008). There are two different pathways that lead to apoptosis: The intrinsic and extrinsic pathways; both converge at the executioner Caspases (Zaman et al.,

2014), from which Caspase-3 is the major effector (Walsh et al., 2008).

The central role that Caspase-3 plays in apoptosis makes it an attractive target in treatment of many diseases. Synthetic inhibitors may be able to be used to treat certain diseases that involve an excess of apoptosis (Alzheimer's and Huntington's). Diseases that involve a lack of apoptosis (Cancer) are more difficult to treat using therapy designed to affect Caspase-3, however it may be possible to selectively activate Caspase-3 in cancer cells only (Boudreau et al., 2019).

Several reports have shown that Caspase-3 expression was associated with a favorable prognosis and was an independent prognostic factor for several cancers of the gastrointestinal tract. However, the conclusions of these studies were controversial (Noble et al., 2013).

Caspase-3 is also often used as a marker for efficacy of cancer therapy, as it is a major mediator of apoptosis activated during cellular exposure to cytotoxic drugs, radiotherapy or immunotherapy. However, recent reports indicate that Caspase-3 has also non-apoptotic roles such as promotion of tumor relapse and tumor angiogenesis. Therefore, the roles of Caspase-3 in tumor progression remain to be defined clearly (Zhou et al., 2018).

Although Caspase-3 activation causes cell death in the host cell, it has been found to stimulate cell proliferation in neighboring, non-apoptotic cells, as the dying cells may send out signals to more resistant cancer cells promoting proliferation and repopulation (Li et al., 2010). Caspase-3 is rarely mutated in CRC; therefore, it is considered an ideal marker for measuring apoptosis in these tumors (Soung et al., 2004). This

study was designed to evaluate Caspase-3 expression and its correlation with the available clinicopathological criteria in patients with CRC.

### Patients and Methods

Fifty formalin-fixed paraffin embedded tissue blocks of 50 cases of untreated CRC were retrieved for this study from archived material in Pathology Department, Sohag University Hospitals, and Sohag Oncology Center during the period from January 2016 to December 2018.

Patients who received preoperative neoadjuvant therapy were excluded from the study. Ethical approval to perform this work was obtained from the Institutional Research Ethical Committee. The tumor samples were collected from colectomy cases. The clinical data of the investigated cases were gathered from patients' clinical files.

### Histological examination

Sections of 4µm thick were prepared from the tissue blocks and stained with hematoxylin and eosin stains (H&E) and reviewed for tumor histological variant, grade, and depth of invasion, lymphovascular invasion (LVI), perineural invasion (PNI), and presence of regional lymph node metastasis. The tumor grade and pathological stage were evaluated according to WHO recommendations (Nagtegaal et al., 2019). TNM pathological staging was carried out according to the AJCC (Brierley, 2017).

### Immunohistochemical staining

Sections of 4µm thick were mounted on pre-labeled poly-L-lysine coated slides. The selected sections were de-paraffinized in xylene for 20 minutes, rehydrated in downgraded alcohol (100%, 80%, 70%, and 50%) 2 min for each, then rinsed in distilled

water. Tissue sections were incubated in 0.5% hydrogen peroxide/methanol for 10 min to block endogenous peroxidase activity followed by washing twice in phosphate buffer saline (PBS). The antigen was retrieved by boiling tissue sections twice in citrate buffer, pH 6.0, using a microwave at mid-high power for 10 min each, followed by cooling down to room temperature for 20 min.

Following washing twice in PBS, tissue sections were incubated with mouse anti-Caspase-3 (active/pro) monoclonal antibody (Clone 31A1067, Catalog # MC0123, Medaysis, USA) at a dilution of 1/200 for overnight at 4°C. Next day, tissue sections were washed twice in PBS, and incubated with Universal Staining Kit EconoTek HRP anti-polyvalent 'DAB' ready to use (ScyTek Laboratories, Inc. Logan, Utah, USA) for 30 min at room temperature.

Excess reagent was thrown off and tissue sections were rinsed twice in PBS, incubated with streptavidin for 10 min at room temperature, washed twice in PBS and exposed to a freshly prepared 3, 3'-diaminobenzidine tetrahydrochloride solution (DAB) solution for 5-10 min until the positive control showed brown staining then tissue sections were washed in distilled water and finally, were counterstained with hematoxylin, washed in running water, dehydrated in upgraded series of alcohol (70%, 80%, 90%, and 100%), cleared in xylene for 5 min then mounted with DPX and cover slipped. Each staining run included sections from tonsil as a positive control. Caspase-3 expression appeared as brownish nuclear staining in the lymphocytic cells of the tonsils. Additional tissue sections were stained in parallel, but with omission of the primary antibody as negative controls for IHC.

### ***Immunohistochemical detection of Caspase-3 and scoring***

Tissue sections were histologically examined by bright-field microscope at low power magnification (X40 and X100) to detect the sites of antibody positivity, then by higher power magnification (X200 and X400) to evaluate immunostaining.

The results of IHC were analyzed independently of the clinico-pathological data. The immunoreaction was considered positive when brownish nuclear staining was demonstrated. The expression of Caspase-3 was evaluated by a semi-quantitative system to calculate the percentage of positive tumor cell expression. The arbitrary range was represented as follows: trace: <5% positive cells, weak: 1+, 5-25% positive cells, moderate: 2+, 26-75% positive cells and strong: 3+, more than 75% positive cells (**Huang et al., 2018**).

### ***Statistical Analysis***

Data were statistically analyzed using IBM SPSS Statistics for Windows version 18. Quantitative data were expressed as mean± standard deviation, median, and range. Qualitative data were expressed as numbers and percentages. The data were tested for normality using the Shapiro-Wilk test, and found that the data were not normally distributed. Chi-Square test, Fissure exact test, Kruskal Wallis test and Spearman's correlation were used to evaluate the statistical significance of various parameters with p-value <0.05 was considered statistically significant and highly significant if <0.001.

### **Results**

#### ***Patients' clinical characteristics***

This study included 50 patients aged between 24-81 years, with a mean± SD and a median of 54.42±

16.2 and 59.5 years, respectively. According to the current data; males were 30/50 and females were 20/50 of cases. The range of tumor size was 1.2-13 cm with a mean $\pm$  SD and a median of 5.36 $\pm$  3.03 cm and 4.4 cm, respectively. Twenty seven out of fifty cases were left-sided tumors whereas 23/50 cases were right-sided (**Table 1**).

#### **Histopathological findings**

The representative H&E stained sections of the studied cases were classified and graded into conventional adenocarcinoma 40/50 cases and mucinous adenocarcinoma 10/50 cases. Most CRC cases were grade II (35/50), 5/50 of cases were grade I, and 10/50 were grade III.

Fourteen out of fifty cases showed LVI and 6/50 cases revealed PNI. Regarding the depth of tumor invasion (T); most of cases 27/50 were T3, 4/50 were T2, and 19/50 of cases were T4. Concerning lymph node status; 24/50 of cases were negative for

tumor metastasis, whereas 26/50 of cases were positive for tumor metastasis (**Table 1**).

#### **Immunohistopathological findings**

Caspase-3 appeared as brownish nuclear staining and was expressed in all cases (100%) of CRCs. None of the studied cases showed trace Caspase-3 expression.

Among different clinico-pathological parameters of the studied cases of CRC, comparative analysis showed that Caspase-3 expression was positively correlated with age ( $p = 0.046$ ) as illustrated in table (2). Significant positive association was found with tumor grade ( $p < 0.001$ ), histological type ( $p = 0.02$ ), depth of tumor invasion; T stage ( $p < 0.001$ ) and lymph node status ( $p < 0.001$ ) as shown in table (1). However, Caspase-3 expression showed no significant correlation with patients' sex, location, LVI, PNI (table 1) or tumor size (Spearman's  $\rho = -0.252$ ,  $p = 0.078$ ).

**Table 1. Correlation between Caspase-3 expression and the studied clinico-pathological parameters**

Clinico-pathological Parameters	No. of cases (50)	Caspase-3 expression			P- value
		Weak No.= 7 (14%)	Moderate No.=15 (30%)	Strong No.=28 (56%)	
<b>Sex</b>					
Males	30 (60%)	5 (71.4%)	10 (66.7%)	15 (53.6%)	0.565 (NS)
Females	20 (40%)	2 (28.6%)	5 (38.5%)	13 (43.3%)	
<b>Tumor Location</b>					
Right	23 (46%)	5 (71.4%)	8 (61.5%)	10 (33.3%)	0.188 (NS)
Left	27 (54%)	2 (28.6%)	7 (46.7%)	18 (64.3%)	
<b>Tumor Grade</b>					
Grade I	5 (10%)	0 (0%)	2 (13.3%)	3 (10.7%)	<0.001**
Grade II	35 (70%)	1 (14.3%)	11 (73.3%)	23 (82.1%)	
Grade III	10 (20%)	6 (85.7%)	2 (13.3%)	2 (7.1%)	
<b>Histological Variant</b>					
Conventional AC	40 (80%)	3 (42.9%)	14 (93.3%)	23 (82.1%)	0.02*
Mucinous AC	10 (20%)	4 (57.1%)	1 (6.7%)	5 (17.9%)	



<b>T Stage</b>					
T2	4 (8%)	1 (14.3%)	2 (13.3%)	1 (3.6%)	<b>&lt;0.001*</b>
T3	27 (54%)	1 (14.3%)	7 (46.7%)	19 (67.9%)	
T4	19 (38%)	5 (71.4%)	6 (40%)	8 (28.5%)	
<b>Lymph node status</b>					
Negative	24 (48%)	3 (42.9%)	4 (26.7%)	17 (60.7%)	<b>&lt;0.001*</b>
Positive	26 (52%)	4 (57.1%)	11 (73.3%)	11 (39.3%)	
<b>LVI</b>					
Present	14 (28%)	4 (57.1%)	4 (26.7%)	6 (21.4%)	0.168 (NS)
Absent	36 (72%)	3 (42.9%)	11 (73.3%)	22 (78.6%)	
<b>PNI</b>					
Present	6 (12%)	2 (28.6%)	1 (6.7%)	3 (10.7%)	0.322 (NS)
Absent	44 (88%)	5 (71.4%)	14 (93.3%)	25 (89.3%)	

\*P- value was calculated by Chi-square test,

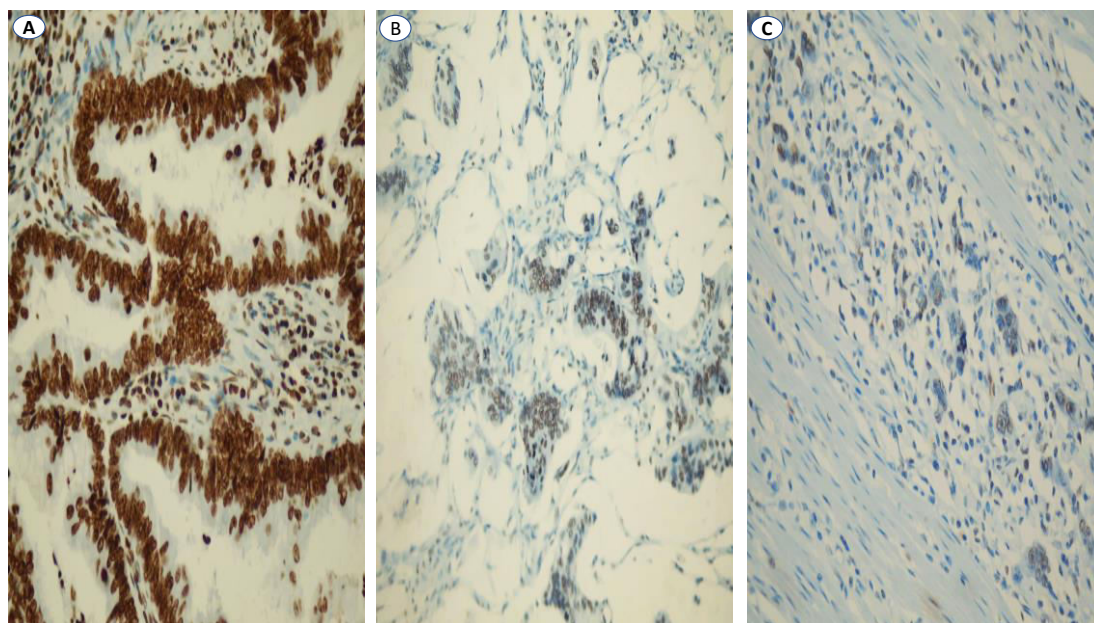
\*\*P- value was calculated by Fisher's Exact test

AC= Adenocarcinoma, No= Number, NS= Non-Significant.

**Table 2. Correlation between Caspase 3 and age of the studied patients**

Parameter	Mean $\pm$ SD	Median (range)	P-value
Age	54.42 $\pm$ 16.2	59.5 (24-81)	0.046*

\* P- value was calculated by Kruskal Wallis Test



**Fig.1. Caspase-3 expression in colon adenocarcinoma; strong expression in grade I (A), moderate expression in grade II (B), and weak expression in grade III (C). Original magnification is 400X**

## Discussion

CRC is the 3<sup>rd</sup> most common cancer in the world and one of the most common causes of death globally. It is a major public health problem because of its asymptomatic nature, so it is frequently diagnosed in its later stages, and approximately 50% of cases develop metastasis and have a poor survival rate (Ferlay et al., 2015). Recently, researches on CRC had led to the development of therapeutic agents that specifically inhibit tumor growth and induce apoptosis (Mazmanian et al., 2005).

Apoptosis is one of the most important factors, which is involved in the etiopathology of several tumors and most therapeutic ways of cancer like chemotherapy and radiotherapy are trying to induce apoptosis in cancer cells to kill them (Johnstone et al., 2002). Previous studies have shown that Caspase-3 expression is a useful prognostic factor in digestive system-related cancers, especially in CRC (Chen et al., 2015).

To date, few studies have discussed the role of Caspase-3 in the progression of CRC. The present study was conducted to evaluate the expression of Caspase-3 and its association with the clinicopathological parameters in CRC.

The current study included 50 specimens of CRC removed by colectomy. The age range of the studied patients was 24-81 years, the mean  $\pm$  SD was 54.42 $\pm$ 16.2 years, and the median age was 59.5 years. These results were close to the findings of de Heer et al. (2007), and de Oca et al. (2007) who reported that the age range of cases in their studies was 26-85, and 30-79 years, with a median age of 65 and 60.2 years, respectively. The male to female ratio was 1.5:1 which was near to the ratios found by de Heer et

al. (2007), Koelink et al. (2009), and Asadi et al. (2018) who reported male to female ratios of 1.37:1, 1.2:1, and 1.7:1 respectively.

The range of tumor size in the studied cases was 1.2-13 cm, with mean  $\pm$  SD and median of 5.36  $\pm$ 3.03 cm and 4.4 cm, respectively which was similar to the findings of de Heer et al. (2007) and Koelink et al. (2009) who reported that the range of tumor size in their studies was 1-11, and 2-12 cm, with a median of 4.5 and 5 cm, respectively.

This study also showed that 46% of cases had a right-sided tumor, while 54% were left-sided, whereas Koelink et al. (2009) showed that 35% of cases were right-sided and 65% were left-sided. This difference could be explained because their studied patients were of the Western European population who have different dietary habits, and also due to heavy alcohol consumption causing a disproportionate increase in tumors of the left colon (Haggard and Boushey, 2009).

In the current study, 80% cases were of conventional adenocarcinoma and 20% cases of mucinous carcinoma. This was near to those reported by Koelink et al. (2009) who found that 75% of cases were conventional adenocarcinomas and 25% of cases were mucinous carcinoma.

Most cases of the current study of CRCs were grade II (70%), this finding was near to those recorded by de Heer et al. (2007), who found that 68% of cases were grade II. Regarding the T stage, most of the studied cases (54%) were T3, near the results found by de Heer et al. (2007) who reported that 65% of cases were T3.

In this study, regional lymph node metastasis was positive in 52% of cases, which was near to what was reported by Meyer et al., (2009) who

found that 50% of their studied cases showed positive regional lymph node metastasis. However, **Hu et al., (2014)** found that 31% of cases showed positive regional lymph node metastasis, this lower percentage may be due to the presence of screening programs for CRC leading to early diagnosis before the occurrence of LN metastasis (**Bevan and Rutter, 2018**).

LVI was observed in 28% of cases in agreement with **de Oca et al. (2007)** who reported that 33% of cases showed LVI. PNI was observed in 12% of cases, near to the results of **Leijssen et al., (2019)** who found that 18% of cases showed PNI.

Caspase-3 is one of the cysteine proteases that are responsible for the morphological changes within the cells during apoptosis (**Kumar, 2007**). It is considered as the point of convergence of the two main apoptotic pathways and cleaves most of the cellular substrates in the apoptotic process (**Zheng et al., 2003**).

Measurement of Caspase-3 activity is therefore the major and the most reliable determinant of apoptosis (**Kaufmann et al., 2008**), and several studies considered it as an independent prognostic factor for several cancers of the digestive system, and a positive indicator of efficacy in cancer treatment. However, the conclusions of these studies were controversial (**Noble et al., 2013 and Feng et al., 2017**).

The present study revealed that Caspase-3 was expressed in all cases of CRCs (100%). It was strongly expressed in 56%, moderately expressed in 30%, and weakly expressed in 14% of cases which were in agreement with **Noble et al. (2013)** who found diffuse Caspase-3 expression in all cases of CRCs (100%); strong expression in 87%, and weak expression in 13% of cases.

The current study showed a significant positive correlation between Caspase-3 expression and patients' age ( $p= 0.046$ ), a finding similar to what was reported by **Perraud et al. (2012)**. In contrast, **Koelink et al. (2009)**, and **Hu et al. (2014)**, reported that Caspase-3 expression did not correlate with patients' age in their studied cases. Their studies included a higher age group of patients than those in the current study.

In the present study, no significant correlation was found between Caspase-3 expression and patients' sex, a finding similar to that found by **de Heer et al. (2007)**, **Noble et al. (2013)**, **Hu et al. (2014)**, and **Asadi et al. (2018)**. However, **Koelink et al. (2009)**, found a significant correlation between Caspase-3 expression and patients' sex. Their studies included a larger number of cases.

The current study showed no significant correlation between Caspase-3 expression and tumor size, a finding which is concordant with that of **de Heer et al. (2007)**, **Koelink et al. (2009)**, and **Hu et al. (2014)**. Also, no significant correlation was found between Caspase-3 expression and tumor location, in agreement with the results of **de Oca et al. (2007)**, **Noble et al. (2013)**, and **Asadi et al. (2018)**.

Our study revealed a highly significant negative correlation between Caspase-3 expression and tumor grade ( $p < 0.001$ ); there was a reduction in nuclear expression in poorly differentiated tumors (grade III) compared to well-differentiated tumors (grade I), thus high expression of Caspase-3 in CRC correlates with good prognosis. These results were in accordance with **Asadi et al., (2018)** who found a negative association between Caspase-3 expression and tumor grade.

In contrast, the studies done by **de Oca et al., (2007)**, **Koelink et al., (2009)**, and **Hu et al., (2014)** did not find any significant association between Caspase-3 expression and tumor grade in CRC. This difference may be attributed to different antibody used which was rabbit polyclonal anti Caspase-3 in their study and showed cytoplasmic expression in contrast to the monoclonal antibody used in our study which showed nuclear expression. Monoclonal antibody is more specific as Caspase-3 is cleaved and activated during apoptosis and translocated into the nucleus resulting in the characteristic apoptotic nuclear changes (**Luo et al., 2010**).

In this study Caspase-3 expression was significantly correlated with a histological variant of the tumor ( $p=0.02$ ); Caspase-3 was strongly expressed in cases of conventional adenocarcinomas, while its expression was reduced in cases of mucinous carcinoma. These results were similar to those reported by **de Heer et al. (2007)**, whereas Caspase-3 was strongly expressed in cases of conventional adenocarcinomas and weakly expressed in cases of mucinous carcinoma.

This study showed a significant negative correlation between Caspase-3 expression and T stage ( $p<0.001$ ), in agreement with **Meyer et al. (2009)**, **Dawson et al. (2013)**, and **Hu et al. (2014)**. Cancer cells in higher tumor stages usually have apoptotic defects and are more difficult to drive into apoptosis (**Brown and Attardi, 2005**), this could explain the reduced expression of Caspase-3 in higher T stages. In contrast, **de Heer et al. (2007)** demonstrated no significant correlation between Caspase-3 expression and T stage. This difference may be due to different method used as they use a colorimetric assay for

measurement of Caspase-3 enzymatic activity.

The present study showed also a significant negative correlation between Caspase-3 expression and lymph node metastasis ( $p< 0.001$ ), whereas Caspase-3 expression reduced with lymph node metastasis in agreement with that reported by **Meyer et al. (2009)**, and **Hu et al. (2014)** who demonstrated a significant correlation between loss of Caspase-3 expression and lymph node metastasis. In contrast, **de Heer et al. (2007)** didn't find a significant correlation between Caspase-3 expression and lymph node status. They also used an enzymatic assay for measuring Caspase-3 activity.

In the current study, no significant correlation was detected between Caspase-3 expression and LVI. In contrast, **de Oca et al. (2007)**, and **Meyer et al. (2009)** found that Caspase-3 expression was correlated with LVI. This disagreement could be due to the different number of cases. Also, no significant correlation was found between Caspase-3 expression and PNI in cases of CRC. To the best of our knowledge, no previous studies have discussed this correlation.

### Conclusion

Decreased Caspase-3 expression is a poor prognostic parameter referring to tumor progression, increasing grade and stage, and occurrence of lymph node metastasis, and may provide a clinically useful biomarker for estimating tumor aggressiveness. In conclusion, our study has shown that high expression of Caspase-3 in CRC patients related to favorable clinicopathological features and could be a potential independent prognostic factor. Caspase-3 may act as a tumor suppressor in CRC.



**List of abbreviations:**

- **AJCC:** The American Joint Committee on cancer
- **Caspase:** Cysteine- aspartic protease
- **CRC:** Colorectal carcinoma
- **DAB:** Diaminobenzidine
- **DPX:** Dibutylphthalate Polystyrene Xylene
- **H&E:** Hematoxyline and Eosin
- **IBM:** International Business Machines Corporation
- **IHC:** Immuno-histochemistry, immunohistochemical.
- **LN:** Lymph node
- **LVI:** Lympho-vascular invasion
- **PBS:** Phosphate buffer solution
- **PNI:** Perineural invasion
- **SD:** Standard deviation
- **SPSS:** Statistical package for social sciences
- **TNM:** T; primary tumor, N; regional lymph nodes, M; distant metastasis
- **WHO:** The World Health Organization

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