How to Cite:
Hussein, T. A., Hameed, A. S., & Amer, M. H. (2022). Role of RASSF1-A gene in early detection of colorectal carcinogenesis. *International Journal of Health Sciences*, *6*(S9), 1908–1917. https://doi.org/10.53730/ijhs.v6nS9.12831

Role of RASSF1-A gene in early detection of colorectal carcinogenesis

Thaer Ali Hussein
University of Southern Technical, Institute of Al-Nasiriya Technical, Department of Nursing, Iraq
Corresponding author email: thaerali@stu.edu.iq

Ahmad Shandookh Hameed
University of Southern Technical, Institute of Al-Nasiriya Technical, Department of Nursing, Iraq

Mohamed Hussein Amer
University of Southern Technical, Institute of Al-Nasiriya Technical, Department of Nursing, Iraq

***Abstract*---**Background: Colorectal cancer (CRC) is a malignant neoplasm originating from the un-controlled cell proliferations in epithelial cells lining of rectum, colon, or appendix. RASSFs wereconcerned in tumourigenesis and numerous members of family are presently supposed to be tumour suppressor, Aim and objectives: Estimate- function of RASSF1-A methylated gene in theCRC pathogenesis, detect RASSF1-A methylated gene cutoff value for diagnosis and using RASSF1-A gene as abio-marker to detect recurrence after surgery, Subjects and methods: This is a prospective observational case-control researchperformed on 50 cases diagnosed as CRC by endoscopy and histopathology and 50 control persons at Aswan university hospital and internal medicine department., Results: there is high significant relation between RASSF 1A and development of colorectal-cancer, Conclusion: Genetical mutations in the Kras genes and epigenetic modifications in RASSF1-A, FHIT and MGMT genes in sporadic CRC are accompanying with the generalprogress of the disorder and can be utilized as diagnostical or prognostically biomarkers in this collection of tumours.

***Keywords*---**RASSF1-A, tumour suppressor gene, Ras-association domain, methylation, colorectal cancer.

Introduction

CRC occurrence and death rate significantlydifferuniversally. Universally, CRC is the 3rd commonest diagnosed tumour in men and the 2nd in women, with 1.4 million new patients and more than 600 thousandrelated mortalities yearly**(1).** Of all CRC patients, 75-80% happensinfrequently as the consequence of complex reactions amongvulner- ability genes and environmentallyinfluences. In spite of the current and nonstopdevelopments in managements, >50% of colon and rectal tumours metastasize to lungs,liver, and lymph node, and the five year survive ratesstill low (nearly 10 percent) for cases with metastatic CRC.There is, consequently, a vitalnecessity for newbio-markers that can be beneficial in diagnosing and canas welladvance prognosis and treatingexpectation. The buildup of in vitro and in vivoevidence proposes epi-genetics applies anessentialfunction in the pathogenesis of CRC**(2)**. The best identified and more common epigenetic modification is DNA methylations, which influencestumour suppressors gene that can be included in cell cycle controlling, DNA repairing, and carcinogensmetabolism, cell-cell interactions, apoptosis and angio-genesis. RAS-associations domain family (RASSF) members were a newly recognized family of putative tumour suppressors RAS effector **(3)**. Loss or altered expression of this gene was also revealed to be connected with the hyper-methylations of its CpG-island promoter area along with pathogenesis of many different cancers **(4)**. RASSF family of proteins includes members from RASSF1 to RASSF10. Among them, RASSF1-A, RASSF2, RASSF4 and RASSF5 were most wildly reported to be associated with colorectal carcinoma. This work aimed to primarily evaluate the methylationscondition of the above genes and to investigatetheir potential predictiveimportancein primarydisorder.

Subjects and Methods

This study was a prospective observational case control study carried out in Tha-Qar Oncology center from March 2019 till March 2020. Fifty patients diagnosed as CRC by endoscopy and histopathology and fifty control persons with the same age and sex were included in the study.

Inclusion criteria: Patients diagnosed recently as CRC and patients with different age and sex.
Exclusion criteria: Patients with malignancies other than CRC, patients with CRC showing metastatic evidence by history, examination or investigations and CRC patients showing recurrence after surgical resection.

Methods

1. Clinical assessment: *Full history* including: history suggesting metastases.*Full examination***:** Pulse and blood pressure measurements, head and neck examination, upper and lower limb examination and chest and heart exam and abdominal examination. With special press on data suggestive of metastases as enlarged tender liver, hemiparesis, hemiplegia, bone pain, pathological fracture, ….etc.
2. Investigations: " End biopsy" three biopsies were taken from colorectal mass, 5 cm proximal, 5cm distal to mass and from healthy persons rectal biopsy will be

taken and examined for: RASSF1-A gene '' methylated gene" by Quantitative PCR, staging of carcinoma by histopathology, metastatic work up whenever indicated as abdominal ultrasound, CT chest, abdomen, mammography, bone scan, … etc. Routine lab.Examinationsinvolving ESR, CBC, liver and kidney functions, prothrombin time, concentration and INR. One portion of the biopsy was conserved in formalin (10%) for histo-pathological examinations, and additionalportion was reserveddirectly at −80°C for DNA extractions, DNA extraction of all samples by Qiagen kit. Collection of tissue DNA samples, DNA from ordinary and anomalous tissues of cases who experiencedcolono-scopy was extracted via the Qia-Amp DNA Mini-Kit rendering to the instructions of producer. The obtainedDNA was quantities by UV spectro-photomer and kept at −20°C, methylations-specific PCR and DNA obtainedfrom tissuesspecimens was exposed to bi-sulfite modifications as definedbefore**(5)**.To change all un-methylated cytosines into uracils leaving methylated cytosines un-modified. The bi-sulfite modificationsweredonevia the EZ DNA methylationsgold kit. Rendering to the protocols of producer, DNA (1–2 μg) was changedby means of sodium bi-sulfite, purified, desalted, and eluted via elution buffer. Centrifuge was performed for the columnthereafter incubations at room temp for 5 minutes to get the adapted DNA. The managed DNA was utilized directly or kept at −20°C tillusage. The bi-sulfiteadapted DNA was exposed to PCR. 2 sets of primers for every gene tested in this work were utilized in discriminationamong methylated and un-methylated alleles as definedbefore**(6)**.In short, the PCR mixcomprising 5μliter of 5X interaction buffer (100-mmol per Liter Tris–HCl {pH8.3}, 0.5-mol per LiterKCl, 15-mmol/L $MgCl_2$), 10-micro liter of adapted DNA, 0.010-mol of every primer, 1 micro liter deoxynucleotide tri-phosphates (0.200-mmol per Liter each, ultimate concentrations), and 1 U of hot start Taq DNA polymerase was accustomed to ultimateamount of 25 micro litervia sterilized water. The cycling condition involved incubation retro at 95 degrees for 15-mins 40 cycles of denaturizing at 94 degrees for 30 sec and 62 degrees for 30 sec (for RASSF1-A genes), 63degreesfor 30 sec (for HIC-1 genes), and 58degrees for 30 sec (for MGMT genes), extensions at 72 degrees for 30 sec and ultimate extensions at 72degreesfor 10 mins. Positive control methylated DNA specimens for every gene examined has been utilized. Water blank has been utilized as negative controls. PCR products have beeninvestigated on 2.5% agarose gel and picturedunderneath UV illuminations.

PCR reaction for the unmethylated *RASF1A* gene

PCR condition is the same as methylated primer.Ethical committee: Consent from the faculty of medicine ethical committee has beenattained and agreement from IRBhas beengotten.

Statistical analysis

data analysis has been performed viaSPSS-20. Quantitative variables have been introduced as mean and SD. Qualitative variables have been introduced as numbers and percentage. For comparing parametric quantitative variables for2 groups, Student t testinghas beendone. Qualitative variables have been compared via chi-square (X2) testing or Fisher's exact testing when frequencies were below 5. Pearson correlations coefficients have been utilized to measure the

associationsamong2 variables with normal distribution. When a variable wasn'tusually distributed, A P < 0.05 wasjudged significant

Results

Table (1): RASSF 1A status in between the studied cases:

Variable	Cases group (n=50)		Control group (n=50)		x^2	p-value
	No.	%	No.	%		
RASSF 1A status:						
Methylated: Unmethylated:	24 26	48.0 52.0	5 45	10.0 90.0	17.53	<0.001 (HS)

Table (2): RASSF 1A status in relation to tumour site:

Variable	5 cm proximal to tumour N=50		5 cm distal to tumour		At tumour		Control		x^2	p-value
	No.	%	No.	%	No.	%	No.	%		
RASSF 1A status:										
Methylated: Unmethylated	18 32	36.0 54.0	20 30	40.0 60.0	24 26	48.0 52.0	5 45	10.0 90.0	18.2	<0.001 (HS)

Table (3): Relation between RASSF 1A status and demographic data in cases group:

Variable	Methylated (n=24)		Unmethylated (n=26)		x^2	p-value
	No.	%	No.	%		
Sex :						
Female Male	9 15	37.5 62.5	11 15	42.3 57.7	0.12	0.728
Smoking history:						
Yes No	12 12	50.0 50.0	8 18	30.8 69.2	1.92	0.165
Family histoy of cancer:						
Yes No	5 19	20.8 79.2	5 21	19.2 80.8	0.02	0.887
Age (years):					T test	p-value
Mean ± SD Range	46.3±11.9 (33-65)		46.7±11.2 (36-67)		0.122	0.903

Table (4): Relation between RASSF 1A status and Charecteristics of colorectal carcinoma:

Variable	Methylated (n=24)		Unmethylated (n=26)		x^2	p-value
	No.	%	No.	%		
Location :						
Right colon	11	45.8	12	46.2	1.35	0.50
Transverse colon	0	0.0	0	0.0		
Left colon	10	41.7	13	50.0		
Recto-sigmoid	3	12.5	1	3.8		
Grades:						
I	10	41.7	9	34.6	1.16	0.654
II	14	58.3	17	65.4		
Tumour size:						
≥5	22	91.7	13	50.0	10.31	0.0013 (S)
<5	2	8.3	13	50.0		
Differentiation:						
Well	6	25.0	9	34.6	0.560	0.755
Moderate	13	54.2	12	46.2		
Poor	5	20.8	5	19.2		

x2 for chi square test
P value is significant if <0.05

Table (5): Relation between RASSF 1A status and Laboratory characteristics of colorectal carcinoma:

Variable	Methylated (n=24)		Unmethylated (n=26)		x^2	p-value
	No.	%	No.	%		
CEA levels:						
≤5ng/ml	6	25.0	9	34.6	0.54	0.458
>5ng/ml	18	75.0	17	65.4		
CA19.9 levels :						
Low (< 37 U/ml)	11	45.8	14	53.8	0.32	0.571
High (> 37 U/ml)	13	54.2	12	46.2		

x2 for chi square test
P value is significant if <0.05

Table (6): Relationship between % methylation change in RASSF 1A and patient characteristics:

		% methylation change in RASSF 1A			
		N=50	Mean± SD	T test	P value
Sex	Male	30	49.29±14.93	0.070	0.944
	Female	20	49.0±13.41		
Smoking	Non	30	45.29±11.91	1.59	0.117
	smokers	20	51.0±13.11		
Family history of cancer	Yes	10	52.22±14.93	0.93	0.35
	No	40	48.21±14.93		

p value statistically significant(<0.05)

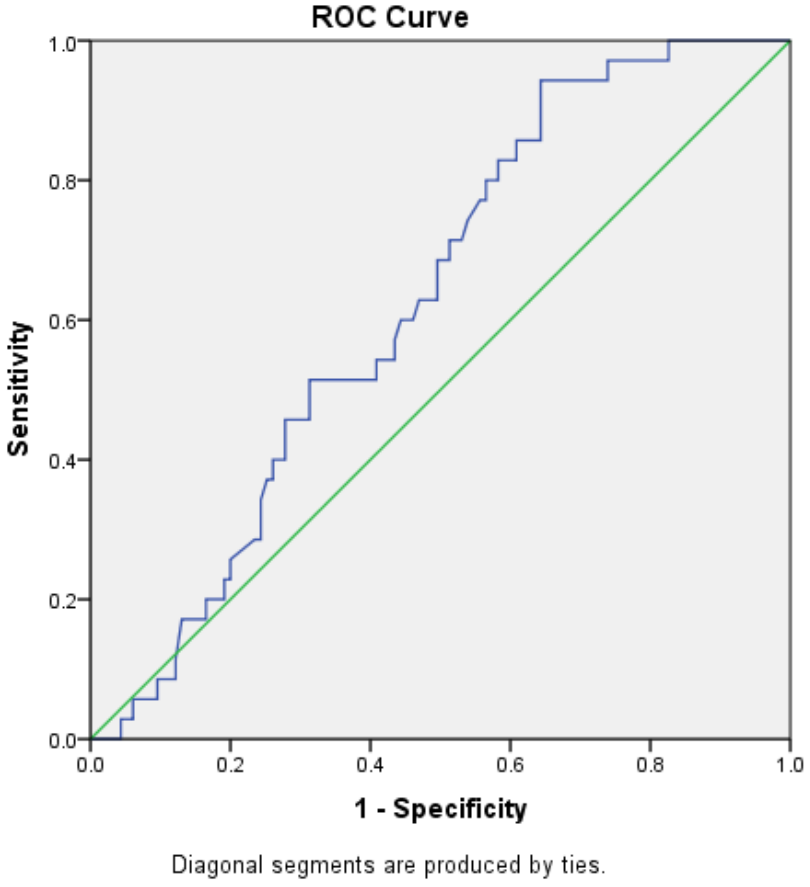

Fig. (1) ROC curve analysis for discrimination of RASSF 1A status between cases and control

Curve characteristics	Values
Area under the curve	0.733
Standard error	0.066
95% confidence interval	0.603 – 0. 864
p-value	0.002*
Sensitivity	83.3%
Specificity	45.0%

Discussion

Lately, anextensivenumber of reports has revealed that lack of expressions of RASSF1-A was revealed to contribute in adversity of human tumourspathogenesis, like lung tumour, breast tumour, in addition to CRC. Growingsuggestion has revealed that lack or aberrant expressions of RASSF1-A is supposed to be significantly connected with methylations of the CpG-island promoter sequence of RASSF1-A, that canchange the role or activity of RASSF1-A, thusdoing a significant function in indorsing the progressions and metastasis of CRC (**7).**This is why this study was selected to be conducted to estimate- function of RASSF1-A methylated gene in the pathogenesis of CRC, to try to detect RASSF1-A methylated gene cutoff value for diagnosis of CRC in microscopy before to be manifested clinically, using the primitive cutoff value of RASSF1-A gene for estimation of distance to detect margins proximally and distally during surgical resection and using RASSF1-A gene as a bio-marker to detect recurrence after surgery.A case control study was held, including fifty patients diagnosed as CRC by endoscopy and histopathology and fifty control persons with the same age and sex. The duration of the study ranged 6-12 months.In the study in our hands, there is high significant relation between RASSF 1A and development of Colo-rectal cancer.Our results are supported by study of **Sinha et al., (7)** as they reported that promoter hyper-methylations in RASSF1-A genes was detected in 47% patients of CRC in the tumourarea and in 13% of analogous controls.Furthermore, **Matthaios et al., (8)** found that APC and RASSF1-A promoters have beendetected to be methylated in 41.9% and33.5% of the 155 colon tumourspecimensinspected, but in no one of the ordinary controls (both P value<0.001).**Leeet al., (9)** demonstrated that RASSF1-A promoter methylations was detected in 47% patients. Other studies have concluded the frequencies of RASSF1-Amehylationfrom 16 to 81% patients in CRC **(10).**RASSF family members belonging to a latelyrecognized kind of putative tumour suppressors RAS effector for which epigenetic silencing via promoter methylationswasrevealed to happenvia the progressions of tumourcounting CRC **(3).**The present study shows that there is high significant relation between RASSF 1A and tumour site. There is no significant Relation between RASSF 1A status and demographic data in cases group. There is significant Relation between RASSF 1A status and tumour size, while there is no significant Relation between RASSF 1A status and Characteristics of colorectal carcinoma. There is no significant Relation between RASSF 1A status and Laboratory characteristics of colorectal carcinoma.RASSF1-A protein is actively concernedin microtubule regulations, genomic constancyconservation, cell-cycle regulations, apoptosis modulations, cells motilities and invasion controls **(11)**. Someauthors have recognized high percentages of RASSF1-Amethylations in CRC specimens. **Wagner et al., (12)** detectedRASSF1-A promoter methylations in 45percent of the primary CRC and

in 80 percent of the CRC cells lines investigated. Disobediently, in a reporton 222 specimens of irregular CRC, **van Engeland et al., (13)**noticedRASSF1-A methylations in 20% of the specimens, and a commonlyrestricted associations with the existence of Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation has beenproposed. Numerous other researchersfound RASSF1-A methylations in 17%, 36% and 47% of the CRC specimens inspected **(14)**. Particularly, various studies revealed thatRASSF1-Amethylations at variousphases, in addition to in the matchingordinary mucosa neighboringto the tumour, point to a function of the tumour suppressors genesRASSF1-A early in the progress of colorectal carcino-genesis. It can mean that methylationsperhapshappens in proximal locations of tumour cells because of field effect phenomena; but, the mechanism still to beresolved. The currentconsequences are in agreement with the afore-mentioned studies, as 25% hyper-methylated RASSF1-A promoter has beennoticed in cases with early oCRC, and 44.8% hyper-methylated RASSF1-A promoter has beennoticed in cases with mCRC, signifying that RASSF1-Amethylations is a common event in CRC, while more obvious at later disorderphases. This elevatedoccurrence of RASSF1-A methylations in metastatic disordercan be revealing of a more belligerenttumour behaviors, that canclarify the associations with poor prognosis detected in the current work. In their precedingreport, they achievedalike analysis of RASSF1-A methylations status in cases with operable stomachtumour**(5)**. In thiswork, a methylations rates of 68.5 percent was noticed, this highlights the variance in the biologic behaviors of stomachtumourcompared to CRC.

The current study shows that there is no relationship between % methylations change in RASSF 1A and sex, smoking status or family history of cancer. There is no Relationship between % methylations change in RASSF 1A and tumour size or differentiation.However, **Balgkouranidou, (6)**revealed that methylated RASSF1-A was accompanied with elevateddisorder stage in cases with early possibleCRC, whereas in cases with metastaticallydisorder methylated RASSF1-A was significantly correlated with elevated levels of CEA and APC hyper-methylated promoter status. Stimulatingly, in other reportslevels of RASSF1-A methylations were significantly elevated in the distal than the proximal CRCs **(15)**in addition to in the normally appeared mucosa, which is as well in agreement to the latestdescription of CRC tumours as left- and right-side tumours with every side having dissimilarprediction and dissimilar responses to therapy **(16)**.In CRC, the methylationsrank of RASSF1-Asignificantlyrelated with the CRC pathogenesis (**17).Nilsson, et al., (18)**concluded that the methylations status of RASSF1-A in 111 CRC samplesdescribes a poor prognosing subdivision of CRC case's non-dependently of both tumour stages and differentiations.**Wagner et al., (12)**studiedifRASSF1-A methylationsassociated with tumour-node-metastasis condition, but didn'tnotice any significance. Correspondingly, in the report by **Van England et al., (13)**RASSF1-A methylationswasn'tcorrelated with sex, Duke's stages or locations of the tumour. The only exclusion was the ages at diagnosing, that was somewhatelevated in RASSF1-A methylated CRC casesin comparison with nonmethylated ones.**Cejas et al., (19)**haven'trevealed any statistical associationsamong the tumour stages and metastasis with Kras mutations. But our findings are agreeable with that detected by **Mannan et al., (20)** where they revealed that Kras mutation has significantcorrelation with lymph nodes metastasis and tumourgrade but not with the growing patterns of colonic

carcinoma. Kras mutation can be has a significant concern in the biologic progress of the disorder, henceforthin flouncing its general behaviors and responding.

Conclusion

The synergistic inter-relationship among the genetically and epigeneticallyissues in colorectal tumourigenesiscanaid in progressingthe generalmethodto this disorder, The highest level of Methylated RASSF 1A was present at tumour itself followed by 5 cm proximal to tumour then 5 cm distal to tumour and the lowest level in control so RASSF 1A may help in microscopic identification of tumour in healthy tissue.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. (2015). Global Cancer Statistics, 2012. CA a cancer J Clin;65(2):87–108. Available from: http://onlinelibrary.wiley.com/doi/10.3322/caac.21262/abstract
2. Bardhan K, Liu K. (2013). Epigenetics and colorectal cancer pathogenesis. Cancers (Basel); 5(2):676-713.
3. Richter AM, Pfeifer GP, Dammann RH. (2009).The RASSF proteins in cancer: from epigenetic silencing to functional characterization. BiochimBiophys Acta;1796:114–28.
4. Fernandes MS, Carneiro F, Oliveira C, Seruca R (2013). Colorectal cancer and RASSF family—a special emphasis on RASSF1-A. Int J Cancer; 132:251–258.
5. Balgkouranidou I, Matthaios D, Karayiannakis A, Bolanaki H, Michailidis P, Xenidis N. (2015). Prognostic role of APC and RASSF1-A promoter methylations status in cell free circulating DNA of operable gastric cancer patients. Mutat Res; 778:46-51.
6. Balgkouranidou, I. (2019). The Methylations Status of RASSF1-A and APC Represents a Valuable Prognostic Indicator in Gastrointestinal Malignancies. EC Gastroenterology and Digestive System; 6, 582-597.
7. Sinha R, Hussain S, Mehrotra R, Kumar RS, Kumar K, Pande P, et al. (2013).Kras gene mutation and RASSF1-A, FHIT and MGMT gene promoter hyper-methylation: indicators of tumour staging and metastasis in adenocarcinomatous sporadic colorectal cancer in Indian population. PLoS ONE 8:e60142.
8. Matthaios, D., Balgkouranidou, I., Karayiannakis, A., Bolanaki, H., Xenidis, N., Amarantidis, K., et al. (2016). Methylations status of the APC and RASSF1-A promoter in cell-free circulating DNA and its prognostic role in patients with colorectal cancer. Oncology letters; 12(1), 748-756.
9. Lee S, Hwang KS, Lee HJ, Kim JS, Kang GH. (2004). Aberrant CpG island hyper-methylations of multiple genes in colorectal neoplasia. Lab Invest; 84(7):884-93.
10. Sakamoto N, Terai T, Ajioka Y, Abe S, Kobayasi O, Hirai S, et al. (2004). Frequent hyper-methylations of RASSF1-A in early flat-type colorectal tumours. Oncogene; 23(55):8900-7.
11. GhazalehHA, Chow RS, Choo SL, Pham D, Olesen JD, Wong RX, et al. (2010). 14-3-3 mediated regulation of the tumour suppressor protein, RASSF1-A. Apoptosis; 15(2):117-27.

12. Wagner KJ, Cooper WN, Grundy RG, Caldwell G, Jones C, Wadey RB, et al. (2002). Frequent RASSF1-Atumour suppressor gene promoter methylations in Wilms' tumour and colorectal cancer. Oncogene; 21(47):7277-82.
13. van Engeland M, Roemen GM, Brink M, Pachen MM, Weijenberg MP, de Bruïne AP, et al.(2002). K-ras mutations and RASSF1-A promoter methylations in colorectal cancer. Oncogene; 21(23):3792-5.
14. Gonzalo V, Lozano JJ, Muñoz J, Balaguer F, Pellisé M, Rodríguez de Miguel C, et al.,2010. Aberrant gene promoter methylations associated with sporadic multiple colorectal cancer. PLoS One; 5(1):e8777.
15. Ahlquist T, Bottillo I, Danielsen SA, Meling GI, Rognum TO, Lind GE, et al. (2008). RAS signaling in colorectal carcinomas through alteration of RAS, RAF, NF1, and/or RASSF1-A. Neoplasia; 10(7):680-6, 2 p following 686.
16. An B. (2010). “Characteristic methylations profile in CpG island methylator phenotype-negative distal colorectal cancers”. International Journal of Cancer; 127(9): 2095-2105.
17. Wang HL. (2014). “Promoter methylations of the RASSF1-A gene may contribute to colorectal cancer susceptibility: a meta-analysis of cohort studies”. Annals of Human Genetics 78.3 208-216.
18. Nilsson TK. (2013). “DNA methylations of the p14ARF, RASSF1-A and APC1A genes as an independent prognostic factor in colorectal cancer patients”. International Journal of Oncology; 42(1): 127-133.
19. Cejas P, López-Gómez M, Aguayo C, Madero R, de Castro Carpeño J, Belda-Iniesta C, et al. (2009). KRAS mutations in primary colorectal cancer tumours and related metastases: a potential role in prediction of lung metastasis. PLoS One; 4(12):e8199.
20. Mannan A, Hahn-Strömberg V. (2012). K-ras mutations are correlated to lymph node metastasis and tumour stage, but not to the growth pattern of colon carcinoma. APMIS; 120(6):459-68.