Nucleus ratio
associated with malignancy
in gastrointestinal stromal tumors
a morphometric study of 39 patients

5-års oppgave i Stadium IV

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Abstract

The goal of our study was to determine if simple morphometric measurements by using only the ratio of longest and shortest nuclear diameter (LS ratio) could complement conventional histopathological analysis in predicting the malignancy potential of GISTs. Previous studies have not been able to establish any such relation.

From slides made for investigation on mesenchymal tumors 40 cases were randomly selected. Of these, 39 stained positive for c-Kit and were designated GISTs. These were the slides of interest. They were studied in a microscope, were number of mitosis was counted. Based on mitotic count and tumor size, the GISTs were classified as very low risk, low-risk, intermediate-risk, and high-risk tumors. Size of the primary tumours was known prior to the investigation. The slides were then examined by semiautomatic morphometry. Largest and smallest diameter of cell nucleus in 100 cells in each case was measured, and the ratio (LS ratio) was calculated. The results were analysed using the SPSS system.

The mean for all LS-ratios was 3.09. For further calculations the cut-off value for LS-ratio was set at 3.03 as this divided the patients into two almost equal groups. When grouping the tumors into risk groups, thirteen were classified as high risk tumors, with size larger than >5 cm and more than 5 mitosis (NIH4) and twenty-six with tumors with very low , low or intermediate risk. 3 out of the patients with high LS ratio were high risk patients versus 10 out of the 20 with a low LS ratio.

This study was limited relatively by sample size with 39 GISTs. However, more than 5 mitosis/50HPF showed strong significance on overall 5 year survival. This is consistent with the finding in larger studies were this is often the most reproducible prognostic marker. Despite the relative small number of cases this study should therefore be considered as representative with intriguing associations between objective nuclear features and malignancy potential.

The current study suggests that nuclear image analysis of GIST may be an important tool for objective pathological analysis. The use of such an approach can overcome the limitations of interobserver agreement and may provide an important supplement to

other procedures for identifying patients with high risk of malignant GIST. Further studies are needed to confirm these findings.

Introduction

In recent years gastrointestinal stromal tumor (GIST) has emerged as an important clinical and pathological entity. Immunohistochemical analysis for KIT has made the once troublesome diagnosis of GIST became relativiely straight forward (1,2). Accurate diagnosis is very important for patients as effective treatment with the kinase inhibitor imatinib mesylate (Gleevec) is available for patients with metastastic and unresectable GIST (3,4). The majority of the tumors present in the stomach (50-70%) or small bowel (20-30%), but they can occur throughout the GI tract (1).

The greater problem today with GIST is determining prognosis and, by extension, need for surgical and medical intervention. A number of approaches have been developed to identify the patients with high risk tumors, and many of these focus on examining the pathological specimens for features that can predict prognosis. There are a number of parameters that have been defined reproducibly as prognostically important, including mitotic index, size, mucosal ulceration, necrosis and site of origin. A consensus guideline for GIST prognosis was developed during a National Institutes of Health/National Cancer Institute-sponsored workshop in April 2001 where size and mitotic index were emphasized as risk factors (2).

One way to overcome the potential problem associated with subjective histological assessments is to develop more objective and reproducible analytical techniques to quantitate key histopathologic features of GISTs, such as nuclear pleomorphism and mitotic activity. Digital analysis can provide an objective assessment of nuclear morphology to complement conventional histopathology analysis. Assessment of nuclear morphometry has been used in several studies in breast cancer for prediction of recurrence (5,6). Recent studies have proved that there is a relationship between morphometric measurements and the prognosis of endometrial hyperplasia, expressed as the D-score (discriminate score) in endometrial hyperplasia for prediction of prognosis (7,8). Partin *et.*al evaluated usefulness of nuclear morphology for prediction of prognosis in stage A2 prostate cancer in 255 patients, and found that an averge nuclear roundness factor provided significant separation of the patients on the basis of outcome, more clearly than the Gleason score did. The best separation was provided by the lower quartile analysis of the ellipticity shape descriptor (9).

Before GIST was established as an entity Cunningham *et. al*, (10) predicted prognosis of 122 gastrointestinal smooth muscle tumors (SMT). From what we know about the distribution of mesenchymal tumors in the GI-tract today many of these tumors are likely to have been GISTs (11). Immunohistochemical assays were used to ascertain that the tumors were not from neurogenic or other non-smooth muscle derivation without further description. By using morphometric imagine techniques they assessed 100 tumor cell nuclei in each case. None of the morphometric measurements (nuclear perimeter, nuclear area, nuclear circularity form factor, longest nuclear diameter, nuclear average ferret diameter and nuclear equivalent diameter) were significant for overall outcome.

The goal of this study was to determine if simple morphometric measurements by only using the ratio of longest and shortest nuclear diameter (LS ratio) could complement conventional histopathological analysis associated with malignancy of GIST. The GISTs are diagnosed using current criterias (including KIT immunohistochemistry). Today mitotic count and size of the tumors are the main features to determine malignancy. The results reported here suggest that image analysis techniques do allow identification of objective nuclear features useful for predicting prognosis of GIST.

Materials and methods

1A. Identification of GIST cases.

From slides made for investigation on mesenchymal tumors 40 cases were randomly selected for morphometric studies. All specimens had been fixed in formalin and embedded in paraffin according to standard procedures. All slides were stained immunohistochemical with primary antibodies. All tumors stained positive for c-KIT were designated as GISTs, and this gave 39 cases for further investigation. (Fig 1)

1B. Diagnostic immunohistochemistry.

The antibodies used were; c-KIT (polyclonal, microwave heating 2 times for 10 minutes followed by cooling for at least 10 minutes, dilution 1:60, Santa Cruz Biothechical); smooth muscle actin (Clone 1A4, no pretreatment, dilution 1:50, Dako A/S); S100 (polyclonal, pretreatment with Ventana kit Protease I for 4 minutes, dilution 1:500, Dako A/S); CD34 (clone NCL-END, pretreatment with microwave heating, 450 W, pH 7 for 3 times in 10 minutes followed by cooling for at least 10 minutes, dilution 1:25, Novacastra medical); desmin (clone DE-R-11, pretreatment with Ventana kit Protease I for 4 minutes, dilution 1:100, Dako A/S), vimentin (clone V9, no pretreatment, dilution 1:50, Dako A/S); CK (clone MNF116, pretreatment with Ventana kit Protease I for 4 minutes, dilution 1:100, Dako A/S) and Ki67 (clone Mib-1, pretreatment with pressure cooker, citrate buffer, pH7 for 5 minutes at full pressure, Immunotech). The immunostaining was performed with an avidin-biotin detection system. As a chromogen diaminobenzidine hydrochloride solution with hydrogen peroxide (Ventana Gen II, Dab basic) was used. Positive controls for all markers were included for every batch.

2. Pathologic prognostic evaluation.

The slides were evaluated microscopically for mitotic activity. The mitotic count was expressed as the number of mitoses pr 50 high-power fields (HPF). In the microscope used (Olympus BX50, WH10X/22, 40X) the area of a single HPF is ~0.2 mm^2. Based on mitotic count and tumor size, the GISTs were classified as very low risk, low-risk, intermediate-risk, and high-risk tumors (2).

One case stained positive for desmin, and did not stain for KIT. The remaining thirty-nine cases were diagnosed as GIST based on previous published criterias (1,2).

3. Morphometry.

4 µm-thick slides were stained with hematoxylin and eosin, and examined by semiautomatic morphometry using a microscope (Leitz Wetzlar Germany, Type 307 – 128.0) coupled to a computerized system using the LEICA Qwin 2.6 Image Processing and Analysis System (Leica Cambridge, Cambridge, England), and a LEICA DC 300F digital camera. Largest and smallest diameter of cell nucleus in 100 cells in each case was measured, and the ratio (LS ratio) was calculated.

The area of interest was marked on the slides to ensure that only nuclei from tumor cells were measured.

4. Statistics.

The results were analysed using the SPSS system. Overall 5 year survival was calculated using Kaplan Meier. Pearson Chi-Square tests were used unless numerical cells had expected count less than 5, in which cases Fisher's Exact Test was performed.

Comment on methods

According to the demands to our essay, personal contributions are here mentioned briefly:

- Prior to starting the practical work background information on the field was necessary. Several papers concerning gastrointestinal stromal tumors and morphometry were studied.
- The first practical part of this study was first to examine histological slides stained with H&E under the microscope. This was useful for getting the experience of handling and viewing slides in a microscope. Mitosis per 50 high power fields was counted.
- The second examining of the slides was a work consuming task using a microscope coupled to a computerized system and doing a semiautomatic measuring of largest and smallest diameter in 100 cells in each slide.
- Average largest and smallest cell-diameter from every case was plotted into a program for statistical analyzes, and LS ratio was calculated. Other patient characteristics as gender and age at the time of diagnosis, death, location of

primary tumor, tumor size and metastasis at the time of diagnosis were incorporated into the same worksheet. Statistical analyzes was challenging as well as time consuming!

- Writing a draft was done simultaneously as the practical work not to forget steps along the way. Discussions with and reviewing of manuscript by the main mentor (SES) was on regular basis.

Results

Basic characteristics are shown in Table 1. The patients were also grouped according to pathologic risk-levels (2) giving 19 patients with very low malignancy potential, 4 with low malignancy potential, and 13 with high malignancy potential.

The mean ratio between longest and shortest diameter of 100 tumor cell nuclei (LS-ratio) was calculated for each case. The mean for all LS-ratios was 3.09 and the mean was 3.03 with range 1.23 - 5.08. For further calculations the cut-off was set at 3.03 as this divided the patients into two almost equal groups; 20 in the group with LS-ratio 3.03 or smaller, and 19 in the group with ratio larger than 3.03.

The patients with mitotic count more than 5/50 HPF had a significant worse outcome than those with less mitosis (p=0.02) on a 5 year overall survival. (Fig 2) Only 2 out of 19 of the tumors with high LS ratio had a high mitotic count versus 7out of 20 for low LS ratio tumors (p=0.056, Fisher exact test). (Fig 3)
For tumors with size >5 cm 3 out of 19 had a high LS ratio versus 10 out of 20 with a lower LS ratio in the tumours with a size<5cm. (p=0.04, Fisher exact test). (Fig 4)

When grouping the tumors into risk groups, thirteen were classified as high risk tumors, with size larger than >5 cm and more than 5 mitosis (NIH4) and twenty-six with tumors with very low, low or intermediate malignancy potential. 3 out of the patients with high LS ratio were high risk patients versus 10 out of the 20 with lower LS ratio (p=0.023, Fisher exact test) (Fig 5).

In the small bowel there were three times as many tumors with LS ratio smaller than 3.03 compared to tumors with larger ratio (9:3) (Fig 6). 15 of the 24 gastric tumors had a larger LS ratio than 3.03 versus 3 out of the 12 tumors in the small bowl (p=0.034, Fisher exact test).

In 37 of the cases the mutation status was known. 27 of the tumors had mutations in KIT (23 in exon 11, two in exon 9 and two in exon 13). Two tumors had mutations in PDGFRA exon 18, and 8 had no detectable mutations. The mutations were equally divided among the tumors having nuclear LS ratio lower and higher than 3.03. The tumors in the stomach had 7 cases with WT, 14 with KIT exon 11 mutations, and one

case with mutation in PDGFRA 18. In the small bowl none of the tumors were WT, 7 had KIT exon 11 mutations, 2 had mutations in KIT exon 9 and 13 while one had mutation in PDGFRA 18. In the large bowl there was one case with WT tumor, and in the omentum both the tumors had KIT exon 11 mutations.

Discussion

The goal of this study was to identify individuals who would have GIST with high malignancy potential. The approach was to use nuclear morphometric image analysis technique to objectively identify the characteristics of lesions that discriminate GIST patients with high malignancy potential from patients with a lower malignancy potential. Computerized morphometry is an objective computer-assisted image analysis to quantify morphologic parameters in every individual cell. The potential diagnostic role of morphometry in discriminating benign from malignant tumors has been investigated by many, but mainly in cytological samples (12,13). In a study by Wang et.al on thyroid histology LS ratio showed a strong statistically difference between follicular carcinoma with a low LS ratio and follicular adenoma with high LS ratio (14). Also Hoque et.al found that low sphericity (elongated nucleus) correlated with lower nuclear grade in ductal carcinoma of the breast (5). This supports that more elongated nuclei (higher LS ratio) can be a sign of less malignant tumors, and the ratio of longest to shortest nuclear diameter might be a useful complement to conventional histopathological analysis in determination malignancy of GIST.

This study was limited relatively by sample size with 39 GISTs. However, more than 5 mitosis/50HPF showed strong significance on overall 5 year survival. This is consistent with the findings in larger studies were this is often the most reproducible prognostic marker (15,16). Despite the relative small number of cases this study should therefore be considered as representative with intriguing associations between objective nuclear features and malignancy potential.

Consensus guidelines for GIST prognosis, assembled during a National Institutes of Health/National Cancer Institutes-sponsored workshop in April 2001 emphasise tumor size and mitotic index for risk stratification of primary tumors (2). This has been supported by others (17), and GIST tumors are now in many studies grouped into the four groups suggested in the consensus guidelines. In an Icelandic study by Tryggvason et.al (18) the GISTs were divided into high risk group (NIH4) versus all the other groups (very low, low and intermediate risk groups). Thirteen out of the 57 tumors in that study were classified as NIH4, and they constituted almost 23% of the patients. Only two of these high-risk tumors were gastric while eleven of them were non-gastric. The patients in the high-risk group had a significant less favourable overall survival. In our study

thirteen of the 39 cases were classified as high-risk versus twenty-six as non-high-risk which is 33% of the cases. This indicates that the tumors in our study might constitute of more high-risk tumors than found in more population based studies.

In the Icelandic study 35 out of the 57 tumors were gastric (61.4%). The non-gastric GISTs were at higher risk of a malignant behavior than gastric GISTs. In our study the distribution of gastric and non-gastric GSITs were comparable with 61.5% of tumors in our study being gastric. We found a significant association between nuclear ratio and gastric versus small-bowel with the gastric tumors having a significant higher LS ratio. This correlates well with also other studies that have noted a significant better prognosis for patients with gastric tumors (19, 20).

In summary the current study suggests that nuclear image analysis of GIST may be an important tool for objective pathological analysis. The use of such an approach can overcome the limitations of interobserver agreement and may provide an important supplement to other procedures for identifying patients with high risk of malignant GIST. Further studies are needed to confirm these findings.

References

1. Miettinen M, Lasota J. Gastrointestinal stromal tumours – definition, clinical, histological, immunohistochemical and molecular features and differential diagnosis Virchows Archive 2001, 438: 1-12

2. Fletcher, CDM, Berman JJ, Corless C, Gorstein F, Lazota J, Longley J. et al. Diagnosis of Gastrointestinal Stromal Tumours: A consensus Approach. *Hum Pathol* 2002;33:459-465.

3. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med.* 2002 Aug 15;347(7):472-80.

4. van Oosterom AT, Judson I, Verweij J, Stroobants S, Donato di Paola E, Dimitrijevic S, Martens M, Webb A, Sciot R, Van Glabbeke M, Silberman S, Nielsen OS; European Organisation for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study.
Lancet. 2001 Oct 27;358(9291):1421-3.

5. Hoque A, Lippman SM, Boiko IV, Atkinson EN, Sneige N, Sahin A, Weber DM, Risin S, Lagios MD, Schwarting R, Colburn WJ, Dhingra K, Follen M, Kelloff GJ, Boone CW, Hittelman WN .Quantitative nuclear morphometry by image analysis for prediction of recurrence of ductal carcinoma in situ of the breast. Cancer Epidemiol Biomarkers Prev. 2001 Mar;10(3):249-59.

6. Tan PH, Goh BB, Chiang G, Bay BH. Correlation of nuclear morphometry with pathologic parameters in ductal carcinoma in situ of the breast.
Mod Pathol. 2001 Oct;14(10):937-41.

7. Ausems EW, van der Kamp JK, Baak JP. Nuclear morphometry in the determination of the prognosis of marked atypical endometrial hyperplasia.
Int J Gynecol Pathol. 1985;4(3):180-5.

8. Baak JP, Orbo A, van Diest PJ, Jiwa M, de Bruin P, Broeckaert M, Snijders W, Boodt PJ, Fons G, Burger C, Verheijen RH, Houben PW, The HS, Kenemans P. Prospective multicenter evaluation of the morphometric D-score for prediction of the outcome of endometrial hyperplasias.
Am J Surg Pathol. 2001 Jul;25(7):930-5.

9. Partin AW, Walsh AC, Pitcock RV, Mohler JL, Epstein JI, Coffey DS. A comparison of nuclear morphometry and Gleason grade as a predictor of prognosis in stage A2 prostate cancer: a critical analysis.
J Urol. 1989 Nov;142(5):1254-8.

10. Cunningham RE, Federspiel BH, McCarthy WF, Sobin LH, O'Leary TJ. Predicting the prognosis of Gastrointestinal Smooth Muscle Tumours The American Journal of Surgical Pathology, 1993, 17(6):588-594

11. Steigen SE, Eide TJ. Trends in incidence and survival of mesenchymal neoplasm of the digestive tract within a defined population of Northern Norway. APMIS. 2006, 114

12. Unger PD, Hoon V, Stone N, Liu Z, Theise N, Stock R, Gil J. Computerized interactive morphometry in the differential diagnosis of irradiated prostates. Anal Quant Cytol Histol. 1995 Apr;17(2):100-8.

13. Koutselini H, Lazaris AC, Kavantzas N, Kiritsi T, Davaris PS. Significance of nuclear morphometry as a diagnostic tool in fine-needle aspirates of the liver. Eur J Gastroenterol Hepatol. 2000 Aug;12(8):913-21.

14. Wang SL, Wu MT, Yang SF, Chan HM, Chai CY. Computerized nuclear morphometry in thyroid follicular neoplasms. *Pathol Int.* 2005 Nov;55(11):703-6.

15. Miettinen M, Sarlomo-Rikala M, Sobin LH, Lasota J. Gastrointestinal stromal tumors and leiomyosarcomas in the colon: a clinicopathologic, immunohistochemical, and molecular genetic study of 44 cases. *Am J Surg Pathol.* 2000 Oct;24(10):1339-52.

16. Singer S, Rubin BP, Lux ML, Chen CJ, Demetri GD, Fletcher CD, Fletcher JA. Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. *J Clin Oncol.* 2002 Sep 15;20(18):3898-905.

17. DeMatteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. *Ann Surg.* 2000 Jan;231(1):51-8.

18. Tryggvason G, Gislason HG, Magnusson MK, Jonasson JG. Gastrointestinal stromal tumors in Iceland, 1990-2003. The Islandic GIST study, a population-based incidence and pathologic risk stratification study. *Int J Cancer* 205;117:289-93

19. Emory TS, Sobin LH, Lukes L, Lee DH, O'Leary TJ. Prognosis of gastrointestinal smooth-muscle (stromal) tumors: dependence on anatomic site. *Am J Surg Pathol.* 1999 Jan;23(1):82-7.

20. Miettinen M, Kopczynski J, Makhlouf HR, Sarlomo-Rikala M, Gyorffy H, Burke A, Sobin LH, Lasota J. Gastrointestinal stromal tumors, intramural leiomyomas, and leiomyosarcomas in the duodenum: a clinicopathologic, immunohistochemical, and molecular genetic study of 167 cases. Am *J Surg Pathol.* 2003 May;27(5):625-41.

Table 1 Basic characteristics

Male	28 (71.8%)
Age years, median	64 (32-93)
Location -stomach -small intestine -other sites -unknown	 22 (56.45) 12 (30.8%) 3 (7.7%) 2 (5.1%)
Size mm, median	40 (7-200)
Mitosis/50 HPF, median	3 (0-49)
Maximum diameter, median	14.95 (8.02-18.56)
Minimum diameter, median	4.74 (3.20-8.51)
Ratio, median	3.03 (1.23-5.08)

Fig 1. Slide with GIST stained with H&E (A), and slide stained with c-KIT (B)
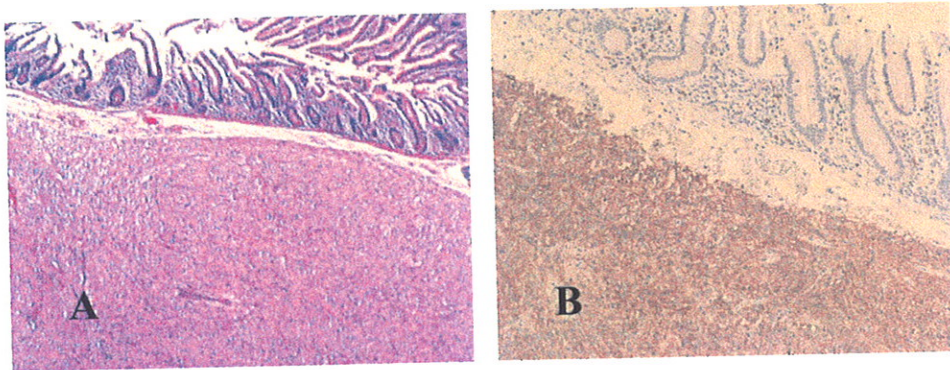
Fig 2. Survival of patients with less than 5 mitosis/50 HPF (green upper line) versus higher mitotic count (bottom blue line)
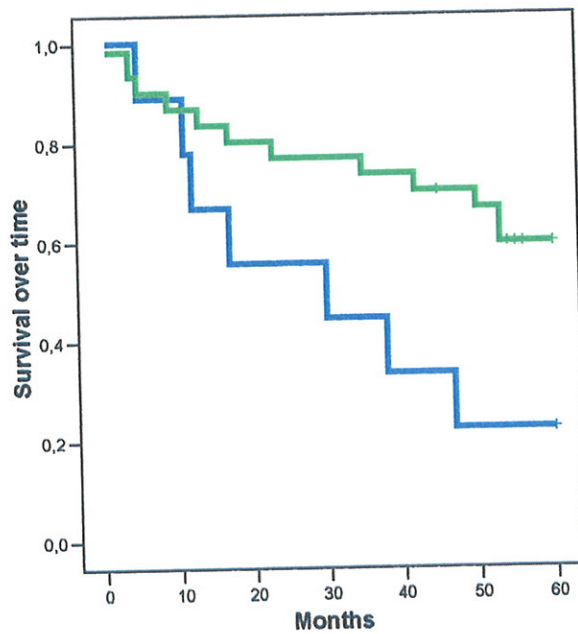
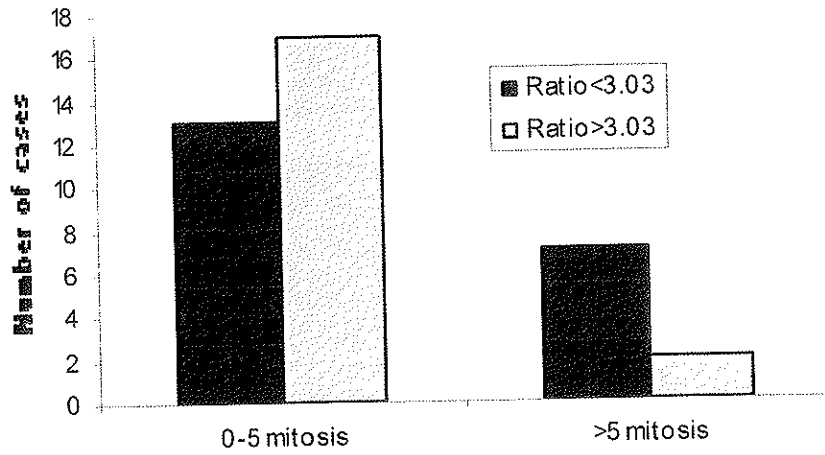Fig 3. Number of cases with 0-5 mitosis versus >5 mitosis in association with high and low LS ratio

Fig 4. Number of cases with tumor size 1-59 mm versus larger tumors in association with high and low LS ratio
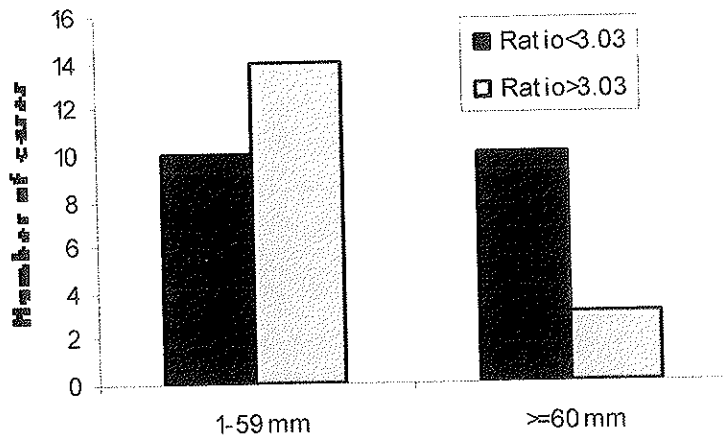
Fig 5. Number of cases with high risk tumors versus tumors lower risk in association with high and low LS ratio
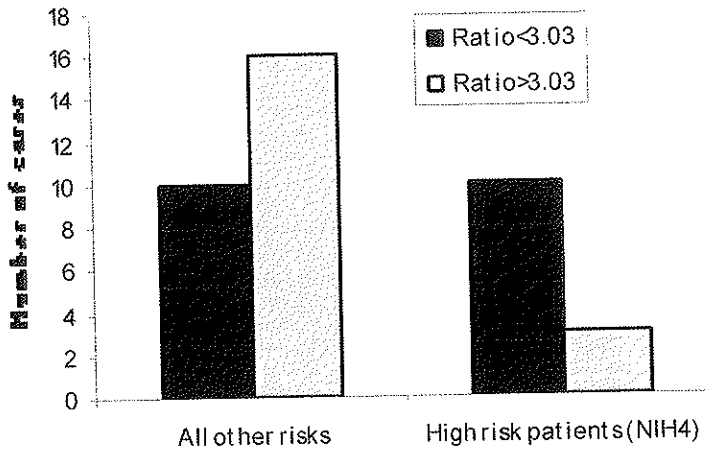

Fig 6. Number of cases with gastric tumors versus tumors in the small bowl in association with high and low LS ratio