# Assessment of Microvascular Density in Cholangiocarcinoma

Neenu Jojan[1], Geetha Sukumaran[2]

[1]Department of State Public Health Laboratory, Trivandrum, Kerala, India.
[2]Department of Pathology, Government Medical College, Kollam, Kerala, India.

**ABSTRACT**

**BACKGROUND**
Cholangiocarcinoma is a highly malignant but rare tumour arising from the bile duct, with a dismal prognosis owing to the advanced stage at presentation. A complete surgical resection is the only curative treatment available till date. There is an increase in the incidence of this tumour, especially intrahepatic cholangiocarcinoma. The objective of the present study was to assess the microvascular density in cholangiocarcinoma and its relationship to grade, which itself is a separate and important prognostic factor.

**METHODS**
All the specimens of cholangiocarcinomas received in the Department of Pathology, Government Medical College, Trivandrum, during a two-year period is included in the study (n = 26). Grading of tumours was done from H & E sections. Microvascular density was assessed using immunostaining with CD34 and counted using the method published by Weidner et al.

**RESULTS**
A total of 26 cases were studied. There was a male preponderance (57.7 %) with a mean age incidence of 55.77 years. Most of the tumours were located extra hepatically. 53.8 % were moderately differentiated tumours. Only 15 % of tumours showed lymph node metastases. Median microvascular density score was 15 vessels per high power field. High microvascular density was found in higher grade tumours. Although 75 % of the cases with lymph node metastases had high microvascular density, there was no significant correlation between microvascular density and nodal metastases, probably due to few cases of nodal metastases in the present study.

**CONCLUSIONS**
This study concludes that microvascular density in cholangiocarcinoma has a positive correlation with the grade of the tumour which itself is a significant prognostic factor. Hence, microvascular density can be used as a prognostic indicator especially in cases of incomplete resection and inadequate lymph node harvest. It may also be helpful in studying the possibility of newer therapeutic measures like anti-angiogenic treatment modalities.

**KEYWORDS**
VD, CD34, Grade, Cholangiocarcinoma

*Corresponding Author:*
*Dr. Geetha Sukumaran,*
*T.C.1/712, R-5, Kesavam,*
*Jai Nagar, Medical College P.O.,*
*Thiruvananthapuram - 695011,*
*Kerala, India.*
*E-mail: gsukumaran08@gmail.com*

*DOI: 10.18410/jebmh/2021/2*

*How to Cite This Article:*
*Jojan N, Sukumaran G. Assessment of microvascular density in cholangiocarcinoma. J Evid Based Med Healthc 2021;8(01):6-11. DOI: 10.18410/jebmh/2021/2*

*Submission 08-09-2020,*
*Peer Review 19-09-2020,*
*Acceptance 19-11-2020,*
*Published 04-01-2021.*

## BACKGROUND

Cholangiocarcinoma is a rare tumour that accounts for less than 2 % of all human malignancies. It is difficult to diagnose and is associated with a high mortality.[1-3] There is an increase in its incidence over the past decade due to unknown reasons. It is second among the primary liver tumours after hepatocellular carcinoma, accounting for 10 % to 15 % of all primary liver tumours. The term cholangiocarcinoma was referred originally to just primary tumours of intrahepatic bile ducts, while now it includes intrahepatic, perihilar and distal extrahepatic tumours of bile ducts.[4] About 15 % of liver cancers are estimated to be intrahepatic cholangiocarcinoma. Incidence of intrahepatic cholangiocarcinoma is increasing worldwide, although the cause is unclear. It could be related to the interplay between predisposing genetic factors and environmental triggers. Hilar cholangiocarcinoma is a specific entity described by Klatskin in 1965, and later on, tumours arising at that particular anatomic location came to be known as Klatskin tumours.[5]

Most cases of cholangiocarcinomas remain asymptomatic till an advanced stage. Once they become symptomatic, presentations vary according to the anatomical site of tumour and the stage. Extrahepatic cholangiocarcinomas present with obstructive jaundice and intrahepatic cholangiocarcinoma causes right upper quadrant abdominal pain. Imaging modalities like ultrasonography, computed tomography scan, magnetic resonance imaging and magnetic resonance cholangiopancreatography are used for clinical diagnosis and staging. Tumour markers like CA19-9, carcinoembryonic antigen, CA-125 may be elevated, although non-specific. CA19-9 is currently the most commonly used tumour marker.[6] Its persistent elevation suggests malignancy in the absence of cholangitis or even after biliary compression.[7] Different modalities of surgeries are done depending on the tumour location and histopathological evaluation gives the absolute diagnosis of cholangiocarcinoma. Complete surgical resection is the only curative option for cholangiocarcinoma, particularly for extrahepatic ones, with results depending on technique and patient selection. Chemotherapy and radiotherapy are ineffective in patients with inoperable tumours and biliary drainage is the mainstay of palliation. Over the last 10 years, considerable effort has been devoted to the search for markers to improve diagnosis and management of cholangiocarcinoma.

Grossly, intrahepatic cholangiocarcinoma is large, very firm, tan-white, and nodular with infiltrating tan-pink areas. Calcification is common, giving a gritty feel on grossing of the tumour. Based on their pattern of spread, carcinomas of the extrahepatic bile ducts have been divided into four types: polypoid, nodular, scirrhous-constricting, and diffusely infiltrative. Microscopically, intrahepatic cholangiocarcinoma most commonly manifests as a well to moderately differentiated adenocarcinoma forming tubules or acini with a prominent intervening desmoplastic stroma.[8] Histologic variants of intrahepatic cholangiocarcinoma include mucinous, adenosquamous, mucoepidermoid, clear-cell, and spindle cell carcinoma. All of these variants are very rare. The vast majority of extrahepatic malignancies are well differentiated, mucin secreting adenocarcinomas. Other types of carcinoma, including intestinal, squamous / adenosquamous, small-cell, signet ring cell, mucinous and undifferentiated carcinomas also occur. More than half the cases stain for TP53 protein.[9] Carcinoembryonic antigen which is limited to the apical membrane of benign cells, is also detected in the cytoplasm of carcinoma cells. Conventional adenocarcinomas are almost always positive for MUC1 and MUC5AC, which are not detected in intestinal type adenocarcinomas. Intracytoplasmic expression of these two markers as opposed to positivity in the apical membrane only of benign epithelial cells, is often seen in higher grade tumours and may serve as a diagnostic aid in small biopsy specimens.

Mutations in codon 12 of the *k-ras* oncogene are frequent in extrahepatic biliary carcinomas but appear to be fewer than in pancreatic ductal adenocarcinomas. Loss of deleted in pancreatic cancer-4 (DPC4) is common in distal common bile duct carcinomas but is significantly less common in proximal cancers. Loss of heterozygosity of chromosomes 8p, 9p and 18q were shown to be relatively common in biliary cancers. HER2 / neu gene amplification was detected in 70 % of biliary cancers. Genetic mutations were not consistent and no correlation with prognosis was identified. The key challenge in diagnosis is differentiating intrahepatic cholangiocarcinoma from metastatic adenocarcinoma. A panel of CK7, CK20, TTF-1, gross cystic disease fluid protein (GCDFP), and prostate-specific antigen (PSA).[7] may help to differentiate tumours such as colorectal, breast, lung and prostate.

The differential diagnosis of adenocarcinoma from benign reactive changes in the extrahepatic biliary tract is one of the most challenging diagnostic problems in surgical pathology. This is compounded by crush artefacts in small biopsies and processing alterations which make differentiation between benign lesions and malignancies a veritable puzzle. Mild injury can cause significant atypia in the biliary epithelium. Conversely, well differentiated adenocarcinomas may appear deceptively benign looking. Perineural invasion, if present, favours a diagnosis of carcinoma. Benign lesions usually show a lobular arrangement of tightly packed or evenly spaced, relatively uniform glands. Malignant glands are more haphazardly distributed with wider, more open lumina which are irregular and angulate. Loss of cellular polarity and nucleomegaly are more in favour of carcinoma.

The presence of intracytoplasmic positivity for carcinoembryonic antigen and MUC1 and nuclear expression of TP53 would clinch the diagnosis of carcinoma. Owing to the dismal prognosis of cholangiocarcinoma, many studies have been conducted on assessment of prognostic factors. The most important prognostic factors include stage, lymph node metastases and grade. This study has assessed the grade of tumour and location. Surgical resection being the only curative treatment available, early diagnosis and newer treatment modalities call for survival betterment.

### Rationale for Assessment of Microvascular Density (MVD)

Tumour growth, survival and metastases are dependent on angiogenesis. The study of blood vessel formation in solid tumours has made considerable progress in recent years.[10] Microvascular density in tumours correlates well with aggressiveness in many cancers. The current area of research in antiangiogenic therapy for cancer began in 1971 with the publication of Folkman's imaginative hypothesis in which he proposed that by cutting off the blood supply of a tumour, cancer cells will be deprived of nutrients and hence die.[11] Tumours induce the sprouting of new blood vessels from the surrounding vasculature (sprouting angiogenesis) which is vital for the growth of tumours beyond 2 - 3 mm in size. Neovascularisation has a dual effect on tumour growth.[12] Blood circulates nutrients and oxygen for tumour cell growth, and newly formed endothelial cells stimulate the growth of adjacent tumour cells by secreting growth factors such as insulin-like growth factors (IGFs) and Platelet derived growth factor (PDGF). The resulting tumour vasculature is leaky and dilated, contributing to metastases. Therefore, angiogenesis is an important facet of malignancy. Vascular endothelial growth factor A (vascular endothelial growth factor A) is a key factor of sprouting angiogenesis and inhibition of vascular endothelial growth factor A can suppress tumour growth in animal models. Based on these postulations, numerous drugs have been developed for blocking the vascular endothelial growth factor A signalling pathway. Taking this as a basis, we have assessed the microvascular density in this study. microvascular density had a positive correlation with disease free survival in extrahepatic cholangiocarcinoma studied by Mobius et al.[13] A study of microvascular density in hilar cholangiocarcinomas studied by Thelen et al. conclude that neovascularization has a crucial role in tumour progression and recurrence.[14]

Grade of the tumour, a prognostic factor, is conventionally assessed by histopathological examination. The relationship between grade and microvascular density is also assessed in this study. A positive correlation between them helps to establish the fact that neovascularisation has a role in tumour progression and hence tumour angiogenesis acts as a prognostic factor and is a potential target for new therapeutic approaches.

This study aims to find out the microvascular density in Cholangiocarcinoma using immunohistochemistry and association of microvascular density with various clinical and biochemical parameters.

## METHODS

This is a descriptive study which was undertaken after institutional ethical clearance. Since, it is a rare tumour, all the 26 cases of cholangiocarcinomas received in the Department of Pathology, Government Medical College, Thiruvananthapuram, over a span of 2 years from January 1, 2014 to December 31, 2015 were assessed.

All specimens were obtained from patients with cholangiocarcinoma who have undergone surgery during the study period. The specimens were fixed in 10 % neutral buffered formalin for 24 hours. On grossing, adequate bits of 4 - 5 mm thickness were taken from normal and abnormal areas and processed. Paraffin embedded blocks were made and tissue sections of 3 - 4 microns thickness were taken. Haematoxylin–eosin-stained slides were studied initially and the tumour was graded as per World Health Organization depending on the tubule / gland formation in the tumour. Paraffin embedded tissue sections were taken on APES coated slides for immunohistochemistry using CD34 (QBEnd/10), to evaluate microvascular density. The slides were incubated and then deparaffinised and dehydrated. Then endogenous enzymes were blocked, antigen retrieval was done, addition of primary antibody-CD34 followed by secondary antibody and chromogen. Sections were counterstained and mounted.

Microvascular density was assessed using a method published by Weidner et al. Initially haematoxylin & eosin-stained slides were studied under low power and "hot spots" were detected which showed maximum number of blood vessels in the tumour. Later corresponding areas of CD34 stained slides were examined under high power. The blood vessels were counted in these areas and an average number was taken on studying two such hotspots. The blood vessels were stained dark brown and those with complete lumen were counted.

### Statistical Analysis

In this study, frequency and percentage are used for expressing qualitative variables, mean and standard deviation for quantitative variables while median as a central tendency measure for microvascular density. The association of microvascular density with various clinical and microscopic parameters was analysed using chi square and Student t-test. The observations and results were tabulated and studied using SPSS version 16.

## RESULTS

### Clinical Data

Of the total 26 cases 57.7 % were males (15) and females accounted for 42.3 % (11). The age of the patients in this study ranged from 37 years to 78 years with a mean age of 55.77 years with a standard deviation (SD) of 9.65.

### Tumour Location

From the radiology and macroscopic assessment 22 cases were shown to arise from extrahepatic bile duct and 4 were from intrahepatic bile duct.

### Microscopy

Of the 26 cases, 25 were adenocarcinomas. Amongst them 11 were well differentiated adenocarcinomas, 14 cases were moderately differentiated adenocarcinoma, and one

was an adenosquamous carcinoma. The microvascular density ranged from 3 vessels per high power field to 45 vessels per high power field with a median value of 15 vessels per high power field.

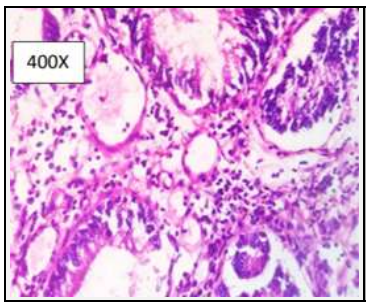


***Figure 1. Well Differentiated Cholangiocarcinoma***

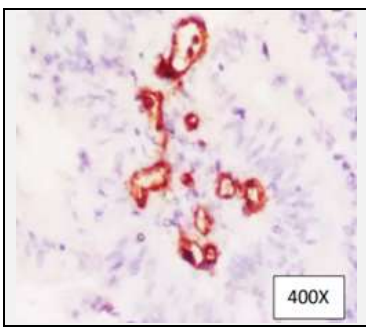


***Figure 2. CD 34, Highlights Blood Vessels in Figure 1***

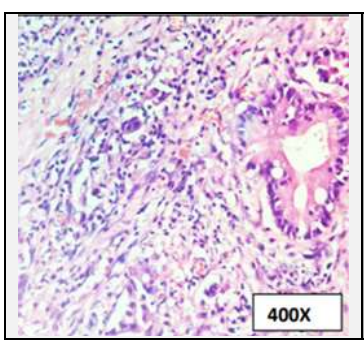


***Figure 3. Moderately Differentiated Adenocarcinoma (H & E)***

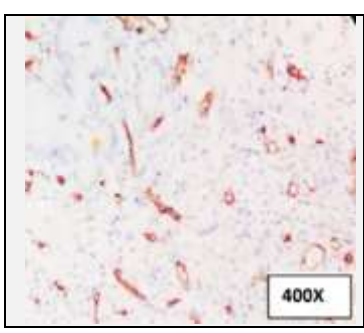


***Figure 4. CD 34 Highlights the Blood vessels in Figure 3***

Microvascular density in cholangiocarcinoma is studied in 26 resection specimens. There were 13 cases with microvascular density less than 15 per high-power field (HPF) and 13 cases with microvascular density more than 15 per HPF. A bivariable analysis is done to find out association with clinical and biochemical parameters. Test used is Pearson chi square for gender, microscopic findings, location and lymph node metastasis with df = 1, p value significance level is < 0.05. Student test-test is used for finding significance in association with age, serum bilirubin, and CA 19-9 tumour marker with df = 1, p value significance level is < 0.05. The values are given in the Table 1 and Table 2.

| Category | Microvascular Density / IHC (< 15) | Microvascular Density / IHC (> / = 15) | P Value (< 0.05 is Significant) |
|---|---|---|---|
| **Gender** | | | |
| Female | 3 (27.3 %) | 8 (72.7 %) | |
| Male | 9 (60 %) | 6 (40 %) | 0.303 |
| **Microscopic Findings** | | | |
| Moderately Differentiated | 1 (6.7 %) | 14 (93.3 %) | 0.042 |
| Well Differentiated | 9 (100 %) | 2 (0 %) | |
| **Location** | | | |
| Extra Hepatic | 9 (40.9 %) | 13 (59.1 %) | 0.1 |
| Intra Hepatic | 3 (75 %) | 1 (25 %) | |
| **Lymph Node Metastasis** | | | |
| Negative | 11 (50 %) | 11 (50 %) | 0.225 |
| Positive | 1 (25 %) | 3 (75 %) | |

***Table 1. Relationship of Microvascular Density with the Various Clinicopathologic Parameters (Test Chi-Square)***

| Variable | Microvascular Density / IHC (< 15) | Microvascular Density / IHC (> / = 15) | P Value (< 0.05 is Significant) |
|---|---|---|---|
| Age Mean (SD) | 58.30 (12.59) | 53.24 (10.23) | 0.158 |
| Serum Bilirubin Mean (SD) | 15.67 (8.88) | 12.29 (5.49) | 0.247 |
| CA19-9 Tumour Marker | 150.83 (331.47) | 61.07 (32.56) | 0.322 |

***Table 2. Relationship of Microvascular Density with the Various Clinicopathologic Parameters (Test Independent Sample T Test)***

There is a significant difference in microvascular density between moderately differentiated cholangiocarcinoma and well differentiated cholangiocarcinoma.

## DISCUSSION

The aim of our study was to assess the microvascular density in cholangiocarcinoma using CD34 and to find its relationship with grading. Cholangiocarcinoma is a very rare tumour accounting for less than 2 % of all human malignancies. In this study, during the period of two years, there were a total of 26 cases. In this study, cholangiocarcinoma affected males (57.7 %) more than females, which is comparable to the study by Wang et al.[15] The age group varied from 37 years to 78 years with a mean age of 55.77 years with a standard deviation of 9.65 and it is similar to the study conducted by Wang et al. (mean age 55 years). Most of the patients presented with jaundice and the rest had abdominal pain, pruritis, fever or tiredness. All of them had high CA 19-9 values. Surgical procedure followed were 7 Whipple's, 11 pylorus preserving pancreaticoduodenectomy (PPPD), 7 hepatectomies, one pancreaticoduodenectomy. Based on the macroscopy and radiology, there were more of extrahepatic cholangiocarcinomas (84.6 %) than intrahepatic cholangiocarcinomas in this study which is comparable to a study by Farhat et al.[16] Microscopically, moderately differentiated adenocarcinomas (53.8 %) topped the list in grade of tumour similar to that in a study by Mao et al.[17] Well differentiated adenocarcinomas were 42.3 %.

Poorly differentiated adenocarcinomas were not obtained in this study. There was one adenosquamous

carcinoma which was not graded. Most of the intrahepatic cholangiocarcinomas (75 %) showed low microvascular density while most of the extrahepatic cholangiocarcinomas (59.1 %) showed high microvascular density. In this study high microvascular density was seen in moderately differentiated cholangiocarcinoma and low microvascular density was seen in well differentiated adenocarcinoma with p value of 0.042. This is similar to that stated in the study by Zhang Chun Yang.[18]

Most of the intrahepatic cholangiocarcinomas (75 %) showed low microvascular density while most of the extrahepatic cholangiocarcinomas (59.1 %) showed high microvascular density but the p value was 0.1 in this study, hence not statistically significant. There was no significant statistical relation of microvascular density with age, sex, tumour marker (CA-19-9) or bilirubin levels. This is at par with the results in study by Zhang Chun Yang.

There was no significant statistical relation of microvascular density with age, sex, tumour marker (CA-19-9) or bilirubin levels which is comparable to the study by Nanashima et al.[19] Though lymph node metastases in this study was low (15 %), 75 % of the nodal metastatic cases fell under the category of high microvascular density which is similar to studies by Thelen et al. but a significant p value was not attained with this respect. This may be due to the smaller number of cases with lymph node metastases encountered in this study.

## CONCLUSIONS

Cholangiocarcinoma, a rare tumour has been increasing over the past decade due to unknown reasons and still has a grave prognosis. The 5-year survival of patients with negative surgical margin is 20 % to 35 % and zero if margin is positive. The most important prognostic factors include stage, surgical margin, lymph node metastasis, and grade of the tumour. Most patients present at an advanced stage precluding curative surgical resection. The tumour progresses rapidly which may be attributed to tumour angiogenesis. In this regard, assessment of microvascular density as a marker of angiogenesis has become a relevant factor. From the literature review it is evident that microvascular density has positive correlation with patient survival and is proposed as an upcoming important prognostic factor.

In this study, microvascular density was assessed and has showed a positive correlation with the grade of the tumour. 75 % of the lymph node positive cases showed a high microvascular density. Grade and lymph node metastasis being important prognostic factors, the study suggests that microvascular density is a useful prognostic factor. The limitation of the study was its small study population owing to its rarity. Further, in this era of personalised medicine, assessment of angiogenesis may help in asserting the prognosis in small biopsies. It is the basis for newer treatment approach under phase I and II trials of antiangiogenic drugs in cholangiocarcinoma. Hence, further studies on both these aspects are recommended and are essential.

Data sharing statement provided by the authors is available with the full text of this article at jebmh.com.
Financial or other competing interests: None.
Disclosure forms provided by the authors are available with the full text of this article at jebmh.com.
We acknowledge gratefully the guidance and useful advice given to us by Dr. Krishna. G and Dr. Santha Sadasivan. We would also like to acknowledge the immense help provided by Sri. Dilip Kumar, Senior Scientific Officer and Smt. Sheela, Scientific Assistant who helped us with the immunohistochemical staining.

## REFERENCES

[1] Lazaridis KN, Gores GJ. Cholangiocarcinoma. Gastroenterology 2005;128(6):1655-1667.
[2] Khan SA, Thomas HC, Davidson BR, et al. Cholangiocarcinoma. Lancet 2005;(9493)366:1303-1314.
[3] Chahal P, Baron TH. Cholangiocarcinoma. Curr Treat Options Gastroenterol 2005;8(6):493-502.
[4] Khan SA, Davidson BR, Goldin R, et al. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. Gut 2005;(51 Suppl 6):7-9.
[5] Blechacz BRA, Gores GJ. Cholangiocarcinoma. Clin Liver Dis 2008;12(1):131-150.
[6] Nehls O, Gregor M, Klump B. Serum and bile markers for cholangiocarcinoma. Semin Liver Dis 2004;24(2):139-154.
[7] Fletcher CDM. Diagnostic histopathology of tumours. Chap-10. Tumors of liver, biliary tree and gallbladder: 4th edn. Philadelphia: Elsevier Saunders 2013: p. 511-512.
[8] Washinton K. Sternberg's Diagnostic surgical pathology. Chapter – 37. Masses of Liver. 6th edn. Philadelphia: Wolters Kluwer 2015: p. 1745.
[9] Adsay NV, Klimstra DS. Odze and goldblum surgical pathology of gi tract, liver, biliary tree and pancreas. Chapter – 38. Benign and malignant tumours of gall bladder and extrahepatic biliary tract. 3rd edn. Philadelphia: Elsevier 2015: p. 1051.
[10] Iakovlev VV, Gabril M, Dubinski W, et al. Microvascular density as an independent predictor of clinical outcome in renal cell carcinoma: an automated image analysis study. Laboratory Investigation 2012;92(1):46-56.
[11] Vasudev NS, Reynolds AR. Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. Angiogenesis 2014;17(3):471-494.
[12] Kumar V, Abbas AK, Aster JC. Robbins and Cotran Pathologic basis of disease. Chapter – 7. Neoplasia. 9th edn. Philadelphia: Elsevier 2015: p. 305.
[13] Mobius C, Demuth C, Aigner T, et al. Evaluation of vascular endothelial growth factor A A expression and microvascular density as prognostic factors in extrahepatic cholangiocarcinoma. Eur J Surg Oncol 2007;33(8):1025-1029.
[14] Thelen A, Scholz A, Benckert C, et al. Microvessel density correlates with lymph node metastases and prognosis in hilar cholangiocarcinoma. J Gastroenterol 2008;43(12):959-966.

[15] Wang K, Zhang H, Xia Y, et al. Surgical options for intrahepatic cholangiocarcinoma. J Hepotobiliary Surgery and Nutrition 2017;6(2):79-90.
[16] Farhat MH, Shamseddine AI, Tawil AN, et al. Prognostic factors in patients with advanced cholangiocarcinoma: role of surgery, chemotherapy and body mass index. World J Gastroenterol 2008;14(20):3224-3230.
[17] Mao Z, Guo X, Su D, et al. Prognostic factors of cholangiocarcinoma after surgical resection: a retrospective study of 293 patients. Med Sci Monit 2015;21:2375-2381.
[18] Yang ZC. Study on the expression of Angiostatin and CD34 in cholangiocarcinoma and clinical significance. China Papers, 2007.
[19] Nanashima A, Shibata K, Nakayama T, et al. Relationship between microvessel count and postoperative survival in patients with intrahepatic cholangiocarcinoma. Annals of Surgical Oncology 2009;16(8):2123-2129.